Regulation of Phosphofructokinase by a New Mechanism

AN ACTIVATION FACTOR BINDING TO PHOSPHORYLATED ENZYME*

(Received for publication, September 5, 1980, and in revised form, October 9, 1980)

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Liver phosphofructokinase has been separated into three fractions by DEAE-cellulose chromatography. Chromatography of $^{32}P$-labeled enzyme reveals that the first fraction contains an average of 1.2 mol of phosphate/mol of enzyme (320,000 daltons), while the second and third fractions contain 3.3 mol of phosphate/mol of enzyme. The high phosphate forms are much more sensitive to ATP inhibition than the low phosphate form.

Recently, during the purification of liver phosphofructokinase, we found an activation factor which is bound to the enzyme and affects the activity (Furuya, E., and Uyeda, K. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5861-5864). When the low phosphate form is subjected to heating followed by gel filtration on Sepharose 4B, the bound activation factor is removed. The ATP inhibition curve of the factor-free low phosphate form is similar to that of the high phosphate form. When the amounts of "activation factor" of the low and high phosphate forms were compared, it was found that the low phosphate form contains 63 milliunits of activation factor/unit of phosphofructokinase activity, while the high phosphate form does not contain any detectable amount of the factor. The $K_{0.5}$ values of the low and high phosphate forms for the activation factor are 10 and 20 milliunits, respectively. Based upon these observations, we suggest that phosphorylation alone does not alter the allosteric properties of the enzyme; however, phosphorylation does decrease the affinity for the activation factor so that the high phosphate form is less active than the low phosphate enzyme at a given inhibitory concentration of ATP. Thus, the binding of the activation factor controls the activity of phosphofructokinase, and the phosphorylation influences the affinity of the enzyme for the factor. Both phosphorylation and an enzyme-bound activation factor may be involved in modulating the role of phosphofructokinase in liver glycolysis.

Phosphofructokinase is a key regulatory enzyme of glycolysis, and its activity is modulated by a variety of metabolites (see review by Uyeda (1)). More recently, muscle and liver phosphofructokinases have been shown to be phosphorylated (2-6). The phosphorylation of liver phosphofructokinase is stimulated by glucagon in vivo (7); the phosphorylated enzyme is strongly inhibited by ATP (7) and its $K_{m}$ for fructose-6-P is increased (8, 9).

In order to understand the mechanism of how the phosphorylation of the enzyme alters its allosteric kinetics, numerous unsuccessful attempts were made to mimic the in vivo observations in vitro. These attempts included phosphorylation of isolated phosphofructokinase using a catalytic subunit of cAMP-dependent protein kinase or the addition of cAMP to crude extracts of rat liver. Although phosphofructokinase was phosphorylated by the protein kinase under these conditions, no change in the allosteric properties was observed. More recently, we observed during the purification of the liver enzyme that an additional factor, which we call "activation factor," is bound to the enzyme fairly tightly and affects the sensitivity of the enzyme to ATP (10).

In this communication, we wish to report: (a) a separation of liver phosphofructokinase into dephospho- and phosphorylated forms according to a modified procedure which we previously employed for muscle phosphofructokinase (5), (b) differences in the allosteric properties of the phosphorylated and dephosphoforms, and (c) the role of the activation factor in determining the kinetics of phosphofructokinase.

MATERIALS AND METHODS

DEAE-cellulose (DE52) was purchased from Whatman Potassium $[^{32}P]P$osphate was obtained from New England Nuclear. All chemicals were reagent grade and were obtained from commercial sources.

Phosphofructokinase Preparations for Column Chromatography—Phosphofructokinase for column chromatography was prepared from fresh liver, freeze-clamped liver, or hepatocytes. Rat liver was homogenized in 3 volumes of 50 mM Tris/phosphate (pH 8), 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 mM ATP using a Polytron homogenizer. The homogenate was centrifuged at 20,000 $g$ for 20 min. Polyethylene glycol (50%) was added to the supernatant solution to give a final concentration of 3.5%. The precipitate was removed by centrifugation, and 50% polyethylene glycol was then added to 8%. After standing 20 min, the precipitate was collected by centrifugation and dissolved in a small volume of the above buffer mixture.

DEAE Cellulose Chromatography—After the polyethylene glycol precipitation, phosphofructokinase was adsorbed onto a DEAE-cellulose (DE52) column (1.5 x 13 cm) which had been equilibrated with the homogenizing buffer. The column was washed with 40 ml of the same buffer, followed with 0.1 M Tris/phosphate (pH 8), 50 mM NaF, 1 mM EDTA, 0.1 M dithiothreitol, and 1 mM phosphate-6-P. The first peak of phosphofructokinase was eluted with 0.2 M Tris/phosphate containing the other compounds as described above, and the second peak of the enzyme was then eluted with 0.3 M Tris/phosphate. A typical elution pattern is shown in Fig. 1.

Preparation of Hepatocytes and $[^{32}P]$-labeled Phosphofructokinase—Hepatocytes were prepared from a rat weighing 180 to 230 g according to the modified procedure of Ishibashi and Cottam (11), which was based on the methods developed by Berry and Friend (12) and Ingebretsen and Wagle (13). The cell viability was determined to be 95% based upon exclusion of trypan blue, and a total of 5.7 x 10⁶ cells (4.9 g wet weight) was recovered. The hepatocytes were incubated in Krebs-Ringer buffer containing a 10-fold lower concentration of phosphate, 20 mM glucose, 1% bovine serum albumin, and $[^{32}P]$-phosphate (1 mcI) at 37°C with shaking (120 cycles/min) in an atmosphere of 95% O₂, 5% CO₂. After 30 min, 0.1 mg of glucagon was added, and the incubation was continued for an additional 8 min. The

* This work supported by a grant from the Veterans Administration Research Service and by Grant 5 RO1 AM16194 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
more than 30% of the total phosphofructokinase. The LP form of rat liver phosphofructokinase, however, can be much higher than the HP form in some of the livers and may constitute as much as 80% of the total. We have observed that the relative proportions of the LP and HP forms of the liver enzyme vary greatly depending upon the physiological state of the liver. The difference between HP1 and HP2 is unclear at present, but they may be identical, as will be discussed later. HP1 is eluted slowly in a broader peak and, when the buffer concentration is increased to 0.3 M, the enzyme is eluted in a very sharp peak.

[^32P]Phosphate Contents of LP and HP Phosphofructokinases—Since the amount of phosphofructokinase in liver is very low (20 μg/g of liver) as compared to that in muscle, it is difficult to obtain sufficient amounts of pure LP and HP forms of the enzyme in order to determine the total phosphate contents chemically. Therefore, it has been necessary to use [^32P]phosphate-labeled enzyme to compare the phosphate contents of LP and HP. Hepatocytes were preincubated with [^32P]Phosphate for 30 min, and then glucagon was added to the incubation mixture. After 8 min, the hepatocytes were collected and homogenized, and [^32P]-labeled phosphofructokinase was fractionated by polyethylene glycol and then separated by DEAE-cellulose chromatography. The[^32P] contents of LP and HP fractions were determined after IgG precipitation and gel electrophoresis of the complex as described under “Materials and Methods.” The phosphate contents were calculated based upon the specific radioactivity of [^32P]ATP, assuming that both β- and γ-phosphates of ATP were equally labeled. The results are summarized in Table I. The phosphate content of the LP fraction was approximately 1.2 mol/mol of enzyme tetramer (M, 320,000) or 0.3 mol/mol of subunit. On the other hand, both HP1 and HP2 contain the same amount of phosphate, 3.3 mol/mol of enzyme, or 0.83 mol/mol of subunit. Thus, the LP form can be considered to be the dephospho-form of the enzyme, and the HP forms can be considered the phospho-form. However, it should be noted that these values are based on [^32P]phosphate content and do not reflect the total phosphate content, since chemical determination has not yet been performed.

ATP Inhibition of LP and HP—Although we have separated LP and HP forms of muscle phosphofructokinase using similar procedures, we have been unable to find any difference in either their catalytic activity or their allosteric properties (5). The ATP inhibition curves of LP and HP forms of liver phosphofructokinase are clearly different, as shown in Fig. 2. The LP enzyme is significantly less sensitive to inhibition by ATP as compared to the HP forms; 50% inhibition by ATP is obtained at approximately 0.2, 0.08, and 0.05 mM ATP for LP, HP1, and HP2 enzymes, respectively.

ATP Inhibition of LP and HP after Heating and Sepharose Treatment—The next question which we asked was how

Table I

| Phosphate contents and the amounts of activation factor in LP, HP1, and HP2 phosphofructokinase |
|---------------------------------------------|
| **[^32P]/PFK** * | **Phosphate/enzyme** | **Activation factor** |
|------------------|----------------------|----------------------|
| cpm/unit         | mol/mol              | milliunits/unit PFK  |
| LP               | 217                  | 1.2                  | 63                   |
| HP1              | 592                  | 3.3                  |                      |
| HP2              | 602                  | 3.3                  | 0                    |

* PFK, phosphofructokinase.

[^32P]ATP was 5,810 cpm/nmol. LP, HP1, and HP2 fractions were eluted from a DEAE column as described in the legend to Fig. 1.

[^1] The abbreviations used are: LP, low phosphate; HP, high phosphate.
the phosphorylation of the enzyme alters its allosteric kinetics. One possibility is that the covalent modification directly alters enzyme conformation. The other possibility is that the activation factor we recently discovered binds to phosphofructokinase and makes the enzyme less sensitive to inhibition. In order to investigate these possibilities, LP enzyme was freed of the factor by heat and then separated by Sepharose 4B gel filtration. This treatment effectively removes the factor from the enzyme as shown in Fig. 2; after this treatment, LP is strongly inhibited by ATP as compared to LP prior to the treatment. The ATP inhibition curve of LP after the removal of activation factor is very similar to that of HP, suggesting that phosphorylation of the enzyme per se does not alter its allosteric properties, since factor-free phospho-HP and dephospho-LP forms exhibit similar inhibition curves.

The Amounts of the Activation Factor in LP and HP—Since the above result favors the possibility that enzyme-bound activation factor is responsible for the enzyme's allosteric properties, the amounts of bound factor in LP and HP phosphofructokinase were compared. Both forms of the enzyme were denatured by heating to 70°C for 5 min, and the concentrations of the released activation factor were determined (10). The results (Table I) show that the LP enzyme contains 6.3 milliunits of activation factor/unit of phosphofructokinase, while HP contains no detectable activation factor. Since the amount of HP is small, it was not examined for the presence of activation factor. Thus, these results strongly suggest that the amount of activation factor which is bound to the enzyme is responsible for the difference in the observed ATP inhibition between the LP and HP enzymes.

Differences in the Affinity of LP and HP for the Factor—In order to obtain additional evidence in support of the above idea, LP and HP phosphofructokinases were freed of the bound factor as described before, and their affinity for the factor was determined. As shown in Fig. 3, the LP enzyme has higher affinity for the activation factor than does HP; the $K_{a5}$ of LP is approximately 10 milliunits, as compared to 20 milliunits for HP enzyme. Furthermore, the saturation curve of LP for the factor is strongly sigmoidal and highly cooperative.

![Fig. 3. Effect of varying activation factor on LP and HP. The activities of LP after heat and Sepharose treatment as described in the legend to Fig. 2 and HP (5 milliunits each) were assayed as in Fig. 2 except that 0.5 mM ATP was used.](image)

Thus, LP shows much higher affinity for the factor than HP, which accounts for the fact that LP is less sensitive to ATP inhibition than HP.

**DISCUSSION**

In this communication, we have shown that rat liver phosphofructokinase can be separated into HP and LP forms using a modification of the procedure which we have employed previously for the separation of rabbit muscle phosphofructokinase according to phosphate content (5). There are essentially two major differences in the results obtained using rabbit muscle or rat liver phosphofructokinases. The two forms of the muscle enzyme contain an average of 0.3 mol of phosphate/mol of enzyme (LP) and 0.8 mol of phosphate/mol of enzyme (HP). On the other hand, the LP and HP forms of the liver enzyme contain 1.2 mol of phosphate/mol of enzyme and 3.3 mol of phosphate/mol of enzyme tetramer, respectively. Thus, there is a 3-fold greater concentration of phosphate in the liver enzyme as compared to the muscle enzyme. The actual phosphate content of the liver enzyme could be even greater since only $^{32}$P incorporation, rather than total phosphate, was determined. The second important difference is that LP and HP forms of liver phosphofructokinase show differences in their sensitivity to ATP inhibition. Previously, we were unable to demonstrate any difference in allosteric properties between the two forms of muscle phosphofructokinase. Furthermore, the HP form of liver phosphofructokinase, which contains approximately 1 mol of phosphate/mol of subunit, is considerably more sensitive to ATP inhibition than the LP form of the enzyme. Thus, these results provide direct evidence for our hypothesis that phosphorylation of liver phosphofructokinase results in increased sensitivity toward allosteric regulators.

We also present in this communication evidence which suggests that phosphorylation of phosphofructokinase is not directly responsible for the changes in its allosteric properties. Factor-free LP exhibits a similar, but not identical, ATP inhibition curve as compared to HP phosphofructokinase (Fig. 2). The difference in allosteric kinetics between the phospho- and dephosphophosphofructokinases is a result of the amount of enzyme-bound activation factor. This has been demonstrated in two ways: (a) the LP form after isolation by DEAE-cellulose chromatography contains a significant amount of the...
factor, but the HP form does not; (b) LP binds the activation factor more tightly than does HP, and the binding is highly cooperative. Thus, these results demonstrate that the dephospho-enzyme binds the activation factor more tightly and, consequently, has a higher activity (or less inhibition) at a given concentration of ATP than the phospho-form of phosphofructokinase. Moreover, these observations provide a plausible explanation for our previous failure to demonstrate changes in allosteric kinetics after in vitro phosphorylation of the enzyme, since the purified phosphofructokinase used in those experiments had already been freed of the activation factor. Further work, however, is necessary to elucidate the exact biochemical mechanism which explains the difference in the interaction of the factor with the LP and HP forms.

There are a number of enzymes whose activity is known to be regulated directly by phosphorylation and dephosphorylation mechanism. We have shown that phosphorylation does not, by itself, regulate phosphofructokinase. It is the activation factor which controls the enzyme activity, and the binding of the activation factor to the enzyme is regulated by phosphorylation. This mechanism represents a new type of control for phosphofructokinase and possibly for other enzymes whose enzyme activity is not influenced by the extent of phosphorylation (15). At the present time, however, we do not know how this mechanism is involved in regulation of phosphofructokinase in liver since we have not yet determined whether the level of the activation factor is controlled by changes in hormone level.

Acknowledgment—We thank Dr. C. Sue Richards for the preparation of 32P-labeled hepatocytes and for her helpful comments during the preparation of this manuscript.

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