Note

Chemical Modification of Potato Phosphorylase by α-Phthalaldehyde

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Potato α-glucan phosphorylase was readily inactivated by incubation with a fluorescent reagent, α-phthalaldehyde (OPA). The inactivation was accompanied by an increase in fluorescence intensity ascribed to the isoindole ring, which was formed by the cross-linking through the ε-amino group of Lys and the thiol group of the Cys residue. Kinetic analysis showed that 1 mol of OPA per mole of enzyme monomer was incorporated, which corresponded closely with the value obtained for the molar absorption coefficient of the isoindole ring measured by UV absorption. A great increase in the pJ from 5.5 to 6.9 was observed upon the OPA modification. Among the various substrate analogs examined, a mixture of soluble starch and P$_i$ gave the best protection of phosphorylase activity from the OPA inactivation.

α-Glucan phosphorylase (EC 2.4.1.1) contains a covalently bound pyridoxal 5'-phosphate (PLP). Recent studies clarified that the catalytic reaction of this enzyme involved a direct interaction between the two phosphate groups of the coenzyme and a substrate, α-D-glucose 1-phosphate.1,2 PLP was known to bind to Lys 680, and Lys 568 and Lys 574, which are located at the catalytic site, are important in the catalytic reaction.3-5

In our previous paper, a fluorescent reagent, OPA, was shown to be useful to modify the essential Lys residue located at the catalytic site of a fungal α-amylase, Taka-amylase A.6,7 OPA modification required a Cys residue near by because the cross-linked product (isoindole ring) was composed of the ε-amino group of Lys and the thiol group of the Cys residue.8 Formation of the isoindole ring could be monitored by UV absorption and fluorescence spectra, and the stoichiometry of the reaction was evaluated by the molar absorption coefficient of the isoindole.9 Potato phosphorylase contained 10 Cys residues per enzyme monomer, and all of them were in the reduced form.9 Some of the Cys residues might be expected to be close to the catalytic site.10

α-Glucan phosphorylase from potato tuber, purified by the method of Kamogawa et al.11 was readily inactivated by OPA at pH 6.0 (10 mM sodium maleate buffer). The inactivation proceeded irreversibly in a time-dependent manner and proceeded more rapidly at pH of 6—9 than at pH of 5—7. A cross-linking of Lys and Cys residues of potato phosphorylase was confirmed by the production of the isoindole ring, which was measured by the fluorescence at λex 338 nm and λem 405 nm. Inactivation of phosphorylase was dependent on the concentration of OPA (Fig. 1a), and a linear correlation between log values of OPA concentration and $k_{app}$, the apparent first-order rate constant for the inactivation, gave a slope of n=1.2 (Fig. 1b). A stoichiometric incorporation of the ligand gave the slope of n=1.0,12 and this also corresponded well to the stoichiometric measurement as described below.

According to the proposed reaction mechanism of OPA,8,14 a fluorescent isoindole ring via the ε-amino group of Lys and the SH group of Cys can be produced, which can also be detected by the UV absorption spectrum. Based on the molar absorption coefficient of the isoindole ring at 337 nm (7.66×10$^{-3}$ cm$^{-1}$),13 it was confirmed that about 1.25 mol of OPA were incorporated into 1 mol of potato phosphorylase monomer. The increase in the UV absorption at 337 nm reached a maximum after 120 min at 30°C (Fig. 2). Polyacrylamide gel electrophoresis showed that OPA-modified phosphorylase had a slightly higher mobility of $R_g$, 0.47 than that of the native enzyme ($R_g$, 0.42). Affinity gel electrophoresis was done with a 10% polyacrylamide gel (pH 8.3) containing 0—0.5% of substrate, soluble starch. Both the native and the modified phosphorylases migrated similarly and gave a $K_{app}$ (apparent dissociation constant) of 0.145% soluble starch. Moreover, the isoelectric point of the modified enzyme (pH 6.9) increased significantly compared with that of the native.

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**Fig. 1.** Effects of OPA Concentration on the Inactivation of Potato Phosphorylase.

The reaction mixture contained 2 mg/ml phosphorylase (50 μl) and 0—3.2 mM OPA (50 μl) in 10 mM sodium maleate buffer (pH 6.0). After incubation at 30°C, 20 μl of the mixture was diluted 20-fold with the buffer containing bovine serum albumin (0.05 mg/ml). Remaining enzyme activity was assayed as follows: the assay mixture of potato phosphorylase contained 1% soluble starch, 10 mM glucose 1-P, and 20 mM sodium maleate buffer, pH 6.0. Inorganic orthophosphate liberated was measured as described previously.13 (a) Inactivation of phosphorylase by 0 ( ), 0.2 (△), 0.4 ( ▽ ), 0.8 ( ▽ ), and 1.6 mM ( ■) of OPA. (b) Relationship between log OPA concentration (mm) and $\log k$ (apparent-first-order rate constant). The values were obtained from the graph (a).

**Fig. 2.** UV Absorption Spectra of Potato Phosphorylase-OPA Complex.

Phosphorylase, 1.25 mg/ml, was mixed with 0.1 mM OPA in 10 mM sodium maleate buffer (pH 6.0). Incubation was done at 30°C in a micro-cell. The spectrum was measured with a Shimadzu spectrophotometer UV-205.

**Abbreviations:** glucose 1-P, glucose 1-phosphate; OPA, α-phthalaldehyde; PLDP-glucose, pyridoxal 5'-diphospho-1-α-D-glucose; PLP, pyridoxal 5-phosphate; SDS, sodium dodecyl sulfate.
enzyme (pH 5.5). These results indicate that modification of potato phosphorylase with OPA is highly specific to give a homogeneous protein with distinctly different characteristics, except for the affinity for the substrate, α-glucan, from the native enzyme.

Although kinetic analysis and UV absorption spectra showed incorporation of one mole of OPA/mole subunit of potato phosphorylase, specificity of OPA modification at the active site should be demonstrated more carefully in a different aspect. Therefore, protective effects of various ligands on the inactivation of potato phosphorylase by OPA were examined (Fig. 3). The inactivation of phosphorylase was markedly inhibited by the substrates, glucose 1-P, soluble starch, and a mixture of soluble starch and P, which gave the best protection of the enzyme. In contrast, phosphate derivatives of glucose and fructose, except glucose 1-P, were less effective. It is interesting to note that pyridoxal 5′-diphospho-1-α-D-glucose (PLDP-glucose) was more effective in retarding the inactivation than the intrinsic substrates. Similar to the case of soluble starch, 10 mM mallose and γ-cyclodextrin gave better protection in the presence of 10 mM P (data not shown).

Inactivation of potato phosphorylase by OPA proceeded faster at pH 5.0 than at pH 6.0, though at pH 7—9 loss of activity increased according to the increase in pH. However, the increase in the fluorescence intensity of the isoindole ring was proportional to the increase in pH from 5 to 9. These observations might be ascribed to the pH stability of potato phosphorylase.

In this study, OPA-inactivation of potato phosphorylase was greatly retarded by substrates, especially by a mixture of soluble starch and P (Fig. 3). About 4.5-fold smaller kcorr obtained with the above mixture of substrates may indicate the specificity of OPA modification for particular Lys residue(s). Lys 574, at the catalytic site, Stoichiometric inactivation of OPA-modified phosphorylase shown in Figs. 1 and 2 also supported the idea of the specificity of OPA modification. Lys 574 is a possible candidate for the current OPA modification of potato enzyme.

References