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Dephosporylation with Alkaline Phosphatase of Histone and Fibrinogen Phosphorylated with Protein Kinase C *in vitro*

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ABSTRACT

Alkaline phosphatase from calf intestinal mucosa dephosphorylated histone H1 and fibrinogen that had been phosphorylated with protein kinase C. The reaction velocity was dependent on the ionic strength of the buffer; decreasing with increasing concentration. The pH optimum was around 7, which is lower than pH-optima described for other kinds of substrates. (^{32}P)phosphorylated fibrinogen was dephosphorylated about 20 times faster than (^{32}P)phosphohistone on a weight basis and the reaction continued linearily with time for the longest time tested (3 hs) even at 37^{0}C. As alkaline phosphatase is present in the blood the possible physiological significance of the dephosphorylation of phosphofibrinogen is discussed.

INTRODUCTION

Some substrates of protein kinase C have been identified, among others membrane-bound EGF and insulin receptors (1,3), and some others only defined by their subunit molecular weight. Little attention has been payed to the dephosphorylation of this group of substrates.

Alkaline phosphatase belongs to a group of phosphatases known to show a broad substrate specificity and the physiological function of which still is unknown. Synthetic substances like dinitrophenyl phosphate, nucleotides and proteins phosphorylated with protein kinase A or tyrosine kinase are some of the known substrates *in vitro* (8,12). Both phosphoester and phosphoanhydrid bonds can be cleaved by the enzyme. As fibrinogen phosphorylated with protein kinase C was found to be dephosphorylated (5) it was of interest to investigate the optimal conditions for this dephosphorylation together with that of another substrate of protein kinase C, histone H1, as alkaline phosphatase could be a useful tool in the investigation of the physiological role of protein kinase C phosphorylation. Also, increased information on the possible role of the phosphatase in biological systems could be obtained by such an investigation.

MATERIALS AND METHODS

Histone H1, 1,2-diolein, L-α-phosphatidylserine were from Sigma (USA). (^{32}P)ATP was a product of NEN (England) and Sephadex G-50 was bought from Pharmacia (Sweden). Alkaline phosphatase was from Boehringer-Mannheim (Germany). All other chemicals were of highest grade commercially available. Protein kinase C and (^{32}P)phosphofibrinogen were prepared as described (5). (^{32}P)orthophosphate was determined by the method of Nimmo *et al* (10).

Preparation of (^{32}P)phosphohistone. Histone H1 (1.0 mg/ml) was phosphorylated with 1500 U of protein kinase C and 0.1 mM (^{32}P)ATP (50 cpm/pmol) in a total volume of 1500 µl containing 42 mM Tris-HCl buffer, pH 7.5, 0.67 mM calcium acetate, 5.0 mM magnesium acetate, 1.3 mg/ml phosphatidylserine, 1.1 µg/ml 1,2-diolein, 0.15 mM dithiothreitol and 0.67 mM EDTA. The lipids were included to activate protein kinase C. After 30 min at 30°C the incubation was interrupted by chromatography on a Sephadex G-50 (75 ml) equilibrated and eluted with 10 mM Tris-HCl buffer, pH 7.0 containing 50 mM NaCl. The radioactivity was measured as Cerenkov radiation (9). The amount of (^{32}P)histone was determined according to Bradford (2) with histone as reference protein and the material was stored at -20°C until used.

Dephosphorylation of (^{32}P)phosphohistone and (^{32}P)phosphofibrinogen with alkaline phosphatase. The dephosphorylation was performed as follows unless otherwise stated. In a total volume of 100 µl the incubations contained 20 mM Tris-HCl buffer, pH 7.5, 0.9% (w/v) NaCl, alkaline phosphatase, and 1-3 µg of (^{32}P)phosphohistone and 20-40 µg of (^{32}P)phosphofibrinogen, respectively. Each sample contained a minimum of 1000 cpm. The reaction was started by the addition of 5000 U of alkaline phosphatase/mg (^{32}P)phosphohistone or 5 U/mg (^{32}P)phosphofibrinogen and was continued for 30 min at 37°C. It was interrupted by the addition of 100 µl 1% (w/v) bovine serum albumin and 2 ml 5% (w/v) trichloroacetic acid (TCA) containing 0.25% (w/v) silicotungstic acid and 50 mM H_3PO_4 for (^{32}P)histone and 10% TCA with 50 mM H_3PO_4 for ^{32}P-labelled fibrinogen. After precipitation for 10 min at 0°C the samples were centrifuged for 5 min at 1000xg. The radioactivity of the supernatant and the pellet dissolved in 0.5 ml 0.5 M NaOH was measured and the percentage of the radioactivity transferred to the supernatant was calculated as a measure of liberated orthophosphate.

The supernatants from the subsequent assays were analysed for orthophosphate. All radioactivity was in the form of orthophosphate indicating that proteases did not contaminate the substrates or the phosphatase.

RESULTS

The effect of buffer substance on the activity of the phosphatase. In the experiments described for (^{32}P)phosphofibrinogen (See Fig. 1) the highest activity was obtained when 20 mM Tris, pH 7.5 was used as buffer. The effect

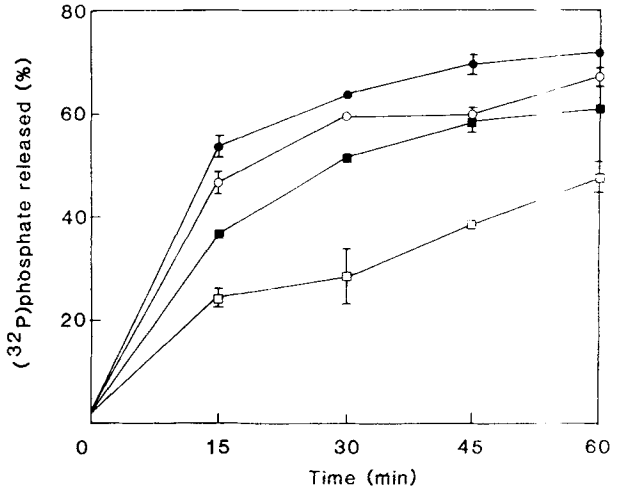

Fig. 1 Dephosphorylation of (^{32}P)phosphofibrinogen in different buffer systems: 0.2 M glycine buffer, pH 9.0 (-o-), 0.4 M dietanolamine buffer, pH 9.0 (-■-) 0.4 M dietanolamine buffer, pH 9.8 (-□-) and 20 mM Tris-HCl buffer, pH 7.5 containing 0.9% (w/v) NaCl (-●-). 50U of phosphatase/mg fibrinogen was used. The conditions were those described in **Material and Methods.**

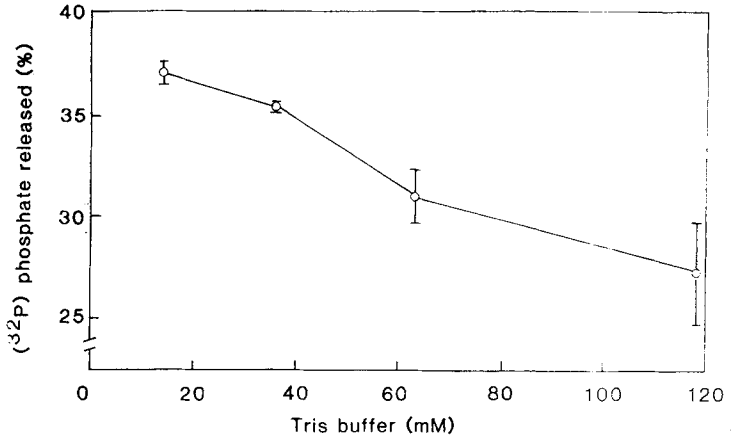

Fig. 2 Dephosphorylation of fibrinogen with varying concentrations of Tris buffer: The buffer used was Tris-HCl, pH 7.5 also containing 0.9% NaCl. The ratio of phosphatase to (^{32}P)phosphofibrinogen was 6.4U/mg. For conditions, see **Materials and Methods.**

of the ionic strenght was studied by varying the concentration of Tris. It was found that the activity decreased around 10% when the concentration of this substance was increased 10 times (Fig. 2) with fibrinogen as substrate.

Therefore, the phosphatase activity seen in Fig. 1 with different buffer substances was, if any, little influenced by the buffer substance itself but was a result of pH and ionic strenght.

The dependence of the phosphatase activity on the pH and divalent cations. The pH-optimum for dephosphorylation was found to be around 7 with a slight decrease in activity towards the alkaline pH-region (Fig. 3). Magnesium^{2+} (1.0 mM) is often used in the reaction mixture when alkaline phosphatase is used, so I tried that cation and also zinc^{2+} (0.1 mM).

This resulted in no effect on the dephosphorylation by these ions when histone was used as substrate. The dephosphorylation of fibrinogen was not effected by magnesium but zinc inhibited the activity by 60%.

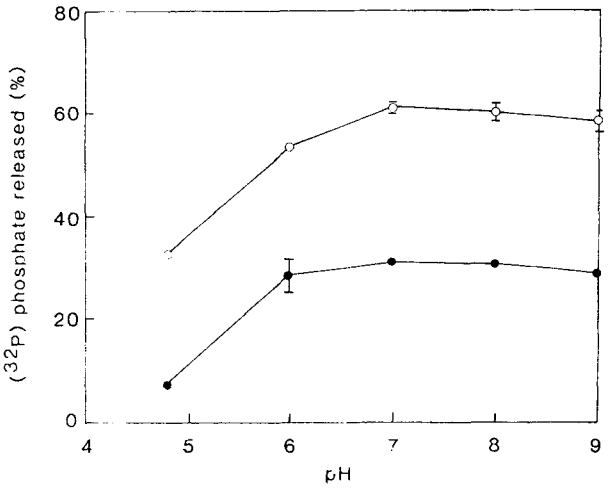

Fig. 3 The dependence of the dephosphorylation on pH: The buffers used were 0.2 M sodium acetate for pH 4.8 and 6.0, 20 mM Tris-HCL for pH 7.0, 8.0 and 9.0. All buffers also contained 0.9% NaCl. The ratio of phosphatase to (^{32}P)phosphohistone was 2500U/mg (-o-). The experiment was otherwise performed as described in **Materials and Methods**. Dephosphorylation of (^{32}P)phosphofibrinogen (-●-).

The phosphatase activity as a function of the amount of enzyme. The amount of phosphatase was varied at a constant concentration of histone and fibrinogen. The result is given in Figure 4. A 30% dephosphorylation is seen at 3900 U of phosphatase/mg histone after 30 min at 30°C (Fig. 4). A similar activity with fibrinogen was obtained with 21 U of phosphatase/mg (^{32}P)fibrinogen after 10 min at 37°C. A comparison between these two substrates showed that alkaline phosphatase dephosphorylated fibrinogen about 20 times faster than histone. The same difference can also be deduced from the results given in Figure 2.

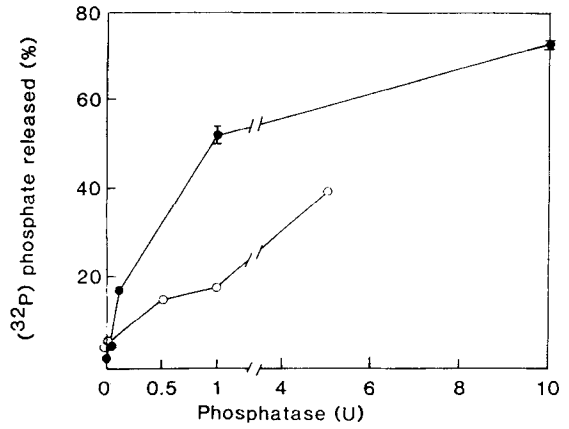

Fig. 4 The dependence on dephosphorylation of (^{32}P)phosphohistone and -fibrinogen on the amount of alkaline phosphatase: The buffer used was 0.2 M glycine, pH 9.0 and the temperature was 30°C. 0.83 μg (^{32}P)phosphohistone (-o-) was added, and the reaction was performed as described in **Materials and Methods**. For (^{32}P)phosphofibrinogen (-●-) the amount was 20 μg and the incubation time 10 min.

The effect of the temperature on the phosphatase activity. The activity increased linearly with temperature (Fig. 5) with no decrease in activity even after a long incubation time (180 min). The increase in the interval tested was 2 μg/ °Cxmin under the conditions used.

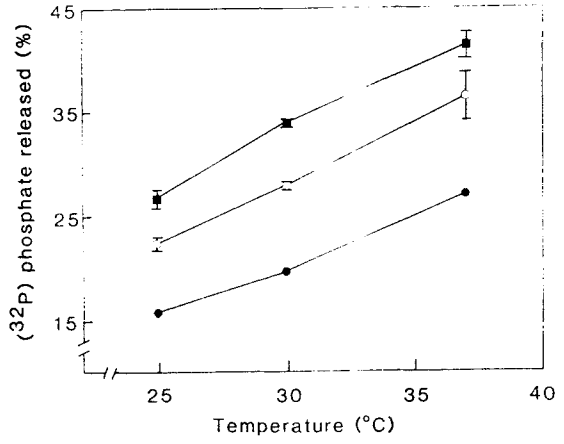

Fig.5 The dependence of dephosphorylation of (^{32}P)phosphofibrinogen on temperature: The incubation times were 60 min (-●-), 120 min (-o-) and 180 min (-■-). The conditions were as described in Materials and Methods except that the ratio of phosphatase to (^{32}P)phosphofibrinogen was 1.3U/mg.

The dependence of the activity of alkaline phosphatase on the (^{32}P)histone concentration: With histone as substrate at different concentrations under optimal conditions the enzyme showed a Michaeli-Menten behaviour (Fig. 6). From a Lineweaver-Burke plot inserted in the figure a K_m of 7.5 μM and a V_{max} of 1.2 nmol/minxmg were obtained.

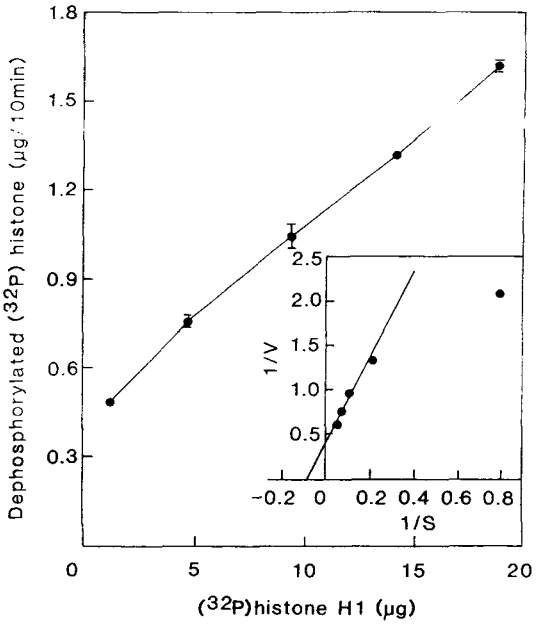

Fig. 6 The dependence of dephosphorylation on amount of (^{32}P)phosphohistone: The amount of enzyme was 47 U, the total volume 80 μl and the incubation time was 10 min. Otherwise the reaction was performed as described under **Materials and Methods**. Inserted in the Figure is the corresponding Lineweaver-Burke plot.

DISCUSSION

It was found that alkaline phosphatase can be used as a tool for dephosphorylation of proteins phosphorylated with protein kinase C. The following parameters are then of value to consider:

The substance buffering the system did not have any pronounced effect (Fig. 1) but with fibrinogen as substrate the activity of the phosphatase decreased with increasing ionic strenght from 100% (at 167mM) to 75% (at 273 mM) (Fig.2).

The optimum pH was 7. The decrease in alkaline phosphatase activity at higher pH values was not more than 5% so the reaction could be performed at higher pH if necessary (Fig. 3). The optimal pH is dependent on the protonised form of the substrate and therefore shifts towards a more acidic pH-optimum at low concentrations of substrates (11).

Cations did not interfere with the reaction, with one exception, $zinc^{2+}$ that inhibited the dephosphorylation of fibrinogen. Zinc is a known inhibitor but is also often used as an activating cation (4,6). It therefore seems, that if possible, no divalent cations should be added.

It was possible to dephosphorylate the substrate linearly for a long time. even at 37°C. This is known and used when alkaline phosphatase is coupled to the secondary antibody in Western techniques. In the case of protein kinase C phosphorylated substrates it was important that they did not contain any contaminating proteases if the reaction was prolonged.

Phosphorylated fibrinogen was a better substrate of alkaline phosphatase than histone. Even so, the K_m of 7.5 µM for phosphohistone indicated a high affinity of the enzyme for this substrate. However, the V_{max} and the turnover number that can be calculated (0.2×10^{-3} mol/mol enzyme x s) are low.

Alkaline phosphatase is present in blood or on the walls of the epithelium, and varies with different diseases. Fibrinogen, a known phosphoprotein, phosphorylated by at least four protein kinases *in vitro* (7), is also present in blood so the dephosphorylation of fibrinogen could be one of the physiological functions of this phosphatase.

In summary, the alkaline phosphatase can be used to dephosphorylate substrates phosphorylated with protein kinase C.

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