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Properties of glutaraldehyde-modified bovine hepatic fructose-1,6-diphosphatase

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SUMMARY

Incubation of bovine hepatic fructose-1,6-diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) with glutaraldehyde, a bifunctional cross-linking agent, alters both the catalytic and allosteric properties of the enzyme. The catalytic activity was decreased at both pH 6.5 and pH 9.0, and the enzyme was desensitized to inhibition by AMP and Fru-1,6-P_2. The pH optimum of the glutaraldehyde-treated enzyme was shifted towards alkaline pH. These changes may be due to the fixing of a neutral activity conformer of fructose-1,6-diphosphatase into a conformer with alkaline activity.

Fructose-1,6-diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) is an important regulatory enzyme in the gluconeogenic pathway[1,2]. The enzyme is inhibited by high concentrations of its substrate, and by the allosteric modifier AMP[3,4]. Current theories on the mechanism of allosteric regulation suggest that allosteric modifiers function by altering conformational states of enzymes[5]. Conformational changes are also believed to occur during catalysis[6]. If conformational changes are important, then a bifunctional cross-linking reagent such as glutaraldehyde might be able to prevent the necessary movement and thereby modify the catalytic and allosteric properties of the enzyme. Glutaraldehyde has been shown to modify the allosteric properties of glycogen phosphorylase *b*[7,8].

In this report, the catalytic and allosteric properties of bovine liver fructose-1,6-diphosphatase have been studied after modification by glutaraldehyde.

Purified fructose-1,6-diphosphatase was prepared either by the method of Byrne *et al.*[9] or by a modified procedure developed by Arneson *et al.* (Arneson, R.M., Geller, A.M. and Byrne, W.L., unpublished results). Enzyme activity was determined either by measuring

the release of inorganic phosphate from Fru-1,6-P_2 (ref. 9) or by measuring the formation of Fru-6-*P* using a coupled spectrophotometric assay. Inorganic phosphate and protein were determined as previously described[9]. The conditions under which fructose-1,6-diphosphatase was treated with glutaraldehyde are described in the legends to Figs 1 and 3.

Bovine fructose-1,6-diphosphatase, which is maximally active at neutral pH, has a double pH optimum, with maxima at pH 6.5 and 9.0. The two activities are associated with a single enzyme, and probably with a single catalytic site[10]. If one assumes that the two activities result from different conformations at neutral and alkaline pH, then treatment with glutaraldehyde at either pH 6.5 or 9.0 might fix the enzyme into a "neutral" or "alkaline" conformer.

After incubation with several concentrations of glutaraldehyde at pH 6.5 (Fig. 1), fructose-1,6-diphosphatase activity at pH 6.2 was found to be considerably reduced. Approximately 50% inhibition of activity was obtained with 5–10 mM glutaraldehyde. Similar results were obtained when the glutaraldehyde-treated enzyme was assayed at pH 8.8, although less inhibition was observed.

When fructose-1,6-diphosphatase was incubated with glutaraldehyde at pH 9.0, and then assayed at pH 6.2 and 8.8, the results were similar to those obtained after incubation with glutaraldehyde at pH 6.2, but the rate of modification was considerably faster. For example after incubation at pH 6.5 with 10 mM glutaraldehyde for 5 min, approximately 60% of the original activity remained when the enzyme was assayed at pH 6.2 and 95% of the original activity remained when it was assayed at pH 8.8. When fructose-1,6-diphosphatase was incubated at pH 9.0 under the same conditions, only 17% of the original activity at pH 6.2 remained and 31% of the activity at pH 8.8 remained. Therefore glutaraldehyde modification at either pH produces the same type of effect, since in both cases the neutral activity is lost relative to the alkaline activity.

The effect of glutaraldehyde modification on the allosteric inhibition by AMP was investigated next. Glutaraldehyde treatment at pH 6.2 was found to decrease the extent of inhibition by AMP when the modified enzyme was assayed at pH 6.5 (Fig. 2). In the neutral pH range, where inhibition by AMP is maximal, untreated bovine fructose-1,6-diphosphatase was inhibited about 95% by 0.25 mM AMP, whereas fructose-1,6-diphosphatase modified with 5 mM glutaraldehyde was inhibited only 55% by the same concentration of AMP. Higher concentrations of glutaraldehyde almost completely abolished inhibition by AMP. Fructose-1,6-diphosphatase was also desensitized to inhibition by AMP after incubation with glutaraldehyde at pH 9.0.

Since glutaraldehyde treatment reduces AMP inhibition, it was of interest to determine if AMP, present during the incubation with glutaraldehyde, would decrease the rate of reaction with glutaraldehyde. When fructose-1,6-diphosphatase was incubated with 20 mM glutaraldehyde at pH 6.2 in the presence of 1 mM AMP, protection against the loss of AMP inhibition was observed.

In the above experiments the incubations were terminated by the addition of Tris. However, in order to obtain a stable preparation of glutaraldehyde-treated fructose-1,6-diphosphatase which could be well characterized it was necessary to remove all excess

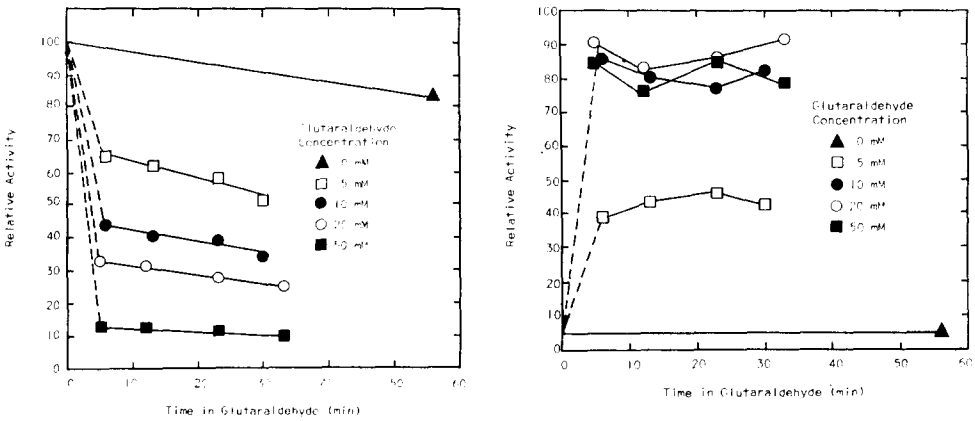

Fig. 1. The effect of glutaraldehyde concentration on fructose-1,6-diphosphatase activity. An aliquot of fructose-1,6-diphosphatase was added to a solution of 25 mM malate–25 mM borate buffer, pH 6.5 at 0 °C. In order to react glutaraldehyde with fructose-1,6-diphosphatase, a solution of glutaraldehyde (one-tenth the volume of the fructose-1,6-diphosphatase solution) was added to the buffered fructose-1,6-diphosphatase at pH 6.5 and 0 °C. At the times indicated, an aliquot of glutaraldehyde-treated fructose-1,6-diphosphatase was removed and added to an equal volume of 100 mM Tris, pH 7.5, to stop the reaction. This solution was incubated at 37 °C for 5 min, and assayed at 37 °C and pH 6.2. The assay solution contained 4 mM Fru-1,6-P_2, 4 mM $MgSO_4$, 0.4 mM EDTA, 10 mM Tris and 90 mM histidine in a final volume of 2.0 ml. The control was treated in an identical manner, but in the absence of glutaraldehyde. Purified glutaraldehyde (70% aqueous solution) was obtained from Ladd Research Industries, Burlington, Vermont.

Fig. 2. Effect of glutaraldehyde on AMP inhibition. Fructose-1,6-diphosphatase was incubated with several concentrations of glutaraldehyde for various lengths of time, and then assayed at pH 6.2 in the presence of AMP as described in Fig. 1. The assay solution contained 0.24 mM AMP.

glutaraldehyde. This was accomplished by passage through a Sephadex G-50 column as described in the legend to Fig. 3.

Some of the properties of the glutaraldehyde-free modified fructose-1,6-diphosphatase were then studied. The pH optimum of the modified enzyme was shifted from pH 6.5 to 9.0, and the ratio of activity at pH 6.5 to the activity at pH 9.0 was decreased from 1.6 to 0.55. Fig. 3 shows the effect of AMP on the control and glutaraldehyde-modified enzymes. The inset shows that the extrapolated maximum inhibition by AMP appears to be the same for both the native and modified enzymes. The $K_{0.5}$ (concentration of AMP required to give 50% inhibition) is approximately 0.03 mM for the control enzyme and approximately 0.10 mM for the glutaraldehyde-treated enzyme. In addition, the modified enzyme no longer exhibits homotropic interactions towards AMP.

Byrne *et al.*[11] have observed that increased concentrations of Mg^{2+} decrease the inhibition of the native enzyme by AMP. However, both native and glutaraldehyde-modified fructose-1,6-diphosphatase showed the same response to varied Mg^{2+} concentrations at pH 6.2 and 8.8; and therefore, changes in the affinity for Mg^{2+} cannot account for the decreased inhibition by AMP.

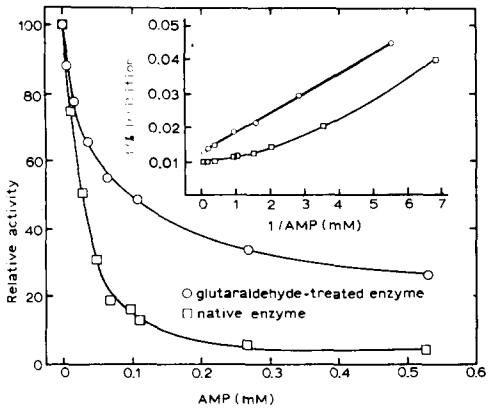

Fig. 3. The effect of AMP on the activity of native and glutaraldehyde-treated fructose-1,6-diphosphatase. Fructose-1,6-diphosphatase activity was determined at pH 6.2 in an assay solution containing 5 mM Fru-1,6-P_2, 5 mM $MgSO_4$, 0.5 mM EDTA, 25 mM Tris, 25 mM histidine and AMP as indicated. The inset is a double-reciprocal plot of percent inhibition *versus* AMP concentration. The glutaraldehyde-treated enzyme used for this experiment was separated from excess glutaraldehyde before use. Purified fructose-1,6-diphosphatase was incubated with 20 mM glutaraldehyde at pH 6.5 and 0 °C for 15 min. The reaction was stopped by addition of an equal volume of cold (0 °C) 100 mM Tris, pH 7.5. The solution was then applied to a 4 cm x 50 cm Sephadex G-50 column previously equilibrated at 5 °C with 50 mM Tris, pH 7.5. The column was eluted with 50 mM Tris, pH 7.5, with a flow rate of 1.0 ml/min. Fractions were assayed for fructose-1,6-diphosphatase activity, and the absorbance at 230 nm was determined to locate the glutaraldehyde.

Bovine fructose-1,6-diphosphatase is inhibited by high concentrations of Fru-1,6-P_2 Glutaraldehyde-treated fructose-1,6-diphosphatase exhibits less substrate inhibition than the native enzyme. The Fru-1,6-P_2 *versus* activity curve is shifted towards higher concentrations of Fru-1,6-P_2, as observed previously for pyridoxal 5'-phosphate modified bovine fructose-1,6-diphosphatase[12].

In summary, incubation of bovine hepatic fructose-1,6-diphosphatase with glutaraldehyde altered several properties of the enzyme. The catalytic activity was decreased at both pH 6.5 and pH 9.0, and there was a decrease in the pH 6.5/pH 9.0 activity ratio. The glutaraldehyde-modified enzyme was desensitized to inhibition by AMP and Fru-1,6-P_2, and the homotropic interactions with respect to AMP were abolished. The properties of glutaraldehyde-modified enzyme resemble those of untreated fructose-1,6-diphosphatase at pH 9.0, and therefore these changes are consistent with the conversion of a "neutral conformer" of fructose-1,6-diphosphatase into an "alkaline conformer". Similar changes in the allosteric properties of fructose-1,6-diphosphatase have been observed after treatment with pyridoxal 5'-phosphate, proteolytic digestion and blockage of tyrosyl residues[11–14].

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