Molecular Properties and Active Form of Nonspecific Acid Phosphatase from *Schizosaccharomyces pombe*

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Equilibrium sedimentation experiments of the native acid phosphatase indicate a dimer-tetramer dissociating nonequilibrating system with a dimer $M_r = 180,000$ g/mol.

The hydrolysis of nitrophenylphosphate was used to determine the sedimentation coefficient of the active species. The $s_{20, w}$ value for the species which degrades nitrophenylphosphate is $13.52 \pm 0.46$ S in 1% sucrose and $13.72 \pm 0.11$ S in 1.5 M sodium chloride, corresponding to the Svedberg value of the tetramer species.

Several lines of evidence are presented which, together with previous data, indicate that the *Schizosaccharomyces pombe* nonspecific acid phosphatase is composed of 4 identical or nearly identical polypeptide chains: a, equilibrium sedimentation analysis of the enzyme in denaturing agents indicates the presence of homogeneous material having $M_r = 90,800$ g/mol; b, digestion with carboxypeptidase A releases 9.82 mol of tyrosine/monomer molecular weight. Concomitant phosphatase inactivation occurred during the splitting off of the tyrosyl terminal residue. Furthermore, a unique NH₂-terminal residue (histidine) was determined.

Our study on the genetic control of the formation of nonspecific acid phosphatase in *Schizosaccharomyces pombe* (1, 2) should be facilitated by the knowledge of the molecular properties of the enzyme. Although nonspecific phosphatases were purified to various degrees from fungi (3, 4) and yeast (5), a complete characterization of their subunit structure has not been reported. The preparation of considerable amounts of stable homogeneous nonspecific acid phosphatase from *S. pombe* (6) has now allowed the study of the molecular properties of the enzyme. The enzyme is a glycoprotein with neutral sugars accounting for ~66% of the total molecular weight.

Previous determinations of the molecular weight of the yeast nonspecific acid phosphatase have indicated that the enzyme is a tetramer of $M_r = 383,000$ g/mol (6).

Results to be published indicate that *S. pombe* acid phosphatase exists in different states of aggregation. Moving boundary velocity experiments showed that the native enzyme sediments as a mixture of two species, dimers and tetramers, with $s_{20, w}$ of 9.9 S and 14.5 S, respectively. Therefore, it seemed of great interest to investigate the molecular properties of the native acid phosphatase by sedimentation equilibrium analysis and to determine which form(s) the enzyme takes under the assay conditions. This last point was attempted by using the active enzyme-substrate sedimentation velocity method.

**EXPERIMENTAL PROCEDURES**

**Materials**

Norleucine was obtained from Mann Research and dansyl chloride was from Calbiochem. Diiisopropyf fluorophosphate-treated carboxypeptidase A was supplied by Worthington. Twice-distilled constant boiling HCl was used for analyses.

**Methods**

Nonspecific acid phosphatase from *S. pombe* was purified by the procedure reported previously (6) to apparent homogeneity, as ascertained from ultracentrifugal, electrophoretic, and chromatographic data. The protein concentrations and the catalytic activity of the enzyme preparations were determined according to the methods previously described (6). Gel filtration and sucrose gradient experiments were performed as reported (6).

**End Group Analyses**—The NH₂-terminal residue was determined by the procedure reported by Gray (7). Following hydrolysis with 5.7 N HCl at 108°C in sealed, evacuated tubes for 16 h, the NH₂-terminal dansyl derivative was separated and identified by this layer chromatography on silica gel or polyamide-coated plates, using chloroform/ethanol/acetic acid (38:4:3) for separation of acid, and butanol/l/ethanol/acetic acid/H₂O (30:20:6:24) for basic amino acid derivatives. COOH-terminal analyses were performed by carboxypeptidase A digestion. Twelve mg of acid phosphatase (~0.4 pmol of protein monomer) dissolved in 0.1 M N-ethylmorpholineacetic acid buffer, pH 8.5, was incubated with 80 μg of diisopropyl fluorophosphate-treated carboxypeptidase A (protein molar ratio, 150:1) at 37°C, with 0.6 mol of norleucine added as internal standard. Immediately after addition of carboxypeptidase and at stated times of incubation, 2-ml aliquots were withdrawn and the protein was precipitated by addition of 1 ml of 50% trichloroacetic acid followed by 1 ml of acetone. After removal of the precipitate by centrifugation, the supernatant was extracted 3 times with an equal volume of ether, dried in a vacuum, and applied to the analyzer. In a parallel experiment, the rate of inactivation of acid phosphatase by carboxypeptidase A digestion was measured. Controls were incubated under the same conditions with boiled carboxypeptidase.

**Ultracentrifugal Analyses**—A partial specific volume of 0.6719 ml/g was determined from the amino acid analysis according to Cohen and Edsall (8) and from carbohydrate content using the value of 0.65 ml/g reported for yeast mannan (9) as $\bar{v}$ of the polysaccharide, calculating the contribution to the partial specific volume from the percentage of each component. No correction of $\bar{v}$ was made for urea denaturation or mercaptoethanol reduction. Ultracentrifugation was performed on a Beckman model E analytical ultracentrifuge equipped with a photoelectric scanner. The absorption scanner was linked to a PDP-12 computer (Digital Equipment Corp.) for data collection and analysis.

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2 The abbreviation used is: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
Active enzyme sedimentation velocity studies were performed and calculated by the methods described by Holcenberg et al. (10) except that the double layering centerpiece was not required. The absorption of p-nitrophenol produced from p-nitrophenylphosphate was monitored at 400 nm. The relative viscosity of 0.2 m sodium succinate, pH 6.2, was determined to be 1.0386 at 20°C. The density at the same temperature was 1.0038 ml/g. The viscosity and density of succinate-buffered sucrose and sodium chloride solutions were calculated from the International Critical Tables. Viscosity and density increments for these solutions were assumed to be additive (10). For active enzyme studies, scans were collected at 2-min intervals after reaching the experimental speed (7 to 9 min). Successive 2-min scans were used to calculate the sedimentation coefficients according to the theory of Cohen and Hahn (11) and Cohen et al. (12) as described by Holcenberg et al. (10).

The active enzyme sedimentation velocity experiments were performed under several solvent conditions and with at least two preparations of enzyme in order to determine the active form(s) of the protein. The calculations were generally made in two ways: a, the rate of movement of the maximum absorbance created by the enzyme between t1 and t0 was measured as described by Holcenberg et al. (10); b, the equivalent activity zone at 2 min differences was calculated according to the theory of Cohen and Hahn (11) as described by Holcenberg et al. (10).

Table 1: Molecular weight of denatured yeast acid phosphatase (8 m urea, 0.1 m mercaptoethanol)

| Initial concentration (mg/ml) | Weight average Mw | z-average Mz | z-1 average Mw+1 |
|-----------------------------|-------------------|-------------|-----------------|
| 0.75                        | 88.8 x 10^3       | 90.7 x 10^3 | 91.3 x 10^3     |
| 0.5                         | 89.0 x 10^3       | 87.6 x 10^3 | 90.3 x 10^3     |
| 0.25                        | 89.3 x 10^3       | 92.2 x 10^3 | 90.9 x 10^3     |
| Average                     | 89.0 x 10^3       | 90.1 x 10^3 | 90.9 x 10^3     |
| ±0.4                        | ±2.4              | ±0.7        |

Fig. 1: Inactivation of S. pombe acid phosphatase following the release of COOH-terminal residue. Time course inactivation of S. pombe acid phosphatase with respect to the control and release of COOH-terminal residues tyrosine and serine on incubation with carboxypeptidase A. Conditions are described in the text.

Fig. 2: Molecular weight distributions observed for yeast acid phosphatase in 0.2 m acetate, pH 4.6. A, number-average data; B, weight-average data. ○, initial loading concentration of 0.71 mg/ml; ▲, 0.48 mg/ml; ◇, 0.23 mg/ml.
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RESULTS

Subunit Structure of Acid Phosphatase

Sedimentation Equilibrium in 8 M Urea—To determine the molecular weight of the constituent polypeptide chains of yeast acid phosphatase, the enzyme was dissolved in 20 mM sodium acetate, pH 7.0, containing 8 M urea and 0.1 M 2-mercaptoethanol. The results, obtained from the three channels and summarized in Table I, indicate the presence of homogeneous material ($M_r = \sim 90,000$) and support our previous results (6) on the homopolymeric structure of this enzyme.

Amino Acid Terminal Residues—For the determination of NH$_2$-terminal residues, 3 samples of acid phosphatase, containing 2, 4, and 10 nmol of protein monomer, were reacted with dansyl-Cl under the conditions described by Gray (7). After acid hydrolysis, the NH$_2$-terminal residue was separated and identified by thin layer chromatography. A single $\alpha$-amino dansyl derivative was detected in each protein hydrolysate, which did not separate from standard dansyl-histidine on bidimensional chromatography.

The COOH-terminal amino acids were determined by following the rate of release with carboxypeptidase A. Only tyrosine and serine were released during the first 30 min of digestion, but the rate of release of the former amino acid was much higher than that of the latter one, as expected from the specificity of this exopeptidase (14). In fact, 0.82 eq of tyrosine/30,000 g of protein was liberated after 30 min of incubation, as compared with 0.19 eq of serine released at the same time. This considerable difference in the rates of release of the COOH-terminal amino acids during the early incubation period allowed us to study the kinetics of inactivation of the enzyme as a function of its digestion by the exopeptidase. As shown in Fig. 1, the rate of inactivation of acid phosphatase incubated with carboxypeptidase closely matches the rate of release of the COOH-terminal tyrosine.

Molecular Properties of the Native Acid Phosphatase

Sedimentation Equilibrium Studies—All the molecular weights were determined in 0.2 M sodium acetate buffer at pH 4.6, in which the protein was enzymatically active. The protein in the peak tube or in the pool of the central part of the peak (6) was analyzed in the equilibrium experiments. Essentially, the same results were obtained in three separate experiments; the results of one of such experiments are presented. Fig. 2A presents number-average molecular weight values obtained from this experiment and Fig. 2B the weight-average molecular weight. From this figure, it will be noted that molecular weight averages vary as a function of both initial concentration and the concentration point within the channel. The strong dependence of molecular weights of yeast acid phosphatase upon initial concentration shown by the lack of superimposability of molecular weight data indicates a dissociating nonequilibrating system (13). Three interpretations are possible: the first, that the system is not in chemical equilibrium; the second, that the system is in chemical equilibrium but contains a finite amount of nonequilibrating, low molecular weight material; and finally, that the system is in chemical equilibrium but the rate of equilibrium is slow in relation to the time required for the ultracentrifugal experiments. If one assumes that the data in Fig. 2 can be described as a dimer-tetramer mixture, it is possible to determine the dimer molecular weight that best fits the data by plotting the average deviations of the predicted and observed molecular weight averages against a dimer molecular weight assigned: a minimum in the curve will be the molecular weight of the dimer that best fits the data experimentally obtained (13). Fig. 3 shows the results of such an analysis of the data from the three cells. The average value of $M_d$ found from the number average and the weight average data is 180,000 ± 9,000 g/mol. Using the methods of Hoagland and Teller (15), as described by Teller (13), it is possible to demonstrate that the system is adequately described as a dimer-tetramer system as shown in Fig. 4. This figure presents a summary of the data from all the

![Fig. 3. Determination of the dimer molecular weight of yeast acid phosphatase from the three-channel data. $M_d$ (assumed) was varied from $155 \times 10^3$ at increments of $5000 \text{ g/mol}$. The figure represents the average deviations of number average molecular weight data and $M_d$ data from the observed data. O, average deviations of $M_d$ data; •, number average molecular weight data.](image-url)
three cells. From these data, we conclude that the tetramer's 
\[ M_t = 360,000 \pm 18,000 \, \text{g/mol}. \]

**Active Form of the Enzyme**—The yeast acid phosphatase 
was stable throughout the course of the velocity ultracentrifugation. Table II presents the results of the active enzyme 
studies. In all cases, the volume of the layering zone was 0.01 
ml. A variety of centrifuge speeds and solvent conditions are 
presented in the table. The light enzyme of the table was the 
trailing edge of fractionation on a Bio-Gel column, as reported 
in a separate paper.\(^1\) In all cases, the buffer was 0.2 M sodium

**Fig. 4.** Allowed space graph (13) of the molecular weight 
averages. The ordinate represents the weight-average molecular 
weight. The abscissa is the reciprocal value of the next lower average 
(number or weight-average, respectively). The hyperbola is the locus of 
ideal homogeneous molecular weight averages. The straight line 
is that calculated for a dimer-tetramer system, with 

\[ M = 180 \times 10^4 \]
and 
\[ 360 \times 10^4 \, \text{g/mol}, \]

respectively. The symbols used are:

| Channel | \( z \)-average versus 1/weight average | Weight average versus 1/number average | 2/weight average - \( z \)-average versus 2/number average - 1/weight average |
|---------|-----------------------------------------|----------------------------------------|-----------------------------------------------|
| 1       | ○                                       | △                                      |                                |
| 2       | ●                                      | □                                      | △                                |
| 3       | ○                                      | ○                                      | *                                |

**TABLE II**

**Active enzyme sedimentation**

| Initial amount of enzyme | Solvent\(^a\) | Speed \( \times 10^{-1} \) rpm | \( s_{max} \) | \( s_{average} \) |
|--------------------------|---------------|-------------------------------|----------------|-----------------|
| pH                       |               |                               |                |                 |
| Intact enzyme            |               |                               |                |                 |
| 0.024                    | 1% sucrose    | 60                            | 13.56          | 13.78           |
| 0.024                    | 1% sucrose    | 52                            | 13.36          | 14.47           |
| 0.048                    | 1.3 M NaCl    | 52                            | 13.88          | 13.92           |
| 0.036                    | 2% sucrose    | 60                            | 13.00          | 13.49           |
| 0.018                    | 2% sucrose    | 60                            | 13.43          | 13.53           |
| Light enzyme             |               |                               |                |                 |
| 1.1250                   | 2% sucrose    | 60                            | 14.38          | 14.59           |
| 1.1250                   | 2% sucrose    | 60                            | 14.40          | 13.62           |
| 1.1250                   | 1.3 M NaCl    | 60                            | 13.63          | 13.17           |
| 1.1256                   | 1.3 M NaCl    | 60                            | 13.76          | 12.52           |
| 1.125                   | 1.3 M NaCl    | 60                            | 13.69          |                 |
| Averages                 | Sucrose       |                               | 13.52 ± 0.46   | 13.91 ± 0.49    |
|                          | 1.3 M NaCl    |                               | 13.72 ± 0.11   | 13.20 ± 0.70    |

\(a\) The buffer was 0.2 M Na-succinate, pH 6.2.

\(b\) Calculated from the peak of the time differences from 2-min intervals.

\(c\) Calculated by the method of Cohen et al. (12).

**Fig. 5.** Absorbance patterns in sedimentation velocity experiments. The profiles in the *lower figure* are from a moving boundary experiment and trace the absorbance at 280 nm at 5 consecutive time points. Scanning intervals shown are at 8 min at 60,000 rpm and 20°C. For the *center figure*, the enzyme was layered on 2% sucrose, 0.2 M Na succinate, and p-nitrophenol phosphate at pH 6.2. The scans shown are at 6-min intervals at 400 nm and 60,000 rpm. The *upper figure* shows the absorbance difference of nitrophenol derived by subtracting the consecutive scans of the *central figure*.
DISCUSSION

The experimental data reported in this manuscript clearly confirm previous evidence indicating that nonspecific acid phosphatase from *S. pombe* is a polymer containing identical or very similar subunits. A single amino acid residue was found at the NH₂-terminal end, the catalytic activity of the acid phosphatase was completely lost with the release of 1 tyrosine residue/protein monomer and 1 mol of Pi/protein monomer was split off. The equilibrium sedimentation experiments in 8 M urea is consistent with the presence of subunits of identical molecular weight. Since the carbohydrate accounts for 66% of the molecular weight, it is assumed that each subunit consists of a polypeptide chain weighing ~30,000 that is bound to a carbohydrate weighing ~60,000. Noteworthy is the loss of catalytic activity of the acid phosphatase with the release of the tyrosyl terminal residue. Some evidences that tyrosyl residues can be involved in phosphatase activities were reported. In fact, prostatic acid phosphatase was irreversibly inhibited by iodine monochloride (16). The ultraviolet spectrum of the iodinated product suggested that tyrosine was modified during the reaction and a competitive inhibitor, tartrate, protected the enzyme from inactivation. Furthermore, fructose-1,6-diphosphatase from both mammalian and yeast source is inactivated by selective modification of tyrosyl residues either by acetylation or by iodination (17). The comparative kinetics of enzyme inactivation and tyrosine splitting by carboxypeptidase A reported in Fig. 1 demonstrated that the COOH-terminal tyrosyl residue is essential for activity of acid phosphatase of *S. pombe*. However, further studies are required to elucidate whether it plays a direct role in enzyme catalysis.

The equilibrium sedimentation analysis on the native enzyme at pH 4.6 revealed a dissociating, nonequilibrating system. But in spite of the lack of chemical equilibria among the molecular species, the molecular weight curves of Fig. 2 can be described as a dimer-tetramer system with a tetramer *M*₄ = 360 ± 18 × 10³. The 2:1 stoichiometry is confirmed by the observed “molecular space” of Fig. 4, which discriminates between monomer-dimer and dimer-tetramer models.

The active enzyme sedimentation velocity experiments reported here do not definitively prove that the tetramer of the subunit is the only active species of the protein assembly. They do establish, however, that this species is the major active form. In order to be definitive, active enzyme sedimentation velocity experiments should be carried under a variety of conditions (19), including the following: 1) a dilution series to establish the range of reliably detectable activity; 2) several solvent conditions to determine whether several active forms may exist under different solvent conditions; 3) several preparations of enzyme to establish that proteolysis during preparation is insignificant; 4) ideally, to perform the same zone sedimentation experiments on the enzyme without substrate to establish that small or large species are inactive; 5) the amount of substrate converted to products should be constant throughout the experiment.

Of these criteria, as well as those mentioned by Holcember et al. (10), we have established most. First, to find the proper conditions for these experiments, we performed a dilution range of at least 100-fold and less than 20% of the substrate was used by the enzyme zone. Second, we have employed several solvents consisting of high salt and high sucrose concentration and found the same active species under both conditions. Third, several enzyme preparations have been employed, including a fraction which would most likely exhibit activity among small species where they do exist (light enzyme of Table II) in significant proportions. This fraction of enzyme also yielded a sedimentation coefficient consistent with the tetramer *M*₄ = 360,000 g/mol. It was not possible to perform zone sedimentation velocity experiments on the native enzyme at the same dilution as the active enzyme sedimentation runs, but the moving boundary experiments at high dilution suggest that the tetrameric species dissociates to dimers in a reversible fashion, implying that the very dilute active enzyme studies may involve a dimer → tetramer shift of this reversible equilibrium. Finally, it should be noted that the amount of substrate converted to p-nitrophenol in each 2-min period in the ultracentrifuge was approximately constant (within 10%), indicating that the enzyme catalyzes substrate hydrolysis and is not inhibited by the reaction product.

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