Effects of Acetylimidazole on the Hydrolysis of Fructose Diphosphate and *p*-Nitrophenyl Phosphate by Liver Fructose Diphosphatase†

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ABSTRACT: Acetylimidazole produces a pseudo-first-order loss of fructose diphosphatase activity with fructose diphosphate as substrate; however, the activity with *p*-nitrophenyl phosphate as substrate is first rapidly stimulated and then slowly inhibited. When the enzyme is incubated with acetylimidazole plus fructose diphosphate the hydrolytic activity with fructose diphosphate as substrate is protected while the stimulation of *p*-nitrophenyl phosphate hydrolysis is inhibited. When the enzyme is incubated with acetylimidazole plus AMP (the allosteric inhibitor) the activity with fructose diphosphate is partially protected and loss of *p*-nitrophenyl phosphate activity is prevented. The results are consistent with the previous suggestion that hydrolysis of *p*-nitrophenyl phosphate is catalyzed at or near the regulatory site of this enzyme. The results also point to mutual interactions between the active site and regulatory site of liver fructose diphosphatase.

The hydrolysis of *p*-nitrophenyl phosphate catalyzed by rabbit liver fructose diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) was first reported by Chou and Kirtley (1971). Based on the mutual inhibitory effects of fructose diphosphate, fructose 6-phosphate, and AMP on *p*-nitrophenyl phosphate hydrolysis it was concluded that this reaction takes place at or near the regulatory AMP site rather than at the FDP catalytic site (Chou and Kirtley, 1973). In order to understand the relation between the hydrolytic activities of this enzyme toward *p*-nitrophenyl phosphate and fructose diphosphate we chose to look at the effect of modification of the enzyme on these catalytic activities.

Pontremoli *et al.* (1966, 1969) have reported that acetylimidazole can block both the FDP catalytic site and the AMP regulatory site of the enzyme. Further, in the presence of FDP the FDP catalytic site is protected and in the presence of AMP the AMP regulatory site is partially protected. This paper reports the results of experiments using acetylimidazole to modify the catalytic activities of fructose diphosphatase.

Materials and Methods

Fructose diphosphatase was purified by a procedure similar to those published (Sarngadharan *et al.*, 1970; Traniello *et al.*, 1972). Frozen rabbit livers were homogenized with an equal volume of 0.25 M sucrose in 1 mM Tris (pH 8.6) for 2 min. After centrifugation, adjustment of pH to 6.0, and dilution with an equal volume of 5 mM malonate (pH 6), the solution was stirred with 1:10 w/v of Whatman carboxymethylcellulose, CM-23. After centrifugation the supernatant solution was passed through a CM-23 column (2.1 × 41 cm) and the pale yellow effluent was diluted with an equal volume of malonate buffer and chromatographed on a column of Cellex-CM (Bio-Rad). The enzyme was eluted with 1 mM FDP in malonate buffer, pH 6.0. On polyacrylamide disc electrophoresis and sodium dodecyl sulfate gel electrophoresis the protein had a single protein band.

Standard assay conditions for FDP hydrolysis were: 0.01 M Tris (pH 9.4 or 7.5), 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM FDP, 1 mM NADP, 0.005 mg/ml of glucose-6-phosphate dehydrogenase and phosphoglucose isomerase in a total volume of 1 ml. For hydrolysis of *p*-nitrophenyl phosphate the cuvets contained 6 mM *p*-nitrophenyl phosphate, 0.05 mM $MnCl_2$, and either 0.04 M glycine (pH 9.4) or 0.01 M Tris (pH 7.5) in a total volume of 1 ml and the increase in absorbance at 410 nm was followed (ϵ 17.1 × 10^3 at pH 9.4).

Incubations with acetylimidazole were carried out as described by Pontremoli *et al* (1966). The enzyme was dialyzed against 50 mM sodium borate (pH 7.5). When FDP or AMP were present these were added in 1 mM Tris giving a final Tris concentration of 0.1 mM and a control was run using Tris alone. An aliquot of enzyme was assayed immediately before addition of acetylimidazole. The reagent (usually at a final concentration of 0.3 mg/ml) was added directly to the enzyme mixture and stirred to ensure its solution. Incubations were at room temperature and samples were removed and assayed at regular intervals following addition of the reagent.

All chemicals were obtained from Sigma. Assay enzymes were from Boehringer and frozen rabbit liver was from Pelfreez.

Results

Acetylimidazole reacting with liver fructose diphosphatase produces a pseudo-first-order loss of activity with FDP as substrate (Figures 1 and 2, solid circles). The original reports of Pontremoli *et al.* (1966) showed a lag of about 20 min before inactivation of the enzyme began. In their experiments aliquots of the enzyme incubation mixture were precipitated with ammonium sulfate and the precipitated enzyme was dialyzed for 90 min before assays were carried out. We use incubation conditions virtually identical with theirs but assay aliquots of the incubation mixture without precipitation or dialysis of the sample. The acetylimidazole

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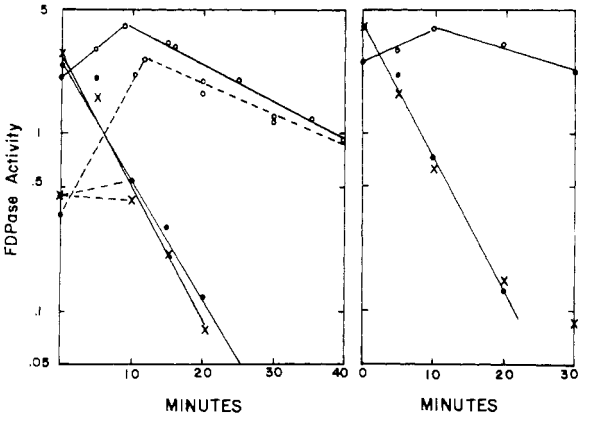

FIGURES 1 AND 2: Effect of acetylimidazole on the hydrolysis of FDP by fructose diphosphatase. Figure 1 (left) shows assays carried out at pH 7.5 and Figure 2 (right) at pH 9.4. (—) Activity measured in the absence of AMP; (- - -) assays in the presence of 0.1 mM AMP. Enzyme activity is expressed as change in absorbance per minute per milliliter of enzyme. The symbols indicate that the enzyme was incubated with acetylimidazole plus: (●) no addition, (○) 1 mM FDP, and (X) 0.1 mM AMP.

carried over into the assay cuvets has no detectable effect on the assays using the coupled enzyme assay system with large excess of the dehydrogenase and isomerase. We observe no lag before inactivation of the enzyme begins.

In all figures the dashed lines show activity measured in the presence of 0.1 mM AMP. The sensitivity of the FDP hydrolysis to AMP inhibition is lost within 10 min of the addition of acetylimidazole under these conditions. In Figure 1 the activity of the unmodified enzyme is about 80% inhibited by 0.1 mM AMP at pH 7.5. After exposure to acetylimidazole for 10 min the enzyme is no longer inhibited by this level of AMP. The rate of loss of AMP sensitivity must be much more rapid than that of the catalytic activity, which decays with a half-time of 4.5 min.

Protection by Substrate. Fructose diphosphate affords protection against inactivation by acetylimidazole. In Figures 1 and 2 (open circles) the FDP concentration which provides protection is 1 mM. This result is similar to that reported by Pontremoli (1966) although the time course differs from theirs. We find very little protection by 0.1 mM FDP. The relatively high concentration of FDP is necessary because it is a reversibly bound ligand which must be present in excess in order to block the irreversible inhibitor significantly.

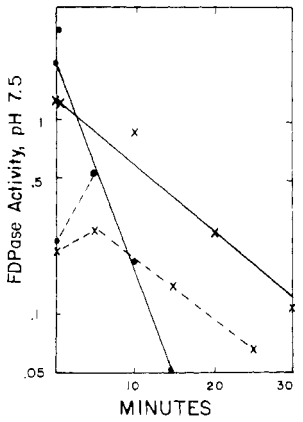

FIGURE 3: Effect of acetylimidazole and AMP on the hydrolysis of FDP by fructose diphosphatase. All assays are of FDP hydrolysis at pH 7.5. Symbols are as described in Figure 1 except that the concentration of AMP in the incubation is 1 mM.

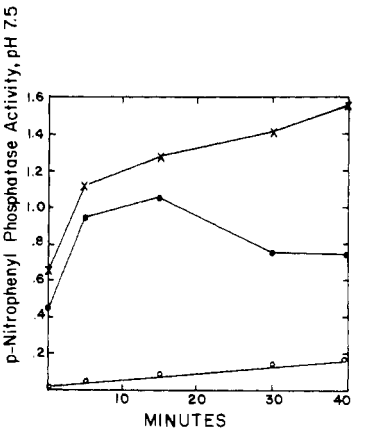

FIGURE 4: Effect of acetylimidazole on the hydrolysis of *p*-nitrophenyl phosphate by fructose diphosphatase. All assays are of *p*-nitrophenyl phosphate hydrolysis at pH 7.5. The symbols are as in Figure 1.

In our experiments we routinely find the activation shown in Figures 1 and 2 during the first 10 min of incubation with acetylimidazole in the presence of FDP. This period coincides with that in which the sensitivity to AMP is lost. It appears that acetylation at the regulatory site of this enzyme not only prevents inhibition by AMP but also stimulates activity at the catalytic site.

Protection by AMP. AMP at 0.1 mM has little effect on the inactivation or desensitization of the enzyme by acetylimidazole (Figure 1, crosses). However, 1 mM AMP not only protects the regulatory site of the enzyme but also provides partial protection of the FDP catalytic site (Figure 3). In the figure the dashed lines trace the activity assayed in the presence of 0.1 mM AMP. In the case of the AMP-protected enzyme the inactivation curves for activity measured in the presence or absence of AMP are nearly parallel. This result shows that the enzyme is not desensitized to AMP when the nucleotide is present during the reaction with acetylimidazole, a result also noted by Pontremoli *et al.* (1969). The partial protection of catalytic activity afforded by 1 mM AMP had not been observed previously.

Hydrolysis of p-Nitrophenyl Phosphate. The effects of acetylimidazole on the hydrolysis of *p*-nitrophenyl phosphate catalyzed by the enzyme are quite different from those described above for the hydrolysis of fructose diphosphate. The hydrolysis rate for *p*-nitrophenyl phosphate more than doubles within the first 10–15 min of incubation of the enzyme with acetylimidazole (Figure 4, solid circles). The increase in activity is followed by a slower decrease of this activity.

AMP protects against the slow loss of catalytic activity toward *p*-nitrophenyl phosphate. When AMP is present during the reaction with acetylimidazole the stimulation of this activity is still observed but the subsequent slow inactivation is prevented. The rapid reaction of acetylimidazole which "desensitizes" FDP hydrolysis (Figures 1 and 2) does not inhibit the hydrolysis of *p*-nitrophenyl phosphate. Thus there appear to be two types of reactive residues which can be protected by AMP: one reacts rapidly and affects the allosteric interconnection between the regulatory and FDP site, the second reacts more slowly and inhibits binding or hydrolysis of *p*-nitrophenyl phosphate.

Similarly, fructose diphosphate "protects" against the rapid stimulation by acetylimidazole of catalytic activity

toward *p*-nitrophenyl phosphate. In the experiment shown the enzyme was incubated with acetylimidazole in the presence of 1 mM FDP. In assaying aliquots of this solution the FDP concentration in the assay cuvet is 0.01 mM, a concentration sufficient to produce strong inhibition of *p*-nitrophenyl phosphate hydrolysis. As is apparent in Figure 4 the rate of stimulation of activity toward *p*-nitrophenyl phosphate is much slower than in the absence of FDP. Fructose diphosphate apparently protects the site which, when acetylated, produces rapid activation of *p*-nitrophenyl phosphate hydrolysis.

All the effects of acetylimidazole on the hydrolysis of *p*-nitrophenyl phosphate by the enzyme apply to assays at pH 7.5. Acetylimidazole has no effect on the hydrolysis of this substrate at pH 9.4. Several experiments have been carried out in which a single incubation mixture of enzyme with acetylimidazole is tested for its ability to hydrolyze FDP and *p*-nitrophenyl phosphate both at pH 9.4 and 7.5. Invariably the activity at either pH with FDP is rapidly lost while the activity toward *p*-nitrophenyl phosphate at pH 7.5 rapidly increases and that at pH 9.4 is practically unchanged. Neither FDP nor AMP alters the insensitivity of the pH 9.4 activity to acetylimidazole.

Discussion

Although there are differences in the kinetic details our results are qualitatively in agreement with those of Pontremoli *et al.* (1966, 1969) with respect to FDP hydrolysis, namely, that acetylimidazole blocks both the FDP catalytic site and the AMP regulatory site of fructose diphosphatase, that the FDP catalytic site is protected by FDP, and that the AMP regulatory site is partially protected by AMP.

The effects of acetylimidazole on *p*-nitrophenyl phosphate hydrolysis in part help to clarify whether this reaction is catalyzed at the FDP catalytic site or at the AMP regulatory site. Acetylation of the FDP site stimulates hydrolysis of *p*-nitrophenyl phosphate while acetylation of the AMP site inhibits this hydrolysis. Thus, FDP "protects" against the stimulation of *p*-nitrophenyl phosphate hydrolysis at pH 7.5 while AMP protects against loss of this hydrolytic activity. By analogy with the results using FDP as substrate these results appear to support the conclusion that *p*-nitrophenyl phosphate hydrolysis at pH 7.5 occurs at or near the AMP site, as was suggested by the earlier experiments (Chou and Kirtley, 1973).

Several interesting results suggest mutual interactions between the FDP catalytic site and the AMP regulatory site. Within 10 min of addition of acetylimidazole to the enzyme the sensitivity of FDP hydrolysis to AMP is lost. This desensitization occurs whether or not FDP is present during the incubation; however, when FDP is present it is possible to observe that the FDP hydrolytic activity is stimulated during this period. The result suggests that desensitization of the regulatory site can also result in stimulation of catalytic activity.

The stimulation of *p*-nitrophenyl phosphate hydrolysis by acetylation at the FDP catalytic site appears to be another example of interactions between the FDP and AMP sites analogous to that described above. In this case "desensitization" of the FDP site results in stimulation of catalytic activity at or near the other site.

A third example of interactions between the sites is the observation that protection of the AMP regulatory site can in part protect the FDP catalytic site. Thus in the presence of 1 mM AMP the rate of inactivation of FDP hydrolytic activity is significantly decreased, as in Figure 3.

The cause of the difference between our results and those of Pontremoli *et al.* (1966) in the time course of inactivation of FDPase remains uncertain. We have reproduced the incubation conditions as described in their paper; however, instead of precipitating and then dialyzing aliquots of the enzyme we assay the samples directly. Our enzyme may differ from theirs in the extent of partial proteolysis. Pontremoli *et al.* suggest that the 20 min lag they observe before loss of FDP hydrolytic activity begins is related to the acetylation of two to six tyrosine residues which are not directly involved in FDP hydrolysis and that "it might be postulated that acetylation of the first six residues alters the tertiary structure of the protein sufficiently as to expose buried tyrosines which subsequently react with the reagent." Possibly these residues are already "exposed" in our enzyme preparation.

References

Chou, T. H., and Kirtley, M. E. (1971), *Biochem. Biophys. Res. Commun. 45,* 98–103.

Chou, T. H., and Kirtley, M. E. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32,* 576.

Pontremoli, S., Grazi, E., and Accorsi, A. (1966), *Biochemistry 5,* 3072–3078.

Pontremoli, S., Grazi, E., and Accorsi, A. (1969), *J. Biol. Chem. 244,* 6177–6181.

Sarngadharan, M. G., Watanabe, A., and Pogell, B. M. (1970), *J. Biol. Chem. 245,* 1926–1929.

Traniello, S., Melloni, E., Pontremoli, S., Sia, C. L., and Horecker, B. L. (1972), *Arch. Biochem. Biophys. 149,* 222–231.