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Evidence for a metalloprotein structure of plasma membrane 5′-nucleotidase

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To point out the metalloprotein structure of bovine liver plasma membrane 5′-nucleotidase, we studied the inhibition mechanism of the purified enzyme by EDTA: this apparently non-competitive inhibition seems to be dependent on EDTA concentration, pH, temperature and incubation time. When the restoration of activity was assayed by addition of divalent cations or by gel filtration, the inhibition became progressively irreversible with time. Incubation of the enzyme with [^{14}C]EDTA allowed us to observe, after gel filtration as well as after sucrose gradient ultracentrifugation, that the chelating agent is bound to 5′-nucleotidase.

5′-Nucleotidase *Metalloprotein* *Chelating agent* *Endogenous cation*

1. INTRODUCTION

5′-Nucleotidase (EC 3.1.3.5) has been shown to be a plasma transmembrane [1] ectoenzyme [2,3]. Although several studies showed that this enzyme is probably implicated in a variety of biological processes [4–7], its role in intermediary metabolism is not well defined [8]. We recently purified 5′-nucleotidase from bovine liver plasma membrane [9] and demonstrated that this enzyme is a dimeric glycoprotein with an apparent M_r of 140000. In addition, we observed an inhibition of the enzyme activity by EDTA; this inhibition was reversed by divalent cations. Similar observations have been reported for 5′-nucleotidase from other tissues [10–12], but only a few studies have been performed on the inhibition mechanism by EDTA, especially the possible binding of the chelating agent to the enzyme. Authors in [13] observed inhibition of 5′-nucleotidase from *Dictyostelium discoideum* by 1,10-phenanthroline; this inhibition was reversed by Zn^{2+}. They concluded that the enzyme is a metalloprotein which specifically requires bound zinc for its activity. Similar observations permitted us to identify a metalloendoprotease from the cytosol of mammalian cells [14].

Here, we studied the effect of time, temperature and pH on the inhibition of 5′-nucleotidase by EDTA. We examined by gel filtration and by sedimentation on sucrose gradients the interactions between [^{14}C]EDTA and 5′-nucleotidase. Our results are in favour of a metalloprotein structure for bovine liver plasma membrane 5′-nucleotidase.

2. MATERIALS AND METHODS

2.1. *Effect of EDTA incubation time*

5′-Nucleotidase from bovine liver plasma membrane was purified as in [9]. Samples containing 20 ng purified enzyme were preincubated with different EDTA concentrations at 37°C in 110 mM NaCl, 40 mM Tris–HCl (pH 7.4, buffer A). The reactions were started at different times by addition of 1 mM AMP, and the specific activity was determined as in [15]. The reversibility of the inhibition was tested by addition of 0.25 mM $MnCl_2$ and preincubation of the samples for 15 min before starting the reaction. The K_i of EDTA for

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the enzyme was calculated by measuring the K_m and V_m at different incubation times.

2.2. *Effect of pH*

The enzyme was preincubated for 30 min at different pH values in the absence or presence of 10 μM EDTA. The buffers used, containing 110 mM NaCl, were 40 mM Mes–Tris (pH 5.5–6.5), Tris–HCl (pH 7–7.5) or glycine–NaOH (pH 8–9.5). The specific activity was measured and the ionisation pK values were determined as in [16].

2.3. *Gel filtration*

The purified enzyme was incubated with unlabelled or radiolabelled [^{14}C]EDTA (52 mCi/mmol, Amersham) in 200 μl buffer A at 37°C; after different times, the mixture was applied to a column (1 × 20 cm) of Trisacryl GF 05 (IBF, France) equilibrated with buffer A containing 0.2% sulfobetaine 14 (SB 14). Fractions were collected at a rate of 14 ml/h and 5'-nucleotidase activity was measured in the absence or presence of 0.25 mM $MnCl_2$.

2.4. *Density gradient ultracentrifugation*

The purified enzyme was incubated with [^{14}C]EDTA in 150 μl buffer A at 37°C for 4 h; the mixture was then layered onto 5–20% sucrose gradients containing 0.2% SB 14 and centrifuged for 20 h at 38000 rpm in an SW 41 Beckman rotor at 4°C. Aliquots were assayed for 5'-nucleotidase activity and radioactivity. Radioactivity was determined in an Intertechnique SL 4000 liquid scintillation spectrometer.

2.5. *Assays*

Proteins were precipitated as in [17] and assayed as in [18]. Inorganic phosphate was determined as in [19].

3. RESULTS

3.1. *Effect of time on 5'-nucleotidase inhibition by EDTA*

The activity of purified 5'-nucleotidase decreased when the concentration of EDTA increased. This phenomenon depended on the incubation time between enzyme and EDTA (fig.1). EDTA at 10 μM inhibited 50 and 85% of the specific activity

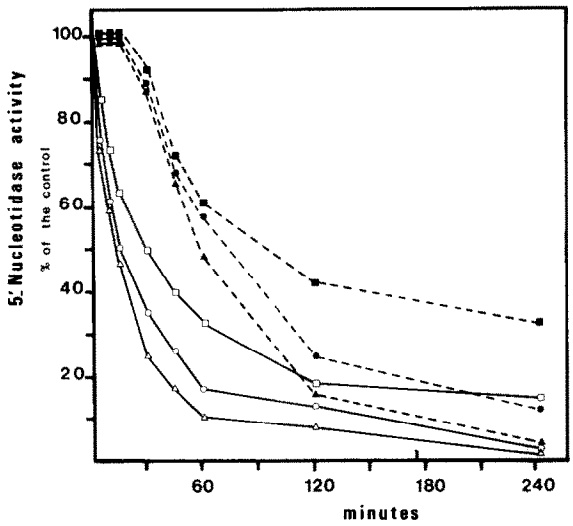

Fig.1. Kinetics of the inhibition of 5'-nucleotidase activity by EDTA. Samples containing 20 ng purified enzyme were preincubated with EDTA at 37°C in buffer A. The reactions were started at different times by addition of 1 mM AMP (——). Reversibility of the inhibition was tested by preincubating the sample with 0.25 mM $MnCl_2$ for 15 min before starting the reaction (– – –). Results are expressed as percentage of the control without EDTA. The 100% value of the control was 300 μmol·min^{-1}·mg^{-1}. (□—□, ■---■) 2.5 μM EDTA, (○—○, ●---●) 10 μM EDTA, (△—△, ▲---▲) 50 μM EDTA.

at 15 and 60 min, respectively. Moreover, this inhibition, which is reversed by addition of 0.25 mM $MnCl_2$ for the first 30 min of incubation, became progressively irreversible for longer incubation times; at this step, increasing the $MnCl_2$ concentration had no effect (not shown). Inhibition was apparently non-competitive and the K_i values decreased with time, indicating a progressive EDTA effect (table 1).

3.2. *Effect of temperature*

The inhibitory effect of 10 μM EDTA on 5'-nucleotidase activity was largely dependent on temperature: the percentage of inhibition after 2 h at 0°C was only about 40% whereas it was 87% at 37°C (fig.2).

3.3. *Effect of pH*

The percentage of enzyme inhibition by EDTA varied with pH. Fig.3 shows that the inhibition in-

Table 1

Variations of the inhibition constant of EDTA for 5′-nucleotidase

	Incubation time (min)			
	10	20	30	45
K_i (μM)	16.4	6.0	5.1	3.3

Samples of purified 5′-nucleotidase (20 ng) were incubated in buffer A at 37°C in the absence or presence of 2.5 μM EDTA (final volume 0.4 ml). At different times, the reaction was started by adding different AMP concentrations (10–1000 μM); the initial activity was calculated by using a short (30 s) incubation period. The K_i was calculated by measuring the V_{max} in the absence or presence of EDTA

creased for pH values nearing the ionisation pK of 5′-nucleotidase (6.2 ± 0.1 and 8.3 ± 0.1). In the presence of EDTA, the ionisiation pK values became 6.9 ± 0.1 and 7.5 ± 0.1, respectively; however, EDTA did not modify the pH optimum of the 5′-nucleotidase.

All these results indicated that interactions occurred between EDTA and 5′-nucleotidase.

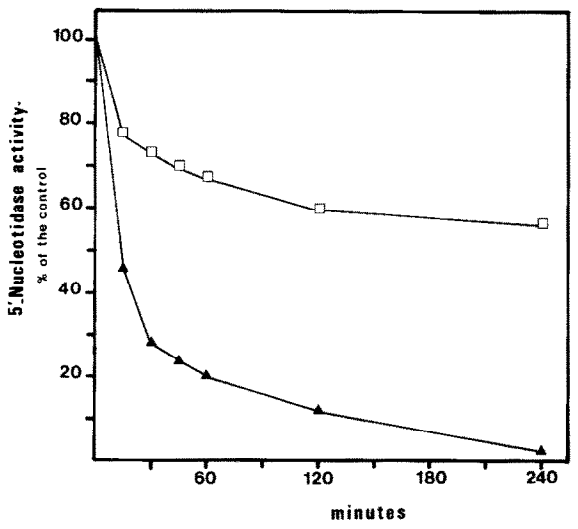

Fig.2. Effect of temperature on the inhibition of 5′-nucleotidase activity by EDTA. The enzyme (20 ng) was incubated at 37°C (▲—▲) or 0°C (□—□) with 10 μM EDTA in buffer A. At different times, the specific activity was measured at 37°C as described in section 2.

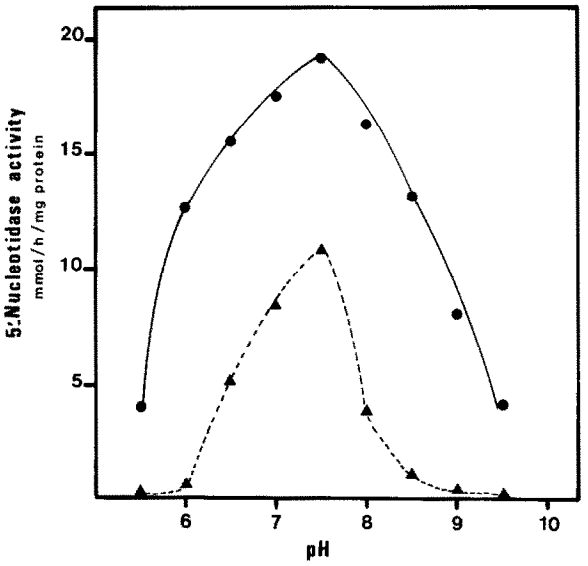

Fig.3. Effect of pH on the inhibition of 5′-nucleotidase activity by EDTA. The enzyme (20 ng) was preincubated at different pH values in the absence (●—●) or presence (▲---▲) of 10 μM EDTA as described in section 2.

However, to understand the inhibition mechanism it remained to be defined whether EDTA was bound to the enzyme or whether it removed the endogenous cations; this was examined by gel filtration.

3.4. *Trisacryl GF 05 gel filtration*

Results obtained from gel filtration of the enzyme preincubated with EDTA exhibited phenomena similar to those obtained from kinetics. After a 30 min incubation period with 50 μM EDTA, 76% of the total activity was inhibited; gel filtration allowed restoration of 88% of this inhibited activity (fig.4); the addition of $MnCl_2$ had little effect on the activity of the eluate (not shown). However, when EDTA and the enzyme were incubated for 2 h, inhibition (90%) was irreversible since it could not be restored by gel filtration (fig.4).

When [^{14}C]EDTA was used, a radioactivity peak was detected in the area of 5′-nucleotidase activity (fig.5); in this experiment, 67 pmol purified 5′-nucleotidase were used and the inhibition of the enzyme was about 55%; since we recovered 30 pmol [^{14}C]EDTA, this proved that the chelating agent is bound to the enzyme.

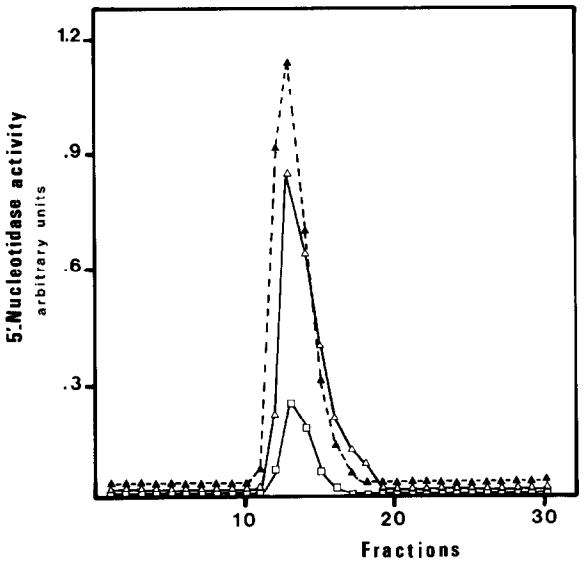

Fig.4. Gel filtration of EDTA-treated enzyme. Samples (200 ng) of purified enzyme were incubated in 200 μl buffer A at 37°C in the absence or presence of 50 μM EDTA. The mixtures were applied to a column of Trisacryl GF 05. Fractions were collected and the 5'-nucleotidase activity measured. (▲---▲) Control without EDTA, (△—△) 30 min incubation with EDTA, (□—□) 120 min incubation with EDTA.

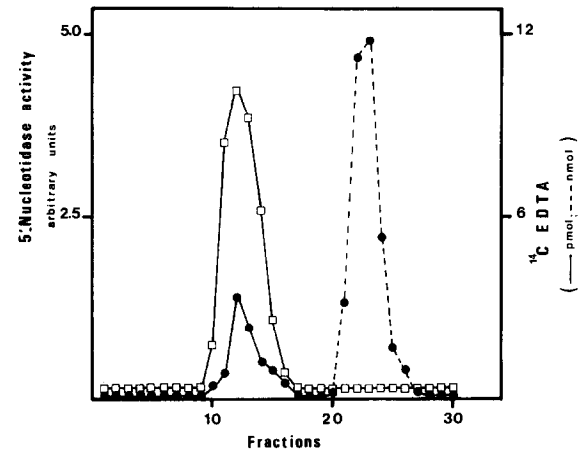

Fig.5. Gel filtration of 5'-nucleotidase preincubated with [^{14}C]EDTA. [^{14}C]EDTA (100 nmol; 52 nCi/nmol) was added to 10 μg (67 pmol) 5'-nucleotidase. The mixture (200 μl) was incubated at 37°C for 2 h and applied to a Trisacryl GF 05 column as described in section 2. Aliquots were assayed for 5'-nucleotidase activity (□—□) and radioactivity (●—●, ●---●).

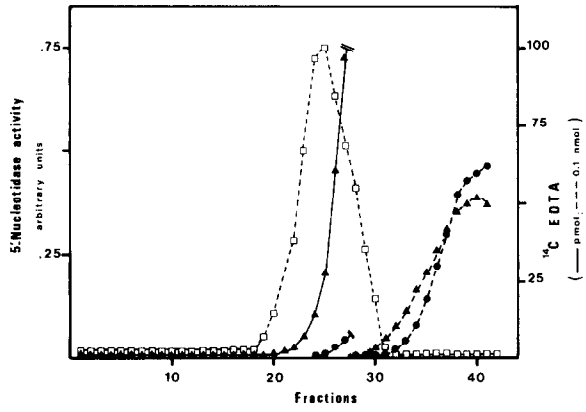

Fig.6. Density gradient ultracentrifugation of 5'-nucleotidase preincubated with [^{14}C]EDTA. Purified enzyme (10 μg; 67 pmol) was incubated with [^{14}C]EDTA (100 nmol; 52 nCi/nmol) in 150 μl buffer A at 37°C for 4 h. The mixture was layered onto 5–20% sucrose gradients as described in section 2. A control of 100 nmol [^{14}C]EDTA incubated without enzyme was conducted. (□---□) 5'-Nucleotidase activity, (▲—▲, ▲---▲) [^{14}C]EDTA incubated with enzyme, (●—●, ●---●) [^{14}C]EDTA control.

3.5. *Density gradient ultracentrifugation*

Sedimentation analysis of the enzyme preincubated with [^{14}C]EDTA showed a shift of the [^{14}C]EDTA peak, as compared to the control. This radioactivity was located in the area of 5'-nucleotidase activity, indicating binding of the chelating agent to the enzyme (fig.6) and confirming the results obtained by gel filtration. Only 7% of the total activity was recovered after ultracentrifugation: the initial activity was not restored by addition of $MnCl_2$.

4. DISCUSSION

In [9], we observed that 5'-nucleotidase purified from bovine liver plasma membrane was inhibited by EDTA; this inhibition, which depended on the EDTA concentration, was reversed by addition of divalent cations (Mn^{2+}, Mg^{2+} and Co^{2+}).

Here, we show that the inhibition of the enzyme by the chelating agent is time-dependent. When incubation was longer than 1 h, restoration of the activity progressively decreased, irrespective of the concentration of exogenous cations added. Authors in [11] could not restore the initial activity of bovine brain 5'-nucleotidase preincubated with

EDTA for periods shorter than 1 h; this was probably due to the fact that the dialysis method used by these authors is a slow process, thus leading to a longer time of contact between EDTA and the enzyme. The variation of the K_i values indicates an increasing affinity of the chelating agent for the enzyme, dependent on time. This phenomenon could account for the irreversibility of the inhibition.

Inhibition of the enzyme by EDTA was also dependent on the incubation temperature; the greater efficiency observed at 37°C than at 0°C could be explained either by a chelation process dependent on temperature or by a critical conformation of the enzyme which would permit chelation of its endogenous cations by EDTA.

The ionisation pK values of the catalytic site of the enzyme, which correspond to the imidazole and sulphydryl groups, varied in the presence of EDTA. This phenomenon was probably due to the binding of the chelating agent on an endogenous cation near the catalytic site. In the pH range 6–9.5, the free carboxyl groups of EDTA [20] should modify the ionisation state of the groups involved in the catalytic site of the enzyme (pK_i = 6.9 and 7.5). However, this hypothesis can be retained only if EDTA is effectively bound to the enzyme through an endogenous cation.

The irreversibility of the activity can be due either to binding of EDTA to the enzyme, or to partial removal of endogenous cations by EDTA, leading to an irreversible conformational change of the enzyme [10]. Indeed, the addition of exogenous cations to the enzyme after gel filtration did not allow the activity to be restored. However, our results obtained by gel filtration and by sucrose gradient ultracentrifugation are in favour of the binding of EDTA to the enzyme. The fact that authors in [10] did not observe binding of [^{14}C]EDTA to the enzyme after gel filtration, was probably due to the low temperature used in their experiment (0°C); such a low temperature was not sufficient to make the EDTA-enzyme link irreversible.

All these results prove that 5′-nucleotidase from bovine liver plasma membrane is a metalloprotein. However, identification of the endogenous cation using physico-chemical methods (X-ray, NMR, EPR) and analysis of the amino acid residues involved in the catalytic site should allow us to propose a hydrolysis mechanism of 5′-AMP by 5′-nucleotidase, as in the case of carboxypeptidase A, a metalloprotein which requires zinc [21].

Furthermore, the metalloprotein nature of membrane-bound 5′-nucleotidase raises the problem of the biosynthesis of this enzyme: rat liver cytosolic 5′-nucleotidase requires exogenous cations for its activity [22]. Thus, if the cytosolic enzyme is a precursor of the membrane-bound enzyme, it would be interesting to elucidate the mechanism of integration of the cation into the enzyme molecule.

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REFERENCES

[1] Zachowski, A., Evans, W.H. and Paraf, A. (1981) Biochim. Biophys. Acta 644, 121–126.
[2] Gurd, J.W. and Evans, W.H. (1974) Arch. Biochem. Biophys. 164, 305–311.
[3] De Pierre, J.W. and Karnovsky, M.L. (1974) J. Biol. Chem. 249, 7111–7120.
[4] Berne, R.M. and Rubio, R. (1979) Handb. Physiol. sect.2: Cardio-Vasc. Syst. 1, 873–952.
[5] Carson, D.A. and Seegmiller, J.E. (1976) J. Clin. Invest. 57, 274–282.
[6] Fisher, D., Van der Meyden, M.B., Snyderman, R. and Kelley, N.N. (1976) J. Clin. Invest. 58, 399–407.
[7] Wolff, J. and Cook, G.H. (1977) J. Biol. Chem. 252, 687–693.
[8] Edwards, N.L., Recker, D., Manfredi, J., Rembecki, R. and Fox, I.H. (1982) Am. J. Physiol. 243, C 270–C 277.
[9] Harb, J., Meflah, K., Duflos, Y. and Bernard, S. (1983) Eur. J. Biochem. 137, 131–138.
[10] Naito, Y. and Lowenstein, J.M. (1981) Biochemistry 20, 5188–5194.
[11] Mallol, J. and Bozal, J. (1983) J. Neurochem. 40, 1205–1211.
[12] Meflah, K., Harb, J., Duflos, Y. and Bernard, S. (1984) J. Neurochem. 42, 1107–1115.
[13] Armant, D.R. and Rutherford, C.L. (1982) Arch. Biochem. Biophys. 216, 485–494.

[14] Kirschmer, R.J. and Goldberg, A.L. (1983) J. Biol. Chem. 258, 967–976.
[15] Zachowski, A., Simonin, G., Aubry, J., Pommier, P., Singh, S.N., Potter, M. and Paraf, A. (1981) J. Recept. Res. 2, 97–118.
[16] Dixon, M. and Webb, E.C. (1979) Enzymes (Longman London ed.) 3rd edn, pp.138–164, Academic Press, New York.
[17] Polacheck, I. and Cabib, E. (1981) Anal. Biochem. 117, 311–314.
[18] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[19] Anner, B. and Mossmayer, M. (1975) Anal. Biochem. 65, 305–309.
[20] Charlot, G. (1974) in: Chimie Analytique Quantitative, vol.1, 6e edn, pp.19–28, Masson, Paris.
[21] Lipscomb, W.N., Coppola, J.C. and Hartsuck, J.A. (1966) J. Mol. Biol. 19, 423–441.
[22] Itoh, R. (1981) Biochim. Biophys. Acta 657, 402–410.