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CHARACTERIZATION OF *CHLORELLA* PHOSPHORYLASE: THE GLUCAN SPECIFICITY AND EFFECT OF TEMPERATURE

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Key Word Index—*Chlorella vulgaris*; Chlorophyceae; phosphorylase; photosynthesis; starch; temperature.

Abstract—The glucan specificity of α-glucan phosphorylase partially purified from *Chlorella vulgaris* was characterized in both directions of glucan phosphorolysis and synthesis. The activity exhibited a pH optimum of 7.0–7.5 for phosphorolysis and 5.1–6.8 for synthesis. The K_m for amylopectin was 0.69 and 0.68 mg/ml for phosphorolysis at pH 7.1 and for synthesis at pH 6.2, respectively. The minimum chain lengths required for efficient activities of glucan phosphorolysis and synthesis were five and four glucose units per maltodextrin molecule, respectively. At saturating substrate concentrations, the apparent K_m for maltodextrins (G5–G8, G17) was higher in the direction of synthesis than in the direction of phosphorolysis, while the apparent V_{max} was higher in the former than in the latter. The Arrhenius plot of phosphorylase activity at pH 6.8 with amylopectin, G5 or G17 showed a break in the slope around 22–24° in both directions. However, the decrease in the slope or activation energy at higher temperature was more significant in the synthesis than in the phosphorolysis especially when amylopectin was used as a glucan primer. Therefore, the ratio of the phosphorolysis activity to the synthesis activity was greatly increased at higher temperatures.

INTRODUCTION

There have been a number of studies suggesting that α-glucan phosphorylase is involved in the degradation of starch produced during photosynthesis in chloroplasts [1–9]. Recently, information on the kinetic properties of starch phosphorylase from a variety of photosynthetic tissues has been published [10–13]. Our previous report [13] has shown that *Chlorella vulgaris* phosphorylase, in contrast to the enzyme from higher plant leaves, exhibits regulatory properties in response to low concentrations of physiologically important metabolites, e.g. ADP, ATP, ADP-glucose and UDP-glucose.

Nakamura and Miyachi [9] suggested that starch phosphorylase plays a principal role in the degradation of starch, which is greatly stimulated at temperatures above 30° in *C. vulgaris* cells. Furthermore, analytical studies by means of gel filtration chromatography and radio-GLC revealed that the temperature-dependent degradation of the total starch fraction is due to the degradation of amylopectin-like polysaccharides (L-starch) [14, 15]. It is assumed that α-glucan phosphorylase, but not α-amylase, is involved in the degradation of L-starch at high temperatures [14]. Therefore, studies on the glucan specificity of glucan phosphorylase are needed for a better understanding of the regulation of starch metabolism in *Chlorella*.

The present paper reports the glucan specificity of a parially purified phosphorylase from *C. vulgaris*. The paper also deals with the effect of temperature on the enzymic activities in both directions when various glucans are used as primer.

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RESULTS AND DISCUSSION

*p*H *Optimum*

Figure 1 shows the pH optimum for activity of a partially purified *Chlorella* phosphorylase in the direction of α-glucan phosphorolysis at a saturating phosphate concentration (7.2 mM) [13]. The optimum was around

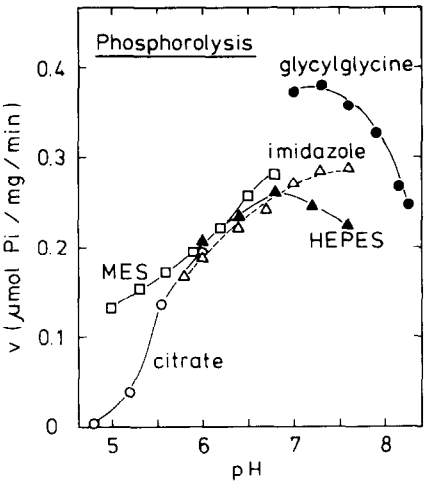

Fig. 1. Effect of pH on the activities of *Chlorella* phosphorylase in the direction of α-glucan phosphorolysis. The assay conditions were the same as described in the Experimental, except that a series of 50 mM citrate, MES, imidazole–HCl, HEPES, or glycine buffer was used as indicated in the figure. The activity (v) is expressed as μmol Pi incorporated into G1P/mg protein per min.

pH 6.8–7.3, but the enzyme exhibited a broad optimum between pH 6.5 and 7.5 which was dependent on the buffer used. The highest activity was observed in glycylglycine buffer at pH values of 7.0–7.5.

The activity of α-glucan synthesis at a saturating glucose 1-phosphate concentration (2.5 mM) [13] had a pH optimum between pH 6.0 and 6.8 (Fig. 2). However, MES buffer gave virtually the same activity at pH values of 5.1–6.5. Lower activity was found in HEPES buffer especially at high pH values above 7.0 as compared to glycylglycine and Tricine buffers.

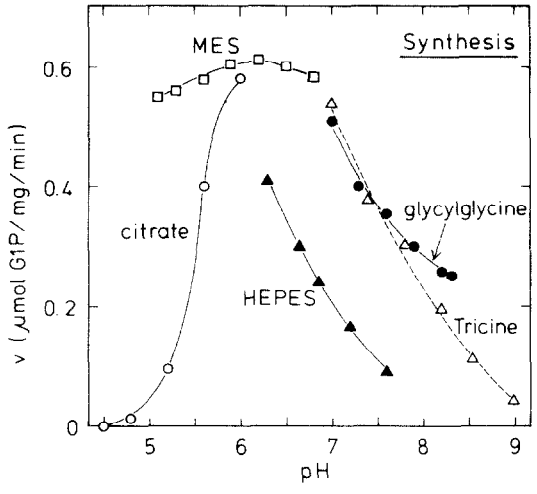

Fig. 2. Effect of pH on the activities of *Chlorella* phosphorylase in the direction of α-glucan synthesis. The assay conditions were the same as described in the Experimental, except that a series of 50 mM citrate, MES, HEPES, glycylglycine, or Tricine buffer was used as indicated in the figure. The activity (v) is expressed as μmol G1P disappeared to form Pi/mg protein per min.

The glucan specificity

Table 1 shows the different activities of *Chlorella* phosphorylase with various glucans as primers in the direction of phosphorolysis or synthesis, in the presence of a saturating amount of phosphate (7.2 mM) or glucose 1-phosphate (2.5 mM), respectively. A considerable activity in either direction was found in the presence of glycogen from oyster. It is interesting that in the presence of glycogen fom rabbit liver the phosphorolysis activity was 60% of that observed in the presence of amylopectin, while the synthesis activity was as low as 5%. The other glycogens from bovine liver and mussel, and pullulan were ineffective glucans in both directions.

Table 2 shows that glucose, maltose, maltotriose and maltotetraose were ineffective primers for *Chlorella* phosphorylase in the phosphorlysis direction. This suggests that the phosphorolysis activity ceased when the maltodextrins were degraded to maltotetraose. A minimum of five glucose units per glucan molecule required for a significant rate of phosphorolysis was also reported with spinach leaf enzyme [11]. The activities with maltopentaose and maltohexaose at 10 mg/ml were higher than that with amylopectin at 10 mg/ml, while those with maltoheptaose and maltooctaose were rather lower (Table 2).

Table 1. The glucan specificity of *Chlorella* phosphorylase

Glucan	Phosphorolysis	Synthesis
Amylopectin (potato)	100	100
Glycogen (oyster)	79	39
Glycogen (rabbit liver)	61	5
Glycogen (bovine liver)	24	5
Glycogen (mussel)	20	0
Pullulan	12	5

The assay conditions were as described in the Experimental. The concentrations of amylopectin, various glycogens and pullulan were 6.4, 12.8 and 12.8 mg/ml, respectively. The rates observed for amylopectin were set at 100, and represent 0.33 μmol Pi incorporated into G1P/mg protein per min for the phosphorolysis and 0.84 μmol G1P disappeared to form Pi/mg protein per min for the synthesis, respectively. The value presented is the mean of duplicate measurements.

Table 2. Phosphorolysis of maltodextrins by *Chlorella* phosphorylase

Glucan	Rate
Amylopectin	100
Glucose	2
Maltose	1
Maltotriose (G3)	1
Maltotetraose (G4)	2
Maltopentaose (G5)	112
Maltohexaose (G6)	125
Maltoheptaose (G7)	35
Maltooctaose (G8)	71

The assay conditions were as described in the Experimental. The concentrations of the glucans were 10 mg/ml. The rate observed for amylopectin (100%) was 0.31 μmol Pi incorporated into G1P/mg protein per min. The value presented is the mean of duplicate measurements.

Steup and Schächtele [11] found that with phosphorylase from spinach chloroplasts maltotetraose was a linear competitive inhibitor with respect to amylose or starch (K_i = 0.1 mM) in the direction of glucan phosphorolysis. However, maltotetraose as well as glucose, maltose or maltotriose at concentrations as high as 2 mM was not inhibitory to the phosphorolysis activity by *Chlorella* phosphorylase even when amylpectin at concentrations as low as 0.4 mg/ml was used as a glucan primer (data not shown).

Table 3 indicates that with glucose, maltose and maltotriose even at high concentrations (25 mg/ml), any significant activity of glucan synthesis from glucose 1-phosphate at a saturating concentration (2.5 mM) was not detectable. This together with the results shown in Table 2

Table 3. Glucan synthesis from maltodextrins by *Chlorella* phosphorylase

Glucan	Rate
Amylopectin	100
Glucose	0
Maltose	0
Maltotriose (G3)	1
Maltotetraose (G4)	132
Maltopentaose (G5)	108
Maltohexaose (G6)	100
Maltoheptaose (G7)	73
Maltooctaose (G8)	69

The assay conditions were as described in the Experimental. The concentrations of the glucans were 25 mg/ml. The rate observed for amylopectin (100%) was 0.91 μmol G1P disappeared to form Pi/mg protein per min. The value presented is the mean of duplicate measurements.

supports the view that *Chlorella* phosphorylase was active to the maltodextrins with a degree of polymerization $\geqslant 4$.

Kinetic properties

Figure 3 shows the Lineweaver–Burk plot for the initial rate of α-glucan phosphorolysis by *Chlorella* phosphorylase at varying concentrations of amylopectin at a series of fixed concentrations of phosphate in glycylglycine buffer at pH 7.1. The apparent K_m for amylopectin was unaffected by the phosphate concentrations and was estimated to be approximately 0.69 ± 0.04 mg/ml. Similarly, a double-reciprocal plot of the initial reaction velocities in the direction of α-glucan synthesis in MES buffer at pH 6.2 against the reciprocal concentrations of amylopectin at a series of fixed concentrations of glucose 1-phosphate also intersected at a point on the horizontal axis, indicating that the apparent K_m for amylopectin was unaffected by glucose 1-phosphate concentrations (data not shown). The K_m for amylopectin was calculated to be *ca* 0.68 ± 0.29 mg/ml. These K_m values for amylopectin are in the same range as those for the enzyme in spinach chloroplasts [16, 17].

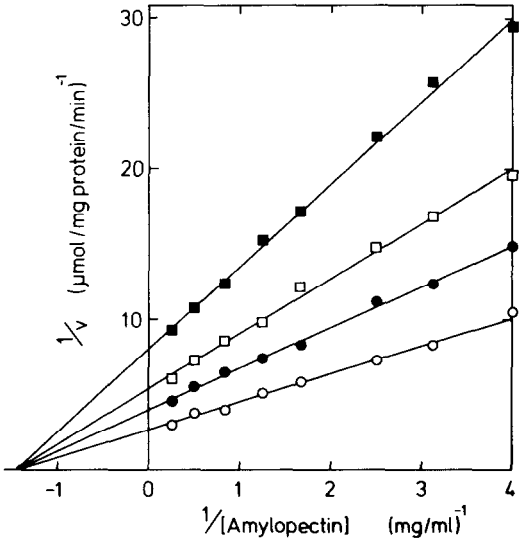

Fig. 3. A double-reciprocal plot of the initial velocity of *Chlorella* phosphorylase against amylopectin at a series of fixed concentrations of Pi. The assay conditions were as described in the Experimental. The Pi concentrations were (mM): 7.2 (○); 2.4 (●); 1.8 (□); 1.2 (■). The activity (v) is expressed as μmol Pi incorporated into G1P/mg protein per min.

By using various concentrations of maltodextrins (degree of polymerization = 4–8 and 17), the initial velocities of *Chlorella* phosphorylase activity in both directions were measured in the presence of saturating concentrations of phosphate (7.2 mM) or glucose 1-phosphate (2.5 mM). Every Lineweaver–Burk plot yielded a straight line (data not shown). Table 4 summarizes the half-saturating glucan concentrations, the apparent K_m values, and the apparent V_{max} values. In the direction of glucan synthesis, the apparent K_m and V_{max} values decreased on the molar concentration basis with the increase of degree of polymerization from 4 to 8. On the other hand, in the direction of glucan degradation such continuity was not observed, in contrast with the report by Steup and Schächtele [11] that both chloroplast and non-chloroplast phosphorylase had lower K_m values for maltodextrins (G5–G11) with increasing degree of polymerization in the direction of phosphorolysis. Especially maltoheptaose (G7) was an ineffective glucan primer for *Chlorella* phosphorylase, since the higher apparent K_m and the lower V_{max} values with G7 were observed compared with those with G6 and G8. The K_m values for maltodecaheptaose (G17) for *Chlorella* enzyme were significantly lower than those for the maltodextrins with the degree of polymerization $\leqslant 8$ in both directions.

The K_m values for maltodextrins used in the direction of phosphorolysis were significantly lower than those in the direction of synthesis, although the K_m values for amylopectin were similar in both directions. On the other hand, the estimated V_{max} values were considerably higher in the direction of synthesis than in the direction of phosphorolysis with all glucans used.

Effect of temperature

The effect of temperature on the *Chlorella* phosphorylase activities was compared in both directions in MES buffer at pH 6.8 in the presence of G5, G17 or amylopectin at a saturating concentration (Figs. 4–6). In the direction of phosphorolysis, the activity with each glucan increased in similar manner with the increase of temperature from 5 to 40° (Fig. 4). When the data were presented as an Arrhenius plot, a break in the slope was observed at *ca* 22° with each glucan(Fig. 4).

By contrast, the temperature-dependent profile in the direction of synthesis was greatly dependent on the glucan used as primer (Fig. 5). The synthesis activity with G5 was very low at temperatures below 10°, but the temperature-dependent increase was steep at higher temperatures. As a consequence, at low temperatures the activity with G5 was far below that with G17, but at high temperatures the former became comparable with the latter. Thus, the

Table 4. Kinetic constants for *Chlorella* phosphorylase

Glucan	Phosphorolysis*		Synthesis*	
	K_m	V_{max}†	K_m	V_{max}‡
Amylopectin	0.69 ± 0.04 mg/ml	0.37 ± 0.08	0.68 ± 0.29 mg/ml	0.93 ± 0.15
Maltotetraose (G4)	—	—	8.3 ± 1.3 mM	1.44 ± 0.24
Maltopentaose (G5)	0.93 ± 0.23 mM	0.38 ± 0.09	3.0 ± 0.5	1.06 ± 0.13
Maltohexaose (G6)	0.68 ± 0.03	0.41 ± 0.08	2.5 ± 0.6	0.81 ± 0.07
Maltoheptaose (G7)	1.22 ± 0.32	0.13 ± 0.01	2.2 ± 0.4	0.68 ± 0.10
Maltooctaose (G8)	0.72 ± 0.18	0.28 ± 0.03	2.0 ± 0.3	0.64 ± 0.07
Maltodecaheptaose (G17)	0.21 ± 0.08	0.57 ± 0.14	0.5 ± 0.1	1.14 ± 0.17

The assay conditions were as described in the Experimental.
*Mean of three replicates ± s.d.
†μmol Pi incorporated into G1P/mg protein per min.
‡μmol G1P disappeared to form Pi/mg protein per min.

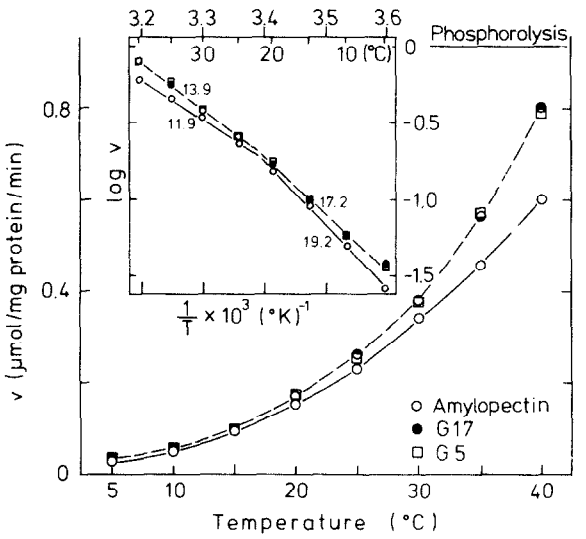

Fig. 4. Effect of temperature on the activities of *Chlorella* phosphorylase in the direction of α-glucan phosphorolysis. The activities (v) expressed as μmol Pi incorporated into G1P/mg protein per min are measured at various temperatures in 50 mM MES buffer at pH 6.8 in the presence of various glucans. The concentration of each glucan was 10 mg/ml. The other conditions were as described in the Experimental. The numbers in the Arrhenius plot are the Arrhenius activation energies (E_a) in kcal/mol (1 kcal is equal to 4.184 × 10^3 J), as calculated from the slopes of the lines.

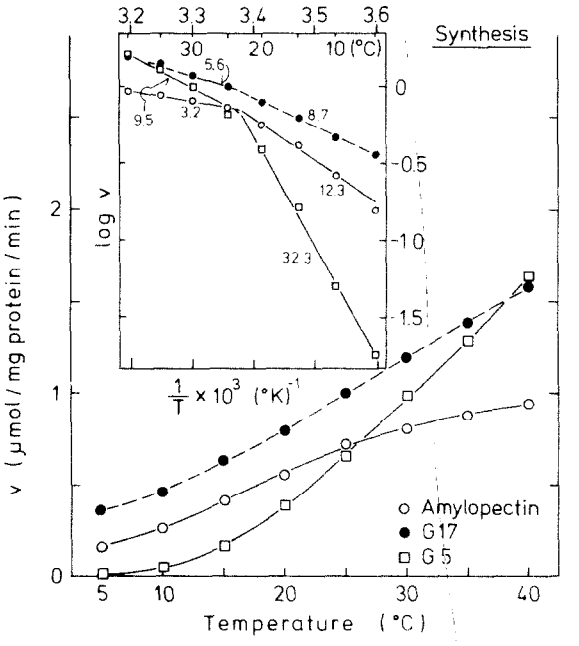

Fig. 5. Effect of temperature on the activities of *Chlorella* phosphorylase in the direction of α-glucan synthesis. The activities (v) are expressed as μmol G1P disappeared to form Pi/mg protein per min. The conditions were the same as in Fig. 4.

Arrhenius activation energy (E_a) for the synthesis activity with G5 was greater than that with G17 or amylopectin especially at low temperatures. On the other hand, it should be noted that the temperature-dependent increase in the synthesis activity with amylopectin slowed down at temperatures higher than 30°. However, it is interesting that the abrupt discontinuity in the slope was also observed in the Arrhenius plot at *ca* 23–24° with either glucan used (Fig. 5). The observed break in the slopes in both directions suggests a conformational change in *Chlorella* phosphorylase around the growth temperature.

The E_a for *Chlorella* phosphorylase activity with amylopectin and G17 was lower in the direction of synthesis than in the direction of phosphorolysis over the whole temperature range tested. The decrease in E_a at high temperatures for the synthesis activity was more pronounced when amylopectin was a glucan primer. Fig. 6 shows that the ratio of the activity of glucan phosphorolysis to that of synthesis increased with increasing temperatures when G17 and amylopectin were used as primers. The increase became significant at high temperatures especially above 30°. This may be related to the previous observations with *Chlorella* cells that degradation of starch was greatly enhanced by high temperatures above 30° due to the degradation of amylopectin-like polysaccharides [14, 15].

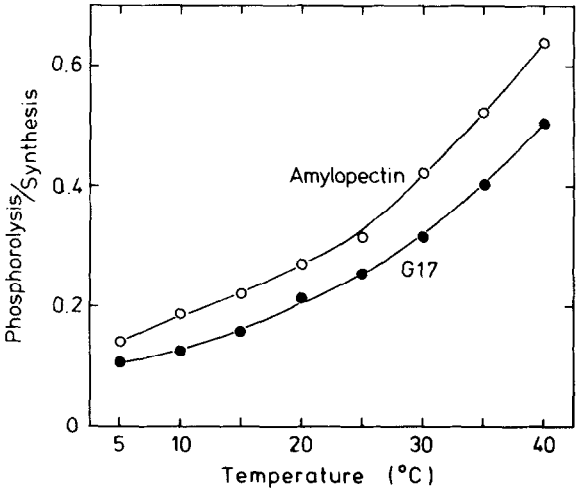

Fig. 6. The ratio of the phosphorolysis activity to the synthesis activity by *Chlorella* phosphorylase at various temperatures. The figure is obtained from the data shown in Figs. 5 and 6.

EXPERIMENTAL

Purification of phosphorylase from Chlorella vulgaris. α-Glucan phosphorylase from *C. vulgaris* 11h was extracted and partially purified as described previously [13]. The partially purified enzyme preparation contained no activities of phosphatase, amylase, starch synthase, branching enzyme and phosphoglucomutase [13].

Assay of phosphorylase. The activity in the direction of glucan phosphorolysis was assayed by incorporation of ^{32}Pi into [^{32}P]G1P, as described previously [13]. The reaction mixture consisted of 50 mM glycylglycine buffer (pH 7.1), 7.2 mM $K_2H^{32}PO_4$, glucan and the partially purified enzyme (4.8 μg protein) in a final vol. of 0.5 ml, unless otherwise described. The reaction was initiated by the addition of the enzyme preparation. The reaction was run for 20 min at 30° and terminated by the addition of 0.3 ml 20% TCA.

The activity in the direction of glucan synthesis was measured by Pi formation from G1P in the presence of glucan by the method of Fiske and Subbarow [18]. The reaction mixture contained 50 mM MES buffer (pH 6.2), 2.5 mM G1P, and the enzyme preparation (4.1 μg protein) in a final vol. of 0.2 ml, unless otherwise described. The reaction was started by the addition of the enzyme preparation, run for 10 min at 30° and stopped by the addition of 0.2 ml 5.0 N H_2SO_4. The experiments presented were generally repeated three times using a single sample of purified enzyme and the data are from single experiments which represent such measurements. The protein content was measured by the method of Lowry *et al.* [19].

Reagents. [U-^{14}C]G1P and [^{32}P]Pi were purchased from New England Nuclear and Japan Atomic Energy Research Institute, respectively. Glucose, maltose, maltotriose, potato amylopectin and the various glycogens listed in Table 1 were obtained from Sigma Chemical Co. Pullulan and maltodecaheptaose (G17) were purchased from Hayashibara Biochemical Laboratories, Inc. (Okayamma, Japan) and the other maltodextrins (G4–8) were the products of Kikkoman Co. (Noda, Japan). All other reagents were commercial samples of reagent grade.

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