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ALTERATION OF THE REGULATORY PROPERTIES OF CHICKEN LIVER FRUCTOSE-1,6-BISPHOSPHATASE BY TREATMENT WITH ASPIRIN

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SUMMARY

Treatment of chicken liver fructose-1,6-bisphosphatase with aspirin at pH 7.8 desensitizes the enzyme toward inhibiiton by AMP. This treatment also reduces the enzyme affinity for substrate and the enzyme sensitivity to high substrate inhibition. These altered properties remain essentially unchanged even after removal of nearly all aspirin by dialysis or ultrafiltration. They are also stable to hydroxylamine. Salicylate is ineffective in inducing these altered properties. Both the reduced affinity for substrate and the reduced sensitivity to high substrate inhibition are largely prevented by substrate, while AMP specifically prevents the desensitization to allosteric inhibition by AMP.

Fructose-1,6-bisphosphatase (Fru-P_2ase) (EC 3.1.3.11) is a key enzyme in gluconeogenesis. Fru-P_2ase isolated from various sources is sensitive to inhibition by excess of substrate and by adenosine-5'-monophosphate (AMP). These two properties have been suggested as the possible mechanisms for the regulation of liver Fru-P_2ase activity under physiological conditions (1). The sensitivity to high substrate inhibition is reduced after treatment with pyridoxal-5'-P (2), while the sensitivity to AMP inhibition is reduced or abolished in the presence of salicylate (3) or after treatment with several reagents, such as fluorodinitrobenzene (4), acetylimidazole (5), diazobenzene sulfonic acid (6), pyridoxal-5'-P (2,7,8), and 2,3-butanedione (9,10). We report here that treatment of chicken liver Fru-P_2ase with aspirin at pH 7.8 desensitizes the enzyme to AMP inhibition. The treatment also reduces the enzyme affinity for substrate and the enzyme sensitivity to high substrate inhibition.

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These altered properties appear to result from the acetylation of the enzyme molecule.

MATERIALS AND METHODS

All the chemicals used in this study were purchased from Sigma. Chicken liver Fru-P_2ase was purified by the method previously described (11). The activity of Fru-P_2ase was assayed at 25° C by 2 different methods. In method I, the activity was assayed by measuring the rate of NADP reduction at 340 nm in the presence of excess phosphoglucose isomerase and glucose-6-P dehydrogenase. The standard assay mixture (1 ml) contained 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.15 mM NADP, 2 mM $MgSO_4$, 2 units each of phosphoglucose isomerase and glucose-6-P dehydrogenase, an appropriate amount of Fru-P_2ase, and various amounts of fructose-1,6-bisphosphate (Fru-P_2). The reaction was initiated by the addition of substrate (Fru-P_2). For cases when very low Fru-P_2 concentrations (10 μM and lower) should be used, the activity was assayed by measuring the rate of DPNH oxidation in a coupling system as previously described (12). In this method II, the assay mixture (1 ml) contained 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.15 mM DPNH, 2 mM $MgSO_4$, 0.15 mM ATP, 0.25 mM P-enolpyruvate, 2 units each of lactic dehydrogenase, pyruvic kinase, and phosphofructokinase, an appropriate amount of Fru-P_2ase, and various amounts of Fru-P_2. The reaction was also initiated by the addition of substrate. Prior to use , ATP was treated with AMP deaminase followed by removal of deaminase through ultrafiltration according to the procedure previously described (13). This process effectively removed the small amount of AMP frequently contaminated in the commercial preparation of ATP. The concentration of purified chicken liver Fru-P_2ase was determined by its absorbancy at 280 nm using the extinction coefficient ($E^{1\%}_{1\,cm}$) of 7.5 (11).

Unless otherwise indicated, treatment of Fru-P_2ase with aspirin was carried out by incubating the enzyme (0.25 mg/ml) at 25° C in 70 mM Tris-HCl (pH 7.8) containing 15 mM Na salt of aspirin (Na acetylsalicylate) and 0.1 mM EDTA. The untreated control was subjected to identical conditions except in the absence of aspirin. Aliquots were removed at the indicated times and immediately diluted 50-fold with cold 50 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA and stored in ice. Then an appropriate amount of this diluted enzyme solution was removed and assayed for Fru-P_2ase activity. The maximum concentration of aspirin introduced into the assay mixture was 15 μM.

RESULTS

Treatment of chicken liver Fru-P_2ase with 15 mM aspirin at pH 7.8 for 4 h enhanced about 60% of the enzyme activity and the enzyme became totally insensitive to 25 μM AMP which inhibited about 75% of the control enzyme activity (Fig. 1). Due to the combined effects of enhanced activity and AMP desensitization, the activity of the treated enzyme increased more than 6-fold when assayed in the presence

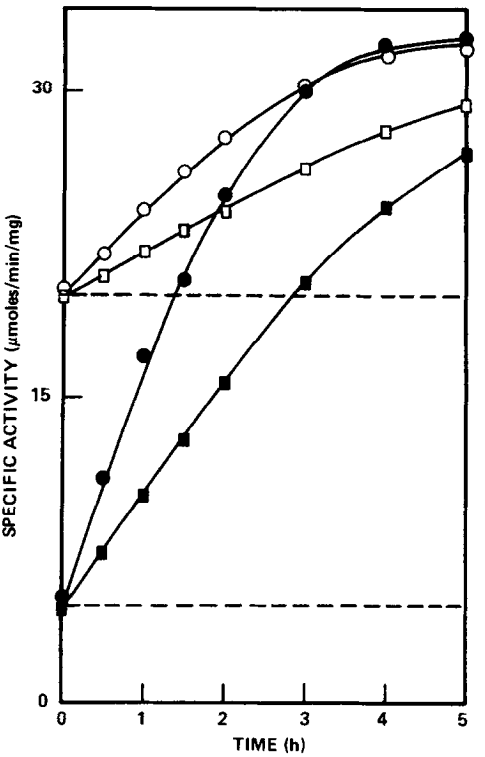

Fig. 1. Time course of changes of chicken liver Fru-P_2ase activity following treatment with aspirin. Fru-P_2ase (0.25 mg/ml) was treated with 7 or 15 mM aspirin at pH 7.8 as described in MATERIALS AND METHODS. The untreated control was subjected to identical conditions except in the absence of aspirin. Aliquots were removed at the indicated times and immediately diluted 50-fold with cold 50 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA and stored in ice. Then 50 μl of the diluted enzyme solution was transferred to a cuvette and assayed for Fru-P_2ase activity at the substrate concentration of 100 μM using assay method I. The following symbols signify: (○) treated with 15 mM aspirin and assayed in the absence of AMP; (●) same as (○) but assayed in the presence of 25 μM AMP; (□) treated with 7 mM aspirin and assayed in the absence of AMP; (■) same as (□) but assayed in the presence of 25 μM AMP. The upper and the lower broken lines represent the activities assayed in the absence and the presence of 25 μM AMP, respectively. These activities remained essentially constant throughout the entire incubation period.

of 25 μM AMP. Both the rates of enhanced activity and AMP desensitization were reduced by a decrease in the concentration of aspirin used. As also shown in Fig. 1, treatment of the enzyme with 7 mM aspirin for 4 h resulted in only about 40% activation, and the

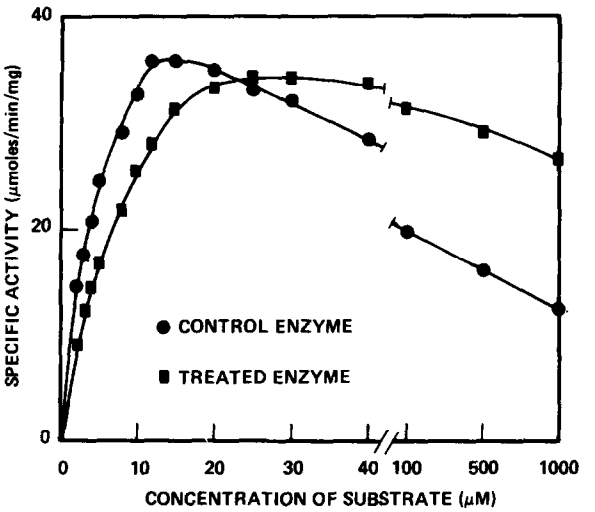

Fig. 2. Effect of substrate concentration on the catalytic activity of chicken liver Fru-P_2ase following treatment with aspirin. Fru-P_2ase (0.25 mg/ml) was incubated with 15 mM aspirin at pH 7.8 as described in MATERIALS AND METHODS. The untreated control was subjected to identical conditions except in the absence of aspirin. After 5 h of incubation, both the control and the treated enzymes were diluted 50-fold with cold 50 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA and stored in ice. Appropriate amounts of these diluted enzyme solutions were then removed and assayed for Fru-P_2ase activities using either method I or method II depending on the concentration of substrate used in the assay (for detail see MATERIALS AND METHODS).

complete desensitization to 25 μM AMP was not yet achieved even after incubation for 5 h.

In Fig. 1, the enzyme activities were assayed with 100 μM Fru-P_2 simply because most of the studies reported in the literature used this substrate concentration to measure Fru-P_2ase activity. Since the activity of this enzyme is sensitive to inhibition by excess of substrate, we measured the specific activities of both the control enzyme and the enzyme treated with 15 mM aspirin for 5 h at various substrate concentrations (Fig. 2). It was found that aspirin treatment markedly reduced the enzyme sensitivity to high substrate inhibition. The % inhibition of the enzyme activity as the result of increasing substrate concentration from 25 μM to 100 μM was about 41

for the control enzyme as compared with only about 8.5 for the treated enzyme. The treatment also significantly decreased the enzyme affinity for substrate. The values of K_m as estimated from the double reciprocal plots using the noninhibitory substrate concentrations were about 4.1 μM for the control enzyme and 7.1 μM for the treated enzyme. The catalytic activity of the treated enzyme, as compared with that of the control, was increased, decreased, or unchanged depending on the concentration of substrate used in the assay. The increased activity when assayed above 25 μM substrate was apparently due to the reduced sensitivity of the treated enzyme to high substrate inhibition, while the decreased activity when assayed below 20 μM substrate probably resulted from the reduced affinity of the treated enzyme for substrate.

Table I shows that the presence of 0.15 mM AMP completely prevented the desensitization to AMP inhibition, but did not prevent the altered responses to substrate (reduced affinity for substrate and reduced sensitivity to high substrate inhibition). Conversely, the presence of 2 mM substrate largely prevented the altered responses to substrate, but did not prevent the desensitization to AMP inhibition. The presence of 0.15 mM substrate provided little protection against altered responses to substrate.

The altered properties induced by aspirin were not reversed by repeated washing on Amicon ultrafilter (XM-50) with 50 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA, nor by dialysis against 1000 volumes of this buffer for 14 h (3 buffer changes). Incubation of the modified enzyme (after dialysis) at 25° C with 1.2 M NH_2OH at pH 7.4 for 5 h also failed to reverse the altered properties. Under the identical conditions, Na salicylate failed to induce the altered properties as observed with aspirin treatment.

Table I

Effect of AMP and Substrate on the Modification of the Catalytic Properties of Fru-P_2ase by Treatment with Aspirin[a]

Enzyme	K_m (μM)	Sp Act. at 25 μM Fru-P_2 (μmoles/min/mg)	% inhibn by[b] 100 μM Fru-P_2	% Inhibn by[c] 50 μM AMP
Control	4.1	33.4	41.3	91.3
Treated	7.1	34.2	8.5	0
Treated in the presence of 0.15 mM AMP	7.1	34.6	8.3	91.8
Treated in the presence of 0.15 mM Fru-P_2	6.7	33.9	10.8	0
Treated in the presence of 2 mM Fru-P_2	4.8	33.6	37.1	0

[a]Fru-P_2ase (0.25 mg/ml) was incubated at 25° C with 15 mM aspirin at pH 7.8 in the presence or absence of AMP and Fru-P_2 as indicated. After 5 h of incubation, both the control and the treated enzymes were diluted 50-fold with cold 50 mM Tris-HCl (pH 7.8) containing 0.1 mM EDTA and stored in ice. Appropriate amounts of these diluted enzyme solutions were then removed and assayed for Fru-P_2ase activity using assay method I except in the determination of K_m where method II was used (for detail see MATERIALS AND METHODS).

[b]Percent inhibition by 100 μM Fru-P_2 was calculated using the relative value of 100 when assayed with 25 μM Fru-P_2.

[c]In the determination of % inhibition by 50 μM AMP, the enzyme activity was assayed with 25 μM Fru-P_2 and a relative value of 100 was given to the enzyme activity assayed in the absence of AMP.

DISCUSSION

Treatment of chicken liver Fru-P_2ase with Na salt of aspirin at pH 7.8 leads to desensitization of the enzyme to inhibition by AMP. This treatment also markedly reduces the enzyme affinity for substrate and the enzyme sensitivity to high substrate inhibition. These altered properties remain essentially unchanged even after removal of nearly all aspirin by dialysis or ultrafiltration. Treatment of this enzyme with Na salt of salicylic acid (deacetylated product of

aspirin) under the identical conditions fails to induce these altered properties. These observations suggest that the aspirin-induced alteration of the enzyme properties may result from the acetylation of the enzyme molecule.

Marcus (3) has recently reported that the sensitivity of pig kidney Fru-P_2ase to AMP inhibition is decreased when salicylate (5 to 20 mM) is present in the assay mixture. He suggests that salicylate interacts at the allosteric site of Fru-P_2ase. Based on our observation that treatment of Fru-P_2ase with salicylate followed by dilution or dialysis fails to alter the enzyme sensitivity to AMP inhibition, it is considered that the effect of salicylate on the allosteric property of Fru-P_2ase as observed by Marcus (3) is reversible. This is in contrast to our observation that the aspirin-induced desensitization to AMP inhibition is not reversed by dilution or dialysis.

Aspirin is known to acetylate human albumin *in vivo* by reaction with the ϵ-amino group of lysine. This may alter the antigenicity of albumin (14). Aspirin is also known to acetylate platelets and hemoglobin (15). The inhibition of prostaglandin synthetase by aspirin is now attributed to the formation of N-acetylserine at the NH_2 terminus of the enzyme (16).

The reduced affinity for substrate and the reduced sensitivity to high substrate inhibition may be related because both are largely prevented when treatment with aspirin was carried out in the presence of 2 mM substrate. These altered responses to substrate, however, can be independent of AMP desensitization since, by manipulating the experimental conditions, the former can occur in the absence of the latter or *vice versa* (Table I). Thus, the altered responses to substrate and AMP desensitization most likely involve the acetylation of 2 distinct regions. The fact that both of these 2 altered

properties are stable to NH_2OH suggests that they are both associated with the formation of N-acetyl linkages (for references see (5) and (16)). Research is now in progress to determine whether the acetylation of the ϵ-amino groups of lysyl residues is responsible for the altered properties observed with aspirin treatment.

Since the desensitization to AMP allosteric inhibition can be specifically prevented by AMP, it is possible that AMP desensitization may result from the acetylation of the AMP allosteric site or the region near this site. The data shown in Table I suggest that high concentration of substrate may prevent aspirin to acetylate the specific site associated with high substrate inhibition. The mechanism of how the substrate exerts this preventive effect remains to be established. Although the inhibition of Fru-P_2ase activity by excess of substrate has been known for at least 15 years (12), the basis for this inhibition is still poorly understood. It is possible that aspirin may become a valuable reagent in elucidating the mechanism of the inhibition of Fru-P_2ase by excess of substrate.

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