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DINUCLEOSIDETETRAPHOSPHATASE INHIBITION BY Zn(II)

José C. Cameselle, María J. Costas, María A. Günther Sillero, and Antonio Sillero

Instituto de Enzimología y Patología Molecular del C. S. I. C., Departamento de Bioquímica, Facultad de Medicina, Universidad de Extremadura, Badajoz, Spain

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SUMMARY: Almost complete inhibition of partially purified dinucleoside-tetraphosphatase (EC 3. 6. 1. 17) was observed with 5 μM Zn(II). The inhibition was reversed by EDTA and was time dependent, reaching a maximum after 5 min of incubation at 37°C. Zn(II) behaved as a non-competitive inhibitor of the reaction, leaving unaltered the K_m value for the enzyme towards diadenosine tetraphosphate. The cellular level of this compound may be directly related to the Zn(II) content since, besides the inhibition here described, Zn(II) has been reported by others to be an activator of the synthesis of diadenosine tetraphosphate by sheep liver lysyl- and phenylalanyl-tRNA synthetases.

Ap_4A[1] has been implicated in the control of cell division. The levels of Ap_4A in cells are inversely related to their doubling time (1) and Ap_4A stimulated DNA synthesis in permeabilized G_1-arrested baby hamster kidney cells, probably through its binding to DNA polymerase α (2, 3, 4). Cellular Ap_4A level results from a balance between its rate of synthesis and degradation. It may be formed by a reversal of the first step catalyzed by some aminoacyl-tRNA synthetases (5). Recently an interesting effect of Zn(II) has been described on the phenylalanyl-tRNA synthetase from Escherichia coli and yeast. This ion drastically inhibited the rate of $tRNA^{Phe}$ aminoacylation (6) but increased the synthesis of Ap_4A by the same system (7). A similar effect of Zn(II) on sheep liver lysyl- and phenylalanyl-tRNA synthetases has been also described (8). On the other hand, a direct correlation has been observed between growth rate of Ehrlich ascites tumor cells and zinc content in the ascites fluid (9),

[1] Abbreviations used: Ap_4A, diadenosine 5', 5'''-P^1, P^4-tetraphosphate or diadenosine tetraphosphate; Gp_4G, diguanosine 5', 5'''-P^1, P^4-tetraphosphate or diguanosine tetraphosphate.

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as well as a zinc-dependent activation of DNA synthesis in cultured lymphoblasts (10). Based on the above reasoning, it seemed to us of interest to test the effect of Zn(II) on dinucleosidetetraphosphatase (EC 3. 6. 1. 17), the enzyme which specifically cleaves Ap_4A to ATP and AMP (11, 12). As shown here, Zn(II) resulted to be a strong inhibitor of the enzyme.

MATERIALS AND METHODS

White female rats weighing about 250 g and fed on a stock diet ad libitum were used. Dinucleosidetetraphosphatase was partially purified as previously described (11). One rat liver (7.1 g) was homogenized with two volumes of 50 mM Tris/HCl buffer, pH 7.5, 0.5 mM EDTA, and 11 ml of a 150,000 x g supernatant were obtained. An ammonium sulphate fraction (0.3-0.6 saturation) was prepared and resuspended in the above buffer to a final volume of 2.9 ml. A 2.5-ml sample of this solution was applied to a Sephadex G-100 column (1.35 x 94 cm) which had been equilibrated with 50 mM Tris/HCl buffer, pH 7.5. The elution was accomplished at a flow rate of 13 ml/h with the same buffer and 2-ml fractions were collected. Fractions 47- 53, containing the major portion of dinucleosidetetraphosphatase activity, were pooled and used in these experiments. The enzyme was assayed by two previously described methods (12). The purification was followed with an inorganic phosphate colorimetric assay applied after the coupling of dinucleosidetetraphosphatase to alkaline phosphatase. All the kinetic experiments which are related below were performed with a spectrophotometric assay without coupling enzymes, taking advantage from the fact that Ap_4A and Gp_4G hydrolysis are accompanied by increments of 5.4 ΔA_{259} and 1.1 ΔA_{252} units per micromol, respectively, when measured in 1-ml reaction mixtures and 1-cm optical path cuvettes.

Gp_4G was obtained from Artemia salina cysts (13) and Ap_4A was purchased from Sigma (No. D-1262, lot 98C-7441). The latter preparation contained a certain amount of adenosine 5'-tetraphosphate, as shown by thin layer chromatography in poly(ethyleneimine)-cellulose (unpublished results). As adenosine 5'-tetraphosphate strongly inhibits (K_i = 48 nM) dinucleosidetetraphosphatase (11), its removal from the commercial preparation, by treatment with alkaline phosphatase, was necessary prior to the use of Ap_4A as substrate. In order to eliminate zinc present in the ammonium sulphate suspension of alkaline phosphatase, 25 µg of enzyme (from Boehringer) were diluted with 2.5 ml of 50 mM Tris/HCl buffer, pH 8.0, and chromatographed in a Sephadex G-25 column (1.5 x 5 cm) previously equilibrated with the same buffer. The second 2.5-ml portion of eluate, containing most part of the desalted phosphatase, was collected. A 0.5-ml sample was mixed with 2 ml of an Ap_4A solution giving an absorbance of 160 (1-cm optical path) at 260 nm and pH 7.0. The whole mixture was incubated overnight at room temperature and chromatographed in a Sephadex G-25 column (1.5 x 5 cm) equilibrated in 50 mM Tris/HCl buffer, pH 8.0. Ten-drop fractions were collected and analyzed for ΔA_{260} and alkaline phosphatase activity (with 1 mM p-nitrophenylphosphate in 25 mM Tris/HCl buffer, pH 8.0, at 405 nm). Fractions with absorbance higher than 6 and no detectable enzyme activity were pooled. The concentration of Ap_4A was determined, with snake venom phosphodiesterase, measuring the increase in absorbance at 259 nm (see above). When the

treated and the untreated Ap_4A were used as dinucleosidetetraphosphatase substrates, the relative initial rates were 100 and 20, respectively (20 μM Ap_4A). Considering the K_m and K_i values for Ap_4A and adenosine 5'-tetraphosphate, respectively (11), the presence of 4-9 % of the latter compound in the Ap_4A commercial preparation was calculated.

Magnesium acetate, EDTA, and Tris were purchased from Merck, and zinc acetate was from Riedel de Haën. The pH values quoted for buffers were adjusted at room temperature, except for the Tris buffer used for dinucleosidetetraphosphatase assay which was adjusted at 37°C.

RESULTS AND DISCUSSION

Preliminary experiments had shown that the inhibition of dinucleosidetetraphosphatase by Zn(II) was time dependent, reaching a constant value after 5 min of the addition of the zinc salt to the reaction mixture (Fig. 1). The effect of the concentration of Zn(II) on the rate of the reaction, in the presence of a fixed concentration of Ap_4A (20 μM), is presented in Fig. 2. Initial velocities were measured at 37°C, 15 min after mixing Zn(II) with the enzyme. The reaction was started by the addition of Ap_4A. Almost complete inhibition was observed at 5 μM Zn(II), and

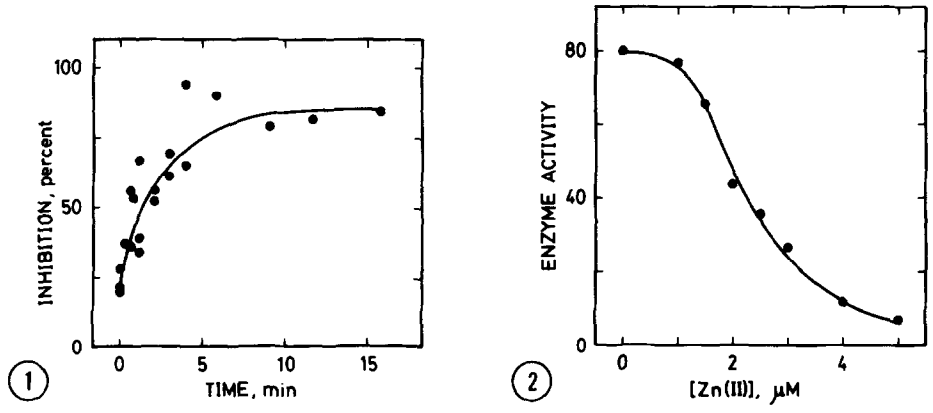

Fig. 1 Time dependence of dinucleosidetetraphosphatase inhibition by zinc. The reaction mixture, carried out at 37°C, contained in a volume of 1 ml the following components: 50 mM Tris/HCl buffer, pH 7.5, 1mM magnesium acetate, 30 μl of dinucleosidetetraphosphatase, and 4 μM zinc acetate. The reaction was started by the addition of Ap_4A (20 μM, final concentration). Time in the Fig. indicates minutes elapsed between additions of zinc and Ap_4A. Initial rates were determined in each case; percent inhibition was calculated in relation to a control without zinc.

Fig. 2 Inhibition of dinucleosidetetraphosphatase by different concentrations of zinc. The reaction mixture was as in Fig. 1, except for the zinc concentration which was as indicated. Reaction mixtures were incubated for 15 min at 37°C, and the reaction was started by the addition of Ap_4A. Enzyme activity is expressed as nmol of substrate transformed per min and mg of protein.

TABLE I

Inhibition of dinucleosidetetraphosphatase by Zn(II). Reversion by EDTA

assay	Preincubation with	Reaction initiated by	Ap_4A hydrolysis nmol x min^{-1} x mg $prot.^{-1}$	Relative activity
a	-	Ap_4A	60 ± 9 (6)	100
b	EDTA	Ap_4A	78 ± 10 (3)	130
c	zinc	Ap_4A	13 ± 1 (3)	22
d	zinc	Ap_4A + EDTA	43 ± 1 (3)	72
e	zinc + EDTA	Ap_4A	71 ± 9 (3)	118

The concentrations of EDTA, zinc acetate, and Ap_4A were 25, 4, and 20 μM, respectively. Preincubation was carried out during 15 min at 37°C, in the presence of 50 mM Tris/HCl buffer, pH 7.5, 1 mM magnesium acetate, 20 μl of dinucleosidetetraphosphatase, and the components indicated in the Table. Results are expressed as average values followed by the standard deviation. Figures in brackets correspond to the number of assays.

half inhibition was attained at around 2 μM Zn(II). The shape of the inhibition curve was sigmoidal, with less relative inhibition at low zinc concentrations. At present, the reason for this behaviour is not clear to the authors. The presence of protein, or other chelating agents for Zn(II), in the enzyme preparation was discarded, since increasing amounts of extract (from 4 to 60 μl) did not produce less percent inhibition at a fixed, 2 μM, Zn(II) concentration. Over this range the reaction was fairly linear both with and without zinc added. The possibility that other components of the reaction mixture, Ap_4A included, could bind Zn(II), or that the phenomenon was due to a specific cooperative effect of Zn(II) on the tetraphosphatase, or to the presence of Zn(II) or other metal in the extract (see below), cannot be ruled out. The pattern of inhibition of the enzyme by Zn(II) when assayed with other natural substrate, Gp_4G, was very similar to that obtained with Ap_4A (results not shown).

The effect of Zn(II) on dinucleosidetetraphosphatase can be reversed by EDTA (Table I). The enzyme was incubated at 37°C for 15 min, in the presence of 1 mM magnesium acetate and 50 mM Tris/HCl buffer, pH 7.5, alone (a) or supplemented with 25 μM EDTA (b), or with 4 μM zinc acetate (c and d), or with both EDTA and zinc (e). The reaction was started by the addition of Ap_4A (a, b, c, e; final concentration 20 μM), or Ap_4A plus EDTA to a final concentration of 25 μM (d). Initial rates were meas-

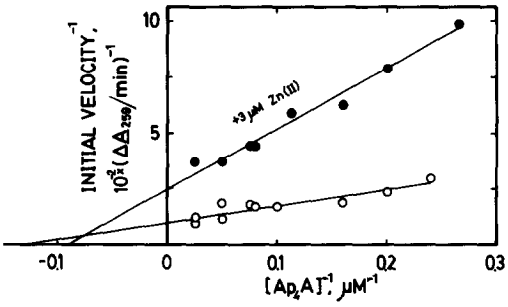

Fig. 3 Effect of Ap_4A concentration, in the presence or absence of zinc, on the initial velocity of dinucleosidetetraphosphatase. Conditions were as described in Fig. 2, except that 20 μl of enzyme preparation were used. The best fitting lines were obtained by linear regression analysis.

ured. Zn(II) inhibited the reaction to 22% of the control value. Preincubation with EDTA in the absence (b) or presence (e) of Zn(II) slightly stimulated the enzyme velocity in relation to the control, pointing to a contamination by Zn(II), or other inhibitory ion, in the reaction mixture. However, when the enzyme was preincubated with Zn(II) alone (d), EDTA did not completely reverse the inhibition caused by the metal in our experimental conditions.

Finally, the effect of Zn(II) was studied in the presence of increasing concentrations of Ap_4A (Fig. 3). In agreement with previous results from this laboratory (11), a K_m value of 7 μM was found for Ap_4A. In the presence of a fixed 3 μM concentration of zinc acetate the K_m value remained nearly unaltered, whereas a decrease was observed in the maximum velocity of the enzyme. This evidence point to a non competitive inhibition of dinucleosidetetraphosphatase by Zn(II) with a K_i value of 2μM.

In summary, in this paper we show that the level of Ap_4A may be related to the zinc content of the cell. This metal favors the synthesis of Ap_4A through the activation of the lysyl- and phenylalanyl-tRNA synthetases (6, 7, 8) and diminishes the rate of hydrolysis of this nucleotide, inhibiting the specific dinucleosidetetraphosphatase. As stated in the Introduction, an increase in the level of Ap_4A may stimulate the cell division, and there is some evidence that Zn(II) is mitogenic (1, 10). Nevertheless many questions remain unanswered and more experimental work is needed to clarify this problem.

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