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PYRIDOXAL 5'-PHOSPHATE IN α-GLUCAN PHOSPHORYLASE FROM THE POTATO

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SUMMARY

1. Some chemical properties of α-glucan phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) from potato were studied, in particular, pyridoxal 5'-phosphate bound to the enzyme protein.

2. The bound pyridoxal 5'-phosphate in α-glucan phosphorylase from potato changes to a form absorbing at 390 mμ at an alkaline pH. Pyridoxal 5'-phosphate was removed from the enzyme protein by incubating in 4.3 M urea, with a concomitant loss of enzyme activity. The presence of 0.02 M $NaBH_4$ protected the phosphorylase from inactivation by urea, but the bound pyridoxal 5'-phosphate was not attached to the enzyme under these conditions.

3. The binding of pyridoxal 5'-phosphate to the enzyme protein was accomplished by reduction with 0.06 M $NaBH_4$ at pH 6.0 ,or with 0.02 M $NaBH_4$ at alkaline pH.

4. The reduced enzyme protein lost its absorption peak at 330 mμ, and, in contrast to the native enzyme, showed a positive blue color in the dichloroquinone chloroimide test and a positive orange color in the *p*-amino acetophenone test. It was concluded that the 3-hydroxyl group of pyridoxal 5'-phosphate was linked to the native enzyme in some way possibly by a hydrogen bond.

5. In the course of reduction, decrease of absorption at 330 mμ was not found to parallel loss of enzyme activity.

6. On reduction with 0.06 M $NaBH_4$, the addition of L-lysine or Tris protected the bound pyridoxal 5'-phosphate against reductive fixation, and caused a loss of enzymic activity by some unknown mechanism.

INTRODUCTION

α-Glucan phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) from patoto is a protein, with an approximate mol. wt. of 207 000 (ref. 1), similar to that of rabbit muscle phosphorylase *b*. However, it is active in the absence of adenosine 5'-phosphate in contrast to the muscle phosphorylase *b*, and does not show the conversion to phosphorylase *a*, a tetramer.

Attempts have been made to obtain information about the structure and function of the phosphorylase by comparing the properties of potato and rabbit muscle phosphorylases. Previous papers[2,3] have reported similarities in amino acid composition, in higher structure, and in the mode of binding of pyridoxal 5'-phosphate and circular dichroism of both enzymes.

Pyridoxal 5'-phosphate is present in the phosphorylase of all species so far investigated, whether from mammals, bacteria or higher plants *etc.* The exact role of this cofactor, however, remains unexplained in spite of various investigations[4]. The binding of pyridoxal 5'-phosphate in rabbit muscle phosphorylase was reported by KENT *et al.*[5] and FISCHER *et al.*[6] as a substituted aldamine or secondary amine derivative.

LEE[7] has demonstrated that potato phosphorylase contains two moles of firmly bound pyridoxal 5'-phosphate per mole of enzyme protein. However, no further study on the binding of pyridoxal 5'-phosphate in the potato enzyme has been published. The present paper describes experimental results on the reaction of potato phosphorylase with $NaBH_4$, as well as some chemical properties of the enzyme.

MATERIALS AND METHODS

Chemicals and enzymes

α-D-Glucose 1-phosphate (dipotassium salt) and AMP were purchased from Sigma Chemical Co. L-Cysteine·HCl, soluble starch, pyridoxal 5'-phosphate, *p*-aminoacetophenone and urea were purchased from Wako Pure Chemical Industries. $NaBH_4$ was purchased from Metal Hydrides Inc. Diazotized *p*-aminoacetophenone was prepared in this laboratory by a standard method. Crystalline α-glucan phosphorylase from potato was prepared by the method described in a previous paper[2]. Crystalline muscle phosphorylase *b* was isolated by the procedure of FISCHER AND KREBS[8].

Assay of phosphorylase activity

The enzymic activity was assayed by determining the amount of P_i liberated from glucose 1-phosphate in the presence of soluble starch. The reaction mixture contained 5–10 μl of enzyme solution (1 mg/ml), 0.2 ml of citrate buffer (0.5 M, pH 6.0), 0.2 ml of soluble starch solution (5%), 0.1 ml of glucose 1-phosphate (0.1 M) and 0.5 ml of water. The reaction was started by the addition of glucose 1-phosphate. After various time intervals at 30°, the reaction was stopped by adding 0.5 ml of 5% trichloroacetic acid solution. After centrifugation, 0.2 to 0.5 ml of the supernatant solution was taken for analysis of P_i by the method of FISKE AND SUBBAROW[9]. Protein concentration was determined by the method of LOWRY *et al.*[10] using bovine serum albumin as standard.

Test for pyridoxal 5'-phosphate

Qualitative tests for pyridoxal 5'-phosphate bound to phosphorylase were carried out using two methods. (1) 2,6-Dichloroquinone chloroimide test. This test was carried out according to the method by HOCHBERG *et al.*[11], boric acid being omitted from the reaction system. (2) Diazotized *p*-aminoacetophenone test. Spots of 10–20 μl of enzyme solution (about 20 mg/ml) were made on filter paper (Toyo No. 51A) which was quickly soaked in a freshly prepared solution made by adding a few drops of

diazotized *p*-aminoacetophenone solution (0.4 M) to 20 ml of 0.5 M Tris–HCl buffer (pH 7.5). After the paper was dry, phenol compounds gave an orange color.

Spectrophotometric measurements were carried out with a Hitachi Perkin–Elmer 139 UV-VIS spectrophotometer.

RESULTS

At a high enzyme concentration (8 mg/ml), α-glucan phosphorylase from potato showed a small absorption peak at 333 mμ. This peak is due to pyridoxal 5′-phosphate bound to the enzyme protein[7], which is also true of phosphorylase from rabbit muscle.

Absorption spectra of α-glucan phosphorylase from potato at alkaline pH

Fig. 1 shows the absorption spectra of α-glucan phosphorylase at various alkaline pHs. On titration to an alkaline pH, a gradual decrease in absorption at 333 mμ

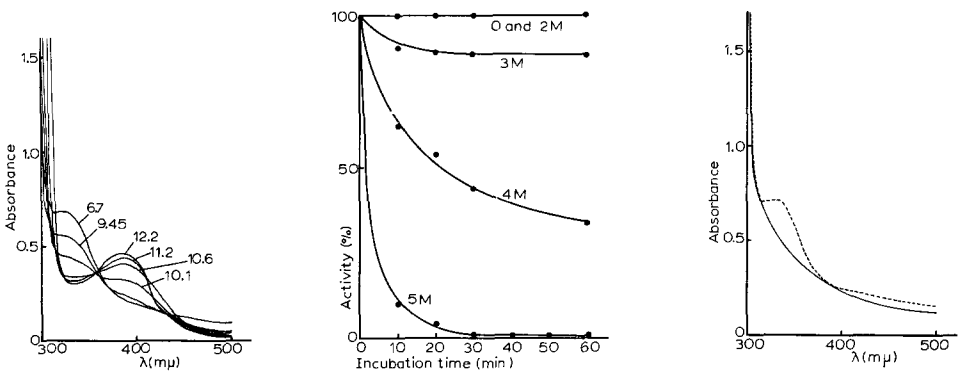


Fig. 1. Absorption spectra of potato α-glucan phosphorylase titrated to alkaline pH values. 8.4 mg/ml of protein solution in 0.005 M citrate buffer (pH 6.7). The pH was first adjusted by adding 1 M NaOH at 0°, and the absorbance measured with a spectrophotometer. The exact pH values were measured with a pH meter soon after the absorbance readings. The changes in volume during titration were negligible. A 1-cm cell was used.

Fig. 2. Effect of urea on the activity of α-glucan phosphorylase from potato. The reaction mixture was as follows; 0.04 ml citrate buffer (0.5 M, pH 6.0), 0.01 ml enzyme solution (24 mg/ml), 0–0.12 ml urea solution (10 M) and water. Total volume 0.2 ml. The reaction mixture was incubated at 30°, and 10-μl aliquots were removed at various time intervals and assayed for phosphorylase activity.

Fig. 3. Absorption spectra of urea-treated and urea *plus* $NaBH_4$-treated α-glucan phosphorylase from potato. The urea-treated enzyme (7.6 mg/ml) and urea *plus* $NaBH_4$-treated enzyme (8.5 mg/ml) were used. Experimental details are given in the text. A 1-cm cell was used. ———, 4.3 M urea-treated enzyme; — — —, 4.3 M urea *plus* 0.02 M $NaBH_4$-treated enzyme.

and an accompanying rise around 390 mμ were observed. At pH 10.6, a peak at 390 mμ became evident. These changes are identical with those obtained with muscle phosphorylase by KENT *et al.*[5], who regarded this shift as being caused by the transformation of the binding of pyridoxal 5′-phosphate from the native aldamine form to a Schiff base structure. The inflection point of the increase of absorption at 390 mμ was at pH 10.2 for the potato enzyme, which agreed with pH 10.6 calculated for the

muscle enzyme from the results of KENT *et al.*[5]. On acidification, potato α-glucan phosphorylase solution became turbid, and the absorption spectrum could not be measured below pH 5.5.

Effect of urea on the enzymic activity and the binding of pyridoxal 5'-phosphate

In order to study the behavior of pyridoxal 5'-phosphate on the denaturation of potato phosphorylase, urea was employed as a denaturing agent. The effect of various concentrations of urea on the activity of potato phosphorylase is shown in Fig. 2. At a concentration of urea higher than 4 M, rapid inactivation was observed. The inactivation was 50% in 30 min at 30° and 3.9 M urea, which is much higher than 2.1 M, the corresponding value for rabbit muscle phosphorylase *b* (ref. 4). To determine whether pyridoxal 5'-phosphate was removed from the enzyme protein by exposure to urea, 20 mg of potato phosphorylase were incubated for 60 min at 30° in 15 ml of 0.01 M Tris–HCl or citrate buffer, pH 6.0, containing 4.3 M urea. The reaction mixture was then passed through a column of Sephadex G-75 (2.5 cm × 65 cm), that had been equilibrated with 0.005 M citrate buffer, pH 6.0. The column was washed with the citrate buffer, and 2-ml fractions were collected. The fractions containing the enzyme protein were combined and concentrated using a collodion bag. The absorption spectrum of this solution (7.6 mg protein per ml) is shown in Fig. 3. The enzyme protein thus treated exhibited no absorption peak at 333 mμ. The protein (0.76 mg) in this solution gave no discernible blue color in the dichloroquinone chloroimide test and was also negative to the *p*-aminoacetophenone test. These results show that exposing potato phosphorylase to a high concentration of urea caused inactivation with a concurrent removal of pyridoxal 5'-phosphate from the enzyme protein.

Effect of $NaBH_4$ on the activity and binding of pyridoxal 5'-phosphate in urea denaturation

FISCHER and co-workers[4,6] showed that treatment of rabbit muscle phosphorylase with $NaBH_4$ under the conditions that structurally altered the enzyme (acid, alkali or urea) promoted the binding of pyridoxal 5'-phosphate to the enzyme protein. The resulting protein retained about 60% of its original phosphorylase activity as well as the absorption peak at 333 mμ. To determine whether the binding of pyridoxal 5'-phosphate by $NaBH_4$ treatment would retard the inactivation of potato phosphorylase by urea, the enzyme activity was measured at various concentrations of $NaBH_4$ in the presence of 4.3 M urea (Fig. 4). Addition of $NaBH_4$ to the reaction mixture resulted in protection of enzyme activity against inactivation by urea. Up to 0.02 M, increasing the concentration of $NaBH_4$ enhanced the protective effect. With concentrations of $NaBH_4$ higher than 0.02 M, however, the protection became less marked. Thus, the protective effect was maximal at 0.02 M $NaBH_4$ under the conditions employed. The enzyme protein (25 mg) was incubated with 4.3 M urea in the presence of 0.02 M $NaBH_4$ for 60 min at 30°. The reaction mixture was then passed through a column of Sephadex G-75 (2.5 cm × 65 cm) which had been equilibrated with 0.005 M citrate buffer (pH 6.0). The column was washed with the citrate buffer, and fractions containing the enzyme protein were combined and concentrated using a collodion bag. The absorption spectrum of this enzyme solution (8.8 mg protein per ml) is given in Fig. 3. The enzyme treated with 4.3 M urea in the presence of 0.02 M

$NaBH_4$ retained the absorption shoulder at 333 mμ shown by the native enzyme. The enzyme treated with 4.3 M urea alone did not show this absorption shoulder. It was therefore presumed that the protection of enzyme activity by 0.02 M $NaBH_4$ against urea inactivation was due to the binding of pyridoxal 5′-phosphate to the enzyme protein, the direct effect of urea on the phosphorylase under the above conditions being the release of pyridoxal 5′-phosphate. In order to test this assumption, the enzyme treated with 0.02 M $NaBH_4$ in the presence of 4.3 M urea was desalted by Sephadex G-75 and concentrated as described before. The concentrated enzyme solution (13.4 mg/ml) was denatured by the addition of perchloric acid up to a final concentration of 10%. The absorbance of the supernatant solution at 295 mμ was nearly equal to that obtained with the untreated enzyme. These results show that binding of pyridoxal 5′-phosphate did not occur under these conditions. The protective effect of $NaBH_4$ against urea inactivation of the phosphorylase could not, therefore, be caused by the reductive binding of pyridoxal 5′-phosphate to the enzyme protein. The possibility that urea concentration was decreased by reacting with $NaBH_4$ was tested by determination of the urea present. This was done by titrating tha ammonia produced by the action of urease. No discernible change was observed in the concentration of urea on incubating with 0.02 to 0.06 M $NaBH_4$ at 30°.

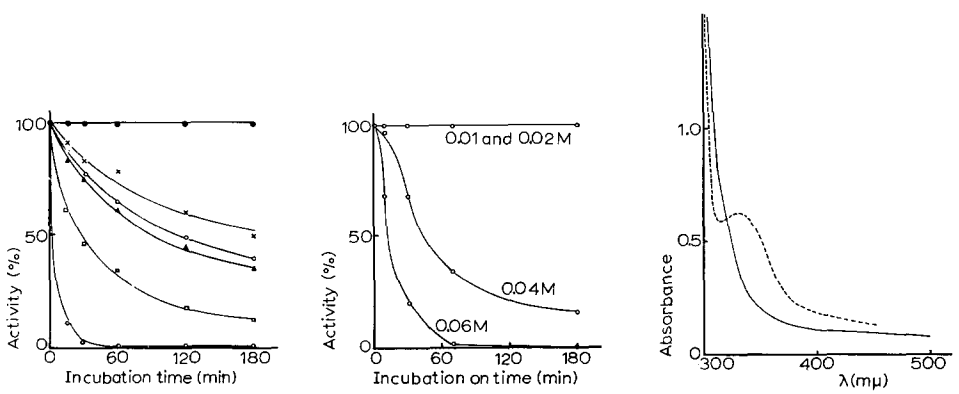


Fig. 4. Effect of $NaBH_4$ in various concentrations on the activity of α-glucan phosphorylase from potato in the presence of 4.3 M urea. Reaction mixture was as follows: 0.04 ml of 0.5 M Tris–HCl buffer (pH 6.0), 0.01 ml of enzyme solution (25 mg/ml), 0.086 ml of 10 M urea, 0–0.06 ml of 0.2 M $NaBH_4$ and water. Total volume was 0.2 ml. Urea and $NaBH_4$ were added at the same time. The reaction mixture was incubated at 30° for various time intervals, and each 10 μl was removed and assayed for phosphorylase reaction by the method described in MATERIALS AND METHODS ●—●, no urea and no $NaBH_4$; ○—○, no $NaBH_4$; □—□, *plus* 0.01 M $NaBH_4$; ○—○, *plus* 0.015 M $NaBH_4$; ×—×, *plus* 0.02 M $NaBH_4$; ▲—▲, *plus* 0.03 M $NaBH_4$.

Fig. 5. Effect of $NaBH_4$ on the activity of α-glucan phosphorylase from potato. Reaction mixture was as follows: 0.04 ml of citrate buffer (0.5 M, pH 6.0), 0.01 ml of enzyme solution (25 mg/ml), various volumes of 0.2 M $NaBH_4$ and water. Total volume 0.2 ml. The reaction mixture was incubated at 30° for various time intervals and each 10 μl was removed and assayed for phosphorylase activity as described in MATERIALS AND METHODS .

Fig. 6. Absorption spectra of potato α-glucan phosphorylase treated with 0.02 M and 0.06 M $NaBH_4$. The 0.02 M and 0.06 M $NaBH_4$-treated enzyme solutions (7.7 mg/ml) were used. Experimental details are given in the text. A 1-cm cell was used. — — —, 0.02 M $NaBH_4$-treated enzyme; ———, 0.06 M $NaBH_4$-treated enzyme.

Effect of $NaBH_4$ on the activity and binding of pyridoxal 5'-phosphate

The above results prompted us to study the effect of $NaBH_4$ itself on phosphorylase activity. The experimental results are given in Fig. 5. Up to a concentration of 0.02 M $NaBH_4$, no effect on the activity was observed, but inactivation became apparent above this concentration. In 0.06 M $NaBH_4$ the enzyme activity was almost completely lost within 1 h. The fate of bound pyridoxal 5'-phosphate on reaction with $NaBH_4$ was investigated by incubating 32 mg of potato phosphorylase with 0.02 M and 0.06 M of $NaBH_4$ in 30 ml of citrate buffer (0.1 M, pH 6.0) for 60 min at 30°. The reaction mixtures were desalted by passing through columns of Sephadex G-75 and concentrated using a collodion bag. The absorption spectra of the concentrated enzyme solutions (8.5 mg protein per ml) are shown in Fig. 6. Qualitative color tests for pyridoxal 5'-phosphate were performed on these solutions. The enzyme treated with 0.02 M $NaBH_4$ showed the absorption shoulder at 333 mμ, and gave a green-yellow color in the dichloroquinone chloroimide test and no color in the *p*-aminoacetophenone test. The enzyme treated with 0.06 M $NaBH_4$, however, lost the absorption shoulder around 333 mμ, and gave a distinct blue color in the dichloroquinone chloroimide test and an orange color in the *p*-aminoacetophenone test. Although the addition of perchloric acid to the 0.02 M $NaBH_4$-treated enzyme solution resulted in liberation of all the pyridoxal 5'-phosphate into the supernatant fluid, the pyridoxal 5'-phosphate in the 0.06 M $NaBH_4$-treated enzyme was retained in the protein.

Effect of pH and selected compounds on the reaction of potato phosphorylase with 0.06 M $NaBH_4$

The effect of pH and certain compounds on the reaction of potato phosphorylase with 0.06 M $NaBH_4$ were examined (Table I). In glycerophosphate buffer a

TABLE I

EFFECTS OF pH AND SOME COMPOUNDS ON THE REACTION OF POTATO PHOSPHORYLASE WITH 0.06 M $NaBH_4$

The enzyme (final concn. 1.4 mg/ml) was incubated in the buffer indicated with $NaBH_4$ (final concn. 0.06 M) at 30°. After 30 min incubation, 10 μl of the reaction mixture were removed and assayed for phosphorylase activity as described under MATERIALS AND METHODS, and the remaining activities were calculated as % of the original activity.

Buffer	*Remaining activity (%)*
Glycerophosphate (0.02 M, pH 6.0)	40
Glycerophosphate (0.02 M, pH 6.8)	7
Glycerophosphate (0.02 M, pH 7.5)	0
Glycerophosphate (0.02 M, pH 6.8) *plus* L-lysine ($5 \cdot 10^{-3}$ M)	41
Glycerophosphate (0.02 M, pH 6.8) *plus* urea (2 and 3 M)	0*
Tris–maleate (0.08 M, pH 7.0)	80
Tris–HCl (0.1 M, pH 7.5)	96
Citrate (0.1 M, pH 6.0)	30

* 10 min incubation.

higher pH caused more inactivation. The enzyme activity was lost completely within 30 min by incubating at 30° in 0.02 M glycerophosphate buffer with 0.06 M $NaBH_4$ at pH 7.5. The addition of Tris and L-lysine to the reaction mixture retarded the inactivation of phosphorylase activity. The addition of low concentrations of urea (2 and 3 M), however, promoted the inactivation.

In order to examine whether the protective effect of Tris against the inactivation of potato phosphorylase by treating with 0.06 M $NaBH_4$ was related to the reduction of pyridoxal 5'-phosphate binding, the absorption change around 333 mμ and enzymic activity were measured. $NaBH_4$ was added to 1 ml of Tris–HCl buffer (0.1 M, pH 6.0) containing 9 mg of potato phosphorylase to give a final concentration of 0.06 M. At various time intervals at 30°, absorbances at 300 to 450 mμ and enzymic activity were measured. No change either in absorbance around 333 mμ or in enzymic activity was observed. After incubation for 12 h at 30°, further $NaBH_4$ was added to a concentration of 0.06 M (it was assumed that all the original $NaBH_4$ had completely decomposed). No change in absorbance around 333 mμ or enzymic activity was observed. A third addition of $NaBH_4$ (0.06 M) after another 12 h again revealed no change in absorption around 333 mμ or in enzymic activity. These results indicate that the presence of Tris in the reaction mixture prevented the reduction of pyridoxal 5'-phosphate binding as well as the inactivation of enzymic activity. However, the exact role of Tris cannot be explained at present.

$NaBH_4$ reduction of potato phosphorylase at alkaline and acidic pH

Phosphorylase solution (9 mg/ml, in 0.1 M citrate buffer) containing 0.02 M $NaBH_4$ was brought to pH 11.0 with 1 M NaOH. After 30 min at 30°, the absorbance was measured at 300 to 450 mμ. The reduced enzyme at pH 11.0 had no absorption peak at 333 mμ in contrast to the reduced enzyme at pH 6.0. The enzyme protein, reacted with 0.02 M $NaBH_4$ at pH 11.0, was collected by passing the solution through a column of Sephadex G-75 and condensing with a collodion bag. The enzyme showed positive blue and orange colors in the dichloroquinone chloroimide and *p*-aminoacetophenone tests, respectively. Adjustment of the enzyme solution to pH 11.0 before the addition of $NaBH_4$ caused the liberation of pyridoxal 5'-phosphate from the enzyme protein. The resulting protein was then negative to both tests. The ad-

TABLE II

DICHLOROQUINONE CHLOROIMIDE TEST, *p*-AMINOACETOPHENONE TEST AND ABSORPTION AT 333 mμ FOR POTATO α-GLUCAN PHOSPHORYLASE TREATED WITH VARIOUS AGENTS

Experimental details are given in the text.

Treatment	*Absorption peak at 333 mμ*	*Dichloroquinone chloroimide test*	*p-Amino-acetophenone test*
Native	+	green-yellow	no color
4.3 M urea	−	no color	no color
4.3 M urea + 0.02 M $NaBH_4$	+	green-yellow	no color
0.02 M $NaBH_4$ at pH 6.0	+	green-yellow	no color
0.02 M $NaBH_4$ at pH 11.0	−	blue	orange
0.06 M $NaBH_4$ at pH 6.0	−	blue	orange

dition of perchloric acid to the enzyme solution containing 0.02 M sodium borohydride evidently caused complete binding of pyridoxal 5'-phosphate, since no absorbance at 295 mμ was observed in the supernatant solution of the perchloric acid-treated enzyme. These results indicate that at alkaline or acidic pH the reductive fixation of pyridoxal 5'-phosphate bound to potato phosphorylase occurs at a lower concentration of $NaBH_4$ than at neutral pH.

The results of the dichloroquinone chloroimide and *p*-aminoacetophenone tests, and the absorbance at 333 mμ of potato phosphorylase treated in various ways, are summarized in Table II. It should be pointed out that fully-reduced potato phosphorylase and the native enzyme exhibit different characteristics for these criteria.

Dichloroquinone chloroimide and p-aminoacetophenone tests with some phenol compounds

To compare the results of the dichloroquinone chloroimide and *p*-aminoacetophenone tests on native and reduced phosphorylase, some phenols were examined as model compounds for these tests. As shown in Table III, saligenin gave a positive

TABLE III

DICHLOROQUINONE CHLOROIMIDE AND *p*-AMINOACETOPHENONE TESTS WITH SOME PHENOL COMPOUNDS

Experimental details are given in the text. 10^{-4} mole of each sample was spotted on the paper.

Compound	*Dichloroquinone chloroimide test*	*p-Amino acetophenone test*
Saligenin	blue	orange
Salicylic acid	no color	no color
Salicylaldehyde	green	orange*
Salicin	no color	no color
Acetylsalicylic acid methyl ester	no color	no color
3-Hydroxy pyridine	blue	orange
0.06 M $NaBH_4$-treated 3-hydroxy pyridine	blue	orange

* Very light color.

blue color with the dichloroquinone chloroimide test and an orange color with the *p*-aminoacetophenone test. Salicylaldehyde and salicylic acid were negative to both tests, possibly because the 3-hydroxyl group is linked to the internal carbonyl or carboxyl groups by a hydrogen bond. Salicin and acetylsalicylic acid methyl ester were also negative to both tests because no free hydroxyl group is present. These results suggest that the free 3-hydroxyl group is essential for a positive reaction to these tests. 3-Hydroxy pyridine treated with 0.06 M $NaBH_4$ was positive to both tests. Therefore, no change was expected in the pyridine ring of pyridoxal 5'-phosphate by reaction with 0.06 M $NaBH_4$.

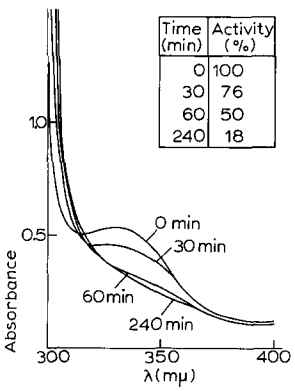
Relationship between the absorption change at 333 mμ and phosphorylase activity during reaction with 0.06 M $NaBH_4$

Potato phosphorylase (9.6 mg) was incubated at 24° in 1 ml of 0.1 M citrate

buffer (pH 6.0) containing 0.06 M $NaBH_4$, and the absorption change was followed. 5 μl of this reaction mixture was removed after various time intervals, and assayed for phosphorylase activity. The results, in which the remaining activity was expressed as percentage of the original activity, are given in Fig. 7. The absorption peak at 333 mμ almost disappeared during the first 60 min of incubation, while 50% of the original enzyme activity still remained after this period. Thus, no parallel relationship between the disappearance of absorption at 333 mμ and enzyme activity was observed. This suggests that the bound pyridoxal 5'-phosphate absorbing at 333 mμ might not be directly involved in the catalytic action of potato phosphorylase. The incubation of the phosphorylase with 0.06 M $NaBH_4$ would result not only in the reduction of pyridoxal 5'-phosphate binding but also in some change to the protein moiety itself. This was supported by the observation, as shown in Fig. 8, that the absorption in a shorter wavelength region, 240 to 300 mμ, changed on incubation with 0.06 M $NaBH_4$. However, this change in absorption was not further characterized.

Trials for resolution of pyridoxal 5'-phosphate from potato α-glucan phosphorylase

Removal of pyridoxal 5'-phosphate from potato phosphorylase was attempted by LEE[7], by precipitating the enzyme with $(NH_4)_2SO_4$ at acidic pH. Resolution,



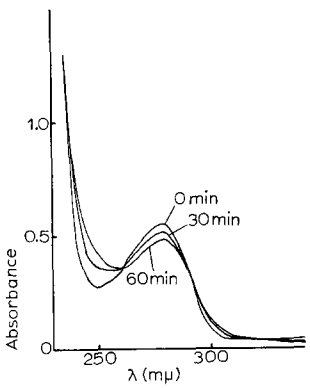


Fig. 7. Time course of the changes in absorbance and enzyme activity of potato α-glucan phosphorylase on reaction with 0.06 M $NaBH_4$. The enzyme (6.6 mg/ml) was incubated in 1 ml of 0.1 M citrate buffer (pH 6.0) containing 0.06 M $NaBH_4$ at 24°, and the absorption spectra followed at various time intervals. A 1-cm cell was used.

Fig. 8. Absorption change, at short wavelengths, of potato α-glucan phosphorylase upon reduction with 0.06 M $NaBH_4$. The enzyme (0.45 mg) was incubated in 1 ml of 0.05 M citrate buffer (pH 6.0) containing 0.06 M $NaBH_4$ at 20° for the time intervals indicated. The absorption spectra were followed at various time intervals. A 1-cm cell was used.

however, was not obtained owing to the instability of the enzyme at acid pH. A mild method for removal of pyridoxal 5'-phosphate from muscle phosphorylase *b* has recently been reported by SHALTIEL *et al.*[12]. According to this method, potato phosphorylase was incubated in 0.1 M citrate buffer (pH 6.0) containing 0.1 M imidazole and 0.1 M L-cysteine, but the treatment had no affect on the enzyme activity. No loss of phosphorylase activity was observed by incubating the enzyme in 0.1 M citrate

buffer (pH 6.0) containing 0.01 M L-cysteine and 0.5 M NaCl; this procedure has been successfully used for rabbit muscle phosphorylase by JOHNSON AND GRAVES[13]. Other attempts to remove pyridoxal 5'-phosphate from the potato enzyme and reconstruct the holoenzyme have been unsuccessful.

DISCUSSION

α-Glucan phosphorylase from potato resembles phosphorylase *b* from rabbit muscle in molecular weight[1], content of bound pyridoxal 5'-phosphate[7], amino acid composition[2] and circular dichroism[3], whereas the regulatory mechanisms of their catalytic actions are quite different. Experimental results described in this paper reveal further chemical properties of potato phosphorylase, which allow a comparison of both enzymes in more detail. Spectral changes were similar for both enzymes upon titrating to an alkaline pH. Much higher concentrations of urea were needed to inactivate potato phosphorylase than to inactivate the muscle enzyme. In a preliminary experiment, removal of the bound pyridoxal 5'-phosphate from the potato enzyme by the same methods as reported for the muscle enzyme[12,13] did not succeed. More rigorous conditions would be necessary for the removal of the cofactor from the potato enzyme.

FISCHER *et al.*[6] reported that the reduction of the muscle enzyme with $NaBH_4$ was not observed between pH 5 and 9.5, and that the fully-reduced enzyme at pH 4.5 showed an absorption maximum at 330 mμ. The present study, however, indicates that the potato enzyme could be reduced at neutral pH, and the resulting protein does not show an absorption maximum at 330 mμ. The reduced enzyme gave a positive reaction in the dichloroquinone chloroimide and *p*-aminoacetophenone tests, whereas the native enzyme was negative to these tests. The same results were obtained with the muscle enzyme (unpublished observation). A comparison of the results with model compounds led to the conclusion that the 3-hydroxyl group of pyridoxal 5'-phosphate in the native enzyme is linked in some way that involves a hydrogen bond. However, any proposal for the structure of pyridoxal 5'-phosphate binding should await further experimental results.

Inactivation of potato phosphorylase by reaction with 0.06 M $NaBH_4$ did not parallel the disappearance of the 330 mμ peak. The reduced enzyme, which showed no absorption maximum at this wavelength, still retained about half its original activity. The results indicate that the 3-hydroxyl group of pyridoxal 5'-phosphate is not involved in the catalytic action of phosphorylase. Pyridoxal 5'-phosphate in the native enzyme might serve to maintain the proper conformation of the phosphorylase. In consequence, the reduction of pyridoxal 5'-phosphate binding would cause transformation of the enzyme protein accompanied by inactivation and a change of absorption around 280 mμ.

The protective effect of Tris and lysine against the reduction of pyridoxal 5'-phosphate binding and the inactivation of enzyme activity cannot be explained at present nor can the protective effect of $NaBH_4$ against urea inactivation. However, the effect of these amino compounds on the phosphorylase protein would be common, since the inactivation of potato phosphorylase by 3 M NaCl was almost completely retarded by the presence of 0.1 M Tris at pH 6.0 (unpublished observation).

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