Functional consequences of treating turkey liver fructose-1,6-bisphosphatase with penicillin G[1]

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Summary. Treatment of turkey liver fructose-1,6-bisphosphatase with penicillin G progressively inactivated the enzyme and desensitized the enzyme toward high substrate inhibition. The treatment also led to reduced sensitivity to AMP inhibition and the loss of cooperative interaction among AMP-binding sites. These altered properties were not reversed by dialysis, but were prevented when treatment with penicillin G was performed in the presence of substrate.

Fructose-1,6-bisphosphatase (Fru-P_2ase) (EC 3.1.3.11) is a key enzyme in gluconeogenesis. The enzyme isolated from various sources is sensitive to inhibition by adenosine-5′-monophosphate (AMP) and high substrate concentration[2–6]. These 2 properties have been suggested as the possible mechanisms for the regulation of liver Fru-P_2ase activity under physiological conditions[2,3]. We report here that treatment of turkey liver Fru-P_2ase with penicillin G (PG) (benzyl-penicillin) partially inactivated the enzyme and altered the response of the enzyme to inhibition by AMP and high substrate concentration.

Materials and methods. All the chemicals used in this study were purchased from Sigma. Turkey liver Fru-P_2ase was purified by the method previously described[7]. Fru-P_2ase activity was assayed at 25 °C by measuring the rate of NADPH formation at 340 nm in a coupling system[3]. The standard assay mixture (1 ml) contained 50 mM Tris-HCl (pH 7.4), 0.1 mM $NADP^+$, 0.1 mM EDTA, 2 mM $MgSO_4$, 2 units each of phosphoglucose isomerase and glucose-6-P dehydrogenase, 0.075 mM fructose-1,6-bisphosphate, and 0.25 µg of purified turkey liver Fru-P_2ase. The reaction was started by the addition of substrate. The enzyme activity was based on the change in absorbancy at 340 nm between 2 and 4 min after the addition of substrate. The concentration of purified enzyme was determined by its absorbancy at 280 nm using the extinction coefficient ($E^{1\%}_{1\,cm}$) of 7.5[6]. Treatment of Fru-P_2ase with PG was carried out by incubating the native enzyme (0.25 mg/ml) at 18 °C in 50 mM Tris-HCl buffer (pH 7.8) containing 20 mM PG (sodium salt) and 0.5 mM EDTA in the presence or absence of 1 mM substrate. The untreated control was subjected to identical conditions but in the absence of PG. Portions were withdrawn at specific times and immediately diluted 10 times with 50 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA. Then 10 µl of this diluted enzyme solution was transferred to a cuvette and assayed for Fru-P_2ase activity.

Results and discussion. Treatment of turkey liver Fru-P_2ase with PG led to a progressive decrease in enzyme activity. The specific activity of the PG treated enzyme decreased about 67% after 80 h of incubation, while the specific activity of the untreated control decreased only about 7% during the same period (figure 1). This decrease in enzyme activity was not observed when treatment with PG was performed in the presence of 1 mM substrate.

Treatment with PG desensitized the enzyme toward inhibition by high concentration of substrate. As shown in figure 2, the specific activity of the untreated control measured at the concentration of 0.075 mM substrate was about 180% higher than that measured with 2 mM substrate. When the enzyme that had been treated with PG for 48 h was tested, the specific activity measured with 0.075 mM substrate was only about 8% higher than that measured with 2 mM substrate. The enzyme activities measured at these 2 substrate concentrations became virtually indistinguishable after 72 h of treatment with PG.

The sensitivity of this enzyme to AMP inhibition was also reduced by treatment with PG. The concentrations of AMP needed to inhibit 50% of Fru-P_2ase activity were about 12 µM for the untreated control and 50 µM for the enzyme treated with PG for 72 h (figure 3A). The treatment also changed the nature of AMP inhibition from sigmoidal to hyperbolic. The Hill plots[3] of $\log[V-v/v]$ vs $\log$ [AMP], where V and v are the uninhibited and the inhibited rates, respectively, yield straight lines for both the treated and the

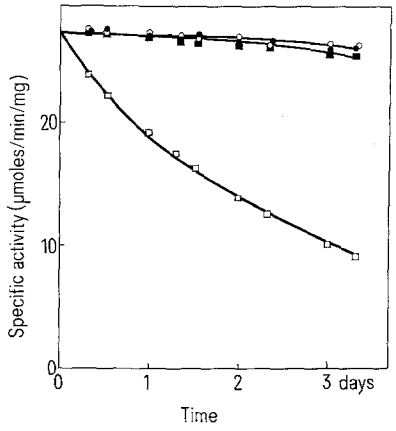

Fig. 1. Effect of PG treatment on the catalytic activity of turkey liver Fru-P_2ase. Fru-P_2ase was incubated: □, with PG in the absence of substrate; ■: in the absence of both PG and substrate; ●: in the presence of both PG and 1 mM substrate; ○: with 1 mM substrate but in the absence of PG. Portions were withdrawn at specific times, diluted, and assayed for enzyme activity as described in the text. Throughout the incubation period, the pH remained constant and no protein precipitation was detected.

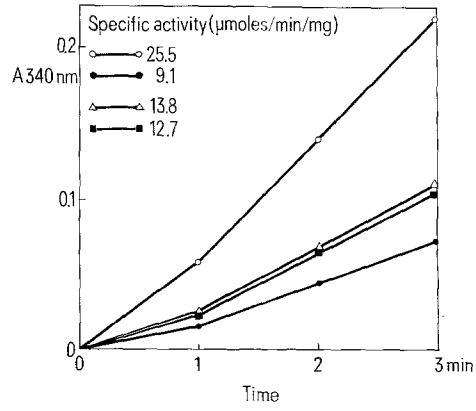

Fig. 2. Effect of PG treatment on the sensitivity of turkey liver Fru-P_2ase to high substrate inhibition. The following symbols signify: ○: untreated control assayed with 0.075 mM substrate; ●: untreated control assayed with 2 mM substrate; △: treated with PG for 48 h and assayed with 0.075 mM substrate; ■: treated with PG for 48 h and assayed with 2 mM substrate. In this experiment, the amount of purified enzyme used to assay Fru-P_2ase activity was 0.5 µg per reaction.

untreated enzymes. The Hill coefficients (n), as estimated from the slopes of the straight lines, are 1.9 for the untreated control and 1.1 for the PG-treated enzyme (figure 3B). Thus, treatment with PG resulted in the loss of cooperative interaction among AMP binding sites.

The presence of substrate not only protected the enzyme against PG-inactivation but also against altered response to inhibition by AMP or high substrate concentration. The mechanism of how the substrate exerts these protective effects remains to be investigated. However, the decreased sensitivity to inhibition by AMP or high substrate concentration is by no means the necessary consequence of enzyme inactivation. We induced about 50% inactivation of this enzyme by incubating it at 25 °C for 3 weeks and found that this partial inactivation was not accompanied by reduction in sensitivity to inhibition by either AMP or high substrate concentration. Partial inactivation of rabbit liver Fru-P_2ase by treatment with ADP or ATP or by incubating the enzyme at acidic pH even led to increased sensitivity to inhibition by AMP[8-10]. The altered properties induced by PG appear to be irreversible since they remained essentially unchanged after extensive dialysis or repeated washing on ultrafiltration membrane (XM-50, Amicon). Incubation of the modified enzyme (after dialysis) with substrate also failed to reverse these altered properties. It is speculated that the altered properties induced by PG might result from the formation of covalent bonds between PG and the enzyme molecule.

The molecule of PG, like those of other penicillins, contains a reactive 4-membered (β-lactam) ring. This β-lactam may be cleavaged at the CO–N bond when reacted with ε-amino group of lysyl residues in proteins to form stable benzylpenicilloyl derivatives of tissue proteins (or enzymes). This has been proposed to be the mechanism for the formation of penicillin antigen[11]. It has been reported that modification of ε-amino groups of lysyl residues of pig kidney Fru-P_2ase with pyridoxal-5'-P partially inactivated the enzyme and markedly decreased the enzyme sensitivity to inhibition by AMP and high substrate concentration[5]. These results are somewhat similar to our observations with PG treatment of turkey liver Fru-P_2ase.

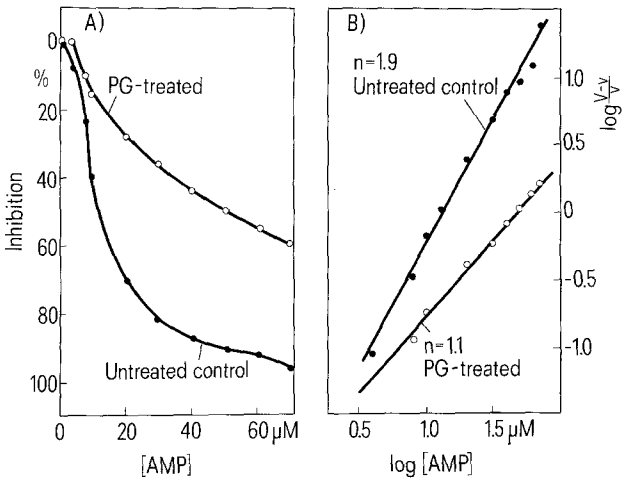

Fig. 3. Inhibition of Fru-P_2ase activity by varying concentrations of AMP (A) and Hill plots of these data performed according to Taketa and Pogell[3] (B). V is the activity without AMP and v is the activity in the presence of AMP. Hill coefficients (n) were calculated from the slopes of the straight lines. The following symbols signify: ●: untreated control; ○: treated with PG for 72 h.

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Synthesis of specific cholinergic inhibitors for affinity chromatography[1]

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Summary. Our first aim was to simplify the spacer-synthesis for affinity-chromatography of cholinergic proteins. Furthermore we synthesized 2 new inhibitors which proved to be useful for purification of acetylcholine-receptor protein.

Specific proteins from the cholinergic nervous system can be highly purified by biospecific adsorption and subsequent desorption (affinity chromatography), as described in our earlier work[2-4]. The best results are obtained when the inhibitor, which has to be bound to a solid support (agarose), adequately fits the receptor, or in case of an enzyme, the active site. However the best inhibitor is useless if bound too close to the solid support. Cuatrecasas[5] first had the idea of separating the inhibitor from the solid support by a 'spacer' of considerable length. In case of acetylcholinesterase, a spacer of about 45–58 Å has been proved to be adequate.

The conventional spacer-synthesis[5], however, is relatively time-consuming, as it requires 5 steps. To overcome this difficulty, we synthesized 1-(N,N,N-trimethylammonium)-10-decylamine bromide hydrobromide (**1**), which already has a considerable length. This substance was first synthesized by Barlow[6], but the description lacks the necessary details. We prepared it (scheme 1) by selective synthesis from 1,10-dibromodecane and potassium phthalimide. The