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BINDING OF ZINC(II) TO β-D-FRUCTOSE 2,6-BISPHOSPHATE

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The formation of a complex between Zn(II) and β-D-fructose 2,6-bisphosphate was shown because the latter compound: (a) activated bis(5'-guanosyl)tetraphosphatase (EC 3.6.1.17) and dinucleoside triphosphatase (EC 3.6.1.29) only to the extent that they could be inhibited by Zn(II); (b) increased the consumption of Zn(II) necessary to titrate to an end point a solution of the metallochromic indicator eriochrome black T; (c) coeluted with Zn(II) in a gel filtration column capable of resolving them if unbound. Neither of those effects was shown by D-fructose 1,6-bisphosphate under the same conditions.

The enzymes which degrade dinucleoside 5',5'''-P ,P^{n}-n-phosphates in rat tissues have been studied for several years in the Sillero's laboratory (1-9). Bis(5'-guanosyl)tetraphosphatase or dinucleoside tetraphosphatase (EC 3.6.1.17) hydrolyzes dinucleoside tetraphosphates like Ap4A[1]/Gp4G to ATP/GTP and AMP/GMP (1,2), and dinucleoside triphosphatase hydrolyzes dinucleoside triphosphates like Ap3A/Gp3G to ADP/GDP and AMP/GMP (3,4). Zn(II) is a strong inhibitor of both enzymes (6,8). In the course of experiments designed to search for other relevant effectors we found that Fru-2,6-P_2 restored the activity of the Zn(II)-inhibited enzymes but had no effect on the uninhibited rates. In this paper these experiments are described together with others showing the formation of Zn(II)-Fru-2,6-P_2 complexes. Similar experiments were carried out with Fru-1,6-P_2 and neither enzyme activation nor Zn(II) binding was seen. The interaction between Zn(II) and Fru-2,6-P_2 could be relevant to the function of the latter compound irrespectively of its effect on dinucleoside polyphosphate metabolism.

MATERIALS AND METHODS

Tris, $MgCl_2$, NH_4Cl, EDTA (disodium salt), and eriochrome black T were from Merck; Fru-2,6-P_2 (sodium salt), Fru-1,6-P_2 (trisodium salt), Ap4A and Ap3A were from Sigma; Fru-6-P (disodium salt) was from Boehringer. Sugar (bis)phosphate solutions were made up in 10 mM Tris/HCl buffer, pH 8.

The abbreviations used are: Ap3A, diadenosine 5',5'''-P^1,P^3-triphosphate; Ap4A, diadenosine 5',5'''-P^1,P^4-tetraphosphate; Fru-1,6-P_2, D-fructose 1,6-bisphosphate; Fru-2,6-P_2, β-D-fructose 2,6-bisphosphate; Fru-6-P, D-fructose 6-phosphate; Gp3G, diguanosine 5',5'''-P^1,P^3-triphosphate; Gp4G, diguanosine 5',5'''-P^1,P^4-tetraphosphate.

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Dinucleoside tetraphosphatase and triphosphatase were purified from rat liver 100,000 x g supernatants by 30-60% ammonium sulfate fractionation, gel filtration on a Sephadex G-100 column and ionic exchange on a DEAE-cellulose column (2,4). The enzymes copurified partially after the gel filtration step and a joint pool was used for the DEAE-cellulose step, where they became resolved. Each enzyme was precipitated with 60% ammonium sulfate, resuspended in 25 mM Tris/HCl buffer, pH 7.5, and desalted on a Sephadex G-25 column of 1.5 x 5 cm (Pharmacia PD-10 prepacked column) equilibrated with the same buffer. The enzymes were assayed by measuring the ultraviolet hyperchromicity accompanying the hydrolysis of substrates Ap4A (2) or Ap3A (3) at 37°C in 1-ml reaction mixtures with 50 mM Tris/HCl buffer, pH 8, 0.5 mM $MgCl_2$. After standing 10 min at 37°C, the reaction was started by addition of substrate (final concentration 30 μM) and a linear increase in absorbance was recorded for 5-10 min.

The binding of Zn(II) to Fru-2,6-P_2 was shown in a 1.5 x 5 cm Sephadex G-25 prepacked column (PD-10, Pharmacia) which was succesively washed with 30 ml each of water, 10 mM EDTA, water again and 100 mM Tris/HCl buffer, pH 8, before use. To equilibrate the bed in Zn(II) an additional wash was performed with 50 μM zinc acetate in 100 mM Tris/HCl buffer, pH 8, well farther the point where a constant Zn(II) concentration was reached in the eluate, which happened within the first 12-ml eluate portion collected. Zn(II) was assayed with the metallochromic indicator eriochrome black T by measuring the absorbance shift at 660 nm (see Fig. 2, inset). Some samples contained Fru-2,6-P_2 or Fru-1,6-P_2 as potential interfering agents, specially the first one (Fig. 2). However, the sugar bisphosphate concentration in the final assay mixture never exceeded 5 μM and standard Zn(II) curves in the presence of up to 50 μM of either sugar bisphosphate showed no significative interference. Different final concentrations of indicator may be and were used depending on the sensitivity required; thus Zn(II) standard curves were prepared for each indicator dilution. All the assays were performed in 100 mM Tris/HCl buffer, pH 8. Indicator was the last addition to the assay cuvette and absorbance readings were taken after a 20 s delay. Sugar bisphosphates were assayed in column fractions with an ascorbate-molybdate reagent (10) measuring P_i after incubation with alkaline phosphatase (Boehringer).

RESULTS

Fru-2,6-P_2 restores the activity of the Zn(II)-inhibited dinucleoside tetraphosphatase and dinucleoside triphosphatase. Zn(II) may play a role in the control of Ap4A and Ap3A turnover because it activates their synthesis by some aminoacyl-tRNA synthetases (11) and inhibits their degradation by the specific tetraphosphatase (6) and triphosphatase (8). For this reason when we tested Fru-2,6-P_2 as an effector of the two latter enzymes we took Zn(II) as an experimental variable. The result was that no effect of Fru-2,6-P_2 (up to 0.5 mM) was seen in the absence of Zn(II) (results not shown), but the Zn(II)-inhibited enzymes were activated by 10-100 μM Fru-2,6-P_2. The effect on dinucleoside tetraphosphatase was dependent on the concentration of Zn(II) in that less Fru-2,6-P_2 was needed to reach the maximal activation at low Zn(II) levels (Fig. 1B). Nevertheless, the maximal rate reached with Fru-2,6-P_2 equalled the uninhibited rate (Fig. 1A) and was independent of the concentration of Zn(II). Fru-1,6-P_2 and Fru-6-P were not effective activators in the presence of Zn(II) (Fig. 1B), whereas in its absence 0.5 mM Fru-1,6-P_2 had no effect and Fru-6-P (0.3 mM) elicited a 20% inhibition. The same experiments were performed with dinucleoside triphosphatase, although the effect of Fru-2,6-P_2 on the Zn(II)-inhibited enzyme was tested only with 10 μM zinc acetate. The results (not shown) were very much the same as those obtained with tetraphosphatase.

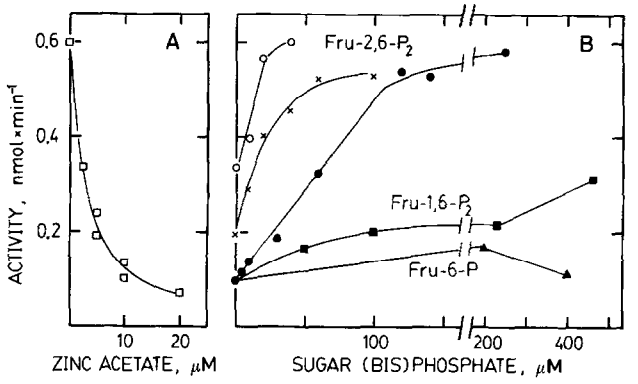

Figure 1. Effect of fructose derivatives on the Zn(II)-inhibited dinucleoside tetraphosphatase. The standard enzyme assay was used with 100 μl of tetraphosphatase (0.6 milliunits). (A) Inhibition of dinucleoside tetraphosphatase by zinc acetate. (B) The activation of the inhibited enzyme was tested at 2.5 μM (o), 5 μM (x), or 10 μM (●) zinc acetate with Fru-2,6-P_2 as indicated; or at 10 μM zinc acetate with either Fru-1,6-P_2 (■) or Fru-6-P (▲).

Fru-2,6-P_2 interferes with the titration of eriochrome black T with Zn(II). The effect of Fru-2,6-P_2 on dinucleoside tetraphosphatase and triphosphatase suggested that the sugar bisphosphate might be an effective Zn(II) ligand. If this is true Fru-2,6-P_2 should compete for Zn(II) with other chelating agents of similar strength. Eriochrome black T is a metallochromic indicator which shows large spectral changes upon Zn(II) binding (12; Fig. 2, inset). A 50 μM solution of this indicator was titrated at pH 8 with zinc acetate measuring the change in absorbance at 660 nm. The presence of 0.5 mM Fru-2,6-P_2 decreased the absorbance shift during the titration, i.e. a higher Zn(II) consumption was necessary to reach an end point (Fig. 2), indicating chelation by Fru-2,6-P_2. This effect was not produced by Fru-1,6-P_2, nor was apparent when Fru-2,6-P_2 and eriochrome black T were at the same concentration (50 μM). The apparently weaker binding of Zn(II) to Fru-2,6-P_2 than to the indicator made easy the use of the latter to assay total Zn(II) in the presence of low levels of Fru-2,6-P_2 (see Materials and Methods).

Zn(II)-Fru-2,6-P_2 complexes can be detected by gel filtration. Metal complexes with relatively small ligands can be studied in gel filtration columns equilibrated with a metal-containing buffer (13). With regard to Fru-2,6-P_2 this is facilitated because it displays an anomalous behavior in gel filtration media, eluting faster than expected from its molecular weight (14). When a pulse of Fru-2,6-P_2 was chromatographed in a Sephadex G-25 column equilibrated with 50 μM zinc acetate, the Zn(II) concentration baseline showed fluctuations; first, a peak coeluting with Fru-2,6-P_2 and, afterwards, an equivalent and transient fall below the baseline level (Fig. 3A). The coelution of Fru-2,6-P_2 and Zn(II) indicates complex formation because the free species would have been resolved. The elution volume of Fru-2,6-P_2 in the absence of Zn(II) was the same as in the

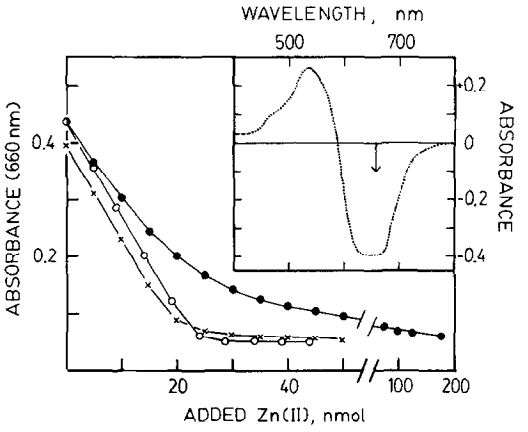

Figure 2. Effect of Fru-2,6-P_2 or Fru-1,6-P_2 on the titration of eriochrome black T with Zn(II). The metallochromic indicator was dissolved (50 μM) in 10 mM Tris/HCl buffer, pH 8, either alone (○) or with 0.5 mM Fru-2,6-P_2 (●) or 0.6 mM Fru-1,6-P_2 (x). Freshly prepared indicator (2-ml samples) was titrated in 5-μl steps with 1 mM zinc acetate and, after having added 50 nmol Zn(II), with 10 mM zinc acetate. The absorbance at 660 nm was measured after each addition. In the inset the difference spectrum of 50 μM eriochrome black T in Tris buffer supplemented with 125 μM zinc acetate, versus an equivalent solution without zinc acetate, is shown. The arrow marks 660 nm, the wavelength selected to follow the titration and to assay Zn(II) in column eluates (see Fig. 3 and Materials and Methods).

presence of metal, but when a 0.2-ml pulse of 0.5 mM zinc acetate was chromatographed in the same conditions of Fig. 3A, the peak of Zn(II) appeared in fractions 22-35, i.e. later than the peak of Zn(II) elicited by and coeluting with Fru-2,6-P_2. The results of Fig. 3B show that Fru-1,6-P_2 did not elicit fluctuations in the Zn(II) baseline, thus confirming the specificity of the interaction between Zn(II) and Fru-2,6-P_2.

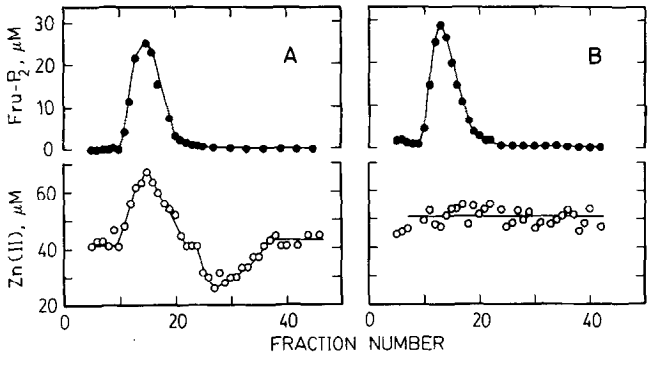

Figure 3. Zn(II)-Fru-2,6-P_2 complexes in Sephadex G-25 eluates. A 1.5 x 5 cm column was equilibrated with 100 mM Tris/HCl buffer, pH 8, 50 μM zinc acetate, as described under Materials and Methods. The samples chromatographed were: (A) 0.2 ml of 0.25 mM Fru-2,6-P_2 or (B) 0.2 ml of 0.3 mM Fru-1,6-P_2, both dissolved in equilibrating buffer. Fractions (0.4 ml) were collected and sugar bisphosphate (●) and Zn(II) (○) were assayed as described under Materials and Methods.

DISCUSSION

From the above results it is concluded that Zn(II) and Fru-2,6-P_2 form a complex in a specific way. The data are compatible with a 1:1 stoichoimetry, because the amounts of Zn(II) and Fru-2,6-P_2 coeluting in the gel filtration experiment (Fig. 3A) are approximately equimolar. Nevertheless other proportions are also possible because it is not known whether in that experiment Fru-2,6-P_2 was saturated with Zn(II). The specific character of the complex is suggested by the facts that the activation of the Zn(II)-inhibited dinucleoside tetraphosphatase was observed even when Mg(II) (required for the enzyme assay) was in a 200-fold excess over Zn(II) (Fig. 1), and that Fru-1,6-P_2 could never substitute for Fru-2,6-P_2. The latter result can be explained by the different conformation of both fructose bisphosphates: Fru-2,6-P_2 is β-D-fructofuranose 2,6-bisphosphate (15,16), mutarotation being impeded by the phosphorylation of the 2-hydroxyl group, and Fru-1,6-P_2 is D-fructofuranose 1,6-bisphosphate, with an anomeric distribution in neutral aqueous solution of 4:1 (β:α) (17). Thus, while the two P_i groups are cis in Fru-2,6-P_2, they predominate in the trans configuration in Fru-1,6-P_2. Apparently, the relative orientation of the P_i groups could be responsible for the binding to Zn(II). This is in contrast to results showing that Mn(II) binds with similar strength to either Fru-1,6-P_2, a stable configurational analog with cis P_i groups, or monophosphate compounds (17). It is likely that the differences between Zn(II) and Mn(II) or Mg(II), as well as those between the cis P_i groups of Fru-2,6-P_2 and α-Fru-1,6-P_2, are responsible for the metal-binding properties of these compounds. Another relevant observation is that in certain conditions Zn(II) binds selectively to NADPH but not to NADH (18). In this case the additional P_i group should be responsible for the different behavior.

Fru-2,6-P_2 is a well known regulator of the 6-phosphofructokinase (EC 2.7.1.11)-fructose bisphosphatase (EC 3.1.3.11) cycle (19), whereas dinucleoside polyphosphates, namely Ap4A, Gp4G, Ap3A and Gp3G are regulatory compounds likely with multiple effects (20,21). An effect of Fru-2,6-P_2 on the turnover of dinucleoside polyphosphates would be interesting and cannot be excluded. It is, however, difficult to extrapolate the activation of dinucleoside tetraphosphatase and triphosphatase to in vivo conditions, because the simplest mechanism for the in vitro effect is Zn(II) sequestration, and the relatively low intracellular concentration of Fru-2,6-P_2 (19) should exert an effective control over free Zn(II) levels that, if also low, could be buffered by other reversibly bound cation pools.

A corollary of the specific binding of Zn(II) to Fru-2,6-P_2 is that the metal could modulate the metabolic role of the bisphosphate either by hindering or facilitating its interaction with specific binding sites.

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