Vol. 93, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 28, 1980 Pages 558-565

EFFECT OF SUBSTRATE AND AMP ON THE REVERSAL OF Zn^{2+} INHIBITION OF TURKEY LIVER FRUCTOSE-1,6-BISPHOSPHATASE BY CHELATORS

P. F. Han*, G. Y. Han+, H. C. McBay+, and J. Johnson, Jr.*

*Department of Chemistry, Atlanta University, Atlanta, Georgia 30314 and +Department of Chemistry, Morehouse College, Atlanta, Georgia 30314

Received February 6,1980

SUMMARY

The ability of chelators to reverse Zn^{2+} inhibition of turkey liver fructose-1,6-bisphosphatase decreases greatly if substrate is first bound to the enzyme. If AMP is also present, chelators become almost completely incapable of reversing Zn^{2+} inhibition when added to the enzyme after substrate. These observations indicate that the prior binding of substrate to this fructose-1,6-bisphosphatase hinders the removal of Zn^{2+} from the inhibitory sites of the enzyme by chelators, especially when AMP is also present. We have also found that the initial rates of the Zn^{2+}-inhibited enzyme activity show a peculiar nonlinearity and the inhibitory effects of Zn^{2+} and AMP are synergistic.

INTRODUCTION

Fructose-1,6-bisphosphatase (Fru-P_2ase, EC 3.1.3.11) is a key enzyme in gluconeogenesis. It has long been known that the activity of this enzyme in the neutral pH range is significantly enhanced by chelating agents (1,2). The basis for this enhancement had puzzled many researchers in this field for more than a decade, but is now attributed to the removal of endogenous Zn^{2+}, a specific and very potent inhibitor of this enzyme (3,4). It has recently been suggested that the inhibition of Fru-P_2ase by Zn^{2+} and its reversal by natural chelating agents such as histidine (4-6) and imidazole pyruvate (7) may play a role in the regulation of gluconeogenesis.

We previously reported that the enhancement of turkey liver Fru-P_2ase activity by chelators was reduced if substrate was first bound to the enzyme (8). This strongly suggests that the prior

0006-291X/80/060558-08$01.00/0

binding of substrate to Fru-P_2ase reduces the ability of chelators to reverse the inhibition of this enzyme by Zn^{2+}. We have confirmed this hypothesis in this study. We have also found that in the presence of adenosine-5'-monophosphate (AMP), a well-known allosteric inhibitor of Fru-P_2ase (9), chelators become almost completely incapable of reversing Zn^{2+} inhibition if added to the enzyme after substrate.

MATERIALS AND METHODS

Chelex 100 was obtained from Bio-Rad. Glucose-6-P dehydrogenase, phosphoglucose isomerase, and other chemicals were all purchased from Sigma. Turkey liver Fru-P_2ase was purified according to the method previously described (10), and the purified enzyme was stored as a suspension in 60% saturated $(NH_4)_2SO_4$. The concentration of the purified enzyme was determined by its absorbancy at 280 nm using the extinction coefficient ($E^{1\%}_{1\,cm}$) of 7.5 (8).

The activity of Fru-P_2ase was assayed at 25°C by measuring the rate of $NADP^+$ reduction at 340 nm in the presence of excess of glucose-6-P dehydrogenase and phosphoglucose isomerase in a Beckman ACTA CIII spectrophotometer. Unless otherwise indicated, the reaction mixture (1 ml) contained 50 mM Tris-HCl buffer (pH 7.4), 2 mM $MgSO_4$, 0.25 mM $NADP^+$, 0.25 mM fructose-1,6-bisphosphate, 2 units each of glucose-6-P dehydrogenase and phosphoglucose isomerase, and an appropriate amount of turkey liver Fru-P_2ase. The reaction was initiated by the addition of substrate.

In order to eliminate the endogenous Zn^{2+} or other metal inhibitors that might be present in the reaction mixture, all the components of the assay system (except $MgSO_4$) were treated with Chelex 100 according to the procedure described by Nimmo and Tipton (3). In addition, all the cuvettes, test tubes, and the beakers used were first washed with 1 mM EDTA and then thoroughly rinsed with Chelex 100-treated buffer or water to remove EDTA.

In assaying the activity of the Zn^{2+}-inhibited Fru-P_2ase, several researchers (4-6) used the phosphate release method rather than the continuous method employed in this study, mainly to "avoid complications arising from inhibition of the coupling enzymes" (4). In our laboratory, we routinely employed a simple test system to ensure that the two coupling reactions catalyzed by glucose-6-P dehydrogenase and phosphoglucose isomerase were indeed the "fast" steps. We simply added fructose-6-P to the complete assay system and observed the rate of increase in absorbancy at 340 nm. In this study, the highest concentration of Zn^{2+} used was 1.8 μM, but addition of 0.15 mM fructose-6-P to the complete assay mixture containing 1.8 μM Zn^{2+} still immediately resulted in stoichiometric formation of NADPH. This indicates that the effect of the added Zn^{2+} on the coupling enzymes can be neglected.

RESULTS AND DISCUSSION

Fig. 1 shows that addition of EDTA still increased the catalytic rate to the extent of approximately 10%. This indicates that either

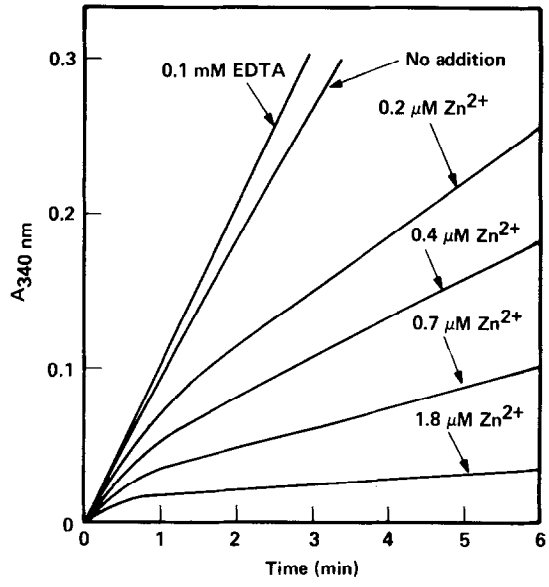

Fig. 1. Effect of EDTA and Zn^{2+} on the catalytic activity of turkey liver Fru-P_2ase. All the components of the assay system (except $MgSO_4$) were treated with Chelex 100 according to the procedure previously described (3). EDTA or Zn^{2+} was incubated with the enzyme (0.98 μg/ml) in the assay mixture for 1 min before the addition of substrate. The activity of Fru-P_2ase was assayed as described in MATERIALS AND METHODS.

the treatment with Chelex 100 (see MATERIALS AND METHODS) failed to remove the endogenous Zn^{2+} completely or that the $MgSO_4$ used was contaminated with a trace amount of Zn^{2+}. Fig. 1 also shows the enzyme activities measured with various concentrations of Zn^{2+}. This enzyme behaved peculiarly in the presence of Zn^{2+}, showing nonlinear initial catalytic rates changing to linear but lower rates. As the Zn^{2+} concentration increased the duration of the nonlinear portion decreased, while the differences in $\Delta A_{340\ nm}$ per unit time between the initial nonlinear portion and the following lower linear portion increased. Thus in determining the enzyme activities of the Zn^{2+}-inhibited enzyme, it is considered necessary to specify whether the initial nonlinear rates or the later lower linear rates are used in calculation. The basis for the peculiar behavior of this enzyme in the presence of Zn^{2+} is not known. It is possible that Zn^{2+} may

not induce significant conformational change until substrate is bound to the enzyme, and the initial nonlinear portion may represent the period of transition from the "more active" form to the "less active" form caused by Zn^{2+} after the addition of substrate.

Fig. 2a shows that incubation of the enzyme with 0.1 mM EDTA for 40 sec before the addition of substrate was sufficient to reverse the inhibition by 1.2 μM Zn^{2+} completely since incubation beyond 40 sec gave the catalytic rates indistinguishable from that represented by line A. Thus, in the absence of substrate Zn^{2+} was rapidly removed by EDTA from the inhibitory sites of Fru-P_2ase. The prior binding of substrate to the enzyme, however, greatly decreased the ability of EDTA to reverse Zn^{2+} inhibition of the enzyme. As also shown in Fig. 2a, if EDTA was added to the enzyme 40 sec after the addition of substrate, the enzyme activity rose slowly

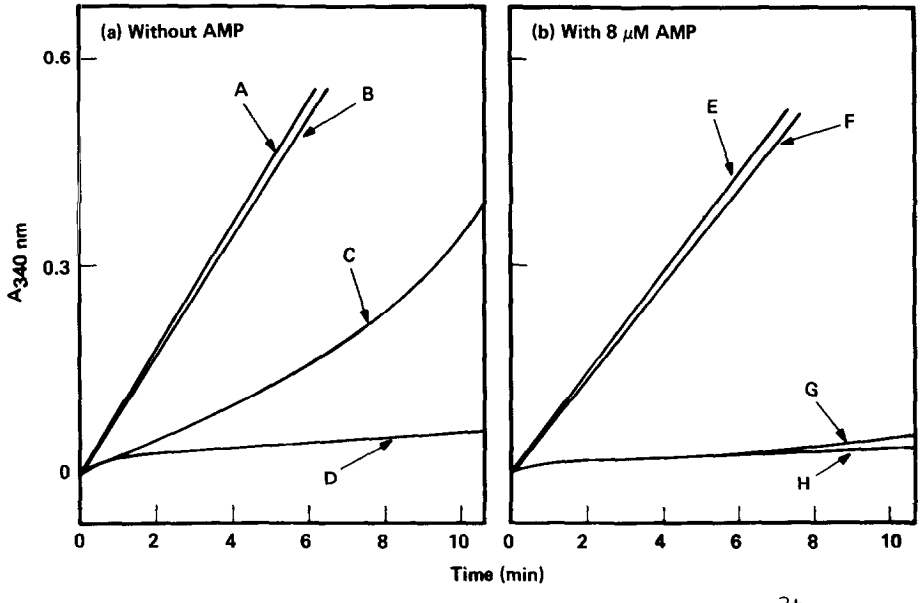

Fig. 2. Effect of substrate and AMP on the reversal of Zn^{2+}(1.2 μM) inhibition of turkey liver Fru-P_2ase by 0.1 mM EDTA. The following symbols signify: A and E, EDTA 40 sec before substrate; B and F, EDTA 20 sec before substrate; C and G, EDTA 40 sec after substrate; D and H, no EDTA. The activity of Fru-P_2ase was assayed as described in MATERIALS AND METHODS, and the concentration of the purified enzyme used in the assay was 0.91 μg/ml.

(see line C) and the maximum rate was not achieved until about 12 min after the addition of EDTA. These data clearly demonstrate that the prior binding of substrate to this Fru-P_2ase greatly hinders the removal of Zn^{2+} from the inhibitory sites of the enzyme by EDTA.

Attempts have also been made to investigate the effect of AMP on the reversal of Zn^{2+} inhibition of this enzyme by EDTA (Fig. 2b). It was found that, in the presence of 8 μM AMP which inhibited about 20% of the catalytic activity, incubation of the enzyme with 0.1 mM EDTA for 40 sec before the addition of substrate was also sufficient to reverse completely the inhibition by 1.2 μM Zn^{2+} since incubation beyond 40 sec gave the catalytic rates indistinguishable from that represented by line E. This suggests that AMP alone probably has no effect on the reversal of Zn^{2+} inhibition by EDTA. However, if EDTA was added to the enzyme 40 sec after substrate when 8 μM AMP was also present, it became almost completely incapable of reversing the inhibition of this enzyme by Zn^{2+} (see line G). This indicates that AMP enhances the ability of substrate to prevent removal of Zn^{2+} from the inhibitory sites of the enzyme by EDTA.

We have also investigated the effect of substrate on the reversal of Zn^{2+} inhibition of this enzyme by other chelating agents such as histidine, imidazole pyruvate, and nitrolotriacetate. As in the case of EDTA, the prior binding of substrate to the enzyme also greatly reduced the ability of all of these chelating agents to reverse Zn^{2+} inhibition of this enzyme, especially when AMP was also present.

The mechanism of how the prior binding of substrate to Fru-P_2ase reduces the ability of chelators to remove Zn^{2+} from the inhibitory sites of the enzyme remains to be established. This might result from any of the following 3 possibilities: (a) Zn^{2+} may bind more tightly to the inhibitory sites of the enzyme in the presence of substrate; (b) the Zn^{2+} inhibitory sites may be located near the

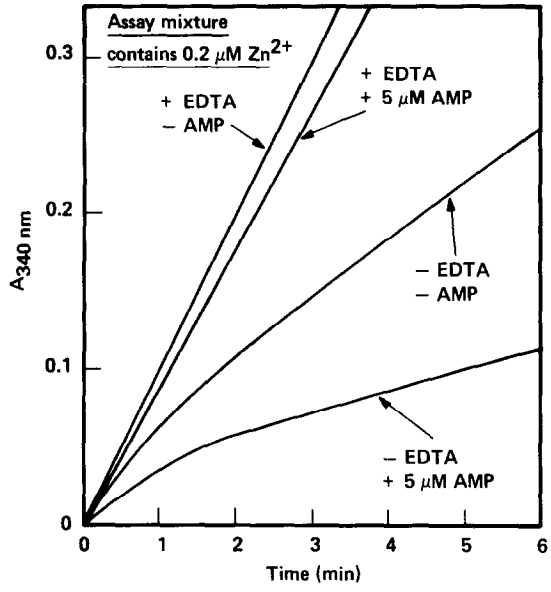

Fig. 3. Synergistic effect of Zn^{2+} and AMP on the inhibition of turkey liver Fru-P_2ase activity. The activity of Fru-P_2ase was assayed as described in MATERIALS AND METHODS. Wherever indicated, EDTA (0.1 mM) or AMP (5 μM) was incubated with the enzyme (0.98 μg/ml) for 1 min in the assay mixture containing 0.2 μM Zn^{2+} before the addition of substrate.

substrate binding site and the binding of substrate to the enzyme may sterically hinder the complexing of Zn^{2+} by chelating agents; and (c) as the result of substrate-induced change in enzyme conformation, Zn^{2+} may become enfolded inside the enzyme molecule, thereby rendering its removal by chelating agents more difficult. Regardless of whichever the mechanism is, the effect of substrate is markedly enhanced by AMP, probably through AMP-induced change in enzyme conformation.

Pontremoli et al. (6) recently reported that the presence of Zn^{2+} enhanced the sensitivity of rabbit liver Fru-P_2ase to inhibition by AMP. We also observed the same phenomenon with turkey liver Fru-P_2ase. In addition, we found that the sensitivity of this avian enzyme to Zn^{2+} inhibition was also enhanced by the presence of AMP, although to a lesser extent (Fig. 3 and Table I). Thus, AMP and Zn^{2+} act synergistically in inhibiting Fru-P_2ase.

Table 1

Synergistic Effect of Zn^{2+} [a] and AMP on the Inhibition of Turkey Liver Fru-P_2ase Activity

Conditions	% Inhibition by 5 μM AMP based on $\triangle A_{340\ nm}$ between	
	0 to 100 sec	100 to 200 sec
0.2 μM Zn^{2+} + 0.1 mM EDTA [b]	10.6	11.7
0.2 μM Zn^{2+}	43.8	63.2

Conditions	% Inhibition by 0.2 μM Zn^{2+} based [c] on $\triangle A_{340\ nm}$ between	
	0 to 100 sec	100 to 200 sec
No AMP	42.5	62.2
5 μM AMP	64.8	84.1

a/ The values shown in this table were calculated on the basis of the data shown in Fig. 3.

b/ EDTA was added to the enzyme 1 min before substrate.

c/ % inhibition by 0.2 μM Zn^{2+} was calculated using the relative value of 100 when assayed in the presence of EDTA.

ACKNOWLEDGMENT

This work was supported by Grant RR-8006 from the General Research Branch, Division of Research Resources, National Institutes of Health.

REFERENCES

1. McGilvery, R. W. (1964) in Fructose-1,6-Diphosphatase and Its Role in Gluconeogenesis, eds. McGilvery, R. W., and Pogell, B. M. (American Institute of Biological Sciences, Washington, DC), pp. 3-13.
2. Hers, H. G., and Eggermont, E. (1964) in Fructose-1,6-Diphosphatase and Its Role in Gluconeogenesis, eds. McGilvery, R. W., and Pogell, B. M. (American Institute of Biological Sciences, Washington, DC) pp. 14-19.
3. Nimmo, H. G., and Tipton, K. F. (1975) Biochem. J. 145, 323-334.
4. Tejwani, G. A., Pedrosa, F. O., Pontremoli, S., and Horecker, B. L. (1976) Proc. Natl. Acad. Sci. USA 73, 2692-2695.
5. Pedrosa, F. O., Pontremoli, S., and Horecker, B. L. (1977) Proc. Natl. Acad. Sci. USA 74, 2742-2745.

6. Pontremoli, S., Melloni, E., Salamino, F., Sparatore, B., Michetti, M ., and Horecker, B. L. (1979) Biochem. Biophys. Res. Commun. 88, 656-660.

7. Han, P. F., Han, G. Y., Cole, T. W., Owen, G. S., and Johnson, J., Jr. (1978) Experientia 34, 704-705.

8. Han, P. F., Owen, G. S., and Johnson, J., Jr. (1975) Arch. Biochem. Biophys. 168, 171-179.

9. Taketa, K., and Pogell, B. M. (1965) J. Biol. Chem. 240, 651-662.

10. Han, P. F., Murthy, V. V., and Johnson, J., Jr. (1976) Arch. Biochem. Biophys. 173, 293-300.