SELECTIVE MODIFICATION OF FRUCTOSE 1,6-BISPHOSPHATASE BY PERIODATE-OXIDIZED AMP

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1. Introduction

The gluconeogenetic enzyme, fructose 1,6-bisphosphatase (FDPase) is composed of 4 subunits [1]. Each subunit has an allosteric site for AMP at neutral pH [2]. The enzyme is also activated by monovalent cations such as K^+ and NH_4^+ [3]. The specific inhibition of the enzyme by AMP is one of the essential regulatory mechanisms of gluconeogenesis [4]. Studies on chemical modification with pyridoxal 5′-phosphate have shown changes in the sensitivity to either allosteric AMP inhibition or to the high substrate inhibition of the enzyme [5,6]. The regulatory properties of the FDPase and the activation by monovalent cations are affected when arginine residues are modified with butanedione [7].

Here, the characteristics of the modification by adenosine 5′-monophosphate 2′3′-dialdehyde (AMP_{ox}) as well as the possible involvement of lysyl groups of the enzyme in the interaction with AMP and the activation by monovalent cations were examined. We show that the AMP analog can be used as an affinity probe for the allosteric site.

2. Methods

Pig-kidney FDPase was isolated as in [8]. The purified enzyme showed a single band on disc polyacrylamide gel electrophoresis with spec. act. 30.5 units/mg protein as measured in the presence of K^+ in the assay system. The enzyme activity was measured by the formation of P_i from the substrate, fructose 1,6-bisphosphate (FDP) as in [9]. Enzyme dilutions were always made in 20 mM Tris–HCl (pH 7.5), 2 mM $MgSO_4$ and 0.1 mM EDTA. FDPase concentrations were determined by A_{280}, using 0.755 for $A.mg^{-1}.ml^{-1}$ [5].

The AMP_{ox} was prepared by oxidation of AMP with $NaIO_4$ [10]. [^{14}C]AMP_{ox} was prepared from [^{14}C]AMP (New England Nuclear, 0.422 Ci/mmol). The oxidized analog was identified by ultraviolet spectroscopy and descending paper chromatography in isobutyric acid/NH_4OH/H_2O/0.1 M EDTA (66:1:32:1).

The stable FDPase–AMP_{ox} complex was formed by incubation of the enzyme with 10 mM AMP_{ox} (unless stated otherwise) at 30°C for 20 min in 60 mM sodium borate buffer (pH 7.5), 0.5 M KCl, with subsequent reduction with solid $NaBH_4$. The enzyme was dialyzed and passed through a Sephadex G-50 column equilibrated in buffer 20 mM Tris–HCl (pH 7.5), 0.1 mM EDTA.

The incorporation of [^{14}C]AMP_{ox} (1.4 mCi/mmol) in the enzyme was measured by precipitation of the modified FDPase with 12% trichloroacetic acid followed by filtration on Millipore filters and counting the radioactivity retained in the filters.

3. Results and discussion

It is known that AMP is a specific allosteric inhibitor of FDPase. However we have found that the oxidized analog is not an inhibitor of the enzyme and does not change the kinetic parameters for the substrate over a wide concentration range (0.005–5 mM). This behavior is probably due to an altered inter-

Table 1
Kinetic characteristics of native and AMP_{ox}-modified fructose 1,6-bisphosphatase[a]

Modification condition	Activity[c] ratio	% AMP[d] inhibition	K_1 AMP (μM)	n_{AMP}[e]
1 Control (treated with $NaBH_4$)[b]	2 62	94	15	2 0
2 Protection with FDP 10 mM	1 35	36	122	1 0
20 mM	2 73	64	52	1 1
3 Protection with 2 mM AMP	1 69	78	34	1 7
4 Protection with 10 mM FDP and 2 mM AMP	2 70	88	17	1 5
5 No protection	1 50	21	187	1 1

[a] The FDPase (4 5 mg/ml) was treated with 10 mM AMP_{ox} in 0 8 ml total vol as in section 2
[b] This enzyme was subjected to the same treatment as the modified enzyme except for the omission of AMP_{ox}
[c] The enzyme assays were performed in the presence and absence of 150 mM K^+ The 'ratio' refers to the relative activity with and without K^+
[d] The enzyme activities were measured at 70 μM AMP
[e] Hill coefficient

action of the analog with the enzyme produced by Schiff-base formation

Treatment of the enzyme with AMP_{ox} followed by reduction with NaBH produced a stable derivative with decreased sensitivity to AMP and decreased activation by monovalent cations (table 1)

Table 1 summarizes the effects of FDP, AMP or both on the modification of the enzyme by AMP_{ox} As shown, the ratio of activities measured in the presence and in the absence of K^+ decreased after the irreversible modification of the unprotected enzyme, indicating a loss of the activation produced by K^+ Such loss of potassium activation was achieved at AMP_{ox} as high as 10 mM, lowering the activity ratio to 1 5 Under these modification conditions the substrate protects the enzyme against the loss of the monovalent cation activation at >20 mM No protective effect of AMP alone was observed but both AMP (2 mM) and FDP (10 mM) prevented the loss of the activation by K^+ after the modification of the enzyme The changes in this enzyme property after the AMP_{ox} modification suggest the association of a lysyl group with the monovalent cation site in the enzyme

The treatment of the unprotected enzyme with AMP_{ox} produced a marked change in the affinity for AMP, decreasing considerably the extent of inhibition and increasing the K_1 value in 1 order of magnitude with respect to the native enzyme (table 1, fig 1A) A decrease in the Hill coefficient (n_H) for AMP is also observed as a result of the modification indicating a loss of cooperativity (table 1, fig 1B) It is shown that AMP has a partial protection against the loss of AMP sensitivity by AMP_{ox} treatment Furthermore, both AMP plus FDP showed a remarkable protective effect on this enzyme property with the K_1 value remaining unchanged after the modification (table 1, fig 1A) On the other hand, there is only partial protection of AMP or AMP plus the substrate against the loss of cooperativity (fig 1B) The additional effect of FDP on the protection of AMP against the loss of AMP inhibition can be explained by the requirement of the substrate for AMP binding [11,12] and this finding is in agreement with that reported [6] on modification with pyridoxal 5'-phosphate

The substrate shows also a slight protection effect of the AMP sensitivity of the enzyme but not of the cooperativity The results of changes in the AMP interaction after the modification suggest the presence of lysyl residues in the enzyme with differential reactivity related to the cooperativity and the sensitivity to AMP

The protection experiments indicate that it is possible to modify selectively reactive lysines involved in the regulatory properties of FDPase using high FDP concentrations during the AMP_{ox} modification Thus the [^{14}C]AMP_{ox} incorporation in the enzyme was measured under these protection conditions and an incorporation of 2 mol analog/mol enzyme tetramer was found (fig 2) When the modi-

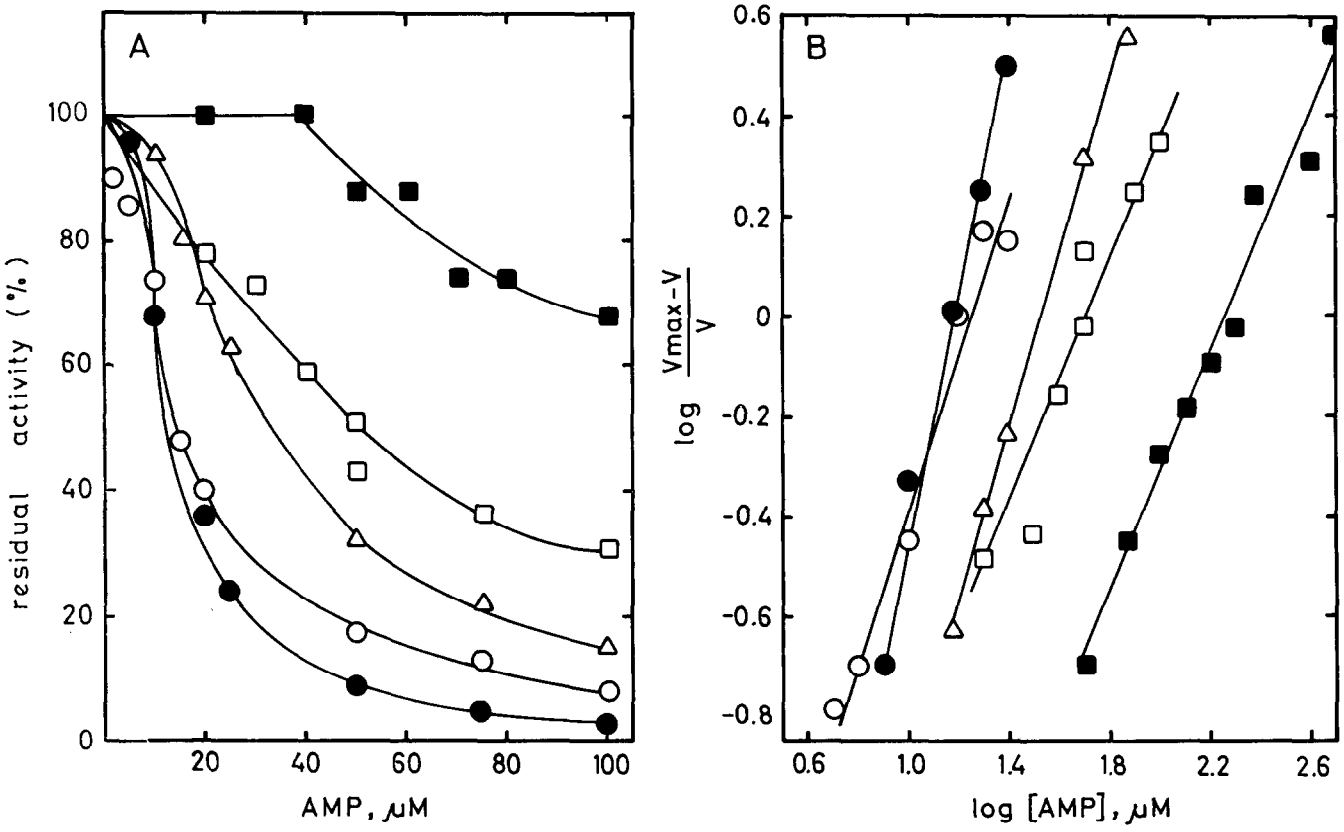

Fig.1. (A) Inhibition by AMP of FDPase control (•) and modified under different site protection conditions: in the presence of 20 mM FDP (□), 2 mM AMP (△), 10 mM FDP plus 2 mM AMP (○) and no protection (■). The modification was performed as in section 2 and the control was subjected to the same treatment except for the omission of AMP_{ox}. (B). Hill plot for AMP inhibition of fructose 1,6-bisphosphatase from the data obtained in fig.1A. The data were plotted according to [13] and the lines were fitted using a least squares program.

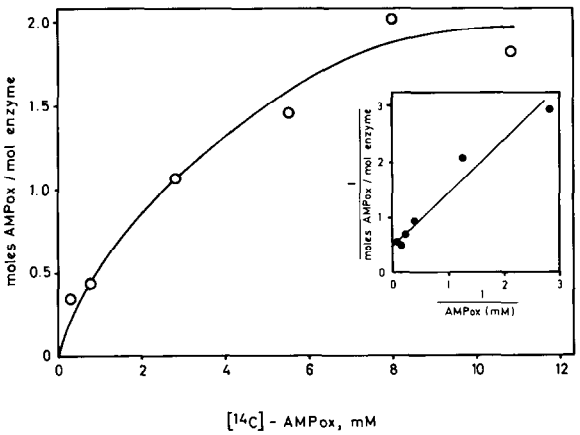

Fig.2. Incorporation of [^{14}C]AMP_{ox} into fructose 1,6-bisphosphatase in the presence of 20 mM FDP, as a function of AMP_{ox} concentration. Different aliquots of 0.2 ml enzyme (4.5 mg/ml) were modified as in section 2 using the [^{14}C]-AMP_{ox} concentrations indicated in the figure. The inset shows a double reciprocal plot of the data.

fication was performed in the absence of either AMP or FDP a covalent coupling of 4.1 mol AMP_{ox}/mol enzyme was observed. Modification studies with a lower concentration of AMP_{ox} (2.5 mM) under the substrate protection conditions produced a stable derivative (1 mol AMP_{ox}/mol enzyme) presenting the

same K_i value of the control enzyme and n_H 1 5 for AMP Additional studies on the characterization of the modified enzyme in non-protective conditions showed a diminished number of exposed sulfhydryl groups titrated with 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) Under these conditions 7 groups were titrated in the native enzyme as compared to 4 groups in the modified enzyme, suggesting a conformational change associated with the modification

The studies presented here indicate that the AMP analog, AMP_{ox}, can be used as an affinity label for the regulatory site of the enzyme

Addendum

While this manuscript was in preparation, we learned that 8-azido AMP appears to be a photo-affinity probe for the allosteric AMP site. We are indebted to Dr F Marcus for sending us a manuscript of this result before publication

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