Significance of Phosphorylation of Phosphofructokinase*

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In order to understand the effect of phosphorylation on phosphofructokinase, the allosteric kinetic behavior, ligand binding at various pHs, and pH-dependent cold inactivation of phosphofructokinase phosphorylated to different extents were studied. A subtilisin-digested phosphofructokinase from which a COOH-terminal peptide containing a phosphorylation site has been cleaved (Riquelme, P. T., and Kemp, R. G. (1980) J. Biol. Chem. 255, 4367-4371) was also included in these studies in order to investigate the possible role of this region of the molecule. Allosteric kinetics and direct binding experiments have shown that increasing phosphorylation of phosphofructokinase results in increased sensitivity to ATP inhibition and strong binding of ATP to the inhibitory site of the enzyme. The subtilisin-cleaved phosphofructokinase is the least sensitive to the inhibition and shows the weakest binding of ATP. The opposite effect is observed with the binding isotherms of fructose-6-P. There is no difference in the binding of fructose-2,6-P₂ among these enzymes. Binding of ATP to the inhibitory site of these enzymes as determined by fluorescence quenching (Pettigrew, D. W., and Frieden, C. (1979) J. Biol. Chem. 254, 1887-1895) is affected by pH; the binding is greatly enhanced at lower pH. Moreover, there is little difference in the binding among the modified enzymes at pH 8, but at lower pHs the binding to the phosphorylated enzyme is much more enhanced than the dephosphoenzyme. A pH-dependent cold inactivation study has shown that the phosphorylation of the enzyme causes an increase in the pH value for the inactivation, and the extent of the pK shift depends upon the degree of phosphorylation. Based on these results, a model originally proposed by Frieden et al. (Frieden, C., Gilbert, H. R., and Bock, P. E. (1976) J. Biol. Chem. 251, 5644-5647) can be applied to explain a possible role for the phosphorylation and the peptide portion of phosphofructokinase in its complex allosteric kinetic behavior.

Muscle phosphofructokinase is phosphorylated in vivo (1-4) and in vitro (5). The site of in vitro phosphorylation by cAMP-dependent protein kinase has been determined near the COOH terminus of the enzyme (6). Riquelme and Kemp (7) have shown that this COOH-terminal peptide is susceptible to proteolysis and a limited proteolysis by subtilisin leads to cleavage of a peptide with 33 to 35 amino acids. Liver phosphofructokinase is also known to be phosphorylated in vivo (8) and in vitro (9).

The effect of phosphorylation of phosphofructokinase on its activity has not been well established. The muscle enzyme has been separated into low and high phosphate forms (2, 4), but the phosphate content of even the high phosphate form is less than a stoichiometric amount. There is no difference in the maximum catalytic activity between these two forms (2, 4). However, in some preparations we have observed differences in the allosteric kinetic properties, but these differences were small (4). We have also separated liver phosphofructokinase into low and high phosphate forms (10). The phosphate contents of these enzymes were much higher than those of muscle phosphofructokinase, and they contain approximately 0.3 and 0.8 mol/mol of subunit (10). Moreover, the high phosphate form of the liver enzyme showed a higher Kₚ₅ for fructose-2,6-P₂ than the low phosphate form as determined by kinetic measurements (10).

More recently Foe and Kemp (11) have prepared a fully phosphorylated muscle phosphofructokinase by treating the "native" enzyme with cAMP-dependent protein kinase and a dephosphorylated enzyme by the action of alkaline phosphatase. They showed that the phosphorylated muscle enzyme is more sensitive to inhibition by ATP and citrate, and it is less sensitive to activation by fructose-2,6-P₂ and AMP than the dephosphoenzyme. We have recently purified the low and high phosphate forms of liver enzyme to homogeneity (12) and shown qualitatively similar differences in their kinetic behaviors as compared to those of muscle enzyme. In addition, we have shown that the high phosphate form of liver phosphofructokinase shows a higher Kₚ₅ for fructose-6-P than low phosphate form and the difference in the Kₚ₅ values becomes greater at lower pHs. These results suggested to us that phosphorylation may induce a change in enzyme conformation as a result of altered ionization of a residue(s) which causes increased binding of negative effector to the enzyme. This explanation is similar to a model originally proposed by Bock and Frieden (13) in which ATP binds to a protonated form of muscle phosphofructokinase preferentially while fructose-6-P binds to an unprotonated form.

In order to obtain additional information on the role of the phosphorylation of phosphofructokinase, the kinetic behavior, direct ligand binding, and cold inactivation of four modified phosphofructokinases were investigated. These enzymes include phospho-, partially phosphorylated, dephospho-, and a subtilisin-cleaved phosphofructokinase. It will be shown that these modifications affect primarily ATP binding to the inhibitory site with little or no effect on the binding of fructose-6-P or fructose-2,6-P₂. It will be further shown that the pH-dependent ligand binding and cold inactivation phenomena differ depending upon the degree of phosphorylation.

MATERIALS AND METHODS

[γ-³²P]ATP (3000 Ci/mmol) was purchased from Amersham Corp. The catalytic subunit of cAMP-dependent protein kinase, bovine intestinal alkaline phosphatase, and subtilisin (Type VIII) were purchased from Sigma. [U-¹⁴C]Fructose 2,6-bisphosphate was prepared...
enzymatically as described previously (14, 15). All other chemicals were reagent grade and obtained from commercial sources. Rabbit muscle phosphofructokinase was purified as described previously (4) and purified further on a N6-[6-aminohexyl]-carbamoylmethyl]-ATP-Sepharose column (16). The phosphate content of the enzyme was determined in an ATP assay containing 0.2 mol of P/mmol of subunit and will be referred to as the "native" enzyme.

**Assay Methods for Phosphofructokinase**—The activity of phosphofructokinase was assayed at optimum (Vmax) assay conditions. The assay mixture contained in a final volume of 1 ml: 50 mM Tris-Cl, pH 8.0, 0.1 mM EDTA, 5 mM MgCl2, 4 mM ammonium sulfate, 2.5 mM dithiothreitol, 0.16 mM NADH, 1 mM each of fructose-6-P and ATP, aldolase (0.4 unit), triose-P-isomerase (2.4 unit), and glycerophosphorylase (0.4 unit). The reaction was initiated by addition of phosphofructokinase, and the decrease in absorbance at 340 nm was measured at 25 °C. One unit is defined as the amount of the enzyme that catalyzes the formation of 1 pmol of fructose-1,6-P2/ min.

Allosteric kinetic properties of phosphofructokinase were determined in 1 ml of a reaction mixture containing 50 mM Hepes, pH 7.4, 0.2 mM EDTA, 5 mM MgCl2, 1 mM NH4Cl, 0.16 mM NADH, 2.5 mM dithiothreitol, indicated amounts of fructose-6-P and ATP, and adenyllyl cyclase (2.4 units), with a final volume of 9 ml: phosphofructokinase (3 mg/ml), the enzyme that catalyzes the formation of ATP and fructose-6-P. All binding experiments were performed in 50 mM Hepes, pH 7.4, 0.2 mM EDTA, 5 mM MgCl2, and 1 mM dithiothreitol with a linear gradient of 0.05 to 0.4 M Tris-Cl, pH 8.0, and the enzyme was subjected to gel filtration on Sephacryl S300 and the phosphorylated phosphofructokinase was 0.7 ± 0.05 mol of P/mol of subunit.

**Dephosphorylation of Phosphofructokinase**—The conditions for the dephosphorylation of phosphofructokinase were the same as those described for the native enzyme without any further treatment. "Phospho-" or "dephospho-" phosphofructokinase was after treatment of the enzyme with protein kinase or alkaline phosphatase, respectively. "Subtilisin-cleaved" phosphofructokinase is referred to the phosphofructokinase as isolated from skeletal muscle without any further treatment. "Phospho-" or "dephospho-" phosphofructokinase was after a limited proteolysis with subtilisin.

**Phosphorylation of Phosphofructokinase**—The reaction mixture contained a final volume of 5 ml: phosphofructokinase (3 mg/ml), 50 mM Tris/P, pH 8.0, 0.5 mM ATP, 5 mM MgCl2, 1 mM EDTA, and 1 mM dithiothreitol. The reaction was initiated with addition of catalytic subunit of cAMP-dependent protein kinase (10,000 units), and the reaction mixture was incubated at 30 °C for 30 min. It was demonstrated that maximum phosphorylation was achieved within 45 min under those conditions. In some experiments [γ-32P]ATP was used. The phosphorylated phosphofructokinase was precipitated with ammonium sulfate (70% saturation) and separated from protein kinase by gel filtration using a Sephacryl S300 column (1.5 × 50 cm) which had been equilibrated with 50 mM Tris/P, pH 8.0, 1 mM EDTA, and 1 mM dithiothreitol. The phosphate content of the phosphorylated phosphofructokinase was 0.7 ± 0.05 mol of P/mol of subunit.

**Cold Inactivation of Phosphofructokinase**—Inactivation of various phosphofructokinases at low temperature was measured by the procedure of Bock and Frieden (13). Phosphofructokinase (0.1 mg/ml) in 0.1 M Na phosphate, 1 mM EDTA, and 1 mM dithiothreitol at pH ranging from 6.0 to 8.0 was incubated at 6 °C for 3 h. The residual activity of the enzyme was determined immediately after diluting the enzyme in 50 mM Tris/P, pH 8.0, 1 mM EDTA, and 1 mM dithiothreitol.

**Polyacrylamide Gel Electrophoresis**—Polyacrylamide slab gel electrophoresis was carried out in a slab gel of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate. Phosphofructokinases are summarized in Table 1. The specific activities and phosphate content of these phosphofructokinases are summarized in Table I. The specific activities of these enzymes are very similar although their phosphate contents differ. The native enzyme contains 0.2 mol of P/mol of subunit which is the same as the so-called higher P form of the enzyme reported previously (4). The phosphate contents of phospho and dephospho-forms are similar to those reported by Foe and Kemp (11). As shown in Fig. 1A, all the enzymes except the subtilisin-digested phosphofructokinase show the same electrophoretic mobility with estimated M, of approximately 80,000 to 82,000. The M, of the subtilisin-treated phosphofructokinase is reduced by approximately 2,000 (lanes 4 and 5) similar to the first cleavage product reported by Rijkel and Kemp (7). When these enzymes were phosphorylated by the catalytic subunit of cAMP-dependent protein kinase in the presence of [γ-32P]ATP, the radioactivity was incorporated into only the native
of the enzyme activity and the phosphate content were as described under "Materials and Methods." The values are an average of three preparations each. ND, not determined.

### Table I

| Phosphofructokinase | Specific Activity | Phosphate Content |
|---------------------|------------------|------------------|
| Native              | 130 ± 10         | 0.2 ± 0.02       |
| Phospho-            | 127 ± 8          | 0.7 ± 0.05       |
| Dephospho-          | 134 ± 3          | 0.06 ± 0.01      |
| Subtilisin-cleaved  | 120 ± 5          | ND               |

The assay mixture and conditions were as described under "Materials and Methods" except in A fructose-6-P (Fru-6-P) was 1 mM, and in B, ATP was 0.5 mM. Phospho- (●), native (○), dephospho- (△), and subtilisin-cleaved (△) enzymes (15 milliunits in A and 8 milliunits in B) were used.

Fig. 1. SDS-polyacrylamide gel electrophoresis of various phosphofructokinases. A, native (lane 1), dephospho- (lane 2), phospho- (lane 3), and subtilisin-digested (lane 5) phosphofructokinases at 1 µg each were analyzed by SDS-polyacrylamide gel electrophoresis. The mixture of the native and the subtilisin-digested enzymes is shown in lane 4. The molecular weight standards on lane 6 are from top to bottom: myosin (200,000), β-galactosidase (116,000), phosphorylase b (92,500), bovine serum albumin (68,000), and ovalbumin (45,000). B, a radioautograph showing the degree of phosphorylation of modified phosphofructokinases by protein kinase. The reaction mixture contained 10 and 40 µg of phosphofructokinase, 0.4 mM [β-32P]ATP (1000 cpm/pmol) and 4 mM MgCl₂ at pH 8 in a final volume of 25 µl. The reaction was initiated with addition of 3000 units/ml of catalytic subunit of cAMP-dependent protein kinase and incubated at 30 °C for 1 h. The phosphorylated enzymes (10 and 40 µg, respectively) were subjected to SDS-gel electrophoresis and the gel was autoradiographed on a Kodak X-Omat AR film. Lanes 1 and 2, dephospho-; lanes 3 and 4, phospho-; lanes 5 and 6, native, and lanes 7 and 8, subtilisin-digested.

Fig. 2. Initial velocity as a function of ATP (A) and fructose-6-P (B). The assay mixture and conditions were as described under "Materials and Methods" except in A fructose-6-P (Fru-6-P) was 1 mM, and in B, ATP was 0.5 mM. Phospho- (●), native (○), dephospho- (△), and subtilisin-cleaved (△) enzymes (15 milliunits in A and 8 milliunits in B) were used.

Phosphorylated and that the phosphorylation site of the latter has been cleaved by subtilisin.

Sedimentation velocity studies of phospho-, dephospho-, and the subtilisin-cleaved phosphofructokinases were performed under the same conditions as ligand binding (protein concentration at 2 mg/ml). The schlieren patterns of these enzymes are very similar, containing three peaks with apparent sedimentation coefficients of 12, 18, and 32 S. However, when the schlieren patterns were analyzed with a curve resolver as described previously (4), it became apparent that a 24 S polymer is present in all the samples and also the phosphoenzyme has somewhat higher concentrations of both 30 S and 24 S polymers compared to the dephospho- or the digested phosphofructokinase. However, these differences in the polymer compositions are small enough so that the interpretation of other results described later would not be affected.

**Allosteric Kinetics with Respect to ATP and Fructose-6-P—**

The effect of increasing ATP concentration on the activities of phosphofructokinase with varying degrees of phosphorylation was compared. As shown in Fig. 2A, the phosphoenzyme is most sensitive to the ATP inhibition and the dephosphoenzyme is the least sensitive among the phosphorylated phosphofructokinases. It is interesting that the native enzyme whose P content falls in between the phospho- and dephospho-forms show an intermediate sensitivity to the ATP inhibition. The difference between the dephospho- and the native enzymes is small, but reproducible. The subtilisin-cleaved phosphofructokinase is the least sensitive among all those enzymes. These results obtained with phospho- and dephospho-forms are similar to those of Fee and Kemp (11) with muscle enzyme. They are also similar to the results obtained with low and high P forms of liver phosphofructokinase (12).

The effect of varying fructose-6-P at constant ATP concentration (0.5 mM) on the activities of these phosphofructokinases is shown in Fig. 2B. The subtilisin-cleaved phosphofructokinase has the lowest Kₜ, for fructose-6-P, and the Kₜ increases with increasing degree of phosphorylation of phosphofructokinase. Moreover, the kinetic behavior of the digested phosphofructokinase is essentially hyperbolic while it becomes increasingly sigmoidal with increasing phosphorylation. The Hill coefficients calculated from the results in Fig. 2B are 1.5, 2.1, 2.1, and 2.6 for the digested, dephospho-, native, and P-phosphofructokinase, respectively.

Thus, increasing phosphorylation of phosphofructokinase results in the enhanced ATP inhibition and their response to fructose-6-P becomes highly cooperative. Since in the above kinetic studies both ATP and fructose-6-P are present in the same reaction, it is difficult to determine whether phosphorylation affects the binding of both ligands or one more than the other. In order to distinguish between these possibilities, direct binding of ATP, fructose-6-P, and fructose-2,6-P₂ to various phosphofructokinases were compared. Fig. 3, A and B, shows Scatchard plots of ATP and fructose-6-P binding, respectively. Kₜ values calculated from the plots are summa-
Phosphofructokinase 13295
BOUND/FREE ATP
FIG. 3. Binding of [γ-32P]ATP (A) and [14C]fructose-6-P (Fru-6-P) (B) was determined in 25 mM glycylglycine, pH 7.4, 25 mM β-glycer-P, 1 mM EDTA, and 1 mM dithiothreitol by the fast-flow equilibrium dialysis method as described under "Materials and Methods". In A 2.4 mg/ml (30 μM) phosphofructokinase protomer and in B 2.0 mg/ml (25 μM) protomer were used, and the results are presented as Scatchard plots. Symbols used are the same as in Fig. 2 except that contained native enzyme and 30 μM fructose-2,6-P₂.

TABLE II
Comparison of ligand binding to various phosphofructokinases
The K_d values were calculated from the data in Fig. 2.

| Phosphofructokinase     | Fructose-6-P | ATP |
|-------------------------|--------------|-----|
| Phospho                 | 12.5         | 26  |
| Native                  | 9.0          | 42  |
| Native plus fructose-2,6-P₂ (30 μM) | 85          |     |
| Dephospho               | 9.0          | 50  |
| Subtilisin-cleaved      | 7.8          | 80  |

rized in Table II. The Scatchard plot in Fig. 3B shows that fructose-6-P binding to all four forms of phosphofructokinase is linear under these conditions, and there is no evidence that the binding is cooperative. The K_d value, however, appears to increase from 8 to 12.5 μM with increasing phosphate content of the enzyme although the difference is small. These differences in the K_d values are consistent with the kinetic results shown in Fig. 2B in which phosphorylation increases the K_d for fructose-6-P.

Phosphofructokinase has been shown to have three binding sites for MgATP or ATP (20, 25). These sites have been identified as an active site, an inhibitory site, and an activator site. According to Pettigrew and Frieden (20) ATP binds to the inhibitory site 10x more strongly than MgATP. Since we are interested in the difference in the inhibitory sites of various phosphorylated phosphofructokinases, the binding of ATP rather than MgATP was studied. Moreover, a complication that the enzyme has an intrinsic ATPase activity which uses MgATP as a substrate (26) can be overcome with the use of ATP. As shown in Fig. 3A, ATP binding to at least two binding sites is clearly seen and possibly a third site in the case of the phospho-phosphofructokinase. This third site ("3") is presumably the catalytic site because it has the highest affinity, and the second and first sites (2 and 1) are probably the inhibitory and the activator sites, respectively. The K_d values for the inhibitory site ("2"), estimated from the plot and summarized in Table II, indicate that subtilisin-cleaved phosphofructokinase has the highest value and the phosphoenzyme has the lowest. The dephosphoenzyme has nearly 2x higher K_d than that of phosphoenzyme. It is also shown that fructose-2,6-P₂ affects the ATP binding although fructose-2,6-P₂ binding to these enzymes is the same. An addition of 30 μM fructose-2,6-P₂ to the native enzyme (Fig. 3A, black square) causes 2x increase in the K_d for ATP. Thus, these results demonstrate that phosphorylation of phosphofructokinase results in an increased affinity for ATP at the inhibitory site and decreased affinity for fructose-6-P.

Interaction with Fructose-2,6-P₂—Fig. 4 shows the binding of fructose-2,6-P₂ to various phosphofructokinases as determined by a column centrifugation method (15). Symbols are the same as in Fig. 2. PFK, phosphofructokinase.

When the binding of fructose-2,6-P₂ to these enzymes was compared in the presence of varying concentrations of ATP,
FIG. 5. Binding of [14C]fructose-2,6-P_2 (Fru-2,6-P_2) in the presence of varying concentrations of MgATP was determined as in Fig. 4. The Mg^{2+} concentration was 1.5 times that of ATP. The binding of fructose-2,6-P_2 in the presence of MgATP was expressed as a percentage, and 100% represents the binding in the absence of MgATP. The actual amounts of fructose-2,6-P_2 bound/mol of protomer for native, phospho-, dephospho-, and subtilisin-digested enzymes, respectively.

as shown in Fig. 5, the differences in the effect of ATP on the fructose-2,6-P_2 binding among these enzymes become apparent. ATP is most effective in inhibiting the binding of fructose-2,6-P_2 to phospho-phosphofructokinase and least effective for the cleaved enzyme. This effect can be explained by the differences in their pH-dependent ATP binding according to the extent of phosphorylation.

pH-dependent ATP Binding—Pettigrew and Frieden (20) demonstrated that ATP binding to the inhibitory site results in quenching of the intrinsic fluorescence of phosphofructokinase and that its binding is increased at lower pH. We have compared the effect of pH on the ATP binding to phospho-, native, and subtilisin-cleaved phosphofructokinase. The results are shown in Fig. 6, and calculated $K_{d}$ values for ATP are summarized in Table III. The results obtained with dephosphoenzyme (not shown) are very close to those of the native enzyme. All these modified enzymes bind ATP more strongly at lower pHs as compared to pH 8.0. For example, the apparent $K_{d}$ for ATP of phospho-phosphofructokinase decreases from 195 μM at pH 8 to 1.5 μM at pH 6.9. Phospho-phosphofructokinase has the highest affinity (1.5 μM at pH 6.9), native phosphofructokinase has intermediate (1.8 μM), and the cleaved enzyme has the weakest apparent affinity (2.5 μM) at all pHs tested. The difference in the binding among these enzymes is very small at pH 8.0 but at lower pHs this difference becomes greater. Thus, these results suggest that the phosphorylation of the protein causes a shift in pK of ionization of specific residue(s) which affects the ATP binding at its inhibitory site.

pH-dependent Cold Inactivation—pH-dependent inactivation of muscle phosphofructokinase at low temperature and the effect of various ligands on this process have been extensively investigated (27, 28). Since the data presented so far suggest that phosphorylation of phosphofructokinase causes

| Phosphofructokinase       | ATP, apparent $K_{d}$ |
|---------------------------|-----------------------|
|                           | pH 6.9    | pH 7.4    | pH 8.0    |
| Phospho                  | 1.48      | 17.8      | 195       |
| Native (O)               | 1.78      | 22.4      | 200       |
| Dephospho (△)            | 1.88      | 24.8      | 200       |
| Subtilisin-cleaved (A)   | 2.51      | 31.6      | 208       |

FIG. 7. pH dependence of the residual activity of modified phosphofructokinases. The enzyme (0.1 mg/ml) was incubated at 6°C for 3 h in 100 mM NaP, 1 mM EDTA, and 1 mM dithiothreitol at the indicated pH. After incubation, suitable aliquots were transferred into 50 mM Tris/P, pH 8, 1 mM EDTA, and 1 mM dithiothreitol, and the residual activity was immediately measured. There was no reactivation of the enzyme during this short period of dilution in the Tris/P buffer. The enzyme activity was determined as described under "Materials and Methods."
a shift in pK, similar to those induced by ATP, it is of interest to determine its effect on the cold lability. We have compared the effect of varying pH on the cold lability of four phosphofructokinases under the conditions similar to those described by Bock and Frieden (28). As shown in Fig. 7, the apparent pK of the dephosphoenzyme at 6.78 is shifted to higher values by Bock and Frieden (28). As shown in Fig. 7, the apparent pK of 6.65 which is lower than the dephosphofructokinase or any of the phosphorylated enzymes. The shape of these curves are similar and the curves are all highly cooperative. These results suggest that the equilibrium between protonated and unprotonated forms of phosphofructokinase is influenced by the phosphorylation as well as the COOH terminus peptide containing the phosphorylation site. The effect of the peptide and phosphorylation tend to shift the equilibrium to a higher pK so that at a given pH, phosphofructokinase occurs as a more protonated form than the dephospho-form.

**DISCUSSION**

The results presented here provide some insight into the roles of phosphorylation and the COOH-terminal peptide containing the phosphorylation site of phosphofructokinase. The kinetic evidence obtained with muscle (11) and liver (12) phosphofructokinase previously as well as in Fig. 2 shows that the phosphorylated phosphofructokinase is more sensitive to inhibition by ATP and less sensitive to activation by fructose-2,6-P2. The direct binding studies presented here demonstrate that the binding of ATP to the inhibitory site is significantly increased by phosphorylation of the enzyme, in agreement with the kinetic data. However, in contrast to the kinetic result, the binding of fructose-2,6-P2 is not affected by the phosphorylation or even by the removal of the peptide containing the phosphorylation site. The difference in the observed K_m, of fructose-2,6-P_2 by kinetic measurements was due to the presence of MgATP in the reaction mixture. Thus, we may conclude that the phosphorylation of phosphofructokinase affects primarily the binding of ATP to its inhibitory site and has little effect on that of fructose-2,6-P_2.

The second important aspect of the effect of phosphorylation on phosphofructokinase is demonstrated in the effect of pH on ATP binding and cold lability. It was shown that the binding of ATP to the inhibitory site is strongly pH-dependent. ATP binding on phospho-phosphofructokinase significantly increased at lower pH in the phosphoenzyme as compared to that of the dephospho-or the cleaved form (Fig. 6). Moreover, phosphorylation of the enzyme results in an increased pK of the inactivation curve at low temperatures, while the cleavage of the peptide from the enzyme causes a shift in pK in the opposite direction (Fig. 7). Thus, the effects of the phosphorylation and the protodetic cleavage of the COOH-terminal peptide of phosphofructokinase on the enzyme are similar to those observed with pH-dependent cold inactivation and ligand binding of phosphofructokinase by Frieden and coworkers (13, 27, 28). Based on their extensive studies on the pH- and temperature-dependent inactivation, ligand binding, and kinetic behavior of phosphofructokinase, they have presented a model to describe the allosteric behavior of the enzyme. They proposed that there are two classes of enzyme conformation, one in which a specific residue(s) is protonated and the other in which it is not. These two forms are in equilibrium and differ in their ability to bind different ligands. ATP binds to the protonated form while fructose-6-P and activators bind to the unprotonated form. It appears that the effect of phosphorylation and the cleavage of the specific peptide on the cold lability and the ATP binding could be also explained by the model. We suggest that the phosphorylation of phosphofructokinase shifts the equilibrium to more protonated forms resulting in the increased binding of ATP at the inhibitory site. Thus, the covalent modification is equivalent to creating a nondiffusible intramolecular regulatory effector.

It should be noted that the above effect is not limited to phosphorylation and that the COOH-terminal peptide portion itself also has influence on ligand binding and cold lability. For example, if one compares the pH-dependent cold lability (Fig. 7), the pK of the cleaved phosphofructokinase is shifted to a lower pH as compared to that of the dephosphoenzyme. The effect of the missing peptide on various properties is opposite to that of phosphorylation. It is, therefore, possible that this peptide portion also influences the ionization of a specific residue(s) affecting the conformational state of the enzyme.

Among many phosphorylated enzymes the best known role of the phosphate group on the enzyme activity is phosphorylation (for a review see Ref. 29). Various studies have shown that the NH2-terminal peptide of phosphorylase b which contains the phosphorylation site is weakly bound to the enzyme surface (30). However, phosphorylation of a serine residue of the peptide (in phosphorylase a) appears to fasten the peptide to the dimer surface via specific ionic interaction (31) and results in stronger interaction between subunits leading to different conformation. It is possible that similar interaction between the phosphate residue of the COOH-terminal peptide and the peptide itself and specific groups on the subunit of phosphofructokinase may occur which result in different conformational states. These interactions are probably very important in promoting more inhibited conformation and also maintaining cooperativity between the subunits. The mechanism by which these changes are accomplished is not known and must await additional information on the nature of intersubunit contact surface, the ionizable group(s), and various conformational states of phosphofructokinase.

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Phosphofructokinase

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