FRUCTOSE 1,6-DIPHOSPHATASE FROM RABBIT LIVER XI. Relation between the adenosine 5'-monophosphate binding and the allosteric inhibition.

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Received September 16, 1968

Adenosine 5'-monophosphate (AMP) is a specific, allosteric inhibitor of fructose 1,6-diphosphatase (Taketa and Pogell, 1965). Four binding sites for AMP are available on the enzyme and the binding, which is independent of the presence of either the substrate or the cations, follows, at pH 7.5, the simple mass action law. When either Mn^{++} or fructose 1,6-diphosphate are added, the binding of AMP takes place with positive interaction between the sites (Pontremoli et al., 1968b). The inhibition by AMP is non competitive with respect to fructose 1,6-diphosphate, competitive with respect to Mg^{++} or Mn^{++}, strongly pH dependent, and has a maximum at pH 7.5 (Taketa and Pogell, 1965; Opie and Newsholme, 1967).

We have now shown that the pH changes affect the inhibition in two ways : (1) up to pH 7.5 the increase of the inhibition is caused by the increase of the binding of AMP to the enzyme (2) from pH 7.5 to 9.5 the binding of the inhibitor is not affected and the decrease in the inhibition is dependent only on changes of the dissociation of groups of the enzyme. From our study it is also apparent that the preferred ionic species of the inhibitor for the binding to the enzyme is the $AMP^{=}$ form.

MATERIALS AND METHODS

Fructose 1,6-diphosphatase was prepared and assayed as previously described (Pontremoli et al., 1965b). Specific activity was 20 IU/mg of protein. The molecular weight was taken to be 130,000 (Pontremoli et al., 1965a). ^{14}C-AMP, specific activity 3×10^5 cpm/μmole, was purchased from the Radiochemical Centre, Amersham, England. Sephadex G50 (coarse) was supplied by Pharmacia, Uppsala, Sweden.

Measurement of AMP binding to fructose 1,6-diphosphatase was made at 2° employing columns of Sephadex G50 which had been equilibrated with 0.02 M acetate - 0.02 M tris buffer, at the desired pH, containing known concentrations of ^{14}C-AMP. The technique followed in these experiments was reported elsewhere (Pontremoli et al., 1968a and b).

RESULTS AND DISCUSSION

As shown in figure 1A, the binding of AMP to the enzyme increases with increasing pH. The increase is large from pH 5.5 to 7.5 and levels off between pH 7.5 and 9.5. The inhibition of the catalytic activity by AMP follows, on the contrary, a different pattern (Figure 1B). It has a maximum at pH 7.5 and it decreases steeply on both the acid and the alkaline sides of the curve. This is in agreement with what was shown by Taketa and Pogell (1965).

Changes in the ionization of groups of both the inhibitor and the enzyme can be responsible for the effect of the pH on both the binding and the inhibition by AMP. In the range of pH studied (5.5 to 9.5) the only important ionic species of the inhibitor are AMP^- and $AMP^=$, the pK of the second hydroxyl of the phosphate group being 6.05 (Alberty et al., 1951). The data of figure 1A seem to suggest that, of these two forms, the most important (or the only important) species for the binding to the enzyme is the dianionic form $AMP^=$. This is supported by the study of the

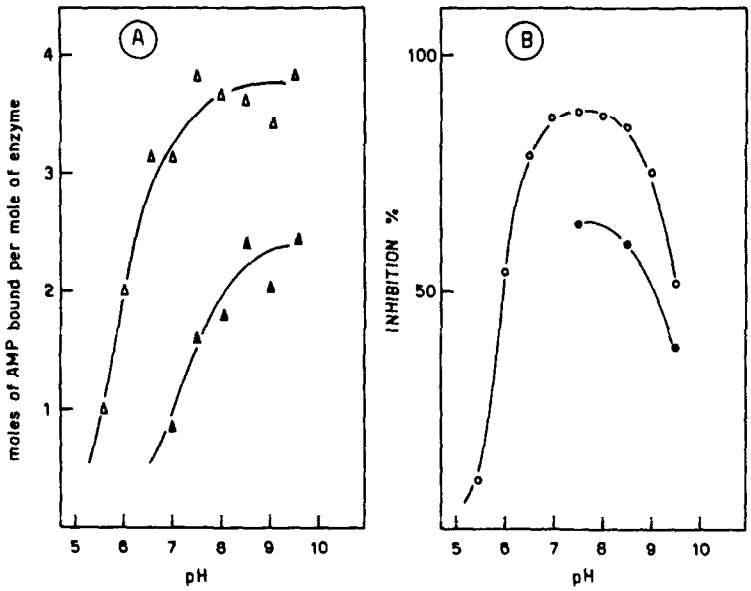

Figure 1) A: Binding of AMP to fructose 1,6-diphosphatase as a function of pH - The binding was studied by filtration of fructose 1,6-diphosphatase (10 mμmoles),dissolved in 0.5 ml of 0.02 M acetate - 0.02 M tris buffer, trough Sephadex G50 columns equilibrated with the same buffer containing 17 μM (Δ) and 7 μM (▲) ^{14}C-AMP. The pH was as indicated in the figure. Temperature was 2°.
B: Inhibition of fructose 1,6-diphosphatase by AMP as a function of pH - The incubation mixtures (0.5 ml) contained fructose 1,6-diphosphatase (0.077 mμmole), 1 mM $MgCl_2$, 1 mM fructose 1,6-diphosphate, 0.02 M acetate - 0.02 M tris buffer and 17 μM (o) or 7 μM (●) AMP. The pH was as indicated. The mixtures were incubated at 2° for 10 min . The reaction was stopped by the addition of 0.1 ml of 5 N sulfuric acid and the orthophosphate formed was determined by the method of Fiske and SubbaRow (1925).

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changes in the association constant for the binding of AMP to the enzyme as a function of pH. In figure 2B the log Kass is plotted against pH using total AMP concentration employed in the experiments. The pH effect is almost eliminated when the above results are plotted using the concentration of the dianionic species ($AMP^{=}$) alone (Figure 2A). This result does not, however, exclude the possibility that the association constant is also affected by changes in the ionization of groups of the enzyme.

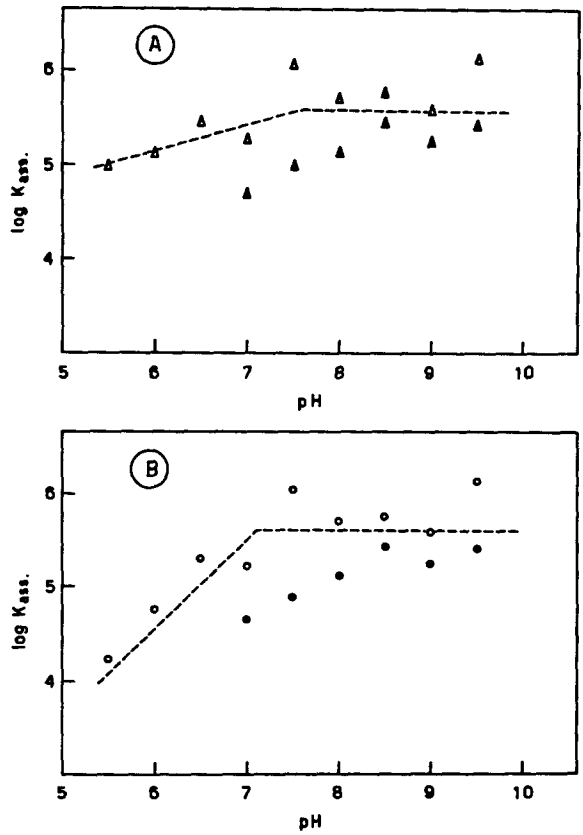

Figure 2) Log Kass for the binding of AMP to the enzyme as a function of pH - The association constants were calculated from the data of figure 1A assuming that, in the entire range of pH explored, pH 5.5 to 9.5, the number of the AMP binding sites is four and the binding follows the simple mass action law. A) Experiments with 17 μM (Δ) or 7 μM (▲) AMP, Kass calculated by using the concentration of the dianionic form $AMP^{=}$. B) Experiments with 17 μM (o) or 7 μM (●) AMP, Kass calculated by using total AMP concentration.

Inspection of figure 1B reveals that the inhibition ofthe catalytic activity by AMP is highest around pH 7.5. The increase in the inhibition from pH 5.5 to 7.5 can be, at least partially, explained by the larger binding of the inhibitor to the enzyme which occurs with the increase of pH. On the alkaline side of the pH curve, the decrease in the inhibition cannot be explained either by the extent of the bin-

ding of the inhibitor, which is essentially unaffected, or by changes in the relative concentration of the ionic forms of the inhibitor since, above pH 8.0, $AMP^{=}$ is practically the only ionic form present. In addition, in separate experiments, we have shown that at pH 9.5 the binding of AMP is not significantly affected by the presence of either 1 mM $MgCl_2$ or 1 mM fructose 1,6-diphosphate. It follows that, at alkaline pH, the decrease of the inhibition by AMP is related only to changes in the dissociation of groups of the enzyme.

Acknowledgement : This work was supported by a grant from the Italian Consiglio Nazionale delle Ricerche, Impresa di Enzimologia, by Grants 12291-03 from the National Institutes of Health, and North Atlantic Treaty Organization Grant 218.

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