Studies on Heart Phosphofructokinase

DESENSITIZATION OF THE ENZYME TO ADENOSINE TRIPHOSPHATE INHIBITION*

(Received for publication, October 28, 1968)

CHARLES F. AHLFORS† and TAG F. MANSOUR

From the Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305

SUMMARY

A procedure has been described for photo-oxidation of crystalline sheep heart phosphofructokinase in the presence of methylene blue. The enzyme after photo-oxidation was insensitive to ATP inhibition. Kinetic studies at pH 8.2 before and after photo-oxidation of the enzyme revealed no significant change in the $K_m$ for either fructose-6-phosphate or ATP. Kinetic studies at pH 6.9 revealed a decrease in both the $K_m$ for ATP and the $K_m$ for fructose-6-P in the photo-oxidized enzyme. The Hill coefficient of the enzyme was 3.15 before photo-oxidation and was decreased to 1.1 after photo-oxidation, indicating disappearance of homotropic interactions between different fructose-6-P sites. The photo-oxidized enzyme was not inhibited by citrate nor activated by AMP. The stability of the enzyme at pH 6.5 was increased after photo-oxidation. ATP protected the enzyme against inactivation during photo-oxidation at pH 8.0 but not at pH 6.9. Fructose-6-P, on the other hand, protected the enzyme at both hydrogen ion concentrations. Neither of the two substrates prevented the desensitization of the enzyme to ATP inhibition following photo-oxidation. The results indicate that desensitization of the enzyme to ATP inhibition is related to the presence of the enzyme in a stable conformation.

Earlier work from this laboratory indicated that heart phosphofructokinase shows cooperative kinetics at slightly acidic pH (1, 2). Reports on phosphofructokinase from other sources described similar kinetic properties (3-8). ATP in high concentration and citrate inhibit the enzyme, while AMP and cyclic 3',5'-AMP relieve such inhibition. These kinetic properties led to consideration of the enzyme as a member of the group of feedback-regulated enzymes termed "allosteric enzymes." A main property for distinguishing an allosteric enzyme is that it should have a site different from the catalytic site, where enzyme modifiers act. The fact that ATP is both an inhibitor as well as a substrate for phosphofructokinase made it imperative to test whether the enzyme is actually regulated by an allosteric mechanism. The existence of special binding sites for modifiers in many allosteric enzymes has been confirmed by desensitizing the enzyme to its inhibitors without destroying the active site (9-11) and also by mutant selection (10, 12, 13). This paper describes a procedure for desensitizing phosphofructokinase to ATP inhibition by photo-oxidation without much destruction of the active site, giving evidence that the inhibitory site is distinct from the substrate site. Some properties of the desensitized enzyme are compared with those of the native enzyme.

EXPERIMENTAL PROCEDURE

Methods

Enzyme Preparation and Photo-oxidation—Sheep heart phosphofructokinase was purified and crystallized by the procedure described previously (14) with a modification in Step III (2). The crystalline enzyme was solubilized by the following procedure. Crystalline suspension (0.1 ml) was centrifuged at 4000 × g for 10 min. The supernatant solution was discarded and the crystals were washed twice with 0.2 ml of 0.05 M phosphate buffer at pH 8.2. After the final wash, the crystals were suspended in 0.2 ml of a solution containing 0.05 M potassium phosphate buffer, pH 8.2; 10⁻⁵ M fructose-1,6-di-P; 10⁻⁴ M ATP; 10⁻⁴ M dithioerythritol; and 10⁻⁴ M EDTA. The tube containing the crystalline suspension was placed in a water bath at 60°, and the solution was stirred for up to 3 min until the enzyme dissolved. Final enzyme concentration was about 2 mg per ml.

The solubilized enzyme was photo-oxidized in the presence of methylene blue and light as follows. Enzyme solution (200 μl) containing 2.68 × 10⁻⁵ M methylene blue was placed in a conical glass centrifuge tube, 1 × 10.5 cm. The tube was mounted in a glass centrifuge holder and placed in a light-tight box. The enzyme solution was exposed to ultraviolet light (2537 A) and room light for 24 to 48 hr. Following irradiation, the tube was removed from the light and allowed to stand in the dark for 24 hr.

1 The term photo-oxidation refers here to irradiation of the enzyme in the presence of methylene blue. Although no direct evidence for oxidation is presented here, the use of this term is in line with the fact that treatment of other enzymes by a similar procedure has always resulted in photo-oxidation.

* This research was supported by United States Public Health Service Research Grant 1AI03214 from the National Institutes of Allergy and Infectious Diseases; United States Public Health Service Career Development Award GM3848 from the Division of General Medical Sciences; and grants-in-aid from the American Heart Association.

† Supported by Public Health Service General Research Support Grant FR6383.
Effect of Photo-oxidation on Kinetics at pH 8.2—Results on the effect of photo-oxidation on kinetics of enzyme activity at pH 8.2 are summarized in Figs. 1 and 2. These results show that, as in the case of native enzyme, the photo-oxidized enzyme exhibits first order kinetics with respect to ATP and fructose-6-P.

Materials

The materials used in this study were obtained from the sources given previously (2). ATP was purchased from P-L Biochemicals and purified by thin layer chromatograms according to the DEAE-cellulose method described by Grippo et al. (17). Elution of ATP from the cellulose was described previously (18). Methylene blue was purchased from Allied Chemicals, Morristown, New Jersey.

RESULTS

Effect of Photo-oxidation on Kinetics at pH 8.2—Results on the effect of photo-oxidation on kinetics of enzyme activity at pH 8.2 are summarized in Figs. 1 and 2. These results show that, as in the case of native enzyme, the photo-oxidized enzyme exhibits first order kinetics with respect to ATP and fructose-6-P.
The $K_m$ for ATP was $7.0 \times 10^{-4}$ M for the native and $6.0 \times 10^{-5}$ M for the photo-oxidized enzyme. The $K_m$ for fructose-6-P was $5.3 \times 10^{-5}$ M for the native enzyme and $4.7 \times 10^{-5}$ M for the photo-oxidized enzyme. When ATP was varied, the absolute maximum velocity ($V_{\text{max}}$) calculated in μmoles of fructose-1,6-di-P formed per min per mg of protein was 209 for the native enzyme and 152 for the photo-oxidized enzyme. The $V_{\text{max}}$, when fructose-6-P concentration was varied, was 101 for the native enzyme and 146 after photo-oxidation. These experiments indicate that the affinity of the enzyme for its substrate was not changed, as shown by the insignificant change in the $K_m$. Maximum velocity of the enzyme at pH 8.2 was only slightly reduced by photo-oxidation, indicating some alteration or destruction of a small fraction of the catalytic sites as a result of photo-oxidation.

**Effect of Photo-oxidation on pH 6.9 Kinetics**—Kinetic studies on the enzyme before and after photo-oxidation are summarized in Figs. 3 and 4. Titration of the native enzyme with ATP shows a typical inhibitory curve (1-3) at high concentrations (Fig. 3). ATP at concentrations above $5 \times 10^{-5}$ M became inhibitory, and the apparent $K_i$ was $1.2 \times 10^{-4}$ M. When the data were plotted on a Lineweaver-Burk plot, the apparent $V_{\text{max}}$ for the native enzyme was 114 μmoles of fructose-1,6-di-P per min per mg and an apparent $K_m$ for ATP from the Lineweaver-Burk plot gave a value of 57 μmoles of ATP per mg. Light were required for desensitization to ATP inhibition. The latter concentration was 34%. Both methylene blue and light were required for desensitization to ATP inhibition. The apparent $V_{\text{max}}$ for the photo-oxidized enzyme as determined in Figs. 3 and 4. The Hill coefficient in the case of the native enzyme was 3.15, while that for the photo-oxidized enzyme was 1.1. These results indicate that photo-oxidation caused a marked decrease in ATP inhibition of the enzyme with a 50% decrease in apparent $V_{\text{max}}$ and apparent $K_m$.

**Fig. 3.** Plot of initial reaction velocity with respect to ATP concentration at pH 6.9. Enzyme assays were carried out in the presence of 4 μM ATP, 10 mM MgCl$_2$, and 0.175 μg of enzyme per ml. Kinetics were studied with the enzyme before (O—O) and after (●—●) photo-oxidation. Other conditions for the assay at pH 6.9 and for photo-oxidation are described under “Methods.” The inset shows a Lineweaver-Burk plot of the data. Photo-oxidation was carried out for 4 min at pH 8.2.

**Fig. 4.** Plot of initial reaction velocity with respect to fructose-6-P at pH 6.9. Enzyme assays were carried out in the presence of 4 × 10^{-4} M ATP, 10^{-3} M MgCl$_2$, and 0.175 μg of enzyme per ml. Kinetics were studied with the enzyme before (O—O) and after (●—●) photo-oxidation. Other conditions for the assay at pH 6.9 and for photo-oxidation are described under “Methods.” The inset shows plots derived from the Hill equation (19, 20). Photo-oxidation was carried out for 4 min at pH 8.2.

Titrations curves for fructose-6-P before and after photo-oxidation are shown in Fig. 4. The titration curve for the native enzyme was sigmoidal with an apparent $V_{\text{max}}$ of 97 μmoles of fructose-1,6-di-P per min per mg and an apparent $K_m$ for fructose-6-P of $1.2 \times 10^{-4}$ M. After photo-oxidation, the curve for fructose-6-P became a rectangular hyperbola. The apparent $K_m$ was reduced to $2.2 \times 10^{-4}$ M fructose-6-P, indicating a 5-fold decrease from the native enzyme, while the $V_{\text{max}}$ was only slightly changed (Fig. 4). The data for titration of fructose-6-P were plotted according to the Hill equation, and the interaction coefficient (Hill coefficient), $n$, was determined from the slopes of the plots (19, 20). These results are shown in the inset of Fig. 4. The Hill coefficient in the case of the native enzyme was 3.15, while that for the photo-oxidized enzyme was 1.1. These results indicate that the cooperative kinetic data shown by the native enzyme was abolished by photo-oxidation.

**Citrate Inhibition and AMP Activation of the Photo-oxidized Enzyme—5-AMP is known to activate phosphofructokinase at pH 6.9, particularly when ATP is present in inhibitory concentrations (1, 2, 5). Citrate is known to inhibit the enzyme in a manner similar to ATP (7, 8). The effects of adenylate and citrate on the photo-oxidized enzyme were tested. The results summarized in Table I show that the enzyme before photo-oxidation showed no activity when the concentration of ATP was $4 \times 10^{-4}$ M, whereas at the same ATP concentration enzyme activity was high in the presence of $10^{-4}$ M AMP. After 15 min of photo-oxidation, the activity of the enzyme with or without AMP present was approximately the same. The inhibitory effect of $10^{-4}$ M citrate in the presence of $8 \times 10^{-4}$ M ATP on the native enzyme is shown in Table I. After photo-oxidation for
TABLE I

Effect of photo-oxidation on citrate inhibition and AMP activation

Phosphofructokinase, photo-oxidized at pH 8.2, was assayed at pH 6.9. The effect of either citrate or AMP was tested in the presence of the indicated ATP concentration. The ratio of the rate in the presence of the added ligand to the rate without the added ligand is expressed as percentage of control.

<table>
<thead>
<tr>
<th>Additions</th>
<th>ATP</th>
<th>Enzyme activity at indicated times of photo-oxidation (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4×10^{-4}</td>
<td>0</td>
</tr>
<tr>
<td>10^{-4} M AMP</td>
<td>4×10^{-4}</td>
<td>19.5</td>
</tr>
<tr>
<td>Percentage of control</td>
<td></td>
<td>135%</td>
</tr>
</tbody>
</table>

None................ 8×10^{-4} 13.8 12.0 7.5
10^{-4} M citrate.... 8×10^{-4} 0 8.2 5.9
Percentage of control | | 68% | 79% |

Results summarized in Fig. 5 show that the photo-oxidized enzyme was less sensitive to inactivation at pH 6.5. These results indicate that the process of desensitization results in an increase in the stability of the enzyme at an acid pH.

The ability of the enzyme to dissociate to subactive protomers at pH 6.5 was tested. The sedimentation velocity of photo-oxidized enzyme which was dialyzed overnight at pH 6.5 was

Fig. 5. Stability of different concentrations of phosphofructokinase at pH 6.5. The crystalline enzyme was washed twice in 0.05 M phosphate buffer (pH 8.0) containing 10^{-5} M dithioerythritol and 2×10^{-4} M EDTA. The solubilized enzyme was incubated for 10 min at 30° in a mixture containing 0.05 M Tris maleate (pH 6.5), 10^{-3} M dithioerythritol, 10^{-3} M EDTA, and the indicated concentration of enzyme. Incubation was carried out with the native enzyme (○—○) or the photo-oxidized enzyme (●—●). Enzyme activity was measured at pH 8.2. Enzyme activity is expressed in the ordinate as a percentage of initial enzyme activity (before acidification). Photo-oxidation of the enzyme was carried out at pH 8.2. Other experimental conditions are given under “Methods.”

10 or 15 min, citrate caused only 20% to 30% inhibition of the enzyme compared to 100% inhibition prior to photo-oxidation. These results indicate that desensitization of the enzyme to ATP inhibition is accompanied by loss of citrate inhibition and AMP activation.

Stability of Photo-oxidized Enzyme at pH 6.5 Solubilized crystalline phosphofructokinase was found to be unstable at an acidic pH. Loss of activity was most marked at pH 6.5 or below (2, 21). The degree of inactivation was found to be dependent on the enzyme concentration. The sensitivity of the photo-oxidized enzyme to mild acid pH (6.5) was tested. Results

Fig. 6. Effect of photo-oxidation on phosphofructokinase freed of bound substrates. The enzyme was freed of ligands at pH 8.0 as described under “Methods” and photo-oxidized. Samples were removed at the indicated time intervals and assayed at pH 8.2 as described under “Methods.”

TABLE II

Effect of different ligands on phosphofructokinase during photo-oxidation

Ligands were removed from the enzyme at either pH 6.9 or pH 8.0, as described under “Methods.” All assays were done at pH 8.2. Photo-oxidation was carried out for 4 min at pH 8.0 and for 12 min at pH 6.9. The activity of the enzyme prior to photo-oxidation at pH 8.0 varied from 15.7 to 37.5 units per ml and 10 to 33 units per ml at pH 6.9. Results are expressed as the percentage of initial enzyme activity. Concentration of additions at pH 8.0 were 10^{-5} M and at pH 6.9 were 10^{-4} M except where noted. Results represent an average of at least two experiments.

<table>
<thead>
<tr>
<th>Photo-oxidation at pH 8.0</th>
<th>Photo-oxidation at pH 6.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addition</td>
<td>Initial activity remaining</td>
</tr>
<tr>
<td>None</td>
<td>30</td>
</tr>
<tr>
<td>ATP</td>
<td>41</td>
</tr>
<tr>
<td>ADP</td>
<td>41</td>
</tr>
<tr>
<td>AMP</td>
<td>30</td>
</tr>
<tr>
<td>Fructose-6-P</td>
<td>43</td>
</tr>
<tr>
<td>ATP + fructose-6-P</td>
<td>60</td>
</tr>
<tr>
<td>ATP + fructose-6-P + MgCl₂</td>
<td>50</td>
</tr>
</tbody>
</table>

* Concentration of MgCl₂ was 6×10^{-3} M.
* Concentration of MgCl₂ was 2×10^{-3} M.
* Concentration of GTP was 2×10^{-4} M.
determined on sucrose gradients as described previously (2, 21). The major part of the enzyme was still in the aggregated form (98, w = 21). The presence of 2 × 10^{-4} M ATP did not shift the equilibrium to the dissociated form of the enzyme, as was shown with the native enzyme (2).

Effect of Different Ligands during Photo-oxidation—Enzyme freed from ligands at pH 6.9 or pH 8.0, as described under "Methods," lost activity very rapidly when photo-oxidized. Fig. 6 illustrates loss of activity during photo-oxidation of enzyme prepared at pH 8.0. Furthermore, the activity could not be restored by adding up to 0.1 M mercaptoethanol to the photo-oxidized enzyme. The enzyme was unaffected by exposure to light in the absence of methylene blue or vice versa.

Various ligands were found to protect the enzyme against inactivation by photo-oxidation (Table II). At pH 8.0, ATP, fructose-6-P, and ADP protected the enzyme, while AMP had no effect. A combination of ATP and fructose-6-P had an additive effect in protecting activity which was somewhat reduced by MgCl_2. At pH 6.9, ATP alone or with MgCl_2 did not protect the enzyme against loss of activity, while fructose-6-P and GTP gave some protection. A combination of GTP and fructose-6-P proved additive in protection of enzyme activity. These results indicate that while some ligands can prevent the loss of catalytic activity at both pH values, ATP loses this property at pH 6.9.

The effect of ligands in preventing desensitization of the enzyme to ATP inhibition was investigated at pH 6.9. None of the following ligands prevented the desensitization of the enzyme to ATP inhibition following photo-oxidation for 12 min: 10^{-4} M fructose-6-P, 10^{-4} M ATP, 5 × 10^{-4} M GTP.

**DISCUSSION**

Variations in the sensitivity of heart phosphofructokinase to ATP inhibition were reported previously (2). A form of phosphofructokinase was isolated, in the absence of stabilizers (ATP and fructose-1,6-di-P), which showed a greater degree of ATP inhibition than native enzyme. Furthermore, after purification and crystallization, the enzyme was found to be less sensitive to ATP inhibition than the partially purified enzyme. These results, when added to the kinetic data reported before (1, 2) suggested the presence of regulatory sites for ATP which are different from the catalytic site. While this paper was being written, a communication by Salas, Salas, and Sols (22) reported the desensitization of yeast phosphofructokinase to ATP inhibition by trypsin digestion. Studies reported with the skeletal muscle enzyme (23) showed that there are three ATP sites for each fructose-6-P site. The fact that maximum activity of the enzyme was affected slightly suggests that both active and allosteric sites might have some identical functional groups. It is also possible that both sites are altered by a conformational change caused by alteration of one group. Desensitization of the enzyme to ATP inhibition resulted in the disappearance of the homotropic interactions between different fructose-6-P sites. This was indicated by the changes of the Hill coefficient from 3.15 for the native enzyme to 1.1 for the photo-oxidized enzyme. The photo-oxidized enzyme also had a greater affinity to its substrate, fructose-6-P, at pH 0.9 (Fig. 4). It therefore appears that photo-oxidation of the enzyme induces a different conformation for the protein, with different kinetic properties.

The enzyme desensitized to ATP inhibition was shown to be less sensitive to inactivation at an acidic pH. It was reported before that conditions which increased enzyme sensitivity to ATP also caused a decrease in the stability of the enzyme to acidic pH (2). The present results further support the hypothesis that phosphofructokinase instability is related to its ability to change its conformation by ATP.

The question arises about the mechanism of desensitization of the protein to ATP. Two possibilities exist: (a) photo-oxidation might affect specifically those groups essential for binding ATP at the allosteric site; and (b) binding of ATP is not affected, but the protein could be incapable of changing its stable active conformation. Sucrose gradient studies indicated that dissociation of the enzyme was hindered after photo-oxidation. Studies on the binding properties of the photo-oxidized enzyme are needed to resolve the above possibilities.

**REFERENCES**

Studies on Heart Phosphofructokinase: DESENSITIZATION OF THE ENZYME TO ADENOSINE TRIPHOSPHATE INHIBITION
Charles E. Ahlfors and Tag E. Mansour

J. Biol. Chem. 1969, 244:1247-1251.

Access the most updated version of this article at http://www.jbc.org/content/244/5/1247

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/244/5/1247.full.html#ref-list-1