Purification of Potato Glycogen Phosphorylase by Affinity Electrophoresis

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ABSTRACT

Potato glycogen phosphorylase (EC 2.4.1.1) was purified through extraction from potato starch grains using β-cyclodextrin followed by the preparative affinity electrophoresis on a slab type apparatus. From 10 kg of potato tubers 2.1 mg of the homogeneous phosphorylase was obtained at a yield of 45%. Its molecular weight was estimated as 18×10^4 from the gel filtration. The enzyme was composed of two identical subunits of molecular weight of 9×10^4. The amino acid composition was distantly related to the potato starch phosphorylase. While the starch phosphorylase has practically no affinity with α-(1,4)-D-glucan, the glycogen phosphorylase has a strong affinity. From electrophoretic and kinetic data, we postulated that the affinity difference between these phosphorylases is due to a difference in their glycogen storage sites.

Key words: glycogen phosphorylase; affinity electrophoresis

INTRODUCTION

Electrophoretic studies of α-glucan phosphorylase (1,4-α-D-glucan: orthophosphate α-glucosyltransferase, EC 2.4.1.1) demonstrated that potato tuber contains one major and several minor phosphorylase fraction[1-3]. The major fraction of phosphorylase was purified by Lee[4] and was crystallized by Kamogawa et al[5]. Its enzymatic and molecular properties have been extensively investigated[4-7]. In regard to the minor fractions, only a few investigations have been reported[1-3,8,9]. Gerbrandy et al[3] demonstrated that one of the minor fractions has a strong affinity with glycogen. They partially purified it through extraction from starch grains[8].

Since 1970, we have been studying distribution of phosphorylase among various plants and animals using affinity electrophoresis[2,10-12].

We demonstrated that the potato tuber contains one minor phosphorylase fraction which has very strong affinity with glycogen or starch. When electrophoresis gel contained glycogen or starch, its mobility retarded strongly. Mobility of the major fraction and the other minor fractions did not change in the presence of glycogen. Based on this electrophoretic property we have tentatively termed the former, glucan dependent phosphorylase, and the latter, glucan independent phosphorylase[11]. The former is identical with the glycogen phosphorylase and the latter with the starch phosphorylase of Gerbrandy[8]. Because of the low content in potato tubers, the enzymatic properties and metabolic significance of the glycogen phosphorylase have not been studied extensively.

Through extraction from potato starch grains using β-cyclodextrin followed by affinity electrophoresis, we obtained a homogeneous preparation of the glycogen phosphorylase. In this paper we have reported the purification procedure of the enzyme. Certain molecular properties, amino acid composition, and some kinetic parameters have also been included.

MATERIALS AND METHODS

Materials.

Fresh potato tubers "May Queen" harvested last spring were obtained commercially. Glc-1-P was purchased from Boehringer-Mannheim; shellfish glucogen, from Sigma Chemicals; potato solube starch, from Wako Pure Chemicals; and β-cyclodextrin, from Nakarai Chemicals. For molocular weight determination by gel filtration and sodium dodocyl sulphate gel electrophoresis, the standard reference of Boehringer-Mannheim protein kit was used. All other reagents were of analytical grade and prepared in Japan.

Methods

Phosphorylase activity was determined by a slightly modified procedure of Lee[14]. The reaction mixture was composed of 0.3 ml of 2.5% potato soluble starch, 0.2 ml of 0.5 M citrate buffer, pH 6.3, 0.1 ml of 0.1 M Glc-1-P, pH 6.3, 0.1 ml of the enzyme solution and 0.3 ml of water. After 5 min preincubation at 30°C, the reaction was started by adding the enzyme. The reaction was stopped after 10 min of incubation by adding 0.5 ml of 5% trichloroacetic acid. The pH was adjusted to about 4 by adding 2.0 ml of 0.1 M sodium acetate. The amount of inorganic phosphate liberated by the enzyme reaction was determined by the method of Fiske-SubbaRow[13]. A control was prepared for each series of measurements by incubating the reactants without Glc-1-P and addition of the latter after addition of the sodium acetate solution. Enzy-

me units were defined as K×1000, where K is the first order velocity constant[14]. Specific activity was expressed as units per mg protein.

Protein was determined by the procedure of Lowry et al[15], using bovine serum albumin as a standard. Extract from polyacrylamide gel contains some substances which interfere with protein determination[16] by the Lowry method and other spectrophotometric methods. In such cases the protein was determined by scanning the density of the stained protein band on disc electrophoresis with bovine serum albumin as a standard.

Electrophoresis.

Analytical polyacrylamide gel electrophoresis was carried out at lower pH[17] using a modified method of Ornstein and Davis [18]. The small pore gel was prepared with the stock buffer solution for the large pore gel at pH 6.7[18] instead of that for the small pore gel at pH 8.9. Protein fractions were stained with 1% Amido Black 10B in 7% acetic acid and phosphorylase activity was determined by the iodine method[10]. Sodium dodecyl sulphate (SDS) gel electrophoresis was performed with the procedure of Weber and Osborn[19], using 10% acrylamide gel.

Sedimentation.

Sedimentation velocity experiments were performed with a Hitachi Model UCA-1A analytical centrifuge at 51,200 rpm at 10.4°C. The sample solution was dialyzed for 1 day against 0.05 M Tris-HCl buffer, pH 7.0, containing 0.01 M 2-mercaptoethanol and 0.01 M EDTA. Sedimentation coefficients were determined for 3 different concentrations of protein: 0.323, 0.645, and 1.29 mg/ml solutions.

Gel filtration.

Estimation of molecular weight was performed by the gel filtration[20]. Sephadex G-200 was equilibrated with 0.05 M Tris-HCl buffer, pH 7.5 containing 0.1 M KCl. A column (2.5×50 cm) was packed and washed with 500 ml of the above buffer. Chromatography proceeded at an elution rate of 45 ml/h and 2.7 ml fractions were collected.

Determination of amino acid compsoition.

The amino acid composition was determined by the procedure of Moore and Stein[21]. Purified phosphorylase was dialyzed against large volume of 5 mM EDTA, pH 6.7 containing 5 mM 2-mercaptoethanol. The dialyzed sample solution was concentrated by ultrafiltration in collogion bag (Sartorius) to about 3 ml and was lyophilized. Hydrolysis was performed in 1.2 ml of 6 N HCl in sealed and evaculated glass tubes at 110°C for 20 and 70 h. The hydrolyzed samples were analyzed

with a Hitachi Amino Acid Analyzer (KLA-5). Tryptophan was determined using the method of Bencze and Schmid[22].

RESULTS AND DISCUSSION

1. Purification of the glycogen phosphorylase.

i) Elution of the glycogen phosphorylase from starch grains of potato tubers by β-cyclodextrin.

Ten kg of potato tubers were washed and sliced. They were homogenized with one liter of 20 mM EDTA solution containing 10 mM 2-mercaptoethanol, pH 7.0. Unless otherwise stated, all purification procedures were carried out at 4°C. The juice was filtered through one sheet of gauze to eliminate peeled skin. The filtered mash was left for about 1 h and the supernatant solution was separated by decantation. The supernatant (S) solution can be used for purification of the major fraction of phosphorylase. The precipitated starch grains were suspended in 1 liter of 0.05 M Tris-HCl buffer, pH 8.0 containing 0.5% β-cyclodextrin. 10 mM EDTA, and 10 mM 2-mercaptoethanol. The suspension was poured into a large Buchner's funnel with a diameter of 21 cm without suction. When the filtration was nearly finished, another 2 l of the same buffer solution was poured carefully in the funnel. This combined filtrate (about 3 l) was concentrated to about 200 ml by ultrafiltration using an Amicon Model DC-2 Follow-Fiber-Concentrator. The concentrated solution was diluted once by adding 1.8 l of 2 mM EDTA solution pH 7.0 containing 10 mM 2-mercaptoethanol, and reconcentrated to about 50 ml. The solution was collected and the concentrator was washed with the minimum volume of the EDTA solution. The combined solution often contained some yellowish turbid materials. They were easily eliminated as precipitate by adding 1 ml of 1 M calcium acetate and centrifuging. One hundred and five ml of clear colorless solution (C) was obtained.

ii) Preparative affinity electrophoresis.

The first electrophoresis: Preparative electrophoresis was carried out as reported previously [23]. The apparatus was composed of a gel chamber and an upper and a lowerbuffer vessels. A gel chamber of 15 cm in length, 18 cm in height, and 3 cm in width was used. The small pore gel was prepared in 3 layers of 5% polyacrylamide gel at pH 6.7 using the same buffer as used in the analytical electrophoresis. The lower small pore gel was prepared to a height of 6 cm without glycogen. The middle small pore gel with 0.4% shellfish glycogen was 3 cm in height, and the upper small pore gel, without glycogen, was 1 cm in

height. The large pore gel at pH 6.7, 1 cm in height was prepared on the upper small pore gel. The sample solution was applied as the mixed solution of the concentrated extract C (104 ml), 10 ml of 0.06 M thioglycolate at pH 6.7, and 12 g of sucrose. Tris-glycine buffer at pH 8.3, with a few drops of 0.001% BPB added, filled the upper and the lower buffer vessels. Electrophoresis was carried out at 450 V and about 70 mA for 16 h. Water at 7°C circulated through the cooling plates of the

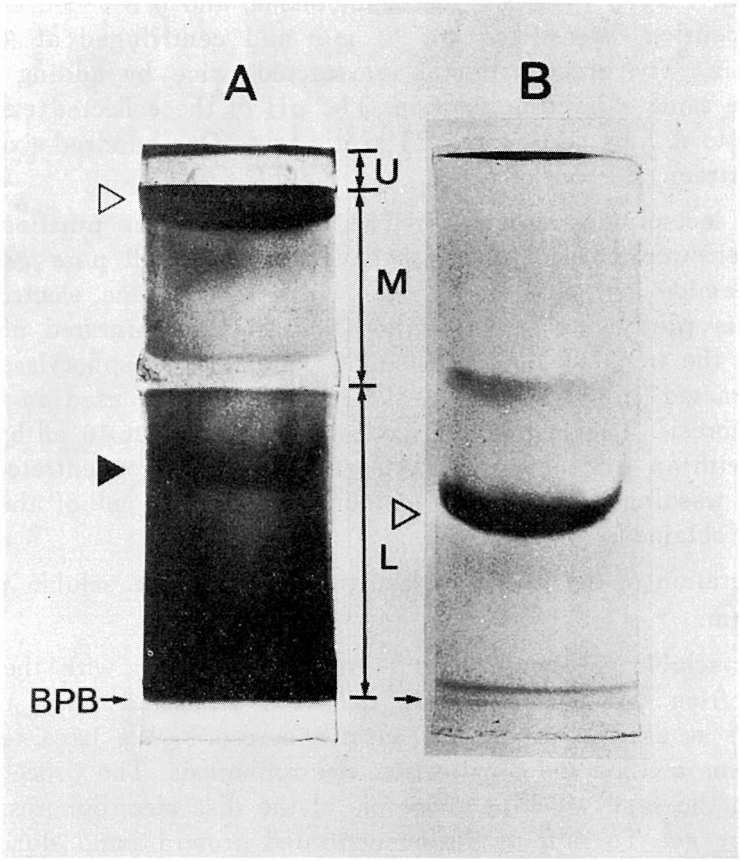

Fig. 1. Protein staining patterns of the preparative affinity electrophoresis. A. The first electrophoresis. The small pore gel was prepared in 3 layers of 5% polyacrylamide; the lower small pore gel (L), 6 cm in height without glycogen; the middle small pore gel (M), 3 cm in height containing 0.4% glycogen; and the upper small pore gel (U), 1 cm in height without glycogen. B. The second electrophoresis. The small pore gel was prepared in one layer of 5% polyacrylamide without glycogen.
▷ indicates the potato glycogen phosphorylase; ▶, the potato starch phosphorylase; and, → the tracking BPB band. Other conditions are seen in the text.

gel chamber. After electrophoresis the gel was taken out and cut in a slice of 2 mm thick parallel to the electric field. The slice was stained with Amido Black 10B. The stained gel is shown in Fig. 1-A. Because of its high affinity with glycogen, the glycogen phosphorylase was retarded in the upper part of the middle small pore gel in a band about 1 cm wide. The phosphorylase fraction, as defined by the stain, was removed from the bulk of the gel and ground as finely as possible in a mortar. It was suspended in 90 ml of the extracting solution at pH 6.7 containing 2 mM EDTA, 10 mM 2-mercaptoethanol, and 0.5% β-cyclodextrin. The suspension was stirred for 30 min and centrifuged at 8000 rpm for 10 min. The precipitate was reextracted twice by adding each 50 ml of the same extracting solution. The pH of the collected extract was adjusted to 6.7 by adding 0.5 M acetic acid. One hundred-sixty-two ml of the extract (E_1) was obtained.

The second electrophoresis: The E_1 fraction was purified by the second electrophoresis using one layer of the small pore gel of 5% polyacrylamide gel without glycogen. In Fig. 1-B, the electrophoresis pattern is pictured. The phosphorylase fraction migrated about two thirds of the tracking BPB as a single band. The phosphorylase fraction was extracted from the gel by the same procedure used in the first electrophoresis. The extract was concentrated to about 10 ml by ultrafiltration with an Amicon Concentrator Type 202. The concentrated sample and the washing solution were combined. Fourteen ml of the extract (E_2) was obtained.

iii) Separation of the phosphorylase fraction from the soluble gel component.

The soluble gel components[16] extracted together with the protein fraction from the polyacrylamide gel were separated using the same apparatus as used in preparative electrophoresis[23]. We have tentatively termed this method the purification electrophoresis. The procedure was based on the same stacking principle of the disc electrophoresis at the large pore gel. To support the concentrated protein band during electrophoresis, a sucrose density gradient of 15 to 45% was used. By this procedure all the protein fractions were concentrated at the position of the tracking BPB band, while the uncharged soluble gel component remained at the same position where the sample had been applied. Hence, the protein fraction can easily be saparated using the tracking BPB band as a marker.

The bottom of the gel chamber was supported by a sheet of a cellophane membrane on a supporting rack. The running buffer solution

was prepared by diluting the stock running buffer solution (Tris 6.0 g, glycine 28.8 g, and water to 1 liter, pH 8.3) 10 fold with water and adding a few drops of 0.001% BPB. The solution was poured into the gel chamber. The supporting buffer solutions were prepared by mixing the stock supporting buffer solution (Tris 5.98 g, 1 N HCl 48 ml, and water 100 ml, pH 6.7), water, and 60% sucrose in proportions of 1:5:2, 1:3:4 and 1:1:6. These solutions which contained 15, 30, and 45% sucrose, were introduced into the gel chamber with a capillary in layers of 1 cm, 1 cm, and 3 cm in height from the bottom of the gel chamber, respectively. Two ml of 60% sucrose was added to the E_2 extract. This solution was layered with a syringe between the running and the supporting buffer solutions. The lower buffer vessel contained the same supporting buffer solution of 45% sucrose. The running buffer solution was poured into the upper buffer vessel.

Electrophoresis was carried out at 440 V for about 2.5 h. Then, the tracking BPB band migrated to the position of the 30% sucrose layer. The BPB fraction, which was about 2 to 3mm wide, was collected using a syringe. The syrupy solution obtained was dialyzed 3 times against 1 liter of 5 mM EDTA solution at pH 6.3 containing 5 mM 2-mercaptoethanol. The dialyzed solution was concentrated by ultrafiltration using a collogion bag (Sartorius). One and a half ml sample (PE) was obtained.

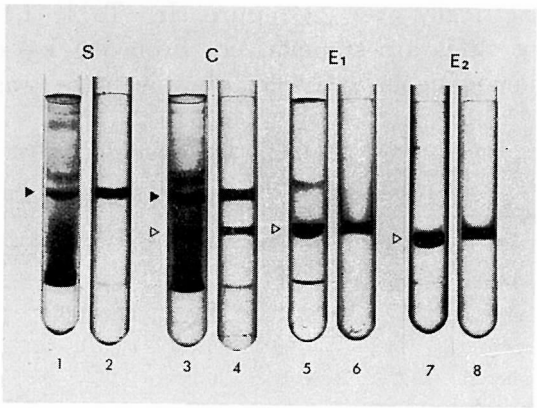

Fig. 2. Disc electrophoresis patterns of each purification step of the potato glycogen phosphorylase.
S: The crude potato supernatant, C: β-cyclodextrin eluate from potato grains, E_1: the extract from the first preparative electrophoresis, and E_2: the extract from the second preparative electrophoresis. Gels 1, 3, 5, and 7 were protein stained with Amido Black 10B and Gels 2, 4, 6, and 8 were stained to demonstrate phosphorylase activity. ▷ indicates the glycogen phosphorylase and ▶, the starch phosphorylase.

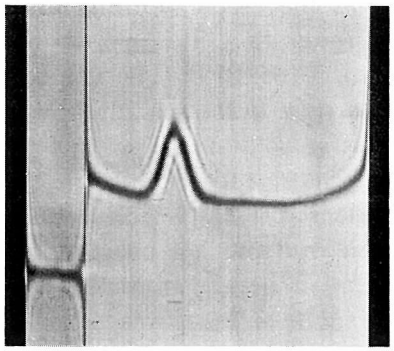

Fig. 3. Sedimentation velocity as determined by the ultracentrifugation of potato glycogen phosphorylase.
Conditions were 1.29 mg/ml in 0.05 M Tris-HCl buffer at pH 7.5 with 10 mM EDTA and 10 mM 2-mercaptoethanol at 51,200 rpm for 46 min at 10.4°C. The bar angle was 55°. The sedimentation was from left to right.

In Fig. 2, the electrophoresis patterns of protein fractions and phosphorylase activity at each purification step are shown. The supernatant of the potato homogenate (S) contained almost exclusively the starch phosphorylase, while the β-cyclodextrin extract from potato grains (C) contains the glycogen phosphorylase in amounts about half of the starch phosphorylase. The phosphorylase preparation obtained from the affinity electrophoresis (E_2) was almost homogeneous. The final sample (PE) is densitometrically over 98% pure. In Table I, the purification and the recovery rates are summarized. From 10 kg of potato tubers 2.1 mg of the homogeneous glycogen phosphorylase was obtained. This

Table I Purification of glycogen phosphorylase from potato tuber.

Purification step	Protein total (mg)	Activity total (U)	Specific activity (U/mg)	Yield (%)	Purification rate
Crude supernatant (S)	31800	3300	0.104		
Eluate from starch grains (C)	945	9510	10.1	100	1
The first electrophoresis (E_1)	7.99	8990	1130	95	112
The second electrophoresis (E_2)	3.69	5670	1540	60	152
Purification electrophoresis (PE)	2.15	4250	1980	45	196

yield amounted to 45% and the purification rate was about 200-fold from the β-cyclodextrin extract. Kamogawa et al[5] obtained 107 mg of the crystalline starch phosphorylase from 5 kg of potato tuber at the yield of 45%. In this respect the content of the glycogen phosphorylase in potato tubers amounted to about 0.1% of the starch phosphorylase.

2. Molecular properties of the glycogen phosphorylase.

Sedimentation. The purified enzyme exhibits a single symmetrical boundary on ultracentrifugation as seen in Fig. 3. The sedimentation coefficient ($s°_{20,w}$) was calculated as 8.19 S.

Molecular weight. In Fig. 4, the calibration curve for the gel filtration are presented. From this plot the molecular weight of the glycogen phosphorylase was calculated as 18×10^4. It coincided with the result of Gerbrandy[8] In Fig. 5, the molecular weight distribution curve obtained by sodium dodecyl sulphate gel electrophoresis are presented. The glycogen phosphorylase migrates in a single band almost the same position as rabbit muscle phosphorylase (9.4×10^4). From the plot the molecular weight of the glycogen phosphorylase subunit was calculated as 9×10^4. From these results it can be concluded that the glycogen phosphorylase is composed of two subunits of identical molecular weight.

Amino acid composition. In Table II, the amino acid composition of the potato glycogen phosphorylase are presented. Also listed in the table are the compositions of the potato starch phosphorylase[5] and

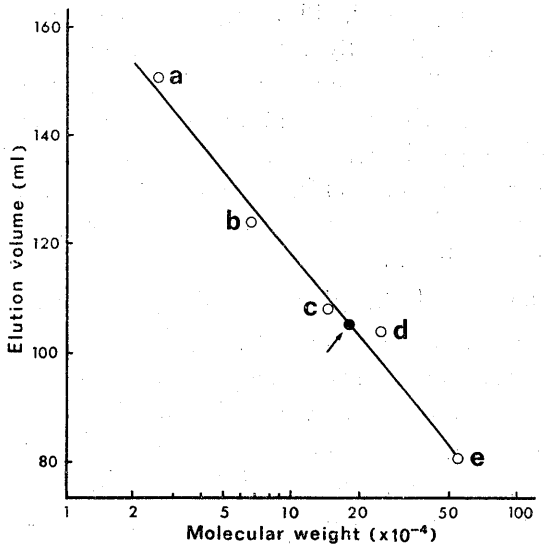

Fig. 4. Calibration curve for Sephadex G-200 gel filtration. A purified glycogen phosphorylase preparation (12μg) was subjected to the gel filtration of a Sephadex G-200 column (2.5×50 cm). Reference proteins (○) are: a) chymotrypsinogen A (4 mg), b) bovine serum albumin monomer (3.9 mg). c) rabbit muscle aldolase (4.4 mg), d) bovine liver catalase (2.9 mg), and e) horse spleen ferritin (1.9 mg). ● indicates the potato glycogen phosphorylase. Phosphorylase activity of the fractions was estimated and the protein fractions were detected by the measurement of the absorbance at 280 nm.

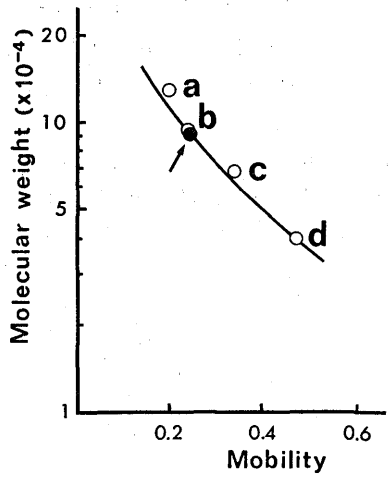

Fig. 5. Calibration curve for sodium dodecyl sulphate gel electrophoresis. Reference proteins (○) are: a) β-galactosidase (E. coli), b) rabbit phosphorylase *a*, c) bovine serum albumin, and d) rabbit muscle aldolase. ● indicates the potato glycogen phosphrylase.

rabbit muscle phosphorylase[24]. The amino acid composition of the glycogen phosphorylase is distantly related to that of the starch phosphorylase. The composition divergence factor of Harris et al[25] was calculated as 0.042 and 0.053 for the potato starch phosphorylase and rabbit muscle phosphorylase, respectively. The closer values were rabbit liver (0.035) or yeast phosphorylase (0.037)[26]. From these data no indications to support the affinity difference between the potato glycogen and starch phosphorylase were evident.

3. Catalytic properties of the glycogen phosphorylase.

The apparent Michaelis constants, Km, with glycogen and starch for the glycogen and the starch phosphorylases were determined from the Lineweaver-Burk plot as shown in Table Ⅲ. For the glycogen phosphorylase, Km with glycogen and starch were calculated as 1.1 and 0.84 mg/dl, respectively in the presence of 10 mM Glc-1-P. The value for the starch phosphorylase were 226 and 8.0 mg/dl, respectively. As also presented in the table, the Km values for the starch phosphorylase nearly coincided with those obtained by the other author[9]. When the affinity is expressed as the reciprocal value of Km or of the dissociation constant, Kd, the glycogen phosphorylase has nearly the same affinity with both glucans, while the starch phosphorylase has a 10-fold stronger affinity with starch than with glycogen.

As previously reported[8,11], the mobility of the potato glycogen phos-

Table Ⅱ Amino acid composition of potatoglycogen phosphorylase.

Amino acid	Percent of amino acid*1			
	Potato glycogen phosphorylase		Potato starch phosphorylase [5]	Rabbit muscle phosphorylase
	20 h hydrolysis	70 h hydrolysis	24 h hydrolysis	
Lysine	6.87	7.05	8.73	6.67
Histidine	2.79	3.03	2.25	3.25
Arginine	6.63	6.79	6.34	10.28
Aspartic acid	11.57	11.65	10.72	11.27
Threonine	4.52	4.40	4.61	3.54
Serine	4.87	4.46	4.09	2.32
Glutamic acid	12.44	12.50	14.63	12.31
Proline	4.23	4.46	3.70	3.61
Glycine	3.58	3.69	3.52	3.14
Alanine	5.62	5.60	5.49	4.91
Half-cystine	0.27	0.35	–	0.96
Valine	4.74	5.23	5.70	6.32
Methionine	2.52	1.91	1.80	2.82
Isoleucine	4.89	5.38	6.32	5.68
Leucine	9.98	10.17	7.88	9.27
Tyrosine	5.42	4.09	5.12	5.75
Phenylalanine	5.31	5.40	5.81	5.61
Tryptophan	(3.78)*2	–	(3.26)*2	2.22

*1 Amounts of each amino acid were expressed in percentage in 100 g of the total amino acid. The data from potato starch phosphorylase [5] and rabbit muscle phosphorylase [24] were converted from the original data.

*2 Determined spectrophotometrically [22].

phorylase is strongly retarded in the gel containing glycogen or starch, while the starch phosphorylase shows no such effect. Kd value of the former, as determined by the affinity electrophoresis, amounted to 1.7 mg/dl with shellfish glycogen and 33 mg/dl with potato soluble starch[11]. Gerbrandy reported similar results[8]. Those results indicated that there are discrepancies between affinity data obtained by affinity electrophoresis and kinetic studies. From the electrophoretic data, glycogen phosphorylase has nearly 20-fold stronger affinity with glycogen than with soluble starch, while from the kinetic data, it has essentially the same affinity with both glucans.

The discrepancies between electrophoresis and kinetic data might be based on the differences in experimental conditions. With electrophoresis, the medium does not contain Glc-l-P, while in the kinetic studies,

Table III Comparison of phsicochemical parameters of potatophosphorylases.

	Glycogen phosphorylase	Starch phosphorylase
Molecular weight ($\times 10^3$)		
SDS-gel electrophoresis	90	108[7]
Gel filtration	180	215[7]
Ultracentrifugation		207[4]
Apparent Km (mg/dl)		
Potato soluble starch	0.84	8.0
		8.1[9]
Shellfish glycogen	1.1	226
		100.1[9]
Dissociation constants calculated by affinity electrophoresis (mg/dl)		
Potato soluble starch	33.0[11]	
Shellfish glycogen	1.7[11]	

Kd was calculated in the presence of Glc-1-P. There could be some change in molecular conformation of the enzyme due to Glc-1-P. It was reported that for rabbit muscle phosphorylase, the Km values with glycogen in the absence and in the presence of Glc-1-P are computed to be 0.56 mM and 0.15 mM, respectively[27]. Hence, the enzyme has nearly 4-fold stronger affinity with glycogen in the presence of Glc-1-P. The same results were reported for rabbit liver phosphorylase[28]. On the other hand, yeast phosphorylase has a stronger affinity with glycogen in the absence of Glc-1-P than in the presence of it[26]. In the absence and the presence of Glc-1-P, the Km values were calculated to be 4.0 mg/dl and 51 mg/dl, respectively. In the case of the potato starch phosphorylase[6], the affinities of the enzyme with amylopectin were nearly equal in the absence and the presence of Glc-1-P. Our preliminary experiment showed that Km values of glycogen for the glycogen phosphorylase determined at lower concentrations of Glc-1-P changed only 2-fold. They amounted to 1.1, 0.93, and 0.53 mg/dl in the presence of Glc-1-P at 10, 5, and 1 mM, respectively.

As recently reported[29], X-ray crystallographic studies of oligosaccharide binding to rabbit muscle phosphorylase *a* demonstrated the presence of a glycogen storage site distant from the active site for glycogen. The affinity with maltoheptaose at the glycogen storage sites is at least 20-fold stronger than that at the active site. The electrophoretic mobility of the muscle enzyme is retarded with the gel containing glycogen[10]. It could be postulated that the potato glycogen phosphorylase also contains a glycogen storage site as does the muscle phosphorylase,

while the starch phosphorylase has no such site. Fischer and Hilpert[30] have previously reported that the potato starch phosphorylase has practically no affinity with shellfish glycogen. To clarify the discrepancies between the kinetic and electrophoretic data, elucidation of the steric conformation of the enyzmes will be awaited.

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