Biochemistry

 Volume 19, Number 11 May 27, 1980

A Comparative Study on α-Glucan Phosphorylases from Plant and Animal: Interrelationship between the Polysaccharide and Pyridoxal Phosphate Binding Sites by Affinity Electrophoresis†

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ABSTRACT: The interrelationship between the cofactor and α-1,4-glucan binding sites in phosphorylases (EC 2.4.1.1) from potato tubers and rabbit skeletal muscle has been investigated by comparing the affinities of the intact enzyme and the enzyme modified at the cofactor site for the glucan. The dissociation constants of the protein–saccharide complexes were determined by affinity electrophoresis in polyacrylamide gel. This procedure allows one to evaluate the extent to which a protein interacts with a ligand present in the gel matrix by the decrease in electrophoretic mobility. The modified phosphorylases were prepared by reconstitution of the apoenzymes with the cofactor (pyridoxal 5′-phosphate) analogues modified at the 5′ position. The affinity of rabbit muscle phosphorylase *b* for glycogen was little affected by the modifications. By contrast, the affinity of potato phosphorylase for amylopectin and maltopentaose was characteristically affected according to the type of modifications: removal of the cofactor from the enzyme resulted in the disruption of the glucan binding site, substitution with a pyrophosphate group at the 5′ position of pyridoxal 5′-phosphate strengthened the affinity, and introduction of a bulky group to the 5′-phosphate group weakened the affinity. These results would mean that the glucan binding site of the potato enzyme is located adjacent to the cofactor site, in contrast to the rabbit muscle enzyme in which the preferential binding site for glucan is separated from the cofactor site. The present findings therefore support the view that the active site is adjacent to the cofactor site. Although both cycloheptaamylose and maltopentaose bind to the potato enzyme competitively with amylopectin, introduction of a bulky substituent on the 5′ position did not interfere with the binding of the former, indicating that cyclodextrin binds at a distance from the cofactor.

α-Glucan phosphorylase (EC 2.4.1.1, α-1,4-glucan:orthophosphate glucosyltransferase) catalyzes the reversible transfer of an α-glucosyl unit between orthophosphate and the nonreducing terminus of an α-1,4-glucan with retention of configuration. Several kinetic studies have shown that the enzyme has a rapid equilibrium random bi bi mechanism (Maddaiah & Madsen, 1966; Chao et al., 1969; Engers et al., 1969, 1970; Gold et al., 1970, 1971). Its activity on glucans depends considerably on the structure of the glucan itself. Rabbit muscle phosphorylase acts on highly branched polysaccharides (e.g., glycogen and amylopectin) rather than linear glucans (e.g., maltodextrin and amylose) (Brown & Cori, 1961; Goldemberg, 1962; Lee et al., 1970a; Smith, 1971). By contrast, phosphorylases from potato and sweet corn have affinities for linear glucans as high as that for amylopectin, while glycogen is a poor substrate when it is compared with amylopectin in spite of their similarity of branched structures (Lee et al., 1970a; Smith, 1971; Lee & Braun, 1973). Phosphorylase from *Escherichia coli* would be classified into another type since the enzyme has the highest affinity for short linear glucans (Schwartz & Hofnung, 1967).

Hu & Gold (1975) confirmed the requirement of muscle phosphorylase for highly branched substrates by using semisynthetic branched saccharides which contained constant numbers of α-1,4-bonded oligosaccharide chains per molecule. They proposed a model in which two chain termini from the same saccharide molecule bind simultaneously to the enzyme molecule, and therefore a highly branched saccharide is a better substrate because of the greater multiplicity of two end-group pairs. Their model does not, however, explain the reason why the affinity of potato phosphorylase for glucan is independent of the multiplicity of two end-group pairs in spite of the existence of probably two active sites per enzyme molecule as in the case of muscle phosphorylase (Lee, 1960; Iwata & Fukui, 1973).

Recent X-ray crystallographic studies might give us a clue to this question of substrate specificity (Fletterick et al., 1976; Sygusch et al., 1977; Kasvinsky et al., 1978; Weber et al., 1978). They have shown two distinct binding sites for substrates per protomer of rabbit muscle phosphorylase. One of them is occupied by an oligosaccharide chain and not by the counterparts of the substrates: orthophosphate and α-D-glucose 1-phosphate. The latter two substrates, and no oligosaccharide chain, bind to another common region adjacent to the cofactor (PLP)[1] site. On the basis of X-ray studies, Kasvinsky et al.

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0006-2960/80/0419-2287$01.00/0

(1978) have explained the substrate specificity of muscle phosphorylase by a model in which the enzyme has two separate binding sites for glycogen per protomer: "a glycogen storage site" providing a point of attachment of the enzyme to the substrate with high affinity and "an active site" with much lower affinity. The X-ray diffraction data have not, however, indicated the exact location of the active site, though they would lead us to suppose that the active site is located adjacent to the cofactor site.

The existence of "a polysaccharide storage site" in potato phosphorylase could not be expected from its kinetic behavior in which this enzyme does not require the branched structure of the substrate, and its proper binding site for the saccharide should therefore be the active site itself. In this sense, the use of potato phosphorylase will be advantageous for elucidation of the topology of the active site. If the active site is adjacent to the cofactor site, especially to its 5′-phosphate moiety, as proposed by several investigators (Parrish et al., 1977; Feldmann & Hull, 1977; Sygusch et al., 1977; Weber et al., 1978; Shimomura & Fukui, 1978; Shimomura et al., 1980), the affinity of potato phosphorylase for glucan should be significantly altered by modification at the 5′ position of the cofactor and, by contrast, the affinity of rabbit muscle phosphorylase should not be changed by the modification.

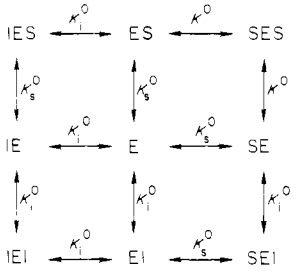
In this work, we have investigated this possibility by comparing the affinities of potato and rabbit muscle phosphorylases and their derivatives modified at the cofactor site for saccharide. The dissociation constants of the enzyme–saccharide complexes have been measured by affinity electrophoresis which has been developed by Takeo & Nakamura (1972) and Hořejší et al. (1977). The modified phosphorylases have been prepared by reconstitution of apophosphorylases with PLP analogues (Shimomura & Fukui, 1977, 1978; Shimomura et al., 1978, 1980).

Materials and Methods

Materials. Shellfish glycogen and dextran (M_r 176 000) were purchased from Nakarai Chemicals. Cycloheptaamylose was a product of Hayashibara Biochemical Laboratories. Maltopentaose was kindly provided by Kikkoman Shoyu Co. Amylopectin was prepared from glutinous rice. Potato phosphorylase and its apoenzyme were prepared as described by Kamogawa et al. (1968) and Shimomura et al. (1980), respectively. Rabbit muscle phosphorylase *b* and its apoenzyme were obtained by the methods of Fischer & Krebs (1958) and Shaltiel et al. (1966), respectively. Phosphorylases reconstituted with PLP analogues were prepared as described previously (Shimomura & Fukui, 1977, 1978; Shimomura et al., 1978, 1980). PLDP, PLDP-Phe, and bis-PLP were used for reconstitution of the potato apoenzyme. PLDP, PLSN, bis-PLP, and pyridoxal were used for the rabbit muscle apoenzyme. These PLP analogues were synthesized as described by Shimomura & Fukui (1978).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel disc electrophoresis was carried at pH 8.0 (Williams & Reisfeld, 1964) with a gel system omitting large-pore gel layers. Separating gels were prepared with the precautions as described by Takeo & Nakamura (1972). The gels (10-cm height in a glass tube of 0.5 × 12 cm) were composed of the indicated concentration of acrylamide [the ratio of *N,N′*-methylenebis(acrylamide) to total acrylamide was a constant value of 0.026], 0.058% *N,N,N′,N′*-tetramethylethylenediamine, 0.07% ammonium persulfate, 70 mM Tris-HCl buffer (pH 7.5), and variable concentrations of polysaccharide. A 10-μL aliquot of 0.5 mg/mL phosphorylase dissolved in 50% glycerol and 15 mM glycerophosphate buffer (pH 7.0) was layered between the running buffer solution (30 mM barbital–Tris at pH 7.0 and 10^{-5}% bromophenol blue) and the separating gel. Electrophoresis was carried out at 2 mA/tube for ~140 min and, unless otherwise noted, at room temperature (26–28 °C) with cooling by an electric fan. The gel was stained by amido black 10-B and scanned with a Fuji Riken densitometer, FD-A IV. In experiments testing the effect of a small competitive inhibitor toward amylopectin (e.g., cycloheptaamylose or maltopentaose), the same concentration of the inhibitor was added to all layers including the protein solution, the running buffer solution, and the separating gel.

Calculation of the Dissociation Constant of a Complex of Protein and Immobilized Ligand or Free Ligand in the Affinity Gel. The acrylamide gel is assumed to contain a protein E, a multibranched ligand S immobilized in the gel matrix (e.g., amylopectin, glycogen, or dextran), and a free ligand I (e.g., cycloheptaamylose or maltopentaose). Assuming that S and I bind to E in a competitive manner and that E has two common binding sites for both ligands per molecule (since phosphorylase exists as a dimer), we can derive the binding model



in which ES, EI, etc. are the species of protein–ligand complexes and K_s^0 and K_i^0 are the intrinsic dissociation constants for the ligands S and I, respectively. The two binding sites are assumed to be identical and independent of each other because potato phosphorylase shows no homotropic cooperativity on polysaccharide kinetics and rabbit muscle phosphorylase shows little homotropic cooperativity. The equilibrium reaction ES (or SE) ↔ SES should be independent of the concentration of a multibranched ligand S because S is immobilized and therefore a second molecule of S cannot come near the ES complex. Thus, the dissociation constant K_s^0 is replaced by the equilibrium constant K^0. Under an electric field, the protein species except for all the complexes with S can move according to their intrinsic electrophoretic mobilities. If all the equilibriums are assumed to be very rapidly established, the observed mobility U_{obsd} of the protein is expressed as

$$U_{obsd} = U_E f(E) + 2U_{EI} f(EI) + U_{IEI} f(IEI) \qquad (1)$$

where U_X and $f(X)$ are the intrinsic electrophoretic mobility and the fractional ratio in a total protein concentration corresponding to species X, respectively. U_{EI} and U_{IEI} were experimentally measured to be the same as U_E, as shown later. The mobility of bromophenol blue (a marker dye) was unaffected by the saccharides used. Equation 1 can be converted to

$$\frac{1}{R} = \frac{1}{R_0}\left(1 + \frac{[S]}{K_{app}}\right) \qquad (2)$$

[1] Abbreviations used: PLP, pyridoxal 5′-phosphate; PLDP, pyridoxal 5′-diphosphate; PLDP-Phe, pyridoxal 5′-diphosphate β-monophenyl ester; bis-PLP, P^1,P^2-bis(5′-pyridoxal) diphosphate; PLSN, 5′-deoxypyridoxal-5′-sulfonate.

where

$$K_{app} = \frac{K_s^0\left(1 + \frac{[I]}{K_i^0}\right)^2}{\frac{1}{K^0} + 2\left(1 + \frac{[I]}{K_i^0}\right)} \quad (3)$$

and R_0 and R are the electrophoretic mobilities of E (relative to that of bromophenol blue) in the absence of S and in the presence of S plus I, respectively.

The equilibrium constant K^0 should relate closely to the structures of both ligand S and protein E. An increase in the multiplicity of the end group of the branched ligand S would decrease the K^0 value, as shown by Hu & Gold (1975) on rabbit muscle phosphorylase. By contrast, the K^0 of potato phosphorylase is considered to be much larger because the potato enzyme shows little discrimination between branched and linear substrates (Lee et al., 1970a). Therefore, in the case of potato phosphorylase, eq 3 is simplified as

$$K_{app} = K_s\left(1 + \frac{[I]}{K_i^0}\right) \quad (4)$$

where K_s ($=K_s^0/2$) is the effective dissociation constant of the complex of potato phosphorylase and immobilized ligand S.

Calculation of Retardation Coefficient. The retardation coefficient K_R of protein was obtained by using the equation (Ferguson, 1964; Rodbard & Chrambach, 1970)

$$\log R = \log R^* - (K_R - K_R^{BPB})T \quad (5)$$

where R and R^* are the mobilities of protein relative to that of bromophenol blue observed in a T% (w/v) acrylamide gel and obtained by extrapolation of the gel concentration to zero, respectively. K_R^{BPB} is the retardation coefficient of bromophenol blue and was determined to be 0.01 by using the 3.1–10.3% gels.

Results

Electrophoretogram of Potato Phosphorylase in the Absence of Ligands. Figure 1 shows the densitometric patterns of potato phosphorylase on polyacrylamide gels after electrophoresis at room temperature (26–28 °C). In the absence of any interacting ligand, native potato phosphorylase showed a single sharp protein band (Figure 1A). Potato apophosphorylase in which the cofactor PLP had been removed showed two broad bands termed I and II in order of mobility (Figure 1B). Every enzyme reconstituted with PLP analogues showed one main sharp band and an additional broad band, termed as I and II, respectively (parts C–E of Figure 1).

Polyacrylamide gel electrophoresis has been both theoretically and experimentally shown to provide physicochemical characteristics of a protein molecule in terms of its size and net charge (Chrambach & Rodbard, 1971). Thus, the mobilities were measured at different gel concentrations from 3.1 to 10.3% to decide whether these protein species observed in Figure 1 are size isomers and/or charge isomers. As shown in Figure 2, good linear Ferguson plots were obtained in every case. Table I summarizes the retardation coefficients K_R and the free electrophoretic mobilities R^* (relative to that of bromophenol blue) calculated from these plots by using eq 5.

Species I of the apoenzyme preparation has K_R and R^* values similar to those of the intact holoenzyme, suggesting that the two proteins resemble each other in both molecular size and net charge. On the other hand, species II has smaller K_R and R^* values than those for the holoenzyme. For globular proteins, the K_R value relates to the molecular radius (Rodbard & Chrambach, 1970). A decrease in the K_R value may reflect a more compact folding of protein or a dissociation of oligomeric protein. Potato phosphorylase is composed of two subunits of the same size and has a sedimentation coefficient,

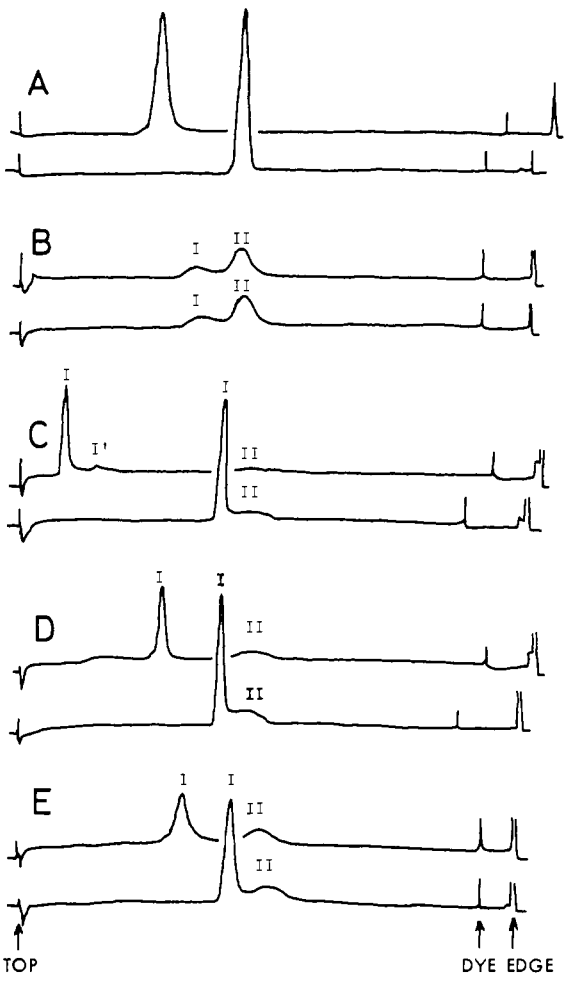


FIGURE 1: Densitometric pattern of potato phosphorylase on polyacrylamide gel electrophoresis. The experiments were carried out at room temperature by using a 5.1% acrylamide gel. (A) Intact holoenzyme; (B) apoenzyme; (C, D, and E) enzymes reconstituted with a slight excess of PLDP, PLDP-Phe, and bis-PLP, respectively. The upper pattern of each pair is the result in the affinity gel containing 0.4% amylopectin, and the lower pattern is the result in the normal gel containing no amylopectin.

Table I: Parameters of Potato Phosphorylase Obtained by Affinity Gel Electrophoresis

enzyme	R^*	K_R	K_s (%) for amylopectin	K_i^0 (mM) for maltopentaose	K_i^0 (mM) for cycloheptaamylose
intact holo	2.2	0.15	0.76	4.6	0.46
PLDP-reconstd I	2.1	0.15	0.13	2.1	0.33
I'	2.5[a]	0.16[a]	0.35	2.5	0.61
II	1.5	0.10	>10		
PLDP-Phe-reconstd I			1.1	22	0.63
II			>10		
bis-PLP-reconstd I	2.6	0.16	1.3	9.1	0.55
II	1.6	0.10	>10		
apo I	2.3	0.16	>10		
II	1.5	0.10	>10		

[a] The electrophoretic mobility at each concentration of gel was measured in gels containing variable concentrations of amylopectin and obtained by extrapolation of the saccharide concentration to zero.

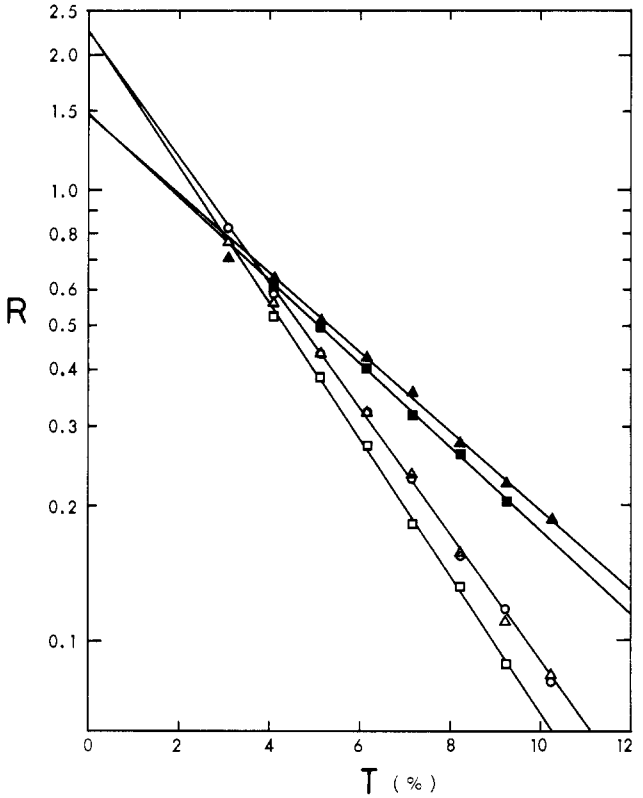


FIGURE 2: Ferguson plot of the relative mobility R of potato phosphorylase vs. the concentration T of acrylamide (w/v, percent) in gel. The ratio of acrylamide monomer vs. N,N'-methylenebis(acrylamide) was constant in every gel. Electrophoresis was carried out at room temperature. (○) Intact holoenzyme; (□) apoenzyme species I; (■) apoenzyme species II; (△) PLDP–reconstituted enzyme species I; (▲) PLDP–enzyme species II.

a molecular weight, and a frictional ratio similar to those of rabbit muscle phosphorylase dimer (Lee, 1960; Iwata & Fukui, 1973). X-ray crystallographic studies have shown that the muscle enzyme is a compact protein (Fletterick et al., 1976). Thus, species II of the potato apoenzyme is considered to be a form which has dissociated to a monomer and not a more compactly folded protein.

The molar ratio between the two species of potato apophosphorylase was observed to depend on temperature. When electrophoresis was carried out at 4 °C, only a single broad protein band having K_R of 0.11 and R^* of 1.3 was detected, which probably corresponds to species II. These results indicate that potato apophosphorylase has a dissociation–association system depending on the temperature; at a lower temperature the protein exists as a monomeric state (species II), and at a higher temperature it exists as a mixture of monomer and dimer (species II and I). The temperature dependence for the potato apoenzyme contrasts with that for the rabbit muscle apoenzyme; the latter enzyme tends to aggregate at a lower temperature (Hedrick et al., 1966). As already shown in the preceding paper (Shimomura et al., 1980), the stability of the potato apoenzyme depends considerably on temperature; it is more stable at lower temperatures. Thus, a monomeric state might be more stable than a dimeric one.

Species I and II of every reconstituted phosphorylase have almost the same K_R and R^* values as those for the intact holoenzyme and apoenzyme species II, respectively (Table I). The molar ratio between the two species of every reconstituted enzyme was unaffected by temperature. The K_R values also did not depend on temperature. In the case of bis-PLP–reconstituted phosphorylase, the values of K_R and R^* were 0.15 and 1.9 for species I and 0.11 and 1.3 for species II at 4 °C, respectively (cf. Table I).

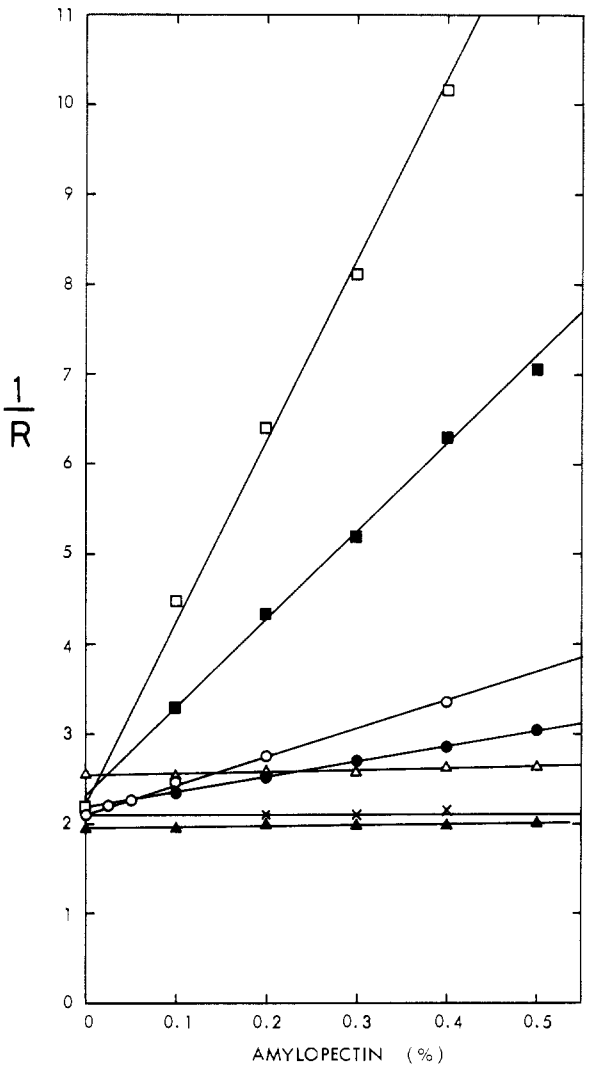


FIGURE 3: Plot of the reciprocal of the relative mobility R of potato phosphorylase vs. the concentration of amylopectin in a 5.1% acrylamide gel. (○) Intact holoenzyme; (□) PLDP–reconstituted enzyme species I; (■) PLDP–enzyme species I'; (●) bis-PLP–enzyme species I; (▲) bis-PLP–enzyme species II; (△) apoenzyme species I; (×) intact holoenzyme plot against glycogen concentration instead of amylopectin concentration.

The apoenzyme preparation used in these experiments is composed of 65% reconstitutable "active" apoenzyme, 30% unreconstitutable "inactive" apoenzyme, and 5% native holoenzyme [cf. Shimomura et al. (1980)]. The reconstitution of the active apoenzyme with PLP analogues is complete under these conditions as judging from titration experiments. The main sharp band I and the minor band II of every reconstituted phosphorylase are therefore considered to correspond to the true reconstituted enzyme and the inactive apoenzyme, respectively. Species II of the apoenzyme preparation should contain both active and inactive apoenzymes.

Affinity Electrophoresis. The electrophoretic mobility of potato phosphorylase decreased by addition of its natural substrate amylopectin to the polyacrylamide gel, as shown in Figure 1A. The relation between the observed mobility and the concentration of amylopectin added to the gels was plotted as in Figure 3. The mobility of potato phosphorylase was not, however, retarded by addition of a poor substrate glycogen up to 1% (w/v) (Figure 3). On the other hand, the mobility

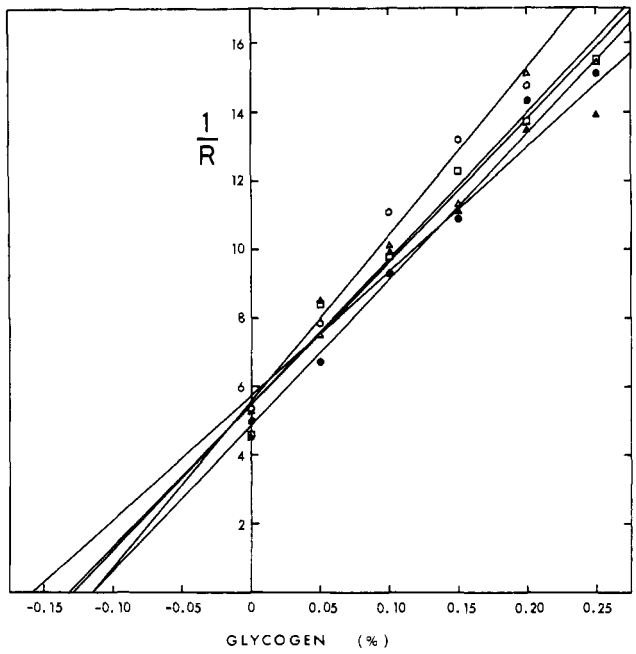


FIGURE 4: Plot of the reciprocal of the relative mobility R of rabbit muscle phosphorylase b vs. the concentration of glycogen in a 5.1% acrylamide gel. (○) Intact holoenzyme; (●) PLDP–reconstituted enzyme; (△) PLSN–enzyme; (▲) bis-PLP–enzyme; (□) pyridoxal–enzyme.

of rabbit muscle phosphorylase b decreased with increasing concentration of its substrate glycogen (Figure 4) and was unaffected by the same concentration of a nonsubstrate dextran. These results are indicative of a specific interaction between enzyme and its natural substrate polysaccharide and rule out the possibilities of a molecular sieving effect produced by such a macromolecule in a way analogous to polyacrylamide and of a nonspecific absorption of protein to polysaccharide.

In the case of potato phosphorylase, degrees of retardation of mobility which is induced by interaction with amylopectin varied significantly according to the modified enzymes, as shown in Figures 1 and 3. For PLDP–reconstituted phosphorylase, the hidden minor band termed I′ was distinguishable from the main band I on the basis of their affinities for amylopectin but not from their molecular sizes or net charges (Figures 1C and 3 and Table I). The species I band of bis-PLP–reconstituted phosphorylase was broadened in the gel containing amylopectin (Figure 1E). This may indicate the presence of several conformational isomers having slightly different affinities for amylopectin, even though the isomers have the same molecular sizes and the same net charges.

A plot of the reciprocal of the observed mobility vs. the concentration of amylopectin (Figure 3) or glycogen (Figure 4) gives a good straight line in every case. These polysaccharides can therefore be regarded as immobilized ligands for phosphorylases in the gel because the plot would give a hyperbolic curve if the protein–polysaccharide complex moved under an electric field.

Competitive Binding Experiments of Affinity Electrophoresis. For further characterization of interactions between phosphorylase and immobilized ligand, we attempted inhibition experiments of free ligand against immobilized ligand. Cycloheptaamylose and maltopentaose were used in these experiments for the following reasons: the cyclodextrin is a strong competitive inhibitor of potato phosphorylase in respect to the substrate amylopectin (Staerk & Schlenk, 1967), and the maltodextrin is a good substrate of the potato enzyme and should therefore be a competitive inhibitor with regard to

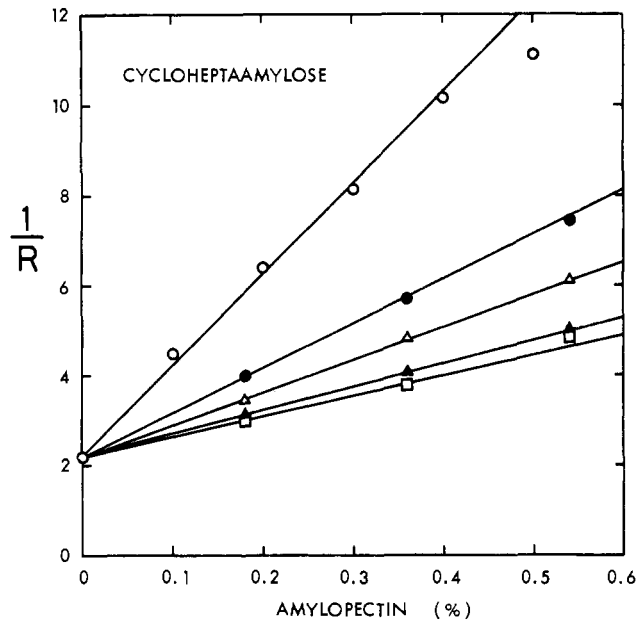


FIGURE 5: Effect of cycloheptaamylose. Relative mobility R of potato phosphorylase reconstituted with PLDP (species I) as a function of amylopectin concentration at five levels of cycloheptaamylose in a 5.1% acrylamide gel. Concentrations of cycloheptaamylose were as follows: (○) 0; (●) 0.24; (△) 0.5; (▲) 0.75; (□) 1 mM.

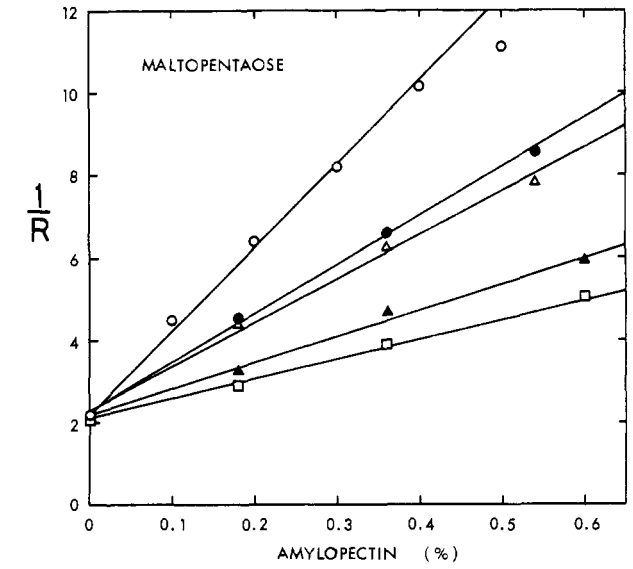


FIGURE 6: Effect of maltopentaose. Relative mobility R of potato phosphorylase reconstituted with PLDP (species I) as a function of amylopectin concentration at five levels of maltopentaose in a 5.1% acrylamide gel. Concentrations of maltopentaose were as follows: (○) 0; (●) 0.5; (△) 1; (▲) 2; (□) 4 mM.

amylopectin (Whelan & Bailey, 1954).

The electrophoretic mobilities of potato phosphorylase and its reconstituted enzymes were little affected by addition of cycloheptaamylose (up to 1 mM) to polyacrylamide gels (cf. Figure 5). The K_R and R^* values of intact holoenzyme and PLDP– and bis-PLP–reconstituted enzymes also were totally unaffected by addition of 1 mM cycloheptaamylose. Under these conditions, more than half of each enzyme forms a complex with the cyclodextrin, judging from their dissociation constants K_i^0's as mentioned later (cf. Table I). Thus, the intrinsic electrophoretic mobility of each protein–cyclodextrin complex is the same as that of free protein.

The maltopentaose–protein complexes also showed the same mobilities as those of free proteins since the K_R and R^* values

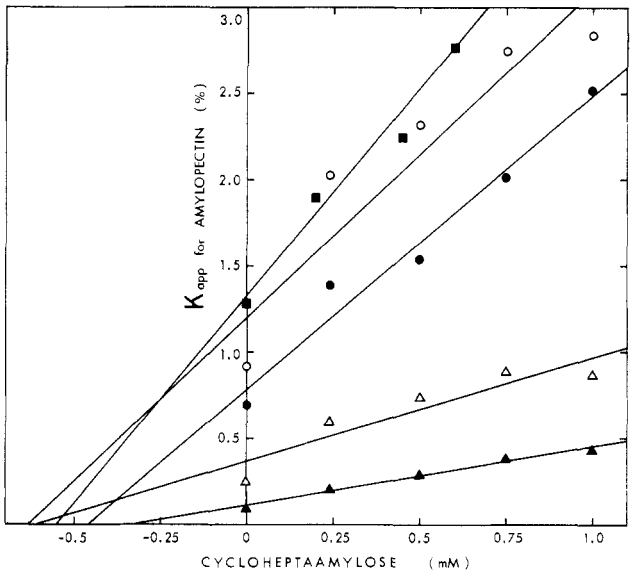


FIGURE 7: Plot of the apparent dissociation constant K_{app} of potato phosphorylase for amylopectin vs. the concentration of cycloheptaamylose. A 5.1% acrylamide gel was used. (●) Intact holoenzyme; (○) PLDP-Phe–reconstituted enzyme species I; (▲) PLDP–enzyme species I; (△) PLDP–enzyme species I'; (■) bis-PLP–enzyme species I.

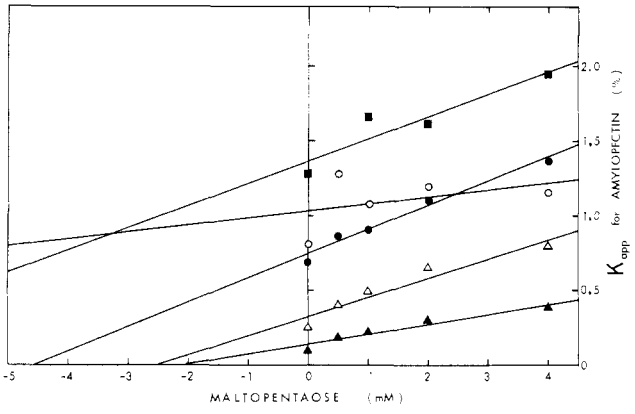


FIGURE 8: Plot of the apparent dissociation constant K_{app} of potato phosphorylase for amylopectin vs. the concentration of maltopentaose. A 5.1% acrylamide gel was used. The symbols are the same as those in Figure 7.

were little affected by addition of 4 mM maltopentaose.

However, addition of these oligosaccharides to the affinity gel containing amylopectin affected the degree of retardation of protein mobility. As shown in Figures 5 and 6, increases in oligosaccharide concentrations abolished the retardation of mobility. At a fixed concentration of cycloheptaamylose (Figure 5) or maltopentaose (Figure 6), a plot of the reciprocal of the observed mobility vs. the concentration of amylopectin gave a good straight line, consistent with the scheme in eq 2. Thus, the apparent dissociation constants K_{app}'s for amylopectin were obtained from the slopes in the figures according to the equation. Although these figures presented only the results of PLDP–reconstituted potato phosphorylase, the data for intact holoenzyme and other reconstituted enzymes also showed good straight lines.

A plot of the dissociation constant K_{app} for amylopectin observed at a fixed concentration of an inhibitor vs. the concentration of the inhibitor also gave a straight line in every case, as shown in Figures 7 and 8. These linear relationships support the proposed binding model in eq 1–4 and demonstrate that these oligosaccharides bind to the same site as amylopectin in potato phosphorylase. Tables I and II summarize the

Table II: Dissociation Constant of the Complex of Rabbit Muscle Phosphorylase *b* and Glycogen Obtained by Affinity Electrophoresis

enzyme	K_{app} (%) for glycogen
intact holo	0.11
PLDP-reconstd	0.11
PLSN-reconstd	0.13
bis-PLP-reconstd	0.16
pyridoxal-reconstd	0.13

dissociation constants calculated from the results shown in Figures 3–8.

Interrelation between the Cofactor and Polysaccharide Binding Sites. Removal of the cofactor PLP from potato phosphorylase considerably diminished its affinity for amylopectin (Figures 1B and 3 and Table I). This finding might be conceivable since potato apophosphorylase and its holoenzyme differ in their secondary structures as judged from their far-ultraviolet circular dichroic spectra (Shimomura et al., 1980) and in their quaternary structures as shown already. However, the apoenzyme from rabbit muscle shows an affinity for glycogen similar to its holoenzyme (Kastenschmidt et al., 1968) in spite of the differences in both secondary (Steiner et al., 1977) and quaternary (Hedrick et al., 1966) structures.

On the other hand, reconstitution of potato apophosphorylase with the cofactor analogues restored its ability to interact with amylopectin as well as its quaternary structure (parts C–E of Figure 1, Figure 3, and Table I). However, there is a characteristic difference according to the type of cofactor analogues used for reconstitution. The dissociation constant K_s for amylopectin decreased 6 times by substituting PLDP for PLP and increased twice by substituting PLDP-Phe and bis-PLP (Table I). Similar phenomena were observed in the affinity for maltopentaose (Table I); substitution of PLDP, PLDP-Phe, or bis-PLP for PLP resulted in a twice as low, a 5 times as high, or a twice as high dissociation constant K_i^0 as that of the native enzyme, respectively. On the other hand, the interaction with cycloheptaamylose was less sensitive to the modification of the cofactor (Table I). These results indicate that binding of the two oligosaccharides to potato phosphorylase results in different local situations, although they both are competitive inhibitors with respect to amylopectin.

In contrast to potato phosphorylase, rabbit muscle phosphorylase *b* showed an interaction with its substrate glycogen which was independent of modifications to the cofactor (Figure 4 and Table II).

Discussion

The binding site detected by affinity electrophoresis should be the highest affinity site for amylopectin or glycogen in the phosphorylase molecule because the method can detect only the first attachment of an enzyme to its immobilized ligand. The interaction between a protein and its ligand in a polyacrylamide gel would differ from that in a free solution. The most probable difference is produced from a diffusional limitation and/or a steric hindrance by the gel matrix. However, the values of the dissociation constants obtained by affinity electrophoresis (Tables I and II) are comparable to the values obtained by enzyme kinetics and other binding experiments in a free solution: from enzyme kinetics, the dissociation constant of the rabbit muscle phosphorylase *b*–AMP binary complex for glycogen is 4.5 mM (~0.08%) (Engers et al., 1969); by direct binding measurements, the dissociation constant of rabbit muscle apophosphorylase *b* for glycogen is 0.067% (Kastenschmidt et al., 1968); by inhibition experiments

of cycloheptaamylose on the trypsinolysis of potato phosphorylase, Iwata & Fukui (1975) obtained a value of 0.23 mM for the dissociation constant of the phosphorylase–cyclodextrin complex. The gel matrix of such a low level of polyacrylamide as 5.1% might therefore make little contribution to the interaction between protein and ligand. We have, however, preliminarily observed that the K_s for amylopectin decreases but the K_i^0 for cyclodextrin increases with increasing concentration of acrylamide in gels (S. Shimomura, unpublished experiments).

On affinity electrophoresis, potato phosphorylase showed a higher affinity for amylopectin than for glycogen and demonstrated competition with maltopentaose and cycloheptaamylose. These phenomena are consistent with the facts previously obtained from enzyme kinetics. The existence of multiple binding sites for polysaccharide per protomer of potato phosphorylase should not be expected. We therefore conclude that the binding site observed here is the active site itself of potato phosphorylase.

For rabbit muscle phosphorylase, the observed binding site (i.e., the highest affinity site) correlates little to the cofactor site since the interaction with glycogen was not affected at all by modification of the cofactor molecule. This finding is reconciled with the recent X-ray crystallographic observation in which the proper binding site for polysaccharide is more than 25 Å distant from the cofactor site (Kasvinsky et al., 1978), as well as with the earlier result obtained by Kastenschmidt et al. (1968) that removal of the cofactor from the enzyme does not injure the glycogen binding site.

On the contrary, here we obtained evidence that the polysaccharide binding site in potato phosphorylase is closely related with the cofactor site: removal of the cofactor resulted in the disruption of the polysaccharide binding site, introduction of an additional phosphate group on the 5′ position of PLP, such as PLDP, strengthened the affinity, and, inversely, introduction of a bulky group on the 5′ position, such as PLDP-Phe and bis-PLP, weakened the affinity. We have already shown in reconstitution studies of the apoenzymes with PLP analogues (Shimomura & Fukui, 1978; Shimomura et al., 1980) that the structural characteristics of the cofactor site are similar in potato and rabbit muscle phosphorylases and that both enzymes have a wide enough space to introduce a large group therein adjacent to the 5′-phosphate moiety of the bound cofactor. For rabbit muscle phosphorylase, this neighboring space has been shown to be occupied by two substrates, orthophosphate and glucose 1-phosphate (Sygusch et al., 1977; Weber et al., 1978). The present results provide stronger evidence for the view that this space is the active site, because we can explain the phenomena observed here in the potato enzyme without any contradiction as follows, assuming that the nonreducing end of an oligosaccharide chain can bind adjacent to the 5′-phosphate group of the enzyme-bound PLP: (1) the bulky group introduced on the 5′ position of PLP should interfere with the saccharide to be bound to the site and therefore result in weakening the affinity of the site; (2) the additional phosphate group introduced on PLP might occupy the orthophosphate binding site and thus cause the affinity to be strengthened since the orthophosphate–enzyme binary complex shows a lower Michaelis constant for amylopectin than the free enzyme does (Gold et al., 1971). In fact, the phosphate group of the enzyme-bound substrate is only 7 Å (center to center) distant from the 5′-phosphate group of PLP in rabbit muscle phosphorylase (Madsen et al., 1979).

The present results show that, although both cyclodextrin and noncyclic dextrin bind to potato phosphorylase competitively with amylopectin, the binding phenomena of these two dextrins differ much from each other; the binding of cyclodextrin is not hindered by introduction of a bulky substituent on the 5′ position of PLP, whereas the binding of noncyclic dextrin is significantly hindered. This difference may indicate that the polysaccharide binding site of potato phosphorylase is separated into two subsites: one is neighboring with the bound PLP in which the nonreducing terminus of saccharide binds; the other is at a distance from the cofactor, situated at the protein surface to interact with macromolecule substrates. Amylopectin and noncyclic dextrin can bind over both subsites, whereas cyclodextrin can bind to only the latter subsite. Thus, cyclodextrin cannot serve as a substrate of potato phosphorylase because of its binding at a distance from the catalytic site.

This idea may resolve the problem of why potato phosphorylase shows a lower affinity for glycogen than for amylopectin. Glycogen has shorter α-1,4 outer chains than amylopectin; the average outer chain lengths are 5.8 for shellfish glycogen and 12.6 for amylopectin (Lee et al., 1970b). Further, glycogen has more α-1,6 branch points than amylopectin, indicating that the available length of the outer chain of glycogen for the interaction with the potato enzyme decreases due to steric hindrance of the neighboring outer chains. Since the affinity of potato phosphorylase for maltodextrins weakens with a decrease in the degree of polymerization of the oligosaccharides (Parrish et al., 1970), the length of the outer chain should be one of the reasons why the enzyme has only a low affinity for glycogen. On the other hand, a longer linear chain portion of branched polysaccharides can easily form a helix; one turn is composed of six to seven glucosyl units (Hollő & Szeitli, 1968). As cyclodextrin is analogous to the helical structure of noncyclic dextrin, potato phosphorylase may discriminate the helical structure from the linear structure. On the contrary, rabbit muscle phosphorylase is less inhibited by cyclodextrin in both the kinetics of glycogen and maltodextrin (Smith, 1971), indicating that the muscle enzyme has no such region discriminating the helical structure at both glycogen storage and active sites as in the potato enzyme.

Nakano et al. (1980) have determined the partial amino acid sequence of potato phosphorylase which can be assumed to correspond to residues 275–289 of the complete sequence of rabbit muscle phosphorylase (Titani et al., 1977). In this sequence, there is no homology in a segment of residues 282–286, which forms just a part of the gate to a cavity of the active site of rabbit muscle phosphorylase (Fletterick et al., 1979). The existence of such a difference in the primary structures may be expected by our present finding that the active site of potato phosphorylase shows a high affinity for α-glucan, while that of rabbit muscle phosphorylase shows little affinity.

A single molecule of potato phosphorylase has two cofactors and is composed of two subunits (Lee, 1960; Iwata & Fukui, 1973), suggesting two active sites per molecule. It therefore will be expected from a model of Hu & Gold (1975) that the increase in multiplicity of the outer chain of a substrate molecule strenghthens the affinity of the enzyme composed of two active sites. However, the experimental results were contrary to the expectation (Lee et al., 1970a). This may reflect an inaccessibility of the second chain terminus from the same substrate to the remaining active site after one of the two active sites has been occupied by the first chain terminus, unless potato phosphorylase has a single active site. For rabbit muscle phosphorylase, two glycogen storage sites and two active sites are located on the same face of its molecule,

indicating that the plural chain termini from the same substrate are able to bind simultaneously (Fletterick et al., 1979). We therefore propose a model to explain the inaccessibility of potato phosphorylase to the second chain terminus in which the two active sites of potato phosphorylase are located on the reverse side from each other. This assumption of the difference in topology of the two active sites in one enzyme molecule between potato and rabbit muscle phosphorylases is not contradictory to our earlier observations which have suggested the difference in structures of the subunit–subunit contact regions between the two phosphorylases; potato phosphorylase shows a secondary structure considerably different from that of rabbit muscle phosphorylase (Shimomura et al., 1980) and cannot be dissociated into monomer by the same procedures as those used for rabbit muscle phosphorylase (Kamogawa et al., 1971; Iwata & Fukui, 1973). The different structures of the contact regions would have been further evidenced by the fact (Lee, 1960) that potato phosphorylase has neither an allosteric nor a covalent regulatory site such as rabbit muscle phosphorylase does have, both sites being located in this contact region (Fletterick et al., 1976).

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