Sensitive, nonradioactive assay of phosphorylase kinase through measurement of enhanced phosphorylase activity towards fluorogenic dextrin

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Glycogen phosphorylase (GP) exists in two interconvertible forms, GP_a (phosphorylated form, high activity) and GP_b (nonphosphorylated form, low activity). Phosphorylase kinase (PhK) catalyses the phosphorylation of GP_b and plays a key role in the cascade system for regulating glycogen metabolism. In this study, we developed a highly sensitive and nonradioactive assay for PhK activity by measuring the enhanced GP activity towards a pyridylaminated maltohexaose. The enhanced GP activity (ΔA) was calculated by the following formula: $\Delta A = A^+ - A^0$, where A^+ and A^0 represent the GP activities of the PhK-treated and PhK-nontreated samples, respectively. Using a high-performance liquid chromatograph equipped with a fluorescence spectrophotometer, the product of GP activity could be isolated and quantified at 10 fmol. This method does not require the use of any radioactive compounds and only 1 μ g of GP_b per sample was needed to obtain A^+ and A^0 values. The remarkable reduction in GP_b concentration enabled us to discuss an interesting new role for glycogen in PhK activity.

Keywords: fluorogenic dextrin/glycogen/glycogen phosphorylase/nonradioactive assay/phosphorylase kinase.

Abbreviations: CD, cyclodextrin; Glc, D-glucose; Glc-1-P, α -D-glucose 1-phosphate; GlcPA, 1-deoxy-1-[(2-pyridyl)amino]-D-glucitol; GP, glycogen phosphorylase; MW, molecular weight; PA, pyridylamino; PhK, phosphorylase kinase; P_i, inorganic phosphate.

Glycogen is a multibranched polysaccharide of D-glucose (Glc) that serves as a form of energy storage in animals (1–3). When the body requires energy, glycogen is rapidly broken down by the combined activity of glycogen phosphorylase (GP, EC 2.4.1.1, MW 1.9×10^5) and glycogen debranching enzyme (EC 2.4.1.25 and EC 3.2.1.33, MW 1.6×10^5) (4–9).

GP catalyses the rate-limiting step of glycogen degradation, in which α -1,4-Glc residues are converted to α -D-glucose 1-phosphate (Glc-1-P) by phosphorolysis (3, 6). The action of GP is essentially reversible, although GP activity in the direction of glycogen synthesis is inhibited in the inorganic phosphate (P_i)-rich environment observed in the body (10).

GP is biologically active as a dimer of identical subunits and exists in two interconvertible forms, GP_a (phosphorylated form, high activity) and GP_b (nonphosphorylated form, low activity) (11). Conversion of GP_b to GP_a is catalysed by a serine/threonine-specific protein kinase, known as phosphorylase kinase (PhK, EC 2.7.11.19, MW 1.3×10^6) (12–14). PhK is a hexadecameric complex comprising four copies of four distinct subunits, α , β , γ and δ (13, 15, 16). Only the γ -subunit is known to possess catalytic activity, whereas the others perform regulatory functions. In the presence of Mg²⁺ and Ca²⁺, the catalytic γ -subunit transfers a phosphate group from ATP to Ser¹⁴ of each GP_b subunit, converting GP_b to GP_a (14, 16).

Therefore, it is believed that PhK plays a key role in the cascade system for regulating glycogen metabolism. However, evaluation of PhK activity is not straightforward.

Edwin G. Krebs and Edmond H. Fischer were awarded the Nobel Prize in Physiology or Medicine in 1992 for their seminal discovery of reversible protein phosphorylation and its importance as a biological regulatory mechanism. In their research, PhK activity was frequently evaluated by measuring the enhanced GP activity (ΔA) for a certain period of time during the enzymatic reaction (12, 17, 18). ΔA was calculated by the formula:

$$\Delta A = A^+ - A^0$$

where A^+ represents GP activity in the PhK-treated sample and A^0 represents that in the PhK-nontreated sample. Before measuring A^+ and A^0 , the conversion of GP_b to GP_a using PhK should be discontinued. Therefore, an excess amount of EDTA was added to the assay solution and PhK was inactivated. Following inactivation, A^+ and A^0 were measured in the direction of glycogen synthesis using the Fiske–Subbarow method (19, 20), where P_i liberated from Glc-1-P was

converted to a molybdenum blue complex formed upon reduction of an ammonium molybdate- P_i complex in acidic solution. Under normal circumstances, the resulting blue chromophore could be quantified at 10 pmol using a spectrophotometer (21); however, under the conditions of Fiske–Subbarow P_i quantification, more than 4% of ATP in the assay solution was hydrolysed to P_i due to catalytic action of the acid and molybdate (22). As 1–3 mM ATP was needed to perform a sensitive PhK assay (12), nanomolar amounts of ‘byproduct P_i ’ were created and converted to the blue chromophore. Therefore, for this traditional PhK assay, the Fiske–Subbarow P_i quantification was lowered to 1/100 of the normal sensitivity, with a large amount of GPb (*e.g.* 6 mg/sample (12), 750 µg/sample (23), 30–300 µg/sample (24)) required to carry out the analysis.

For the alternative method, GPa formation from GPb was monitored using radioactive [γ - 32 P]ATP as a substrate (25, 26). The amount of 32 P incorporated in the GP protein could be measured by autoradiography or scintillation counting. Although this method using a radioactive substrate was sensitive, special facilities and technical skills were required for using radioactive materials (27). As such, wide and routine use of this method for measuring PhK activity was difficult. In addition, it should be noted that 32 P decays to 32 S with a short half-life of 14.3 days (decay rate of approximately 5% per day) (28, 29), with the high-energy β -rays emitted from 32 P causing significant radiolytic decomposition of surrounding compounds (30–32). Thus, commercially available [γ - 32 P]ATP is presumably contaminated with significant amounts of nucleotide derivatives from the β -decay and radiolytic decomposition. As PhK is known to have several types of nucleotide-binding sites (33–35), PhK activity evaluation may be complicated by these nucleotide derivative contaminants.

In this study, we intended to develop a sensitive and nonradioactive method for assaying PhK activity by evaluating ΔA through the use of fluorescence techniques. A^+ and A^0 could be assessed using our previous method of analysing the chain-lengthening action of GP on pyridylaminated (PA-) maltohexaose (Glc α 1-4Glc α 1-4Glc α 1-4Glc α 1-4GlcPA, where GlcPA = 1-deoxy-1-[(2-pyridyl)amino]-D-glucitol residue) in a Glc-1-P-rich medium (36). Using a high-performance liquid chromatograph equipped with a fluorescence spectrophotometer, the resulting PA-maltoheptaose (Glc $_6$ -GlcPA) was isolated and quantified at 10 fmol. ATP and EDTA did not affect the HPLC analysis of PA-maltoheptaose. This sensitive method for analysing GP activity using a fluorescent indicator was successfully applied to the PhK assay.

Materials and Methods

Materials

Maltohexaose, γ -cyclodextrin (γ -CD), 2-aminopyridine, oyster glycogen, gelatin and Wakosil-II 5C18 HG column (10.0 × 250 mm) were purchased from Wako Pure Chemicals (Osaka, Japan). AMP, ATP, Glc-1-P and rabbit muscle enzymes

(GPa, GPb and PhK) were purchased from Sigma (St. Louis, MO, USA).

Preparation of dextrans

Glc α 1-4Glc α 1-4Glc α 1-4Glc α 1-4Glc α 1-4GlcPA (PA-maltohexaose) was prepared as previously reported (36).

γ -CD was recrystallized twice to remove maltooligosaccharide contaminants.

Oyster glycogen was dialysed against water to remove short-chain glycogen and nucleotides. The external water was replaced every 12 h. After 36-h dialysis, the remaining glycogen was lyophilized.

Standard conditions for the assay of PhK activity

A mixture (60 µl) containing 50 mM Tris–HCl buffer (pH 8.2), 3.0 mM ATP, 10 mM MgCl $_2$, 0.3 mM CaCl $_2$, 40 mM sodium fluoride, 3.0 mM β -mercaptoethanol, 0.05% gelatin, 1.0 µg of rabbit muscle GPb and 90 µU of rabbit muscle PhK was incubated at 37°C for 15 min. To stop the kinase reaction, 400 mM EDTA solution (10 µl, pH 6.1) was added. A mixture (5 µl) containing 320 mM Glc-1-P (pH 6.1) and 16 µM AMP was then added. Subsequently, to measure GP (GPa and GPb) activity in the direction of glycogen synthesis, 500 µM PA-maltohexaose solution (5 µl) was added. The mixture (pH 6.8) was incubated at 37°C for 40 min. After incubation, the mixture was heated at 100°C for 5 min to inactivate GPa and GPb. The chain-lengthened product (PA-maltoheptaose, Glc $_6$ -GlcPA) was then isolated and quantified by reversed-phase HPLC on a Wakosil-II 5C18 HG column as previously reported (36). The GPa formation was evaluated by the enhanced GP activity (ΔA). One unit of GPa was defined as the amount of enzyme that produces 1 nmol of PA-maltoheptaose per minute under the employed conditions. One unit of PhK was defined as the amount of enzyme that produces 1 unit of GPa per minute under the employed conditions. Although the GPb concentration in this study was low, it was considered to be constant, as only a very small amount of GPb (less than 5%) was consumed during the kinase reaction.

Results and Discussion

Effects of ATP and EDTA on the activity measurement of rabbit muscle GPa

Muscle GPb is dynamically and allosterically activated by AMP and inhibited by the addition of ATP (37, 38). In contrast, AMP-induced activation of muscle GPa is smaller than that of GPb (39, 40), and muscle GPa is not affected by ATP (37, 38). Note that muscle GPa has a very strong affinity for AMP (K_d approximately 0.3 µM) compared with muscle GPb (K_d approximately 100 µM) (41). Commercially available ATP is reported to be contaminated with trace amounts of AMP (approximately 0.03%) (42), which is difficult to completely remove. Therefore, muscle GPa activity at millimolar concentrations of ATP should be measured carefully. To circumvent this difficulty, Childress and Sacktor proposed, in the case of insect muscle GP, that at millimolar concentrations of ATP, GPa should be assayed with a preliminary addition of AMP (10 µM), ensuring full activation of GPa but not of GPb (42). Here, we investigated whether the AMP contaminant influenced the activity of rabbit muscle GPa. In Fig. 1, the dotted line shows that rabbit muscle GPa was significantly activated at millimolar concentrations of commercially available ATP. As in the case of insect muscle GPa (42), rabbit muscle GPa exhibited high sensitivity towards AMP, whereas rabbit muscle GPb needed a nearly 300 times higher concentration of AMP to bring about similar acceleration (Fig. 2). When 1 µM AMP was added to the assay solution first, the rate of the GPa-catalysed reaction increased approximately 3-fold and the effect of

Fig. 1 Effect of commercially available ATP on rabbit muscle GPa activity. GPa activity in the direction of glycogen synthesis was assayed at various concentrations of commercially available ATP. The dotted and solid lines indicate the absence and presence of a preliminary addition of AMP (1 μ M), respectively.

the AMP contaminant was negligible (Fig. 1). In contrast, under the same conditions, the increase in rabbit muscle GPb activity was very small (Fig. 2). These results indicate that the strategy of Childress and Sacktor is also applicable to the rabbit muscle GPa assay at millimolar concentrations of ATP (42).

Next, to ensure that EDTA did not affect the actions of the GPa catalytic sites, rabbit muscle GPa was assayed with 0–75 mM EDTA (Fig. 3). The results showed that EDTA at concentrations less than 75 mM did not significantly affect the actions of the GPa catalytic sites.

Effects of ATP and EDTA on the activity of rabbit muscle PhK

As ATP is a substrate for the PhK-catalysed phosphorylation reaction, it is presumed that higher concentrations of ATP enhance the rate of this reaction. However, Krebs *et al.* (12) reported that PhK showed substrate inhibition at high ATP concentration. To determine the optimum ATP concentration for PhK assay, rabbit muscle PhK activity was assayed at various concentrations of ATP (Fig. 4). The maximum rate was observed when ATP concentration was 3 mM, which is consistent with the results shown by Krebs *et al.* (12).

To stop PhK activity and evaluate A^+ or A^0 , EDTA was added to the solution ([EDTA] = 50 mM), which corresponded to 6.5 equivalents of Mg^{2+} and Ca^{2+} involved in the assay solution. EDTA formed stable complexes with Mg^{2+} and Ca^{2+} and, consequently, PhK activity was completely lost (data not shown).

Method for the sensitive and nonradioactive assay of PhK activity

Our PhK assay comprised two steps: (i) conversion of GPb to GPa and (ii) measurement of the enhanced GP activity. Step (i) was carried out in a mixture (60 μ l) containing 50 mM Tris–HCl buffer (pH 8.2), 3.0 mM ATP, 10 mM $MgCl_2$, 0.3 mM $CaCl_2$, 40 mM sodium

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Fig. 2 Effects of AMP on the activities of GPa and GPb from rabbit muscle. GP activity was assayed in the direction of glycogen synthesis: (●) GPa; (○) GPb.

Fig. 3 Effect of EDTA on the fluorescence assay of GPa activity, performed at various concentrations of EDTA. The rate of formation of PA-maltoheptaose at 0 mM EDTA was taken as unity.

fluoride, 3.0 mM β -mercaptoethanol, 0.05% gelatin, 1.0 μ g of GPb and 90 μ U of PhK. After 15 min, 400 mM EDTA (10 μ l, pH 6.1) was added. The ability of PhK to convert GPb to GPa was completely lost, whereas the GP function was not significantly affected. Step (ii) was then carried out by adding a mixture (5 μ l) containing 320 mM Glc-1-P (pH 6.1) and 16 μ M AMP, followed by a solution (5 μ l) of 500 μ M PA-maltohexaose. The chain-lengthened product (PA-maltoheptaose, Glc₆-GlcPA) was isolated and quantified by reversed-phase HPLC (36). It was possible to detect the chain-lengthened product with a fluorescent indicator as low as 10 fmol (36).

The time course of the action of PhK on GPb is shown in Fig. 5A. This demonstrates that the amount of GPa increases linearly with time. The relationship between PhK concentration and the resulting GPa is also linear under the conditions used (Fig. 5B).

Fig. 4 Relationship between ATP concentration and the amount of GPa formed by the catalytic action of PhK, examined at various concentrations of ATP. The rate of GPa formation at 3 mM ATP was taken as unity.

Fig. 5 GPa formation by PhK. (A) Time course of GPa formation. (B) Relationship between PhK concentration and amount of GPa formed. Each value shows the mean $\pm$ SD ($n = 3$).

In the traditional PhK assay, the enhanced GP activity was evaluated in the direction of glycogen synthesis using the Fiske–Subbarow method (19, 20), where P_i liberated from Glc-1-P was converted to a molybdenum blue complex formed upon reduction of

an ammonium molybdate– P_i complex in acidic solution. In the presence of 3 mM ATP, the sensitivity of P_i quantification by the Fiske–Subbarow method was lowered to 1/100 of the normal value, as more than 4% of the ATP was hydrolysed to P_i by the catalytic actions of the acid and molybdate, with the resulting ‘byproduct P_i ’ being converted to the blue chromophore (22). However, in our method, ATP did not affect the HPLC analysis of PA-maltoheptaose. In addition, this method requires only a small amount of GPb (1 μ g/sample), which is much less than that required for the traditional PhK assays (*e.g.* 6 mg/sample (12), 750 μ g/sample (23), 30–300 μ g/sample (24)).

Glycogen has been classified as an activator of PhK (Table I); thus, in some cases, it is added to the PhK assay solution to enhance the sensitivity (24). However, in our method, a highly sensitive assay was achieved without the addition of glycogen.

Application of the present method for analysis of the stimulatory effect of glycogen on PhK activity

Previously, Krebs and Fischer (12) reported that glycogen exhibited a marked stimulatory effect when added to the PhK assay solution. The main cause of this phenomenon has been considered to be the conformational changes caused by the interaction of glycogen with PhK, glycogen with GPb, or both (43). To gain information on this phenomenon, Tabatabai and Graves (43) analysed the effects of glycogen on PhK activity using the synthetic tetradecapeptide (corresponding to Ser⁵–Leu¹⁸ of the rabbit muscle GPb protein) instead of the native GPb protein. It should be noted that the short-chain synthetic peptide does not have a glycogen-binding site and that interaction of the synthetic peptide with glycogen is not present in this assay. As significant acceleration of the peptide phosphorylation was not observed in the presence of glycogen, it was hypothesized that GPb might be stimulated by glycogen. Conversely, at a later date, the same research group reported that glycogen exhibited a direct effect on PhK activity and that the PhK α -subunit might play an important role in the recognition of glycogen (14). This was because in the latter study, glycogen stimulated the activity of the synthetic $\alpha\gamma\delta$ -complex towards GPb, but not in the case of the synthetic $\gamma\delta$ -complex. Subsequently, in 1997, Shmelev and Serebrennikova (44) proposed a new theory in which a conformational change of GPb occurred upon binding with glycogen that induced a conformational change of PhK. However, in 2003, crystallographic binding studies indicated that the conformational change of GPb upon binding of maltooligosaccharides or cyclodextrins (CDs) was very small (45).

To investigate whether the small structural change in GPb caused by dextrin binding influenced PhK activity, the effects of maltohexaose and γ -CD on PhK activity were investigated using our newly developed method. CD has been used to investigate the functions of glycogen binding sites of glycogen-metabolizing enzymes, as it has a structure that resembles the helical structure of the maltooligosyl- (Glc_{*n*}-) branches of

Table I. Stimulatory effects of glycogen on PhK reported previously

	Step (i) ^a			Step (ii)
	GPb concentration (mg/ml)	Glycogen concentration inducing half-maximal PhK activation (%)	Maximal PhK activation (fold)	P _i quantification method used
Ref. 12	10	0.30	2.4	Fiske–Subbarow method
Ref. 14	6.42	0.35	1.8	[γ - ³² P]ATP method
Ref. 24	0.65	—	2.5 ^b	Fiske–Subbarow method
	6.5	—	1.5 ^b	Fiske–Subbarow method

^aStep (i) was performed at pH 8.2.^bThe value was obtained at a glycogen concentration of 3%.

Table II. Effects of dextrans on the activity of PhK

Entry	Additive in step (i)	Additive in step (ii)	PA-maltoheptaose produced by GP _a (pmol) ^a	Enhanced PhK activity in step (i) (fold)
1a	None	0.075% Oyster glycogen	40 ± 2 ^b	1.33
1b	0.10% Oyster glycogen	0.075% Oyster glycogen	53 ± 3 ^b	
2a	None	0.075% Maltotetraose	45 ± 2	0.98
2b	0.10% Maltotetraose	0.075% Maltotetraose	44 ± 2	
3a	None	0.075% γ -CD	48 ± 2	1.00
3b	0.10% γ -CD	0.075% γ -CD	48 ± 1	
4a	None	15 mM γ -CD	46 ± 2	0.98
4b	20 mM γ -CD	15 mM γ -CD	45 ± 2	
5a	None	0.075% Oyster glycogen + 15 mM γ -CD	44 ± 2	1.05
5b	0.10% Oyster glycogen + 20 mM γ -CD	0.075% Oyster glycogen + 15 mM γ -CD	46 ± 3	

^aEach value shows the mean ± SD ($n = 4$).^bValues were significantly different at the $P < 0.05$ level.

glycogen. These results are summarized in Table II. As previously reported by Krebs *et al.* (12), acceleration of the kinase reaction was observed in the presence of 0.1% oyster glycogen (Table II, entry 1); however, no significant effect was observed when maltotetraose or γ -CD was introduced to the PhK assay solution (Table II, entries 2–4). Next, we investigated the effect of the mixture containing 0.1% oyster glycogen and 20 mM γ -CD on PhK activity (Table II, entry 5). To our surprise, no significant acceleration of the kinase reaction was observed, even though 0.1% glycogen was involved in the reaction mixture. These results indicate that the conformational changes of GP_b and/or PhK speculated previously might not be the primary cause of the glycogen-induced acceleration of the kinase reaction, *i.e.* glycogen may not be a true activator of PhK.

Recently, we introduced the notion of ‘advantage on the glycogen surface’ to the field of GP_b (46). GP_b is biologically active as a dimer of identical subunits, with each subunit having two distinct maltooligosaccharide binding sites: the storage site and the catalytic site (45, 47). The storage site has approximately 20 times higher affinity for maltooligosaccharide (K_d approximately 1 mM) than the catalytic site (47). Due to the very large size and highly branched structure of the glycogen molecule, distribution of nonreducing end Glc residues is not uniform; rather, there is a high density region of nonreducing end Glc residues localized on the surface (1–3). Therefore, by binding to the

glycogen surface at the storage sites, GP_b is most likely surrounded by nonreducing end Glc residues (46). The term ‘advantage’ infers that in the glycogen solution, glycogen-bound GP_b produces Glc-1-P more efficiently than free GP_b. Similar to the case of GP_b, PhK in the glycogen solution may also obtain ‘advantage on the glycogen surface (more precisely, advantage on the surface of the GP_b–glycogen complex)’, as many of the GP_b molecules are most likely on the glycogen surface. A comparison of Fig. 6A and B may help to understand how PhK obtains ‘advantage’ in the glycogen solution. Under conditions of high ionic strength and high ATP concentration in the body, direct binding of PhK to glycogen was reported to be very weak (44). However, GP_b molecules located on the glycogen surface might serve as good connecting molecules between PhK and glycogen and, consequently, promote indirect binding of PhK to the glycogen surface (44, 48). Similar to our hypothesis, Vénien-Bryan *et al.* (16) reported that GP_b recognition by PhK can involve not only the local epitope around GP_b Ser¹⁴ but also other regions of GP_b by other sites on the catalytic γ -subunit as well as other possible subunits, such as the α -subunit. By binding to the glycogen surface directly or through GP_b, PhK is localized in close proximity to GP_b molecules. The term ‘advantage’ for PhK infers that the ternary complex (*i.e.* the PhK–GP_b–glycogen complex) produces active GP_a very efficiently compared with PhK either alone or in the binary complex (*i.e.* the PhK–GP_b complex). As we recently reported, γ -CD acts as an

Fig. 6 Advantage of forming the ternary complex in the kinase reaction. In the glycogen solution, GPb binds to the glycogen surface through the two storage sites. It might be beneficial for PhK to form the ternary complex (PhK–GPb_n–glycogen complex), as PhK in the ternary complex is expected to produce GPa very efficiently, compared with PhK either alone or in the binary complex (PhK–GPb complex). Schematic representation of (A) PhK assay solution without glycogen, (B) PhK assay solution with a low concentration of glycogen, (C) PhK assay solution with glycogen and an excess amount of γ -CD and (D) PhK assay solution with a high concentration of glycogen. Dashed circle represents an imaginary sphere in which PhK is expected to easily capture and phosphorylate GPb. The addition of reasonable amount of glycogen accelerates PhK activity by the ‘advantage on the glycogen surface’ (panel B), whereas the addition of γ -CD (panel C) or large amount of glycogen (panel D) reduces the acceleration by separating PhK from GPb.

inhibitor of the GPb storage site, with an apparent K_d value for GPb– γ -CD binding of 2.2 mM (46). Therefore, in the 20 mM γ -CD solution, most of the GPb storage sites should be saturated with γ -CD, preventing GPb from forming the GPb_n–glycogen complex. In this case, distribution of the GPb– γ -CD₂ complex is nearly uniform; therefore, it is reasonable to believe that PhK cannot obtain ‘advantage on the glycogen surface’ in the presence of excess amounts of γ -CD (Table II, entry 5; compare Fig. 6B and C). In addition, it should be noted that the previous findings by Graves and coworkers (14, 43) can also be explained by our notion of ‘advantage on the glycogen surface’, though they assumed conformational changes of PhK and/or GPb by glycogen. The PhK α -subunit might have GPb-binding site and/or glycogen-binding site, as previously discussed in the cryo-electron microscopic studies (16).

The half-maximal value for oyster glycogen-induced PhK stimulation was reported to be approximately 0.30% (Table I); however, this value was evaluated without considering the glycogen to GPb concentration ratio. For example, Krebs *et al.* (12) and Chan and Graves (14) used GPb concentrations of 10 mg/ml and 6.42 mg/ml, respectively. Due to steric hindrance caused by the high density of maltooligosyl-

(Glc_n-) branches, oyster glycogen molecules, which have a spherical shape with 6–8 concentric tiers, cannot bind GPb molecules on their inner tiers (49–51). It is unlikely that 3 mg of oyster glycogen has enough surface area to bind as much as 6.42–10 mg of GPb. Therefore, the effect of glycogen on PhK activity must be analysed at GPb concentrations much lower than the glycogen concentration. Thus, it cannot be concluded from Table I that PhK is stimulated by the presence of glycogen. Using our newly developed method, the GPb concentration in step (i) could be decreased to 17 μ g/ml (0.0017%). Even in 0.05% glycogen solution, the concentration of GPb was much lower than that of glycogen and, consequently, there was enough glycogen surface area to bind all 0.0017% GPb. Based on these discussions, the effects of glycogen concentration on PhK activity should be reevaluated using our method (Table III). In contrast to the reports by Krebs and Chan (12, 14), acceleration of the kinase reaction was not significant under high concentrations of glycogen. This indicated that when the concentration of glycogen was too high, the glycogen in the solution existed largely as the GPb free molecule or with one GPb attached to the surface. The GPb density on the surface of the glycogen

Table III. Effect of glycogen concentration on the PhK activity

Glycogen concentration in step (i) (%)	PA-maltoheptaose produced by GP _a (pmol) ^{a,b,c}	Enhanced PhK activity in step (i) (fold) ^d
0.00	40 ± 2 ^c	
0.05	48 ± 2	1.20
0.10	53 ± 3 ^{c,f}	1.33
0.30	49 ± 2	1.23
1.00	45 ± 2 ^f	1.13

^aStep (ii) was performed at a glycogen concentration of 0.075%.

^bEach value shows the mean ± SD (*n* = 4).

^cValues followed by the same letter are significantly different (*P* < 0.05).

^dPhK activity without glycogen was taken as unity.

molecule decreases with increasing glycogen concentration, resulting in the disappearance of 'advantage on the glycogen surface' for PhK (compare Fig. 6B and D).

Recently, nonradioactive methods for assays of various kinds of protein kinases are gaining interest with organic chemists. In particular, various fluorescence techniques have emerged as alternatives to radioactive methods (Supplementary Discussion). In the present study, we have developed a highly sensitive and nonradioactive assay method for PhK activity by analysing the enhanced GP activity towards a fluorogenic dextrin. This method does not require the use of any radioactive compounds. As well, only 1 µg of GP_b per sample is required as the substrate. The remarkable reduction of GP_b concentration enabled us to discuss a new role for glycogen in PhK activity. We believe that the glycogen molecule provides a surface where PhK and GP_b can come in close proximity, resulting in acceleration of the phosphorylation rate.

Supplementary Data

Supplementary Data are available at *JB* Online.

Conflict of Interest

None declared.

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