Non-enzymic protein phosphorylation

Phosphorylation of 6-phosphogluconate dehydrogenase by acyl phosphates

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Incubation of 6-phosphogluconate dehydrogenase from *Candida utilis* with either acetyl phosphate, 1,3-diphosphoglycerate or carbamoyl phosphate results in the phosphorylation of the protein. The binding of one phosphate residue per enzyme subunit does not affect significantly the kinetic properties, but makes the enzyme less reactive toward thiol reagents, trypsin and pyridoxal 5'-phosphate. We suggest that the group involved in the binding of phosphate is a histidine residue. These results indicate that: (1) 6-phosphogluconate dehydrogenase from *C. utilis* is phosphorylated non-enzymically by physiological acyl phosphates and (2) the phosphorylation of the enzyme modifies the rate of protein inactivation.

Protein phosphorylation is a widely occurring process. The best known phosphoproteins can be divided in two groups: the first comprises the enzymes which catalyse the transfer of phosphoryl groups, where the phosphoprotein occurs as catalytic intermediate (Knowles, 1980); the second group comprises the proteins which are phosphorylated by protein kinases (Krebs & Beavo, 1979). This latter group is continuously increasing, and a great deal of work has been performed in this field, since the protein-kinase-dependent phosphorylation of the enzymes appears as one of the major routes of metabolic control. We now report that some physiological acyl phosphates can directly phosphorylate, under mild conditions, the 6-phosphogluconate dehydrogenase from *Candida utilis*.

Materials and methods

6-Phosphogluconate dehydrogenase [6-phospho-D-gluconate:NADP⁺ 2-oxidoreductase (decarboxylating), EC 1.1.1.44] was prepared and assayed as described (Rippa & Signorini, 1975). The crystalline enzyme was freed from (NH₄)₂SO₄ by dialysis against 50 mM-triethanolamine/HCl (pH 7.5)/0.1 mM-EDTA. Acetyl phosphate, carbamoyl phosphate, 6-phosphogluconate, NADP⁺, ATP, 3-phosphoglycerate, 3-phosphoglycerate kinase and creatine phosphate were purchased from Boehringer. Anion exchange resin PBE 94 and buffer PB 74 were from Pharmacia.

Enzyme phosphorylation

The enzyme (160 μ M in subunits) dissolved in

50 mM-triethanolamine buffer, pH 7.5, was treated with 2 mM-acetyl phosphate or carbamoyl phosphate. After a short incubation time (10 min) at room temperature, a small sample of the incubation mixture (20 nmol of subunits) was passed through a column (1.4 cm $\times$ 25 cm) of Sephadex G-25 equilibrated in 25 mM-triethanolamine buffer, pH 7.5, to remove the excess reagents. The protein was collected and precipitated with trichloroacetic acid [final concn. 5% (w/v)]. The precipitated protein was washed three times with 5% (w/v) trichloroacetic acid and assayed for phosphate content as described (Tashima & Yoshimura, 1975).

Phosphorylation by 1,3-bisphosphoglycerate was carried out by adding to 1 ml of enzyme solution (160 μ M in subunits) in 50 mM-triethanolamine buffer, pH 7.5, the following components: phosphoglycerate kinase (5 units), ATP (50 mM), 3-phosphoglycerate (10 mM) and MgCl₂ (10 mM). After 1 h of incubation, the enzyme was isolated by the procedure described above. Experiments lacking 3-phosphoglycerate, or 3-phosphoglycerate kinase, or ATP, were run in parallel as controls.

Separation of phosphorylated from non-phosphorylated enzyme

The native and the phosphorylated enzymes were separated on a column (0.4 cm $\times$ 2 cm) of PBE 94 equilibrated in 25 mM-triethanolamine (pH 7.5)/0.15 M-NaCl, and elution was performed with PB 74 buffer, pH 5, diluted 1:8, and containing 0.15 M-NaCl, to give a linear pH gradient from pH 7 to pH 5. A small sample of the phosphorylation mixture (corresponding to 0.3 mg of protein) was

chromatographed and the eluate was assayed for enzymic activity.

Properties of the phosphorylated enzyme

The untreated and phosphorylated enzymes were inactivated, by the described procedure, with periodate (Rippa *et al.*, 1978a), 5,5'-dithiobis-(2-nitrobenzoate) (Rippa *et al.*, 1978b), pyridoxal 5'-phosphate (Rippa *et al.*, 1967), diethylpyrocarbonate (Rippa *et al.*, 1972), and trypsin (Rippa *et al.*, 1981).

Results and discussion

Incubation of 6-phosphogluconate dehydrogenase with either carbamoyl phosphate, acetyl phosphate or 1,3-bisphosphoglycerate results in the incorporation of a phosphate group into the protein (Table 1). The rate of phosphorylation is time dependent; when the protein was tested for phosphate content immediately after addition of acyl phosphate, the phosphate assay was negative; however, after 10 min of incubation a nearly stoichiometric amount of organic phosphate is bound to the protein. The time required for the assay of the bound phosphate (see the Materials and methods section) does not allow a detailed kinetic analysis of the process. One molecule of phosphate is incorporated per subunit of enzyme; neither higher concentration of acyl phosphates, nor longer incubation times, nor a second treatment with a different acylphosphate, yielded higher incorporation. However, when the phosphorylation was performed with 1,3-bisphosphoglycerate, a lower incorporation of phosphate was observed. This result could be due to the low concentration of 1,3-bisphosphoglycerate in the reaction mixture, or to the presence of high concentrations of organic phosphates, which could have a protective effect toward phosphorylation. The phosphoenzyme

releases the bound phosphate in about 1 week at 4°C. The possibility of a non-covalent tight binding of the reagents to the protein was ruled out by the following findings: (a) the incorporation of phosphate requires a short but measurable time of incubation; (b) the phosphate group is still bound to the protein after denaturation with 5% (w/v) trichloroacetic acid; (c) the protein retains the bound organic phosphate under conditions that totally hydrolyse the acyl phosphate used. Further evidence that the phosphate is covalently bound to the protein comes from chromatography on PBE 94. Enzyme incubated with acyl phosphates elutes at a significantly lower pH than does native enzyme (Fig. 1). This indicates that the modified enzyme has an isoelectric point lower than the native enzyme, as would be expected from the presence of a negatively charged phosphate group.

Control experiments have shown that the presence of phosphate does not affect the chromatographic behaviour of the protein. The elution pattern of the modified enzyme is asymmetric. This could be explained by the presence of two species: one with a single phosphate group, the other, preponderant, with two phosphate groups per dimeric enzyme. Other high-energy phosphates, as ATP, GTP and creatine phosphate, under our experimental conditions, are unable to phosphorylate the enzyme. This indicates a chemical specificity for the reaction, and also rules out a possible contamination of the enzyme preparations by a protein kinase. Our data indicate a direct phosphorylation of an enzyme by physiologically occurring metabolites.

Table 1. Phosphorylation of 6-phosphogluconate dehydrogenase by high energy phosphate compounds

Enzyme (160 μ M) dissolved in 50 mM-triethanolamine buffer, pH 7.5, was incubated for 10 min with a 10-fold excess of each phosphoryl donor. The protein was then passed through a Sephadex G-25 column, precipitated with 5% (w/v) trichloroacetic acid and assayed for phosphate content.

Phosphoryl donor	Phosphate bound (mol/mol of subunit)
None	0
Acetyl phosphate	0.98
Carbamoyl phosphate	0.96
1,3-Bisphosphoglycerate	0.56
ATP	0
Creatine phosphate	0

Fig. 1. Separation of phosphorylated from non-phosphorylated enzyme

Native and acetyl phosphate-treated enzyme were separated on PBE 94 as described in the Materials and methods section. O, native enzyme; ●, phosphorylated enzyme.

Properties of the phosphorylated enzyme

The effect of phosphorylation on 6-phosphogluconate dehydrogenase has been investigated for its kinetic and chemical properties. The phosphorylation has no effect on the $V_{\max}$ and on the K_m for NADP⁺; the most significant difference between native and phosphorylated enzyme is the increased value (from 100 μM to 200 μM) of the K_m for the substrate 6-phosphogluconate. These results make rather unlikely a role of the phosphorylation in the modulation of enzymic activity. Marked differences have instead been observed in the behaviour of native and phosphorylated enzyme toward some inactivating agents. The rates of inactivation by the thiol reagents, periodate and 5,5'-dithiobis-(2-nitrobenzoate), are greatly decreased upon phosphorylation, although the number of titratable thiol groups is unchanged (Fig. 2). Another inactivator of 6-phosphogluconate dehydrogenase, pyridoxal 5'-

phosphate, shows a lower rate and extent of inactivation with the phosphoenzyme as compared with the native enzyme (Fig. 3a). Moreover, the inactivation of the enzyme by tryptic digestion is slowed by phosphorylation (Fig. 3b). These data indicate that phosphorylation increases the stability of the protein, most likely via a conformational change.

Identification of the amino acid phosphorylated

At the present we have only indirect evidence for the site of phosphorylation. The phosphoenzyme is stable in neutral and basic solution, but is hydrolysed in acid solutions (Table 2). These findings indicate a basic amino acid, i.e. histidine, lysine or arginine, as the site of phosphorylation. Histidine residues were titrated with diethylpyrocarbonate in the native and phosphorylated enzyme, as shown in Fig. 4; in the phosphorylated enzyme only 10 of the

Fig. 2. Inactivation of 6-phosphogluconate dehydrogenase by sulphydryl reagents

Native and acetyl phosphate-treated enzyme were inactivated (a) with 5,5'-dithiobis-(2-nitrobenzoate) and (b) with periodate. ○, native enzyme; ●, phosphorylated enzyme.

Fig. 3. Reactivity of native and acetyl phosphate-treated enzyme

(a) Enzyme inactivation by pyridoxal 5'-phosphate; (b) enzyme inactivation by trypsin. ○, native enzyme; ●, phosphorylated enzyme.

Table 2. Hydrolysis of the phosphorylated enzyme
The phosphorylated enzyme (10 nmol of subunit) was treated as indicated in the Table. Inorganic phosphate was assayed in the supernatant after neutralization of the solution and precipitation of the protein.

Experimental conditions	pH	Phosphate released (%)
1 h, 38°C	5	10
1 h, 38°C	8.6	—
1 h, 110°C	0.5 M-HCl	100
1 h, 110°C	0.5 M-NaOH	Trace
5 min, 100°C	1 M-HCl	100

Fig. 4. Titration of histidine residues with diethylpyrocarbonate

Native and acetyl phosphate-treated enzyme were treated with diethylpyrocarbonate as described (Rippa *et al.*, 1972). ○, native enzyme; ●, phosphorylated enzyme.

12 residues present were titratable, with a loss of one histidine residue per subunit. These data indicate that the amino acid phosphorylated could be a histidine residue. However, the isolation and characterization of the amino acid phosphorylated is needed for an unequivocal identification.

Phosphorylation of other enzymes

Aldolase, glycerol-3-phosphate dehydrogenase and glutamate dehydrogenase were treated with acetyl phosphate and carbamoylphosphate as described for 6-phosphogluconate dehydrogenase. Only traces of phosphate were recovered with the proteins, indicating that, under our experimental conditions, these enzymes were not phosphorylated. Thus the phosphorylation is not a widespread, non-specific reaction of acyl phosphates, but a characteristic of the 6-phosphogluconate dehydrogenase from *C. utilis*.

Conclusions

We have shown that *C. utilis* 6-phosphogluconate dehydrogenase is phosphorylated non-enzymically by some acyl phosphates. All cells

contain acyl phosphates; it might well be that these compounds also have the role of phosphorylating some proteins and thus modifying their properties. Studies are needed to check which other proteins can be phosphorylated non-enzymically and to investigate a possible physiological role of this phosphorylation.

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