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PHOTOOXIDATION OF α-GLUCAN PHOSPHORYLASES FROM RABBIT MUSCLE AND POTATO TUBERS

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Summary

Photooxidation of α-glucan phosphorylases from rabbit muscle and potato tubers in the presence of rose bengal leads to a rapid loss of enzymatic activity which follows first-order kinetics. The process is pH dependent, being more rapid at higher pH. The inactivation is closely related to the destruction of histidine residues in the enzyme. It is suggested that histidine residues are largely responsible for the loss of enzymatic activity in the photooxidation. The inactivation of potato phosphorylase is retarded by substrates, whereas that of the muscle enzyme is not. The rate of photoinactivation of muscle phosphorylase *b* is increased with AMP, and decreased with ATP, ADP, IMP and glucose-6-*P*. This finding is considered to be closely related to the allosteric transition of phosphorylase.

Introduction

α-Glucan phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) has been well studied by numerous investigators. However, previous information concerning the mechanism of its action is rather limited. Although bound pyridoxal-5′-*P* is known to be essential for the action of phosphorylase [1,2], whether or not it is directly involved in the mechanism of action is still obscure. Phosphorylase is also known to be inactivated upon reaction with various chemical reagents; the sulfhydryl reagents [3—7], cyanate [8], dinitrofluorobenzene [9,10], glyoxal [11] and the carbodiimide reagent [12]. It is deduced from the results of these studies that the sulfhydryl, amino and carboxyl groups play important roles in phosphorylase action. From pH kinetic studies by Holló et al. [13—15] and by Chao and Graves [16], the involvement of a group with a pK_a of about 6 was suggested in the function of phosphorylase, although unambiguous assignment of the group was not made.

The present report is concerned with dye-sensitized photooxidation of

phosphorylase from rabbit muscle and potato tubers, which catalyze the same reaction but have different regulatory properties. The results provide chemical evidence for the involvement of histidine residues in the action of phosphorylase. An intriguing observation that the rate of photoinactivation of rabbit muscle phosphorylase *b* is increased by AMP and decreased by ATP, ADP, IMP and glucose-6-*P* is also described.

Materials and Methods

Potato phosphorylase was purified according to the method previously reported [17]. Rabbit muscle phosphorylase *b* was prepared according to the method of Fischer and Krebs [18] and was recrystallized five times. Before use, phosphorylase *b* was passed through a charcoal column to free it from AMP. Phosphorylase activity was assayed in the direction of polysaccharide synthesis, as described in a previous paper [11], and is expressed as μmol of P_i liberated per min. Protein concentration was determined spectrophotometrically using $E^{1\%}_{1\,cm}$ values at 280 nm of 11.7 and 13.2 for the potato and muscle enzymes, respectively.

Photooxidation was carried out in a darkroom as follows. A buffered enzyme solution was mixed in a Pyrex tube (0.8 × 12 cm) with an aqueous solution of rose bengal at final concentrations of 6 mg/ml of the enzyme and 0.001% of the dye in a total vol. of 0.2 ml. The tube was cooled in a water bath at a constant temperature of 1 °C, and illuminated at a distance of 55 cm with a 300-W tungsten lamp. Aliquots were removed at various time intervals and used for determination of enzymatic activity after appropriate dilution. The rate of photoinactivation was graphically obtained from a semilogarithmic plot of residual activity vs time of illumination. Samples of the photooxidized enzyme for amino acid analyses were prepared similarly but in a total volume of 2 ml. They were subsequently precipitated with 5% trichloroacetic acid, washed four times with ether/acetone (3 : 1, v/v), and dried in vacuo over conc. H_2SO_4. The native enzyme was treated without illumination. The samples were then hydrolyzed in sealed, evacuated tubes with 6 M HCl for 24 h at 110 °C and amino acids were analyzed on a Hitachi 034 liquid chromatograph according to the procedure of Spackman et al. [19].

Titration of cysteine residues with 5,5′-dithiobis-(2-nitrobenzoic acid) was carried out in 0.2 M Tris · HCl buffer, pH 8.0, with or without 8 M urea, according to the method of Ellman [20]. Fluorescence emission spectra were measured with excitation at 330 nm on a Hitachi MPF 2A fluorospectrophotometer.

Results

Inactivation of phosphorylase by photooxidation

Phosphorylase was found to be rapidly inactivated by photooxidation in the presence of rose bengal. The loss of enzymatic activity of the enzymes from rabbit muscle and potato tubers follows first-order kinetics down to nearly complete inactivation (Figs 1A and 1B, controls). The molar ratio of the enzyme monomer to rose bengal is of the order of 6 : 1 in these experiments.

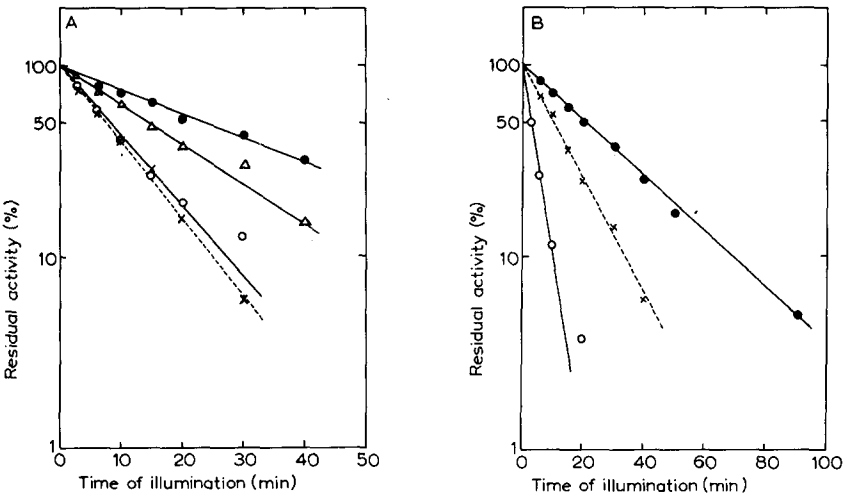

Fig. 1. Photoinactivation of phosphorylases from potato tubers and rabbit muscle in the presence of substrates and allosteric modifiers. The enzyme at a concentration of 6 mg/ml in 70 mM glycerophosphate buffer, pH 6.8, was illuminated at 1 °C in the presence of 0.001% rose bengal. Substrate or modifier was mixed with the enzyme solution prior to the addition of rose bengal. (A) Potato phosphorylase: ×, control with no addition; ○, 20 mM Pi; ●, 20 mM glucose-1-*P*; △, 1% soluble starch. (B) Muscle phosphorylase *b*: ×, control with no addition; ○, 10 mM AMP; ●, 10 mM ATP.

Decreasing the enzyme concentration to one-eighth with constant dye concentration results in approximate doubling of the rate of photoinactivation. Methylene blue is much less effective and lumichrome does not function as a photosensitizer for phosphorylase.

Photoinactivation in the presence of substrates and allosteric modifiers

When potato phosphorylase is photooxidized in the presence of substrate, protection against inactivation is observed clearly with glucose-1-*P* and starch, and slightly, if at all, with P_i (Fig. 1A). In muscle phosphorylase *b*, none of the substrates affects the rate of inactivation. However, conspicuous results were obtained with allosteric modifiers (Fig. 1B); the rate of photoinactivation is increased more than three times with 10 mM AMP, which is the essential activator for this enzyme, and is decreased to about one-half with 10 mM ATP, an allosteric inhibitor acting competitively with AMP. Other inhibitors, ADP and glucose-6-*P*, and an activator, IMP (all at 10 mM), cause a decrease in the inactivation rate similar to that caused by ATP. IMP has been known to be a poorer activator than AMP, and the kinetic behavior of its activation differs from that of the activation by AMP in several respects [21,22]. Simultaneous addition of AMP and ATP, both at 10 mM, results in the same degree of acceleration as in the case of AMP alone. Inclusion of substrate in the system with AMP does not produce any additional effect on the rate of photoinactivation. Even if the enzyme were further purified, prior to photooxidation, to remove any trace of contaminating nucleotides, similar results would be obtained.

Effect of allosteric modifiers on photoinactivation

The effect of allosteric modifiers on photoinactivation was observed only

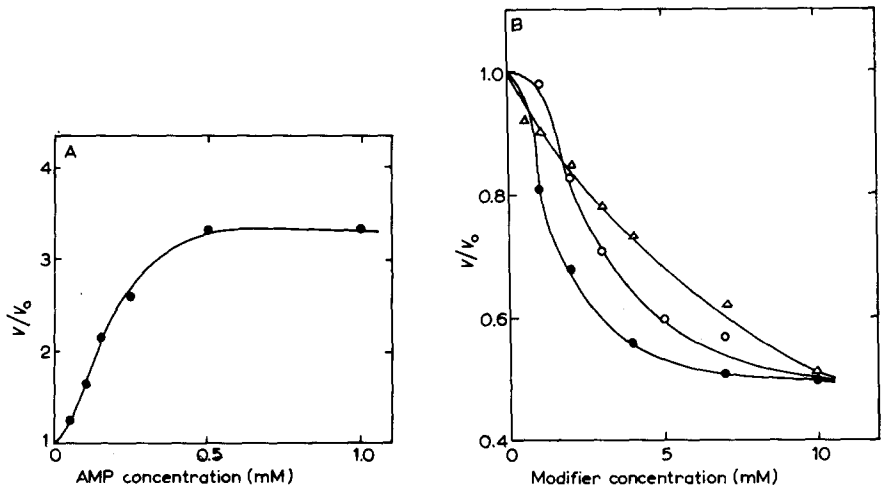

Fig. 2. Effect of allosteric modifiers on the rate of photoinactivation of rabbit muscle phosphorylase *b*. Experimental conditions were the same as in Fig. 1. v/v_0 is a ratio of the rate of photoinactivation in the presence of modifier to that in the absence of modifier. (A) The effect of AMP. (B) The effect of: ○, glucose-6-*P*; ●, ATP; △, IMP.

with muscle phosphorylase; the rate of photoinactivation of the potato enzyme whose activity is unaffected by AMP [23] is not altered by the addition of these nucleotides. The results suggest that the effect of these compounds observed in photoinactivation correlate directly to their function as allosteric modifiers. In order to test this supposition, the rate of inactivation of muscle phosphorylase *b* was determined in the presence of various concentrations of AMP. A plot of the relative rate of photoinactivation vs the concentration of AMP gives a sigmoidal curve (Fig. 2A). The effect is saturated at an AMP concentration of 0.5 mM , and the nucleotide concentration giving 50% of the maximal stimulation is about 0.2 mM. The dissociation constant of AMP and rabbit muscle phosphorylase *b* has been determined as around 0.1 mM by various investigators [5,24,25]. Ho and Wang [26] have recently reported that the high-affinity AMP binding sites are essentially filled at 0.3 mM AMP, as studied by means of calorimetric titration of the enzyme with AMP.

Fig. 2B shows the retarding effects of ATP, IMP and glucose-6-*P* on the photoinactivation of muscle phosphorylase *b* as a function of the concentrations of the modifiers. Their effects are all saturated around 10 mM, giving the same degree of retardation with all the compounds used on the rate of inactivation, one-half their control values. At 20 mM, the degrees of retardation are approx. the same as those at 10 mM. The apparent K_i values for ATP and glucose-6-*P* have been reported to be 2 mM and 0.3 mM, respectively [27], and the apparent dissociation constant of ATP 1.2 mM [26]. It seems reasonable to assume from these results that the effects of AMP, ATP, IMP and glucose-6-*P* in the photooxidation of muscle phosphorylase *b* are closely related to the allosteric transition of phosphorylase effected by these compounds.

Effect of pH on photoinactivation

The rate of photoinactivation of phosphorylase in the presence of rose

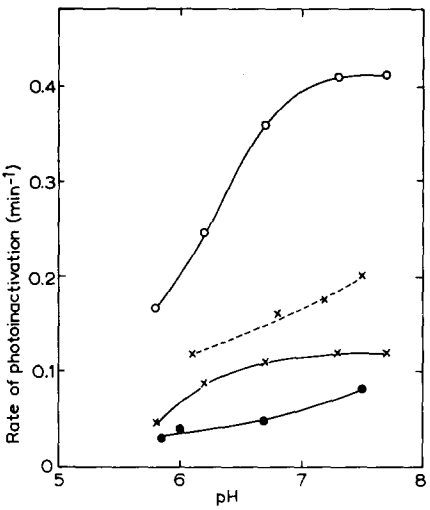

Fig. 3. Effect of pH on the rate of photoinactivation of phosphorylase. The enzyme at a concentration of 6 mg/ml in 0.1 M phosphate buffer (for the muscle enzyme) or in 70 mM glycerophosphate buffer (for the potato enzyme) was illuminated at 1 °C in the presence of 0.001% rose bengal. ×, no addition; ○, with AMP; ●, with ATP. Solid and dashed lines indicate the results with phosphorylases from muscle and potato, respectively.

bengal is affected by pH (Fig. 3). The enzymes from potato tubers and rabbit muscle in the presence and absence of allosteric modifiers all show the same tendency towards higher rates of inactivation at higher pH, within the range of pH applied. Determination at lower pH is difficult because of the lability of the enzyme. The results correspond to that expected for the photooxidation of the imidazole group [28].

Amino acid destruction in photooxidation

Table I represents the results of amino acid analysis of photooxidized potato phosphorylase and rabbit muscle phosphorylase *b*. It appears that approx. 2 and 4 histidine residues per subunit molecule of the potato and muscle enzymes, respectively, are degraded during the photoinactivation. Although there are some experimental deviations, especially in the case of the latter enzyme, apparently no significant difference was observed in other amino acids. Table II shows the results of the remaining activity and histidine content of the muscle enzyme photooxidized under various conditions. In spite of a wide range of illumination times in these experiments, the number of histidine residues lost is roughly parallel to the extent of inactivation, regardless of the kind of allosteric modifiers added or their absence. Complete loss of the enzymatic activity of rabbit muscle phosphorylase *b* accompanies the destruction of 4—5 histidine residues.

The content of cysteine residues of proteins was determined by the method of Ellman [20]. Essentially the same number of sulfhydryl groups in the native and photooxidized enzymes are titrated with 5,5′-dithiobis-(2-nitrobenzoic acid) in the presence of 8 M urea; 8.4 (native muscle), 8.2 (irradiated muscle), 8.2 (native potato) and 8.2 (irradiated potato), all in mol/100 000 g

TABLE I

AMINO ACID CONTENTS OF PHOTOOXIDIZED POTATO AND RABBIT MUSCLE PHOSPHORYLASE

The values (mol/100000 g protein) were calculated by assuming that glutamic acid and arginine residues were not altered during photooxidation. Photooxidized potato and muscle phosphorylase had residual activities of 4 and 27%, respectively.

	Potato		Rabbit muscle *b*	
	Native	Photooxidized	Native	Photooxidized
Lys	57.4	58.2	46.7	45.5
His	14.1	12.1	20.3	16.1
Arg	35.7	35.4	64	64
Asp	88.6	87.4	103	97.1
Thr	41.2	41.1	35.4	33.4
Ser	43.4	41.8	25.9	24.5
Glu	109	109	102	102
Pro	31.0	32.3	36.3	38.3
Gly	53.4	52.0	48.3	47.5
Ala	67.0	66.0	64.9	61.3
Val	53.8	53.2	60.1	58.2
Met	13.2	12.7	21.1	20.9
Ile	53.0	53.2	46.1	43.9
Leu	64.9	64.9	84.8	85.5
Tyr	30.6	31.1	37.1	35.0
Phe	36.6	37.3	37.9	36.2

protein. The content of tryptophan residues was determined according to the method of Matsubara and Sasaki [29]. The number of tryptophan groups is essentially the same in the native and irradiated muscle enzyme: 12 (native), 13 (irradiated without addition), 11 (irradiated with AMP) and 11 (irradiated with ATP), all in mol/100 000 g protein. Further, the irradiated muscle enzyme having 10% residual activity regains less than 6% of the original activity upon prolonged incubation with 0.1 M 2-mercaptoethanol in a nitrogen atmosphere.

TABLE II

HISTIDINE CONTENTS OF RABBIT MUSCLE PHOSPHORYLASE *b* PHOTOOXIDIZED UNDER VARIOUS CONDITIONS

The values were calculated by assuming that arginine residues were not altered in the photooxidation. See text for detailed procedure.

Addition	Illumination time (min)	Remaining activity (%)	Histidine content (mol/100000 g protein)
None	0	100	20.3
None	7	64	19.8
	15	44	19.1
	45	27	16.1
AMP	3	62	19.6
	5	46	19.3
	20	18	16.8
ATP	15	67	19.3
	35	48	18.5
	90	33	17.1

In the case of the photoinactivated potato enzyme, no restoration of the enzymatic activity was observed. These results indicate that the photoinactivation is not caused by oxidation of the cysteine, methionine and tryptophan residues in phosphorylase.

Other changes in photooxidation

In the photooxidation of muscle phosphorylase *b*, a gross change of its conformational structure is obvious, since the photooxidized enzyme tends to form aggregates and gives a fluorescence emission spectrum with a decrease at 525 nm and an increase at 410 nm as compared with that of the native enzyme, indicating the partial release of bound pyridoxal-5′-*P*. On the other hand, the photooxidized potato phosphorylase still shows the same fluorescence spectrum as that of the native enzyme, and its sulfhydryl groups all remain unreactive with 5,5′-dithiobis-(2-nitrobenzoic acid), as in the native potato enzyme [7]. It is likely that the photooxidation of potato phosphorylase is not accompanied by gross conformational change, in contrast to the case of the muscle enzyme.

Discussion

The present results indicate, together with the previous information obtained through pH kinetic studies [13—16] and the suggested preferential oxidation of histidine on photooxidation in the presence of rose bengal [28], that histidine residues are involved in the action of phosphorylase. This is further supported by the fact that potato phosphorylase is also rapidly inactivated by reaction with ethoxyformic anhydride and the original activity is completely restored on the addition of hydroxylamine to the alkylated enzyme (Kamogawa, A., unpublished results).

Although photoinactivation occurs at a comparable rate in phosphorylases from both rabbit muscle and potato tubers, the features of the oxidized products are not quite the same. The inactivation of the muscle enzyme which is not protected by substrates is accompanied by the destruction of more histidine than in the case of the potato enzyme, and by gross conformational change of the enzyme protein. The differences between both phosphorylases upon photooxidation may be attributed to the fact that the potato enzyme has more rigid structure as compared with the muscle enzyme [30,31]. On the other hand, they still have many similarities; they have approximately the same molecular weight and exist as dimers [30] having similar amino acid composition [17]. The enzymes are also similar in their kinetic mechanisms [32], a rapid equilibrium random bi bi mechanism [33]. It should be emphasized that the enzyme from potato tubers, which behaves like the muscle enzyme stripped of its regulatory apparatus, is more suitable for studies on the mechanism of action of phosphorylase, although most of the previous work on phosphorylase has been carried out only with the enzyme from rabbit muscle.

Several hypotheses can be formulated to explain the present results of the effect of allosteric modifiers on the rate of photoinactivation of rabbit muscle phosphorylase *b*. The addition of the modifier may cause a change (a) in the degree of exposure or the environment of the specific histidine residues essen-

tial for the enzyme action, or (b) in the binding of the photosensitizer, rose bengal, to the enzyme. Regarding the second hypothesis, Ullman et al. [34] showed that the binding of bromothymol blue to phosphorylase *b* was enhanced by AMP and suggested that this is indicative of a conformational change in the enzyme. It is possible that the destruction of specific amino acid residues in dye-sensitized photooxidation is mediated only by the dye which is bound to the active site of enzyme, as revealed by Rippa and Pontremoli [35] with 6-phosphogluconate dehydrogenase. Even if either of these possibilities is true, these changes may occur in association with an alteration in the quarternary structure of phosphorylase. Binding of AMP to phosphorylase *b* is accompanied by conformational changes, as well as by an enhanced tendency of the enzyme to associate into a tetramer [36—38]. IMP, unlike AMP, does not cause phosphorylase *b* to associate into a tetrameric species [20,21]. Although there is no further supporting evidence at present, the observations reported here might be important for elucidation of the mechanism of allosteric transition in phosphorylase.

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