THE SUBUNIT STRUCTURE OF α-GLUCAN PHOSPHORYLASE FROM POTATO

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1. Introduction

α-Glucan phosphorylases (EC 2.4.1.1) from various mammalian sources, particularly from rabbit skeletal muscle, have been extensively studied [1]. Information on the enzymes from plant tissues, on the contrary, is rather limited. Phosphorylase from the potato exists only in an active form, the activity of which is independent of AMP. Although Lee [2] has reported that the potato enzyme has a mol. wt. of 207 000 and contains 2 moles of pyridoxal-5′-P per mole of enzyme, unambiguous evidence for the subunit structure has not been obtained. We have investigated the subunit structure of potato phosphorylase in order to elucidate the structural relationship between phosphorylases from various origins.

2. Materials and methods

Phosphorylases from potato and rabbit muscle were purified according to the methods of Kamogawa et al. [3] and Fischer and Krebs [4], respectively. Sodium dodecyl sulfate (SDS) was purchased from Wako and recrystallized in ethanol. Urea, formamide, glucose-1-P and guanidine hydrochloride (Gu·HCl) were the products of Nakarai. Cyclodextrins, maltotriose and amylose (DP 30) were the products of Hayashibara. Phosphorylase activity was assayed as in the previous paper [3]. Protein concentration was determined either spectrophotometrically using $A^{1\%}_{278nm} = 11.7$ [3] or by the method of Lowry et al. [5]. SDS-gel electrophoresis was performed according to the method of Weber and Osborn [6]. Split gels were made as described by Dunker and Rueckert [7]. Gels were usually 5% acrylamide in 0.1% SDS–0.1 M sodium phosphate buffer, pH 7.2. Electrophoresis was run at 8 ma per tube for 5 hr at room temperature. The gels were stained with Coomassie brilliant blue. The proteins were pretreated with 1% SDS at 100°C for 3 min to eliminate possible proteolysis [8].

Gel filtration in 6 M Gu·HCl was carried out according to Fish et al. [9]. Protein (10 mg) was dissolved in 0.5 ml of 6 M Gu·HCl–0.1 M 2-mercaptoethanol–5 mM EDTA in 0.1 M Tris–HCl buffer (pH 8.5) at 37°C for 3–4 hr and then alkylated with iodoacetamide. The solution (0.1 ml) was applied on a Sepharose 6B column (1.6 × 50 cm) equilibrated with 6 M Gu·HCl–20 mM Tris–HCl buffer (pH 7.3) at 15°C. Flow rate was maintained at 5 ml/hr with an LKB perpex pump. All the effluents were monitored by measurement of absorption at 230, 280 and 340 nm in a Hitachi UV-VIS effluent monitor (220 nm was used for an extremely diluted protein solution). The large zone technique [10] was applied for a low concentration of protein. A Sephadex G-200 column (1.6 × 36 cm) was equilibrated with 0.1 M KCl–20 mM Tris–HCl buffer (pH 7.2) at 20°C and eluted by the up-flow manner at 6.2 ml/hr. The protein solution (23 ml) equilibrated with the same buffer at 20°C overnight was applied to the column. Gel filtration of potato phosphorylase in the presence of ligands and denaturants was performed on a Sephadex G-200 column (1.6 × 39 cm) at 20°C and 14.8 ml/hr. Prior to loading, the column was permitted to flow 3-fold bed volumes of each solvent and the sample (1 ml containing 0.13 mg protein) was equilibrated in the solvent at 20°C overnight. Ultracentrifugal measurements were carried out in a Beckmann Spinco ultracentrifuge model E using an An-E rotor (12-mm double sector cell) or an An-D (30-mm double sector cell) with schlieren optics. A partial specific volume value was calculated to be 0.735 from amino acid composition [3].

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3. Results and discussions

Fig. 1 shows the results of SDS polyacrylamide gel electrophoresis. Freshly prepared potato phosphorylase gave a single band on the gel. Omission of the reduction and the following alkylation did not affect the pattern and the mobility, indicating that disulfide linkage is not involved in the subunit interaction. The subunit molecular weight of this protein in 0.1% SDS was determined to be 108 000, by using a calibration curve (fig. 2A). The value is larger than that of rabbit muscle phosphorylase *b* (100 000; ref. [11]). This result was confirmed by the split gel method (fig. 1B); the mobility of potato phosphorylase was definitely smaller than that of muscle phosphorylase, whereas the bovine serum albumin and the alcohol dehydrogenase in the both halves coincided quite well. To further establish the subunit molecular weight of potato phosphorylase, chromatography on Sepharose 6B in 6 M Gu·HCl was performed. Potato phosphorylase which was eluted as a single peak had a smaller elution volume compared with rabbit muscle phosphorylase *b*. A value of 112 000 for the subunit molecular weight of potato phosphorylase in 6 M Gu·HCl was obtained from a calibration curve (fig. 2B).

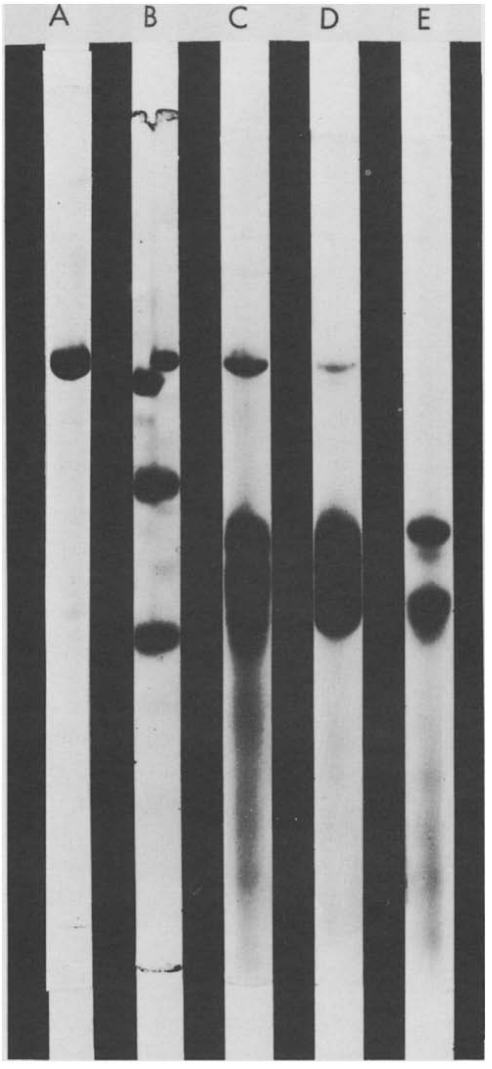

Fig. 1. SDS gel electrophoresis: A) a fresh preparation of potato phosphorylase; B) split gel, a fresh preparation of potato phosphorylase on the right; rabbit muscle phosphorylase *b* on the left; bovine serum albumin and yeast alcohol dehydrogenase on both sides; C–E) aged preparations of potato phosphorylase.

During these experiments, we have found that some limited proteolysis occurred on the prolonged storage on the enzyme in solution in the cold, while the specific activity did not change appreciably. Fig. 1C–E show the typical SDS-gel electrophoretic patterns of potato phosphorylase stored at 4°C in Tris–HCl buffer, pH 7.5. In the first stage (several weeks to several months depending upon different samples), approximately four bands with mol. wt. 40 000–60 000 emerged with concomitant decrease of the intact band (C). Upon further storage, the original band mostly disappeared and the bands with larger mobilities became eminent (D). It should be noted that the specific activity remained essentially unaltered during these stages. After nearly one year, the original band completely disappeared and the two bands, of each mol. wt. 40 000 and 60 000, resided (E). This unusually aged preparation had still about 35% of the activity compared to that of a fresh sample.

Franken et al. [12] has recently reported that the molecular weight of potato phosphorylase decreased at a protein concentration below 0.5 mg/ml and became a half of the original at 0.1 mg/ml. To test this finding, the analytical gel filtration on Sephadex G-200 was performed according to the large zone method [10]. A profile at 0.504 mg/ml revealed that 23 ml of the protein solution was enough to obtain a plateau region under these experimental conditions (inset of fig. 3). In a protein concentration range of 0.008–0.504 mg/ml, the leading and trailing boundaries gave nearly the same profile. The weight-average partition coefficients,

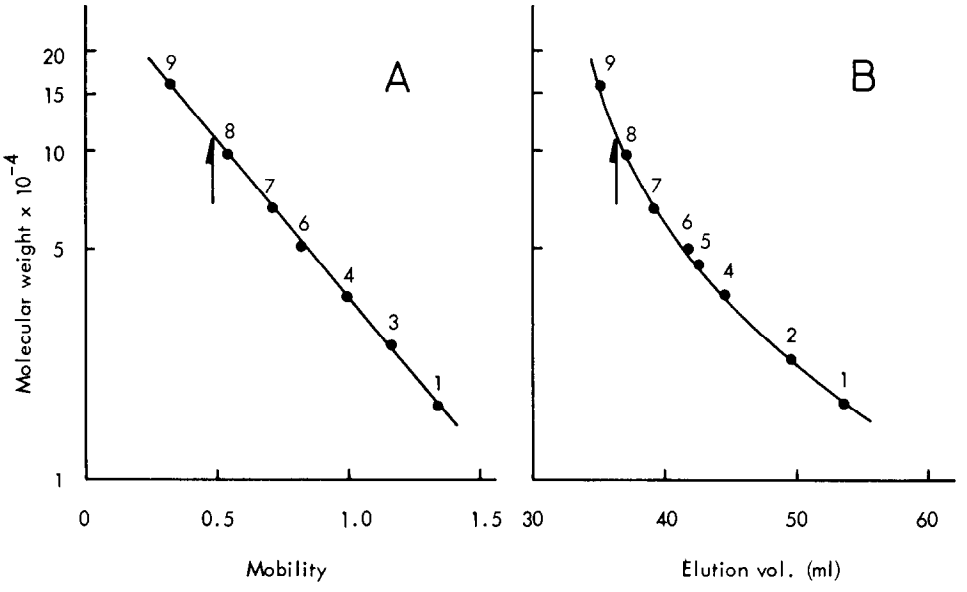

Fig. 2. Calibrations of the SDS-gel electrophoresis system (A) and of the Sepharose 6B gel filtration in Gu·HCl system (B): 1, myoglobin; 2, trypsin; 3, chymotrypsinogen A; 4, yeast alcohol dehydrogenase; 5, ovalbumin; 6, Taka-amylase A; 7, bovine serum albumin; 8, rabbit muscle phosphorylase *b*; 9, immunoglobulin G. Arrows indicate the position for potato phosphorylase.

$\bar{\sigma}_w$, were calculated from a centroid elution volume of both boundaries. A constant $\bar{\sigma}_w$ value, 0.112, was obtained throughout the protein concentrations tested (fig. 3). It corresponds approximately to a molecular weight of 215 000, calibrated by immunoglobulin G (IgG) bovine serum albumin and ovalbumin. Further, a linear relationship between the sedimentation coefficeints and protein concentrations was obtained in a concentration region from 0.321–8.6 mg/ml (fig. 4). An $\underline{s}^0_{20,w}$ value of potato phosphorylase was calculated to be 8.43 S, which is slightly higher than the previous values [2, 12]. It was concluded that potato phosphorylase exists as dimer and no dissociation occurred at low protein concentrations tested. Reason for the disagreement with the results by Franken et al. [12] is not clear.

The effect of ligands and denaturants on the molecular weight of potato phosphorylase was studied by gel filtration on Sephadex G-200. The molecular weights were all calculated by using a linear calibration plot which had been made with IgG, bovine serum albumin and ovalbumin in each solvent. A value of 220 000 ± 10 000 was obtained for the enzyme in 30 mM glycero-phosphate buffer–1 mM EDTA, pH 6.5 with any of the following additions: maltotriose; cyclohexaamylose; cycloheptaamylose; glucose-1-P (all 10 mM); amylose (0.1%) and a mixture of 6 mM glucose-1-P, 40 mM sodium phosphate and 0.1% amylose. Presence of moderately high concentrations of denaturants caused a decrease in the apparent molecular weight, while the activity did not change appreciably. Mol. wt. $\times 10^{-3}$: 192 and 180 in 2 and 3 M NaCl; 205 and 195 in 2 and 3 M urea; 197 and 200 in 2 and 3 M formamide, respectively. Higher concentrations of these substances brought about inactivation of the enzyme. The enzyme was completely inactivated with 1 M $CaCl_2$ or 2 M Gu·HCl, and was eluted at or near the void volume. These results indicate that substrates and inhibitors did not affect the dimeric structure of potato phosphorylase, whereas denaturants caused dissociation and association of the enzyme. The subunit interaction in potato phosphorylase appears to be considerably stronger than that in rabbit muscle phosphorylase *b*.

The limited proteolysis observed in aged preparations of potato phosphorylase would be of interest in con-

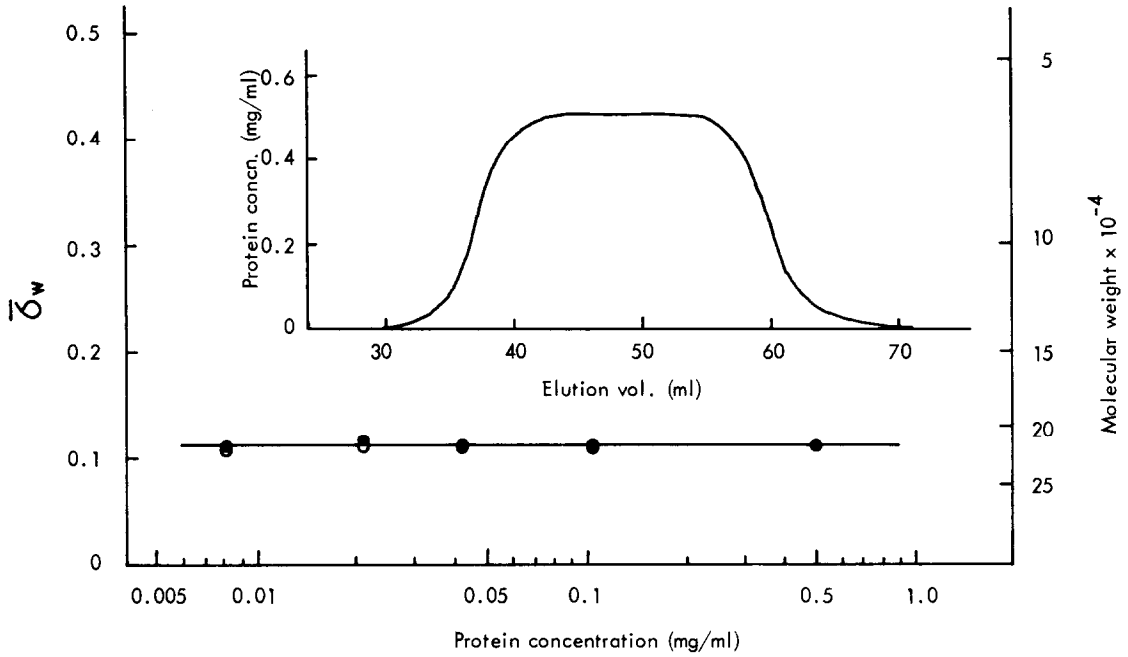

Fig. 3. A plot of partition coefficients ($\bar{\sigma}_w$) *versus* protein concentrations of potato phosphorylase, as determined by the large zone method of Sephadex G-200 gel filtration. (● and ○) Calculated from the leading and trailing boundaries, respectively. The inset: an elution profile at 0.504 mg/ml.

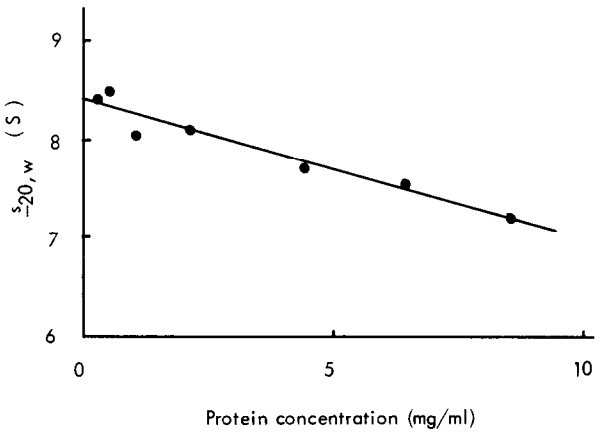

Fig. 4. A plot of sedimentation coefficients ($s^{\circ}_{20,w}$) *versus* protein concentrations of potato phosphorylase. The $s^{\circ}_{20,w}$ values measured in 0.1 M NaCl–5 mM Tris–HCl buffer, pH 7.5, at 20°C and 47 660 rpm.

nection with its tertiary structure. We assume that the subunit has two distinct regions of about the same size linked by a peptide bond. This linkage might preferentially be hydrolyzed by protease without significant change in the molecular weight and the specific activity; an aged preparation (fig. 1D) with the same specific activity as the fresh showed about 10% decrease in the apparent molecular weight. We have recently found that phosphorylase from sweet potato is composed of four polypeptide chains of mol. wt. 46 000 [13]. It might be reasonable to assume that phosphorylases from both plant tissues have similarly four separate regions of similar sizes, and that the two of the potato enzyme are linked by a peptide bond and those of the sweet potato enzyme are not.

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