DESENSITIZATION OF YEAST PHOSPHOFRUCTOKINASE TO ATP INHIBITION BY TREATMENT WITH TRYPSIN*

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Yeast phosphofructokinase (PFK) is inhibited by ATP and citrate and deinhibited by fructose-6-phosphate, AMP, and NH_4^+ ions (Sols and Salas, 1966). Phosphofructokinases from a great variety of sources are also subject to a multiplicity of regulatory effects. The results of kinetic and binding studies do not permit to ascertain how many different regulatory sites are involved, particularly in regard to the adenyl nucleotides (Lowry and Passonneau, 1966; Ramaiah, Hathaway and Atkinson, 1964; Kemp and Krebs, 1967).

It is shown here that incubation of purified yeast PFK with trypsin can lead to desensitization to inhibition by ATP. This result supports the conclusion that the enzyme has a regulatory site for ATP different from the nucleotide substrate site. Moreover, the effects of ATP, AMP, NH_4^+ ions, and mixtures of them on the desensitization by trypsin treatment suggest that yeast PFK has a regulatory site for AMP different from the ATP inhibitory site.

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Experimental. Desensitization of PFK to inhibition by ATP, without loss of activity, was achieved by incubation of the enzyme with trypsin in the conditions described in Figure 1. It can be seen in this figure that the activity of the enzyme at a low, non-inhibitory concentration of ATP was

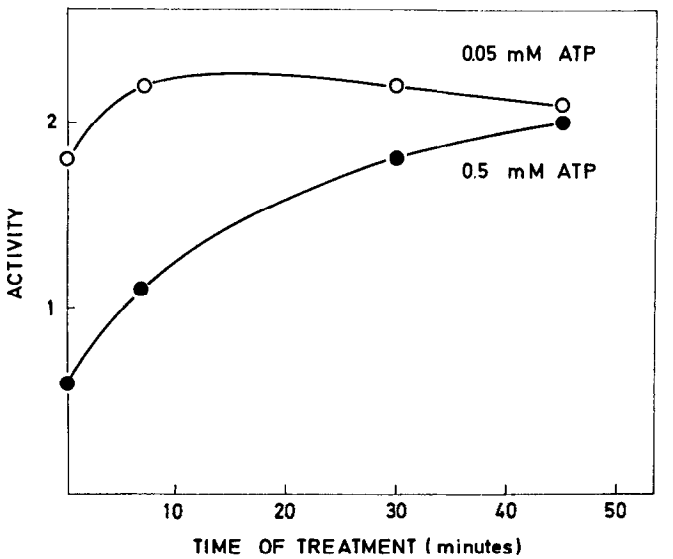

Figure 1. Desensitization of PFK to ATP inhibition by treatment with trypsin. 0.2 mg of a 150 fold purified preparation of PFK (Sols and Salas, 1966) were incubated at 22° with 0.14 mg of trypsin (Armour) in a final volume of 0.2 ml of 5 mM potassium phosphate, pH 6.5, 5 mM $MgCl_2$, and 1 mM ethanethiol. 5 µl aliquots were taken at the indicated times and PFK activity was assayed in a total volume of 2 ml, in the presence of 0.1 mM NADH, 5 mM ethanethiol, 5 mM $MgCl_2$, 25 mM potassium phosphate, pH 6.5, 0.035 mg of soybean trypsin inhibitor, 0.5 mM fructose-6-phosphate, 0.2 units each of aldolase, triose phosphate isomerase and glycerol-3-phosphate dehydrogenase and 0.05 mM ATP (open circles) or 0.5 mM ATP (dark circles). The reaction was started by the addition of PFK and followed at 340 mµ. Activity is expressed as µmoles of substrate transformed per minute per mg of protein.

essentially unaffected by trypsin. In contrast, the activity at an inhibitory concentration of ATP increased with time of incubation. At 45 minutes, both activities were similar, indicating that the enzyme had lost its sensitivity to inhibition by ATP. The effect of the concentration of ATP on the native and trypsin-treated enzyme is shown in Fig. 2. No inhibition by

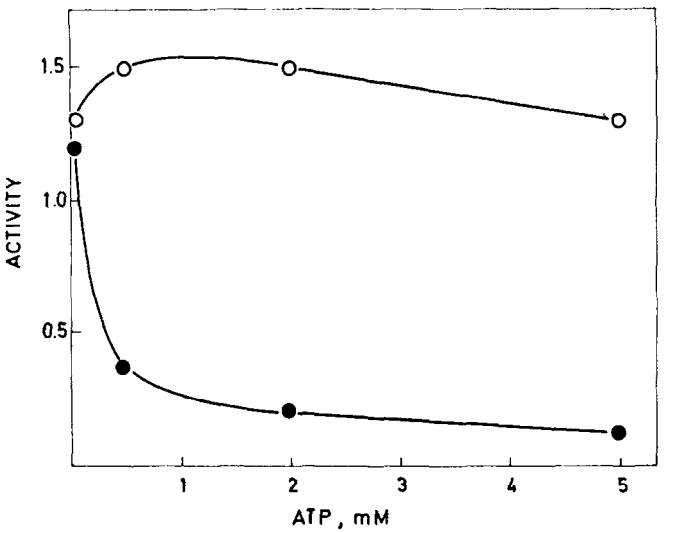

Figure 2. Effect of ATP concentration on the activity of native and trypsin-treated PFK. O—O, 0.1 mg of the preparation of PFK used in Fig. 1 were incubated at 22° for 60 minutes with 0.069 mg of trypsin in a total volume of 0.1 ml as indicated in Fig. 1. PFK activity was assayed with 5 μl of the incubated mixture as described in Fig. 1 with the exception that the concentration of ATP was varied as indicated. ●——●, the same preparation of PFK was assayed without previous incubation with trypsin.

ATP was observed with the trypsin-treated enzyme, even at concentrations of ATP that inhibited 90% of the native enzyme.

The possible effects of substrates and modifiers of PFK on its desensitization by trypsin was then tested. As shown in Table I, the presence of ATP during the incubation of PFK with trypsin prevents the desensitization to ATP inhibition. ITP, which is a good substrate of PFK, but does not inhibit the enzyme (Sols and Salas, 1966) did not replace ATP in this protective effect. Also, AMP and NH_4^+ ions, which in kinetic studies were shown to counteract the inhibition by ATP of PFK (Ramaiah et al., 1964; Sols and Salas, 1966) did not prevent the desensitization by trypsin. Nevertheless when both ATP and AMP are present during the incubation of PFK with trypsin, AMP counteracts the protective effect of ATP, so that the enzyme is desensitized to ATP inhibition. In contrast, NH_4^+ ions did not prevent the protective effect of ATP.

Several other treatments were unsuccessful in achieving desensitization of the enzyme to these regulatory effects. By incubation with 2 M urea at 22° the enzyme was progressively inactivated, whereas the sensitivity to inhibition by ATP and deinhibition by AMP and NH_4^+ ions remained unchanged. The presence of ATP or FDP during the incubation with urea had no protective effect on this inactivation. However, in the presence of ATP, the sensitivity to ATP inhibition tended to increase. p-Chloromercuriphenylsulfonate and heating as potential desensitizing agents were also largely unsuccessful.

Discussion. The desensitization of PFK to inhibition by ATP by trypsin treatment, without marked loss of activity, provides additional evidence for the existence of an inhibitory site for ATP different from the substrates sites (Viñuela, Salas and Sols, 1963).

The desensitizing effect of trypsin can depend mainly on an attack either at the inhibitor binding site itself or at another point in the enzyme molecule, in such a way that the allosteric interaction between the inhibitor and substrates sites is prevented. In this last case (Fig. 3, model A), the observed protection by ATP against trypsin action could be explained by a conformational change of the protein, induced by the binding of ATP to its inhibitory site (ATP_r), that prevents the attack by trypsin. Moreover, it could be expected from this model that both

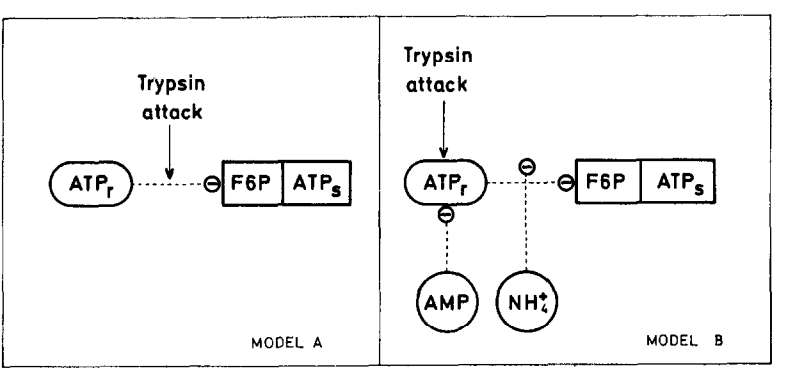

Figure 3. Models for trypsin attack and binding sites on yeast PFK. ATP_r stands for ATP regulator, and ATP_s for ATP substrate.

Table I. Effect of substrates and modifiers of PFK on its desensitization by treatment with trypsin

Additions during treatment	Activity (μmoles/minute/mg protein) 0.05 mM ATP	0.5 mM ATP	Inhibition by 0.5 mM ATP, per cent
Untreated control	3.6	0.95	74
None	2.4	2.7	-
5 mM ATP	3.1	1.3	58
5 mM ITP	2.0	2.8	-
2 mM AMP	2.3	3.0	-
100 mM NH_4Cl	1.6	2.2	-
5 mM ATP + 2 mM AMP	3.0	3.2	-
5 mM ATP + 100 mM NH_4Cl	3.0	1.4	54

0.1 mg of the preparation of PFK used in Figure 1 were incubated at 34° for 45 minutes, in a total volume of 0.1 ml of a solution as indicated in Fig. 1, with 0.07 mg of trypsin and the indicated additions. After incubation, the mixtures were diluted 10 times with 10 mM potassium phosphate pH 6.8, 10 mM $MgCl_2$, and 2 mM ethanethiol, and PFK activity was assayed with 5 μl aliquots as in Figure 1 with 0.05 mM or 0.5 mM ATP as indicated.

AMP and NH_4^+ ions would counteract the protective effect of ATP by preventing either the binding of ATP to its inhibitory site or the conformational change induced by ATP. The fact, however, that NH_4^+ ions do not counteract the protective effect of ATP makes this model improbable.

The experimental results are compatible with Model B of Fig. 3. In this model, the desensitizing effect of trypsin would be due to a direct attack at the ATP regulatory site. Binding of ATP to this site prevents this attack. The finding that NH_4^+ ions do not protect the enzyme against trypsin action suggests that the binding site for NH_4^+ ions is not in the ATP_r area. On the other hand, the inability of NH_4^+ ions to counteract the protective effect of ATP is compatible with the view that the effect of NH_4^+ ions as deinhibitor is at the level of the allosteric interaction

between the inhibitor and substrates sites. Deinhibition of PFK by AMP could be either at the ATP_r site, as an inert analogue of ATP, or at a different site. The observation that AMP, in contrast to ATP, does not protect the enzyme against desensitization by trypsin suggests the latter hypothesis. Moreover, the fact that, in contrast to NH_4^+, AMP can prevent the protective effect of ATP against desensitization by trypsin suggests that binding of AMP at its regulatory site allosterically interferes with binding of ATP at the ATP_r site. If this were the case, AMP would be a primary allosteric effector of PFK, although its main potential effect is secondary to the inhibition by a different allosteric effector, ATP, since AMP strongly counteracts the latter inhibitory effect. This postulate is consistent with the fact that the apparent affinities of yeast PFK for adenyl nucleotides as effectors is in the order AMP > ATP > ADP (Sols and Salas, 1966). Two dissimilar sites for allosteric regulation by adenyl nucleotides can of course allow for differences in structural requirement, as suggested by the observations of Uyeda and Racker (1965) with the muscle enzyme.

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