EFFECT OF METAL IONS AND EDTA ON THE ACTIVITY OF RABBIT LIVER FRUCTOSE 1,6-DIPHOSPHATASE

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The properties of fructose 1,6-diphosphatases (D-fructose 1,6-diphosphate 1-phosphohydrolase, E C 3.1.3.11) from a variety of sources are very similar. Thus, they are activated by divalent metal ions (Mg^{2+} or Mn^{2+}), stimulated by EDTA, and inhibited by excess substrate and low concentrations of AMP. Rabbit liver FDPase* has been purified and crystallised and its molecular weight determined (Pontremoli et al., 1965a, b). It has been shown that reaction of either one or two sulfhydryl groups per mole enzyme with FDNB or PHMB, respectively, results in considerable stimulation of activity when assayed in presence of Mn^{2+}, although no such effect was observed when Mg^{2+} was used (Pontremoli et al., 1965b, c).

Activation of rabbit liver FDPase by Mg^{2+} and Mn^{2+} has been re-investigated and the effect of chelating agents studied. It has been shown that low concentrations of Mn^{2+} bring about a time-dependent inhibition which may be reversed by EDTA or excess Mg^{2+}. FDP prevents such reactivation.

Experimental

Buffers: TEA (pK_a = 7.76 at 25°; Bates and Allen, 1960) and DHEG (pK_a =

*Abbreviations: FDP, D-fructose 1,6-diphosphate; FDPase, fructose 1,6-diphosphatase; TEA, triethanolamine; DHEG, N,N-di(2-hydroxyethyl)glycine; FDNB, 2,4-dinitrofluorobenzene; PHMB, p-hydroxymercuribenzoate.

8.33 at 25°; Datta et al., 1964) buffers were prepared by adjusting 0.2 M solutions of TEA-HCl or DHEG to the desired pH with 1 N-NaOH, and diluting to give solutions 0.1 M in TEA or DHEG.

FDPase: Rabbit liver FDPase was purified as described by Pontremoli et al.(1965a). The eluate from the second CM-cellulose column was concentrated by dialysis against 5mM-malonate buffer, pH 6, under reduced pressure; solution containing 0.4 mg. enzyme per ml. was obtained. One mg. of this preparation released 8.8 μmoles P_i/min./ml. at 25° in DHEG buffer, pH 7.5, in presence of 0.8 mM-FDP and 5 mM-$MgCl_2$.

Assay of FDPase activity: Standard assay mixture contained, per ml.: 0.5 ml. buffer and approx. 8 or 16 μg. FDPase; and the following at concentrations shown: 1 mM-$MnCl_2$ or 5 mM-$MgCl_2$ and 0.8 mM-FDP. Suitable portions were withdrawn immediately and then at noted times and pipetted into equal volume of 10% trichloroacetic acid. The P_i released was estimated by the method of Chen, Toribara and Warner (1956).

EDTA-treated FDPase: A 0.4 ml. portion of the enzyme was diluted with 0.4 ml. TEA buffer, pH 7.5 and 0.2 ml. 0.01 M-Na_2EDTA about 30 min. before use and stored in ice.

Results and Discussion

The pH-activity curves of FDPase are shown in Fig.1. EDTA and DHEG (which is a good chelating agent for transition metals but not Mg^{2+}) greatly enhanced the activity in presence of $MgCl_2$. Treatment of the enzyme with EDTA prior to the addition of $MgCl_2$ and FDP was necessary to achieve this. EDTA had very little effect on the activity in presence of Mn^{2+}. Preincubation of FDPase with as little as 0.02 mM-EDTA gave maximum activation in presence of 5 mM-$MgCl_2$ (Fig.2A); however, treatment of the enzyme with 0.05 mM-$MnCl_2$ increased the concentration of EDTA required to give comparable activity under similar conditions. It appears, therefore, that the FDPase preparation contained an inhibitory

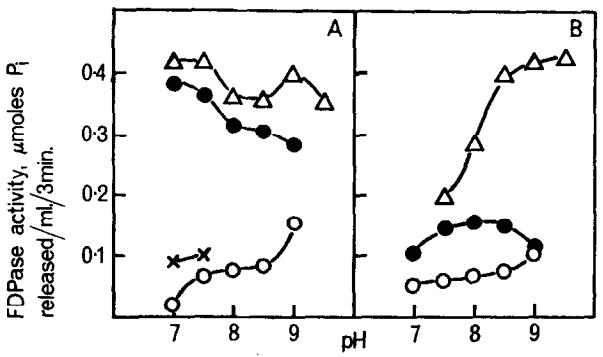

Fig.1. Effect of pH on the activity of FDPase. A, Assay in presence of 5 mM-$MgCl_2$ and B, in presence of 1 mM-$MnCl_2$. O, Δ, TEA and DHEG buffers respectively; FDPase incubated for 4 min. with the metal. Reaction initiated by addition of FDP (0.8 mM). ●, TEA buffers; FDPase incubated with 0.2 mM-EDTA prior to the addition of metal and FDP. ×, TEA buffers; FDPase added last to a complete reaction mixture plus 0.2 mM-EDTA. FDPase in all experiments was 16 μg./ml.

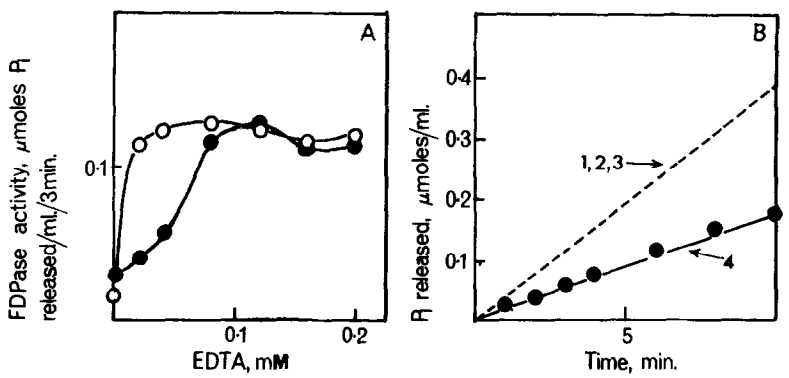

Fig.2. Effect of EDTA and $MnCl_2$ on the FDPase activity in presence of 5 mM-$MgCl_2$, TEA buffer, pH 7.5.

Fig.2A: O, FDPase incubated with EDTA for 10 min. ●, FDPase incubated with 0.05 mM-$MnCl_2$ for 5 min. followed by addition of EDTA and further incubation for 10 min. Reactions initiated by simultaneous addition of $MgCl_2$ and FDP.

Fig.2B. Experiments with EDTA-treated FDPase (see text). Curves 1 and 2, FDPase added last to a reaction mixture which finally contained 5 mM-$MgCl_2$, 0.8 mM-FDP and 0.1 mM-EDTA. Soln.2 also contained 0.1 mM-$MnCl_2$. Curve 3, FDPase incubated with $MgCl_2$ + $MnCl_2$ for 5 min. before the addition of FDP. Curve 4, FDPase incubated with $MnCl_2$ for 5 min. followed by simultaneous addition of $MgCl_2$ and FDP. FDPase used was 8 μg./ml. in all experiments.

metal removable by EDTA or DHEG and that Mn^{2+} may partly replace this inhibitory metal. Preincubation of the EDTA-treated FDPase with 0.1 mM-$MnCl_2$ alone was necessary to depress its activity in presence of 5 mM-$MgCl_2$ (Fig.2B). Simultaneous presence of 5 mM-$MgCl_2$ and 0.1 mM-$MnCl_2$ gave complete protection.

The inhibition by low Mn^{2+} appears to be a secondary time-dependent process. That this is so is further illustrated by results shown in Fig.3A. Preincubation of EDTA-treated enzyme with 0.1 mM-$MnCl_2$ resulted in a slow loss of activity in presence of 5 mM-$MgCl_2$. It should be noted that a major portion of Mn^{2+} would be chelated to EDTA which was 0.1 mM also. The much slower inhibition by preincubation with 0.1 mM-$MgCl_2$ in TEA buffer is probably due to trace amounts of transition metals in reagents since the inhibition was much reduced in DHEG buffer. An odd result was that increasing $MnCl_2$ to 0.2 or 0.4 mM in TEA buffer resulted in a rapid drop to about 80% activity in presence of 5 mM-$MgCl_2$, but no further change was observed.

Treatment of Mn-inhibited enzyme with 5 mM-$MgCl_2$ prior to the addition of FDP or with more EDTA (0.3 mM, final concentration) followed by simultaneous addition of $MgCl_2$ and FDP partially restored the activity. These reactivation processes were time-dependent (Fig.3B).

It is interesting to note that FDP prevents reactivation of Mn-inhibited enzyme by $MgCl_2$. This is obvious from the fact that the curve 4 in Fig.2B is linear with time; otherwise, the rate of P_i formation would tend to increase as the reaction progresses due to progressive reactivation.

Inhibition by Mn^{2+} but not Mg^{2+} indicates at least two metal-binding sites on FDPase - (i) the active metal site, where both Mg^{2+} and Mn^{2+} may bind; and (ii) a site which has affinity for Mn^{2+} but not for Mg^{2+}. Such a site may consist of the amino or imidazole groups of the enzyme since these are known to bind Mg^{2+} very weakly. The Mn-inhibited

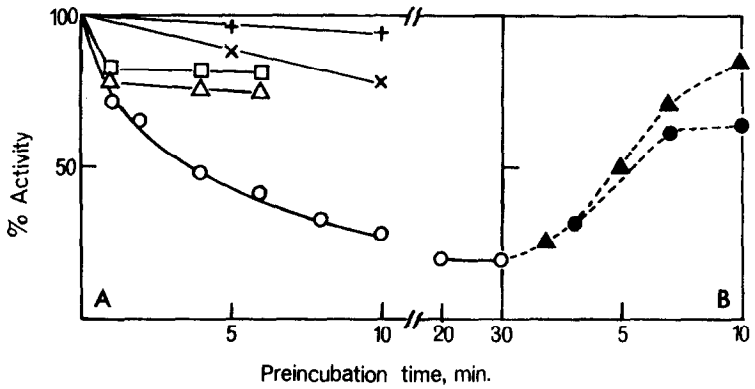

Fig.3A. Effect of preincubation with low concentrations of $MnCl_2$ or $MgCl_2$ on the activity of EDTA-treated FDPase. Activity measured at pH 7.5 in presence of 5 mM-$MgCl_2$, 0.8 mM-FDP and a final EDTA concentration of 0.1 mM. FDPase was treated with EDTA as described in the text; 8 ug./ml. used. ○, △, □, TEA buffer, pH 7.5. FDPase incubated with 0.1, 0.2 and 0.4 mM-$MnCl_2$, respectively, prior to the simultaneous addition of $MgCl_2$ and FDP. ×, TEA buffer; preincubation with 0.1 mM-$MgCl_2$. +, DHEG buffer; preincubation with 0.1 mM-$MgCl_2$.

Fig.3B. Reactivation of Mn^{2+}-treated FDPase by $MgCl_2$ or EDTA. FDPase assayed in TEA buffer, pH 7.5. EDTA-treated FDPase was incubated with 0.1 mM-$MnCl_2$ for 30 min. (Fig.3A). The activity in presence of 5 mM-$MgCl_2$ fell to about 20% (○). This enzyme was either incubated with 5 mM-$MgCl_2$ prior to the addition of FDP at times shown (●), or with more EDTA (0.3 mM, final concentration) before simultaneous addition of $MgCl_2$ and FDP (▲).

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enzyme may be a chelate in which these two sites are bound simultaneously to a central Mn-atom. Reactivation by Mg^{2+} may then involve undoing this chelate, Mg^{2+} replacing Mn^{2+} at the active metal site such that a mixed chelate (Mg-E-Mn) results. Less inhibition by high Mn^{2+} may reflect the formation of a 2:1 Mn:FDPase complex in which each of the two metal sites binds an Mn-atom. In presence of Mg^{2+} alone, an active 1:1 Mg:FDPase complex is assumed with an Mg-atom bound at the active metal site.

The changes involved in these activation processes, the stoichiometry of the metal complexes of this enzyme, and the relation of the sulfhydryl group, whose modification leads to stimulation of activity in presence of Mn^{2+} to these changes, require further study.

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