POTATO PHOSPHORYLASE

II. PHOSPHATE AND SULFHYDRYL GROUPS

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SUMMARY

Potato phosphorylase contains two moles of firmly bound organic phosphate/mole of enzyme which has been identified as pyridoxal-5-phosphate. Resolution of the enzyme and recombination with pyridoxal-5-phosphate were only partially successful because of denaturation of the enzyme at pH values below 5.5. In contrast to the corresponding mammalian enzymes, potato phosphorylase contains no serine phosphate. The specific phosphatase and the kinase for the serine phosphate groups of the mammalian enzyme had no effect on the potato enzyme.

Potato phosphorylase was found to contain 6 moles of sulfhydryl groups per mole of protein. The enzyme is inhibited by *p*-chloromercuribenzoate and this inhibition is only partially reversed by the addition of cysteine.

INTRODUCTION

The isolation of pyridoxal-5-P from muscle phosphorylase[1] and the demonstration that it functions as the prosthetic group of the enzyme[2] raised the question whether this vitamin was also an essential component of the plant phosphorylase system. Muscle phosphorylase *a* also contains serine phosphate[3], which can be split by a specific phosphatase, formerly called PR enzyme[4], and which can be rephosphorylated by a special kinase in the presence of ATP and Mg^{++}[5]. Here again it was of interest to compare the animal and plant phosphorylase systems. In a preliminary report[6] it has been shown that potato phosphorylase contains pyridoxal-5-P, whereas serine phosphate was found to be absent. The experimental evidence for these findings is reported in this paper.

MATERIALS AND METHODS

The preparation of potato phosphorylase and the measurement of its concentration and activity are described in the preceding paper[7]. PR enzyme[4] and phosphorylase *b* kinase[5] were prepared from rabbit skeletal muscle and the corresponding liver enzymes[8,9] from beef liver. A purified preparation of glutamic-aspartic transaminase from heart muscle was kindly supplied by Dr. S. VELICK.

Abbreviation used: pyridoxal-5-P, pyridoxal-5-phosphate.

Pyridoxamine phosphate was obtained from the California Foundation for Biochemical Research. Pyridoxal-5-P and other derivatives were purchased from Mann Research Laboratory, Inc. The amount of pyridoxal-5-P in solution was calculated from measurements in the Beckman spectrophotometer, based on the values for absorbancy established by PETERSON AND SOBER[10].

Phosphate determinations were carried out by the sensitive micromethod of CHEN *et al.*[11].

RESULTS

Identification of pyridoxal-5-P

According to PETERSON AND SOBER[10], pyridoxal-5-P in 0.1 *N* HCl shows a peak at 295 mμ ($E^{m}_{1\,cm}$ = 6700). In 0.1 *N* NaOH a new peak appears at 388 mμ ($E^{m}_{1\,cm}$ = 6600). A perchloric acid extract of the enzyme undergoes the same spectral changes when it is made alkaline. This is illustrated in Fig. 1. From the extinction at 295 and 388 mμ one calculates 1.9 and 2.1 moles of pyridoxal-5-P/mole of enzyme, respectively. The enzyme itself in 0.01 *M* phosphate buffer pH 6.8, when examined in sufficiently high concentration, shows a broad peak around 335 mμ; in 0.1 *N* NaOH this peak is replaced by a broad peak around 390 mμ (Fig. 2).

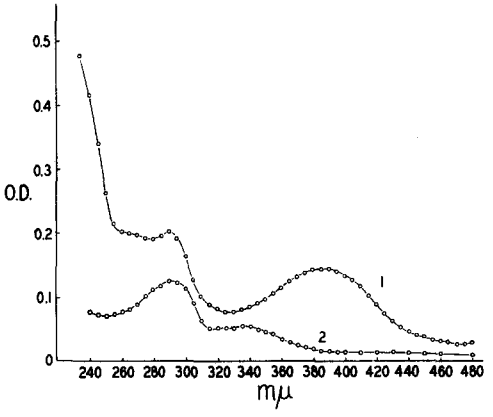

Fig. 1. Absorption spectra of the extract of 2.29 mg of potato phosphorylase. Curve 1, in 0.1 *N* NaOH; Curve 2, in 6% perchloric acid.

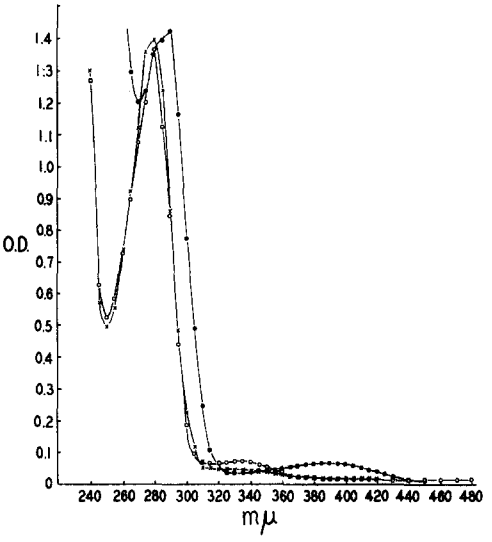

Fig. 2. Absorption spectra of potato phosphorylase, 1.055 mg/ml. ○, in 0.01 *M* potassium phosphate pH 6.8; ×, in 0.1 *N* HCl; ●, in 0.1 *N* NaOH.

Pyridoxal-5-P was isolated as the alcohol insoluble barium salt from a perchloric acid extract of the enzyme, as described by BARANOWSKI *et al.*[1]. After removal of barium ions with $SO_4^{=}$, the sample was used for paper electrophoresis, paper chromatography and enzymic tests with the glutamic-aspartic transaminase (a preparation consisting largely of apoenzyme). A part of the isolated material was also hydrolyzed for 6 h in 1 *N* HCl at 100°.

Paper electrophoresis was carried out in acetate buffer pH 5.1, ionic strength 0.05 in the Model R, Series D Spinco apparatus. A current of 3.5 mA was applied

for 6 h. The bands appeared as blue fluorescence under u.v. light, which changed to yellow fluorescence on exposure to ammonia vapor. In a given experiment, an authentic sample of pyridoxal-5-P, a mixture of it with the isolated sample and the isolated sample alone all migrated the same distance. The acid hydrolysate of the isolated sample migrated at the same rate as pyridoxal.

Ascending paper chromatography on Whatman No. 1 filter paper was carried out at room temperature for 22 h in *n*-butanol–ethanol–glacial acetic acid–H_2O (5:3:0.5:1.5) solvent. The material isolated from the enzyme migrated at the same rate as pyridoxal-5-P, while the hydrolysate migrated like pyridoxal.

The activation of glutamic-aspartic transaminase, in an assay system similar to that of BARANOWSKI *et al.*[1] is shown in Fig. 3. Without the addition of pyridoxal-5-P, the reaction was slow. The material isolated from the enzyme, as well as heated potato phosphorylase, stimulated the transamination reaction to a similar extent as authentic pyridoxal-5-P.

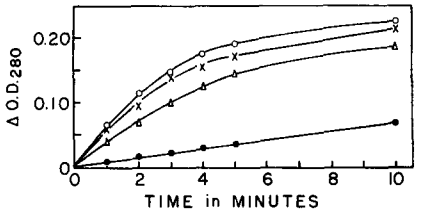

Fig. 3. Activation of glutamic-aspartic transaminase. ●, without the addition of pyridoxal-5-P; ○, with the addition of pyridoxal-5-P ($1 \cdot 10^{-5}$ *M*); ×, with the addition of the isolated material; △, with the addition of heated potato phosphorylase.

Resolution of enzyme

The apoenzyme of muscle phosphorylase *a* could be prepared in crystalline form[12]; it was completely inactive, but when pyridoxal-5-P was added, recombination took place and full activity was restored. The method of resolution consisted of lowering the pH of the enzyme solution with citrate buffer to about 4 in the presence of an excess of cysteine, followed by precipitation with ammonium sulfate. Muscle phosphorylase *b* could be resolved under milder conditions; incubation with 0.1 *M* Na glycerophosphate–0.08 *M* cysteine pH 6.0 at 0° for 12 h resulted in the complete resolution of the enzyme.

The difficulty encountered in attempts to resolve potato phosphorylase is that the enzyme is very unstable at pH values below 5.5 and that this pH is insufficient to liberate pyridoxal-5-P. When the enzyme was dialyzed against 0.1 *M* citrate–0.1 *M* cysteine, pH 5.5, no resolution occurred even in 24 h. The use of carbonyl reagents (semicarbazide, 0.1 *M* or hydroxylamine, 0.1 *M*) in place of cysteine was also unsuccessful.

Pyridoxal-5-P bound to the potato enzyme could be liberated below pH 4.5, but at the same time some unavoidable enzyme inactivation took place. One of the experiments is shown in Table I. To 0.5 ml of enzyme solution in an ice bath was added 0.2 ml of 0.1 *M* citrate–0.1 *M* cysteine, pH 4, 4.5 or 7.0. After 30 sec 3 ml of saturated ammonium sulfate and 1 drop of 0.1 *M* ammonia were added. The precipitate was washed once with alkaline ammonium sulfate solution and was dissolved in water. Addition of pyridoxal-5-P to the enzyme solution treated at pH 4

TABLE I

EFFECT OF PYRIDOXAL-5-PHOSPHATE ON ENZYMIC ACTIVITY

After the treatment (for details see text), the enzyme solutions were incubated for 30 min at 30° in $5 \cdot 10^{-2}$ *M* citrate buffer, pH 7 and $1.6 \cdot 10^{-4}$ *M* pyridoxal-5-phosphate before being assayed for enzymic activity.

Treatment	*Activity in units/mg protein*	
	Without P-5-P	*With P-5-P*
None	1820	1780
pH 4, 30 sec	0	153
pH 4.5, 30 sec	233	433
pH 7, 30 sec	1673	1673

restored some activity, but the recovery was less than 10 %. Addition of 5'-AMP or cysteine did not improve the recovery. At pH 4.5 the enzyme was incompletely resolved, but a stimulation of enzyme activity by pyridoxal-5-P could nevertheless be demonstrated. Pyridoxine, pyridoxamine, pyridoxamine phosphate and pyridoxal could not be substituted for pyridoxal-5-P.

Phosphate content

A solution of potato phosphorylase was dialyzed against 0.1 *M* KCl–0.01 *M* $KHCO_3$, pH 8 for 3 days with 3 changes of buffer. A sample was ashed and analyzed according to the method of CHEN *et al.*[11]. The total phosphorus content was found to be 2 moles/mole of enzyme, that is, no organic phosphate except pyridoxal-5-P could be detected. The phosphorus content of the protein residue after precipitation with perchloric acid was analyzed, but no significant amount could be detected.

The absence of serine phosphate from potato phosphorylase is of interest. Unlike muscle phosphorylase *b* which is formed from phosphorylase *a* by the enzymic hydrolysis of serine phosphate and which requires 5'-AMP for activity, the potato enzyme is fully active in the absence of 5'-AMP. As might be expected, the specific phosphatase from muscle (PR enzyme) and from liver does not inactivate potato phosphorylase. There was also no inactivation during incubation with crude potato juice.

The question whether a serine group of potato phosphorylase can be phosphorylated by the muscle phosphorylase *b* kinase or kinase from beef liver in the presence of ATP and Mg^{++} has also been examined. An indirect coupling system was used to detect the formation of ADP by the phosphorylase *b* kinase reaction. This was based on the addition of phosphoenolpyruvate kinase and phosphoenolpyruvate to form pyruvate and ATP and on the addition of lactic dehydrogenase and DPNH to form lactate and DPN. The system worked satisfactorily with phosphorylase *b* or inactive liver phosphorylase as substrate. With potato phosphorylase no phosphorylation could be detected.

Sulfhydryl groups

The number of SH groups in potato phosphorylase was determined as follows. The enzyme solution was dialyzed extensively against 0.1 *M* Tris buffer pH 7.5 and 10 mg of the protein was titrated amperometrically with $1 \cdot 10^{-3}$ *M* $AgNO_3$ or $1 \cdot 10^{-3}$ *M*

$HgCl_2$. The values obtained were 5.69 and 6.0 moles of SH groups/mole of protein with $AgNO_3$ and 5.4 moles with $HgCl_2$. Titration of the SH groups with *p*-chloromercuribenzoate in 0.04 *M* glycerophosphate buffer pH 7.0 according to BOYER's[13] spectrophotometric method yielded a value of 6.8 moles/mole of enzyme. Muscle phosphorylase *a* contains 18 moles of sulfhydryl groups/mole of enzyme on the basis of amino acid composition and titration with *p*-chloromercuribenzoate[14]. The potato enzyme differs from the muscle enzyme in having fewer sulfhydryl groups per unit weight of protein.

Potato phosphorylase is not inhibited by iodoacetate ($1 \cdot 10^{-2}$ *M*). Heavy metals (Ag^+, Cu^{++}, Hg^{++}) are inhibitory, as shown in the preceding paper. Preincubation of the enzyme in glycerophosphate buffer pH 6.5 with a 100 fold excess of *p*-chloromercuribenzoate for 1 h at 30° gave a 60% inhibition and preincubation with a 10 fold excess for 5 h gave a nearly complete inhibition of enzyme activity. Contrary to what has been observed with muscle phosphorylase[14], it was not possible to restore completely the activity of the inhibited enzyme by the addition of an excess of cysteine. Experiments were also conducted at low temperature (2°), but here again there was only a partial reversal of the inhibited enzyme activity by cysteine (Fig. 4).

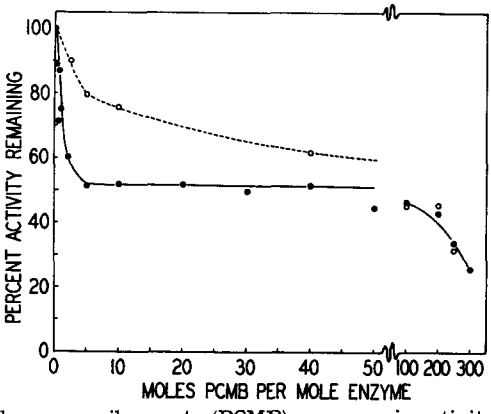

Fig. 4. Effect of *p*-chloromercuribenzoate (PCMB) on enzymic activity of potato phosphorylase. The enzyme, at a concentration of $1 \cdot 10^{-6}$ *M*, was incubated for 10 h at 2 ± 1° with PCMB in 0.04 *M* glycerophosphate buffer pH 6.5. The enzymic activity which remained was determined. ●—●, without further treatment; O---O, after treatment with $5 \cdot 10^{-3}$ *M* cysteine for 3 h.

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STUDIES ON THE ELECTRON TRANSPORT SYSTEM

XXVI. SPECIFICITY OF COENZYME Q AND COENZYME Q DERIVATIVES

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SUMMARY

In succinic dehydrogenase complex there are three alternative pathways by which quinones mediate the oxidation of succinate by cytochrome *c* or oxygen. The first, mediated by menadione, 2,3-dimethoxy, 5-methyl benzoquinone and the short chain coenzyme Q analogues terminates at external cytochrome *c* and is antimycin insensitive. The second, mediated only by lipophilic homologues of coenzyme Q (Q_2, Q_3, Q_6, Q_{10}) terminates at external cytochrome *c* and is antimycin sensitive. The third, mediated by coenzymes Q_2, Q_3, Q_6, Q_{10} and in part by Q_0, Q_1, plastoquinone and heptyl and heptadecyl coenzyme Q terminates at molecular oxygen, involves bound cytochrome *c* and is antimycin sensitive. There is a close correlation between the effects of Q analogues on the succinoxidase activity of acetone extracted SDC and the effects on the succinoxidase activity of acetone extracted electron transport particle ETP. This suggests that the reconstituted succinoxidase system of succinic dehydrogenase complex closely resembles that of electron transport particle from which it was derived. Succinic cytochrome *c* reductase activity of succinic dehydrogenase complex, in principle, is a measure of the segment of the electron transfer chain which has been detached from the cytochrome *a* terminus of the chain. This segment by virtue of changes induced by the cleavage process shows a new pattern of quinone specificity. Only derivatives of 2,3-dimethoxy, 5-methyl benzoquinone with two or more isoprenoid units in the side chain at carbon 6 are able to serve as mediators in the antimycin sensitive oxidation of succinate by cytochrome *c*. The antimycin insensitive pathway involving cytochrome *c* *via* other quinones involves transfer of electrons from a site in the electron transfer chain prior to the locus for antimycin action.

Abbreviations: ETP, electron transport particle; SDC, succinic dehydrogenase complex.

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