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Electron microscopic and biochemical evidence that proline-β-naphthylamidase is composed of three identical subunits

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Electron microscopy of pig intestinal proline-β-naphthylamidase revealed that the enzyme is composed of 3 subunits, which are assembled in a trifoliolate shape. At pH 4.5 and 4°C, the enzyme dissociates reversibly into active subunits in 4 h. Dissociation also occurs at higher pHs when the enzyme concentration is very low. The activity per mg protein of the native, trimeric enzyme is about 2.5-fold higher than that of the dissociated enzyme.

Proline-β-naphthylamidase; Electron micrograph; Trimer protein; Subunit dissociation

1. INTRODUCTION

Mammalian tissues contain a variety of enzymes that hydrolyze synthetic substrates for aminopeptidases [1]. Recently we have purified from the homogenate of pig intestinal mucosa an enzyme capable of hydrolyzing the substrate, L-proline-β-naphthylamide and characterized it [2,3]. The enzyme which we called proline-β-naphthylamidase gave a single band corresponding to $M_r = 58\,000$ in SDS-PAGE under both reducing and nonreducing conditions. The molecular weight of the native enzyme was estimated to be 240 000 in gel filtration experiments, while it was calculated to be 169 000 ± 9000 in sedimentation studies [2]. Now we demonstrate the geometry of the enzyme structure with a clear view of triangularly laid out subunits, as revealed by electron microscopic and biochemical studies. Dissociation of the enzyme into active subunits at acidic as well as alkaline pHs was also examined.

2. MATERIALS AND METHODS

2.1. *Proline-β-naphthylamidase*

Proline-β-naphthylamidase was purified from pig intestinal mucosa according to the method of Takahashi et al. [2]. The purified enzyme had a specific activity of 0.66 μmol/min/mg protein when assayed using proline-β-naphthylamide as substrate. The enzyme activity was assayed at pH 8.0 as described [2]. Molar concentration of proline-β-naphthylamidase was calculated assuming that the molecular weight of the enzyme is 169000.

2.2. *Electron microscopy*

Electron micrographs were taken on an Hitachi H7000 electron microscope (Hitachi, Japan). Purified protein samples were passed through a TSK G3000SW gel permeation column (Tosoh, 0.75 × 60 cm) mounted on an Applied Biosystems 150A HPLC system with 50 mM sodium phosphate (pH 7.0) as elution buffer. The final concentration of protein samples was adjusted to 50 μg/ml. A drop of protein solution was placed on a glow-discharged carbon-coated Formvar film formed on a copper grid and excess solution was blotted. A staining solution of 2% phosphotungustate was then applied to the membrane and blotted. The resulting stained sample was dried under air and used as a negatively-stained specimen for electron microscopy. Photographs were taken at the nonimal magnification of × 50000 and enlarged in printing when necessary. Negatively-stained images at a high magnification were scanned by a Shonic GA graphic analyzer (Showa Denko, Tokyo) and relative brightness in each image was digitized as 10000 data points in a 100 × 100 mesh over 3 cm × 3 cm on a photograph. Digitized images were averaged by rotational superposition.

2.3. *Gel filtration*

For dissociation study, gel chromatography of proline-β-naphthylamidase was carried out at room temperature on a TSK G3000SW column (0.75 × 60 cm) with 0.1 M Tris-HCl, pH 8.0), containing 0.2 M NaCl. Prior to column chromatography, samples were treated at 4°C in 0.1 M Tris-HCl, pH 8.0, containing 0.2 M NaCl, or 0.1 M potassium phosphate (pH 6.0) containing 0.2 M NaCl, or 0.1 M sodium acetate (pH 4.5) containing 0.2 M NaCl for periods shown in the legend to Fig. 3. Fifty microliters of the treated samples were applied. The column was eluted at a flow rate of 0.2 ml/min, and was monitored at 230 or 280 nm with a range setting of 0.01 absorbance.

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Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

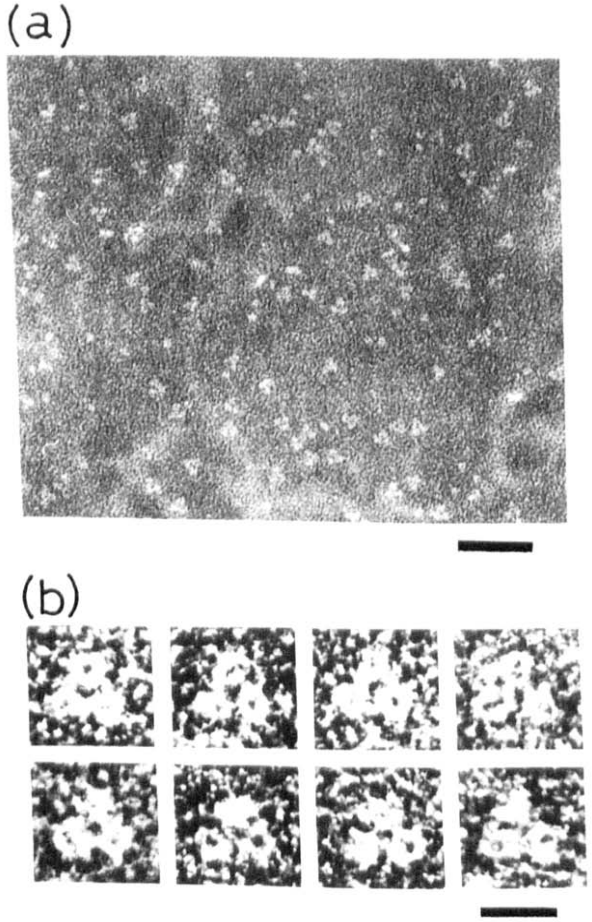

Fig. 1. Electron micrographs of negatively stained proline-β-naphthylamidase. Scale bars = 50 nm (a) and 10 nm (b).

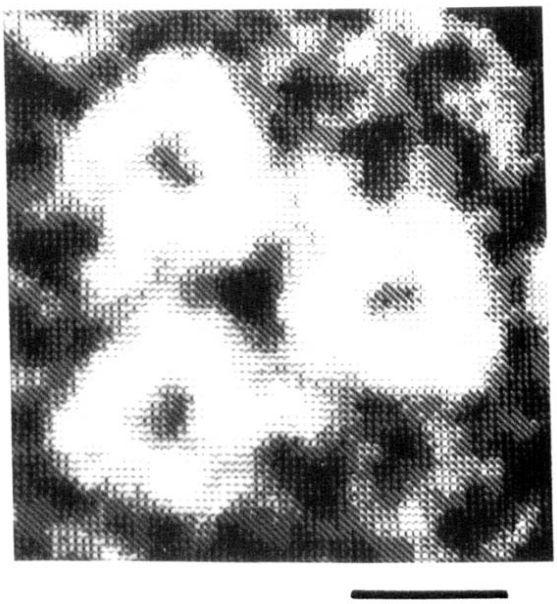

Fig. 2. Rotationary averaged image of negatively stained molecules after superimposition of images each with 120° rotation from the others. Scale bar = 3 nm.

3. RESULTS

3.1. *Electron micrograph of proline-β-naphthylamidase*

Fig. 1a and b show low and high magnification photographs of proline-β-naphthylamidase after negative staining with phosphotungustate. The low manification photograph shows uniformly sized particles corresponding to the expected size of the enzyme on a relatively clear background. When enlarged in printing in Fig. 1b, proline-β-naphthylamidase showed distinct structural characteristics resembling a trifoliolate shape, each subunit looking like either a closed or a slightly open ring. The diameters of the whole molecule and the subunit are 10 ± 2 nm ($n = 30$) and 4.5 ± 1 nm ($n = 30$), respectively. The features become more obvious when a digitized image was superimposed after 3-fold rotation in a computer (Fig. 2). It is clear from the figure that subunits make contact with each other at two peripheral sites leaving the center of the molecule empty. It is also to be noted that the

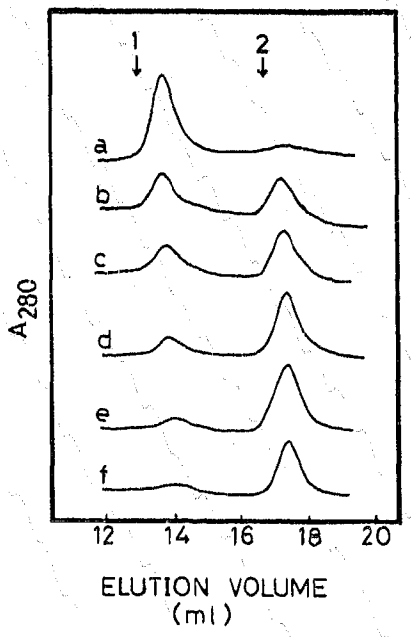

Fig. 3. Elution patterns of proline-β-naphthylamidase from TSK G3000SW at room temperature. The enzyme sample was treated at pH 4.5 at a final enzyme concentration of 0.17 μM, and the durations of treatment were 1(a), 15(b), 30(c), 60(d), 150(e), and 230 (f) minutes. Fifty microliters of each of the treated samples was injected into the column which had been previously equilibrated in 0.1 M Tris-HCl, pH 8.0, containing 0.2 M NaCl. Peaks eluted at 14 and 17.5 min contained undissociated whole molecule and dissociated subunit molecule, respectively. The elution positions of pig intestinal prolyl aminopeptidase ($M_r = 300\,000$, arrow 1) and bovine serum albumin ($M_r = 68\,000$, arrow 2) are also shown.

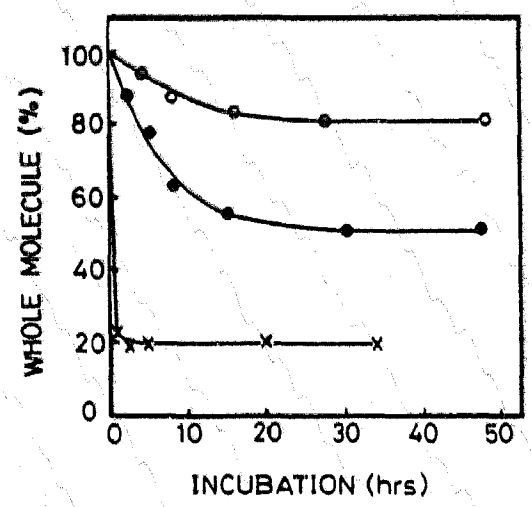

Fig. 4. Equilibrium for dissociation into subunit molecules. Enzyme samples were treated at pH 4.5 (×) or pH 6.0 (•) or pH 8.0 (○) for the indicated periods, and were injected into the gel filtration column. The ratios of undissociated whole molecule and the dissociated subunit molecule were determined from the chromatograms.

center of each subunit allowed penetration of staining material.

3.2. *Dissociation studies by gel chromatography*

The amount of enzyme dissociated into subunits was estimated by chromatography on TSK G3000SW. The native enzyme was preincubated at a pH of 4.5, 6.0, or 8.0 prior to gel filtration analysis. Fig. 3 shows the elution profile of the enzyme after preincubation at pH 4.5. Judging from the changing elution profiles, the native, trimer enzyme was converted to subunits with $M_r = 58000$ with time, attaining the dissociation equilibrium after 4 h. Fig. 4 shows that an increase in preincubation pH from 4.5 to 6 or 8 decreased the equilibrium concentration of dissociated enzyme. An approximate value of the equilibrium constant for dissociation (eq 1 and 2) was calculated from the ratios of the areas in the two peaks at equilibrium. In eq 2, [S] and [W] refer to the molar

$$\text{Native molecule} \rightleftharpoons 3\ \text{Subunits} \qquad (1)$$

$$K_{eq} = \frac{[S]}{[W]} = \frac{27[E_0]}{(A_w/A_s) + 2(A_w/A_s)^2 + (A_w/A_s)^3} \qquad (2)$$

concentrations of dissociated subunit and undissociated whole molecules, A_w and A_s refer to the measured area in the first and second peak, respectively, and $[E_0]$ is the initial molar concentration of undissociated enzyme. The validity of this calculation depends on the assumption that the equilibrium position is not perturbed by chromatography on TSK G3000SW. Based on the results given in Fig. 4, the calculated values of K_{eq} are shown in Table I. There is an approximately 1300-fold increase in the dissociation constant as the pH is decreased from 8.0 to 4.5. The addition of the substrate in the preincubation at each pH caused no significant change in the dissociation. We also observed that the dissociated subunits slowly re-associated to a trimer enzyme when the pH was raised from 4.5 to 8.0.

In a separate experiment, the undissociated and dissociated enzyme fractions were obtained through the gel filtration column chromatography of the sample which had been treated at pH 4.5 at 4°C. The two fractions were immediately assayed for the activity by adding the substrate. The specific activities of the undissociated and dissociated enzyme were 0.61 and 0.24 μmol/min/mg protein, respectively. The former value was very close to the specific activity (0.66 μmol/min/mg protein) of untreated proline-β-naphthylamidase.

4. DISCUSSION

The electron micrographs of proline-β-naphthylamidase showed that the native enzyme is arranged in a trifoliolate configuration. From the results of Edman degradation and SDS-PAGE analyses with the purified enzyme [2], it is clear that the subunits are identical and are associated non-covalently. Each subunit appears to have two peripheral sites for interacting with neighboring subunits. The driving force for the subunit assembly is not known at present, but the rate and equilibrium for dissociation into subunits are greatly enhanced at acidic pH. It should be noted that the presence of substrate did not affect the dissociation.

Table I

Equilibrium distributions of enzyme forms

Conditions	Initial enzyme concentration, E_0 (M)	Whole (%)	Area as subunit (%)	K_{eq} (%)
0.1 M sodium acetate (pH 4.5) containing 0.2 M NaCl	1.7×10^{-7}	19	81	1.3×10^{-5}
0.1 M potassium phosphate (pH 6.0) containing 0.2 M NaCl	4.3×10^{-8}	52	48	2.8×10^{-7}
0.1 M Tris-HCl (pH 8.0) containing 0.2 M NaCl	4.3×10^{-8}	81	19	9.8×10^{-9}

This fact suggests that the active site is located on the surface of each subunit in such a way that it is not directly involved in the interaction among subunits. Another interesting finding is that the specific activity of native proline-β-naphthylamidase is about 2.5-fold greater than that of the dissociated enzyme. This means that the subunit association makes the enzyme a more efficient catalyst.

2-Keto-3-deoxy-6-phosphogluconate aldolase from *Pseudomonas putida* [4–7], a bacteriochlorophyll protein from *Chlorobium limicola* [8] and influenza virus hemagglutinin [9,10] are well established to be a homotrimeric protein by physical, chemical, and X-ray crystallographic studies. The homotrimeric structure of proline-β-naphthylamidase was also demonstrated in the present study. This is undoubtedly a characteristic feature of the enzyme since trimeric proteins are rare in occurrence as compared with even-numbered ones [11]. Such structural information should help us conduct further structure/function studies with the protein.

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