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Desensitization of kidney fructose-1,6-diphosphatase by acetic anhydride

The inhibitory effect of AMP on the activity of fructose-1,6-diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) from several sources has been explained by the existence of different sites, concerned with catalytic activity and AMP inhibition, in the enzyme molecule (for review on fructose-1,6-diphosphatases, see ref. 1). The involvement of different amino acid residues at the two sites has been conclusively demonstrated for *Candida utilis* fructose-1,6-diphosphatase, by means of chemical modification with fluorodinitrobenzene[2], and for rabbit liver fructose-1,6-diphosphatase, by treating the enzyme with *N*-acetylimidazole[3] or with diazobenzene sulfonic acid[4].

The AMP inhibition of purified pig kidney fructose-1,6-diphosphatase was first shown by MENDICINO AND VASARHELY[5], and more extensively studied by MARCUS[6]. The latter report showed that the sensitivity of kidney fructose-1,6-diphosphatase to allosteric AMP inhibition was not suppressed, without loss of enzymic activity, by using a variety of treatments usually employed for desensitization of allosteric enzymes.

In the present study, it has been found that treatment of kidney fructose-1,6-diphosphatase with acetic anhydride, under conditions favouring the selectivity of the reagent for amino groups[7,8] largely desensitizes the enzyme against allosteric inhibition by AMP.

Fructose-1,6-diphosphatase activity was measured by the formation of inorganic phosphate from fructose 1,6-diphosphate, as previously described[6]. 1 unit of fructose-1,6-diphosphatase activity was defined as that amount of enzyme catalyzing the formation of 1 μmole of inorganic phosphate per min under the conditions of the assay. Pig kidney fructose-1,6-diphosphatase was prepared as previously described[6]. 20 ml of enzyme thus obtained (0.35 mg/ml) were dialyzed twice for 16 h at 4°, each time against 1000 ml of 0.5 M KCl, containing 0.1 mM EDTA. Under these conditions the enzyme is stable and this preparation can be kept at 4° for at least a month without appreciable change in either fructose-1,6-diphosphatase activity or sensitivity to AMP inhibition. Dilutions of fructose-1,6-diphosphatase solutions were made in 0.1 M KCl, containing 0.01 mM EDTA.

Acetylations were performed in 50 mM Tris–HCl, pH 7.5. All other conditions were as described by COLMAN AND FRIEDEN[7], using fresh dilutions of acetic anhydride (Merck AG, Darmstadt, Germany) with distilled water. As expected, the concentration of Tris, as well as the speed of preparation of acetic anhydride solutions, were critical in order to obtain reproducible results. Since the effect of acetylation, as judged by the observed changes in fructose-1,6-diphosphatase activity and AMP inhibition, was complete in 15 min and no further changes occurred in 24 h at 4°, acetylation times of at least 15 min (usually between 15 and 60 min) were used in the experiments reported below.

As shown in Fig. 1, treatment of fructose-1,6-diphosphatase with acetic anhydride results in a concentration-dependent loss of sensitivity of the enzyme to its allosteric effector, AMP. While catalytic activity is not affected at the lower concentrations of the reagent (up to 0.8 mM), it is reduced at higher concentrations. Since no

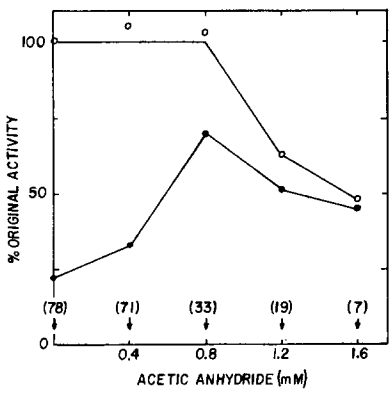

Fig. 1. Effect of acetic anhydride on activity of kidney fructose-1,6-diphosphatase and on its inhibition by AMP. The incubation mixture (1.0 ml) contained 0.23 mg of fructose-1,6-diphosphatase (specific activity 12.9), 50 mM Tris–HCl buffer at pH 7.5 and freshly prepared acetic anhydride at the concentrations shown in the abscissa. After at least 15 min at 0°, aliquots were removed, diluted (in 0.1 M KCl, 0.01 mM EDTA) and assayed for residual activity with 0.5 mM fructose 1,6-diphosphate in the presence (●—●) and absence (○—○) of 0.11 mM AMP. The values in parentheses indicate the percentage inhibition by 0.11 mM AMP after treatment by acetic anhydride at the concentration shown by the arrows.

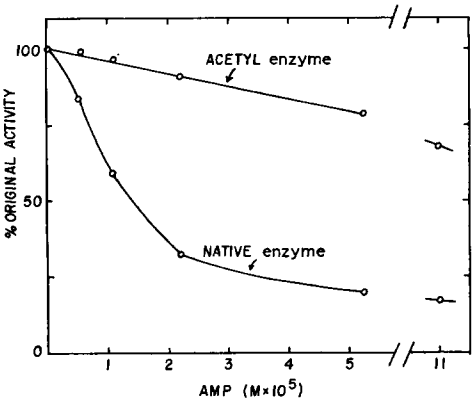

Fig. 2. Effect of AMP on the activity of native and acetylated fructose-1,6-diphosphatases. Fructose-1,6-diphosphatase was acetylated as described in the legend to Fig. 1, except that acetic anhydride was fixed at 1.2 mM. The specific activities were 12.2 for the native enzyme and 11.3 for the acetylated fructose-1,6-diphosphatase.

alterations of either fructose-1,6-diphosphatase activity or AMP inhibition were observed when fructose-1,6-diphosphatase was incubated with acetic acid, up to 5 mM, under the conditions described in Fig. 1, the effect can be attributed to acetic anhydride. As a first approximation the result can be interpreted as acetylation of functional groups responsible for allosteric inhibition, as well as acetylation of groups involved in catalytic activity.

That two different sets of functional groups are involved is clearly demonstrated by the results shown in Table I. While the presence of fructose 1,6-diphosphate or Mg^{2+} is not affecting the desensitization of the enzyme, the allosteric effector AMP, affords complete protection of groups responsible for allosteric inhibition. The observed

TABLE I

EFFECT OF SUBSTRATE, EFFECTOR AND COFACTOR ON INACTIVATION AND DESENSITIZATION OF FRUCTOSE-1,6-DIPHOSPHATASE BY ACETIC ANHYDRIDE

The experiments were performed as described in the legend to Fig. 1, except that acetic anhydride was fixed at 1.2 mM, and other additions were made as indicated.

Additions to acetylation mixture	*Specific activity (units/mg)*	*AMP inhibition (%)*
None	11.2	31
7.5 mM $MgSO_4$	11.0	26
2.0 mM fructose 1,6-diphosphate	11.3	31
0.1 mM AMP	9.3	81

TABLE II

EFFECT OF HYDROXYLAMINE ON NATIVE AND ACETYLATED FRUCTOSE-1,6-DIPHOSPHATASES

Except for the presence of 45 mM $MgSO_4$, fructose-1,6-diphosphatase was treated with 1.1 mM acetic anhydride as described in the legend to Fig. 1. Samples of native and acetylated enzymes were assayed, before and after treatment with 1.1 M hydroxylamine at pH 7.5 for 10 min at 25° (ref. 10). Hydroxylamine treatment was performed in a reaction mixture containing 1.1 M NH_2OH, 11 mM $MgSO_4$, 0.1 mM EDTA, and 10 mM Tris buffer.

Enzyme	*Specific activity (units/mg)*	*AMP inhibition (%)*
Native	10.4	74
Native + NH_2OH	9.6	77
Acetylated	10.4	19
Acetylated + NH_2OH	11.0	13

decrease in activity when AMP was added to the acetylation mixture was not due to AMP inhibition, since its final concentration in the assay system was 1.4 μM, a concentration which causes no inhibition of fructose-1,6-diphosphatase activity. Whether the AMP protection against desensitization is due to blocking of a group responsible for its binding, or of a group involved in allosteric interaction between sites, remains to be established.

For comparison, a detailed analysis of the effect of AMP concentration on the native and desensitized forms of fructose-1,6-diphosphatase is shown in Fig. 2.

Although acetylation by acetic anhydride in Tris buffer, as used in the present study, appears to exclude the acetylation of several amino acid reactive groups, including tyrosyl residues[8,9], it was important to obtain preliminary evidence in relation to the nature of the groups acetylated, especially since tyrosyl residues are involved in the allosteric regulation of rabbit liver fructose-1,6-diphosphatase[3,4]. As shown by the negative results of reversal of desensitization after treatment with 1.1 M neutral hydroxylamine (Table II), the participation of tyrosyl, as well as hystidyl or sulfhydryl residues, could be preliminarily excluded in the desensitization herein reported.

Further investigations are now in progress to establish the exact nature and number of the groups which on acetylation lead to the loss of the allosteric properties of kidney fructose-1,6-diphosphatase. However, the fact that desensitization occurs under conditions that have been shown to be more selective for the acetylation of amino groups[7,8], the negative results of reversal by hydroxylamine, together with the recent finding that kidney fructose-1,6-diphosphatase can be desensitized by pyridoxal 5'-phosphate (F. MARCUS, unpublished results), strongly suggest the involvement of amino groups in the allosteric regulation of kidney fructose-1,6-diphosphatase.

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Laboratory of General Biochemistry,
Faculty of Chemistry and Pharmacy,
University of Chile,
Santiago (Chile)

FRANK MARCUS

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