Biochimica et Biophysica Acta, 571 (1979) 55—62

BBA 68839

DEPHOSPHORYLATION OF PURINE MONONUCLEOTIDES BY ALKALINE PHOSPHATASES

SUBSTRATE SPECIFICITY AND INHIBITION PATTERNS

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(Received February 8th, 1979)

Key words: Alkaline phosphatase; Purine mononucleotide; Dimethyl xanthine; Nitrophenol phosphate

Summary

Three purine mononucleotides, adenosine-, inosine- and guanosine monophosphate, were used as substrates at pH 7.4 and at 10.4 for three alkaline phosphatases (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.1) containing similar phosphate-binding serine groups at their esteratic sites.

Substrate specificity was found for the enzymes from calf intestine and bovine liver. Alkaline phosphatase from *Escherichia coli* was nonspecific. A substrate-dependent and pronounced inhibition with the purine analogue 1,3-dimethyl xanthine was found for the enzymes from intestine and liver, but not for alkaline phosphatase from *E. coli*. A substrate-independent and pronounced inhibition was found for all three enzymes with the phosphomonoester *p*-nitrophenol phosphate as the inhibitor.

Alkaline phosphatases may play an important role in the regulation of the intracellular content of purine mononucleotides.

Introduction

Buhl and Jensen attempted to reduce the loss of purine compounds from ischaemic kidneys with various potential 5′-nucleotidase inhibitors, among these Ni^{2+} and 1,3-dimethyl xanthine [1]. The inhibiting effect on the phosphomonoesterase activity in rat kidney homogenates using adenosine monophosphate as the substrate was greater with 1,3-dimethyl xanthine than with Ni^{2+} in equimolar amounts.

The loss of dephosphorylated purine compounds from the intact ischaemic

kidney was also significantly smaller when 1,3-dimethyl xanthine was added to the perfusion fluid than when using Ni^{2+}.

Due to this study, the question of the role of the different phosphomonoesterases arose. The subdivision of phosphomonoesterases into nonspecific alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.1) and specific 5'-nucleotidase (EC 3.1.3.5) is mainly the result of the work of Reis [2]. Ahmed and Reis [3] described a specific inhibition pattern of 5'-nucleotidase by means of Ni^{2+}. It is generally accepted that both 5'-nucleotidase and nonspecific alkaline phosphatase are able to dephosphorylate adenosine monophosphate, while only nonspecific alkaline phosphatase is able to dephosphorylate unphysiological phosphomonoesters, such as *p*-nitrophenol phosphate.

The object of the present investigation was to study the dephosphorylation of the three purine mononucleotides, AMP, IMP and GMP, by different alkaline phosphatases. The inhibition patterns with the purine analogue 1,3-dimethyl xanthine and with the phosphomonoester *p*-nitrophenol phosphate were also studied. Alkaline phosphatase from *Escherichia coli*, from calf intestine and from bovine liver were selected for this study as the esteratic sites of these three enzymes are known to be identical, containing the so-called 'active' serine group [4–6].

Materials and Methods

The enzymes employed were alkaline phosphatases from *E. coli* (Sigma cat. No. P-4250), from calf intestine (Sigma cat. No. P-4502) and from bovine liver (Sigma cat. No. P-5760). One unit of all three enzymes will hydrolyze 1.0 μmol *p*-nitrophenol phosphate/min at pH 10.4 and 37°C. The enzymes were dissolved in 10 ml Tris buffer (5 mM, pH 7.4) with $MgCl_2$ (5 mM) to a calculated activity of 200 units/l, and in Tris buffer (5 mM, pH 10.4) with $MgCl_2$ (5 mM) to a calculated activity of 50 units/l.

The substrates used were adenosine, inosine and guanosine monophosphate and the inhibitors 1,3-dimethyl xanthine and *p*-nitrophenol phosphate.

All the incubations were carried out at 37°C at pH 7.4 and 10.4. The incubation times varied from 30 to 120 min. Enzyme solutions containing 200 units/l were used at pH 7.4 and 50 units at pH 10.4. The concentration of the substrates was maintained at a constant 15 mM in the incubation solutions; a maximum of 10% was dephosphorylated. The substrates were dissolved in Tris buffer (50 mM, pH 7.4 or pH 10.4) with $MgCl_2$ (5 mM). 1000 μl of the substrate solution plus 1000 μl of Tris buffer (5 mM) with $MgCl_2$ (5 mM) (or 1000 μl of an inhibitor solution, see below) were incubated with 250 μl of alkaline phosphatase solution in each experiment. In the inhibition experiments, the 1,3-dimethyl xanthine and the *p*-nitrophenol phosphate were dissolved in the Tris buffer with $MgCl_2$ to the calculated concentrations of 1, 5, 7.5 and 12 mM and 0.5, 1.0, 2.5 and 5.0 mM, respectively. After the desired incubation time, the reaction was stopped by the addition of 250 μl of 30% trichloroacetic acid. The amount of liberated phosphate in the incubation mixtures was determined colorimetrically [7].

The experiments with *p*-nitrophenol phosphate were carried out by the

determination of the amount of dephosphorylated purine compounds. 5 min after the reaction had been stopped the incubation mixture was neutralized to pH 7.4 by 400 µl Tris buffer (0.5 M, pH 7.4), to which was added NaOH (1.2 M). Then the purine compounds were sequentially enzymically transformed to uric acid which was measured using the uricase method [8].

Statistics. The slope of the lines in Fig. 1 and the inhibition percents in Fig. 2 were compared by means of the *t*-test for paired data. The percentages of inhibition of the dephosphorylation of the three purine mononucleotides in Fig. 3 were compared for each inhibitor concentration by means of analysis of variance.

Results

Fig. 1 shows the relationship between the incubation time and the amount of liberated phosphate, when AMP, IMP and GMP are incubated with alkaline phosphatases. A comparison of the coefficients of slope for the lines showed that IMP and GMP were dephosphorylated at the same rate by both the intestinal and liver enzymes. For both these enzymes the dephosphorylation of AMP was slower than of IMP and GMP at pH 7.4, but more rapid than the dephosphorylation of IMP and GMP at 10.4. Whereas there was no significant difference between the rates of dephosphorylation of AMP, IMP and GMP when using alkaline phosphatase from *E. coli* at pH 7.4 or 10.4.

Fig. 2 shows the inhibiting effect of 1,3-dimethyl xanthine on the dephosphorylation of AMP, IMP and GMP, when incubated with alkaline phosphatases for 90 min at pH 7.4 and at 10.4. The inhibition of the dephosphorylation of IMP and GMP was similar for the liver enzyme as well as the intestinal enzyme. For the liver enzyme the inhibition of the dephosphorylation of AMP at 7.4 was less than the inhibition of the dephosphorylation of IMP and GMP, but the opposite was the case at 10.4. With regard to the intestinal enzyme, the inhibiting effect on the dephosphorylation of AMP was greater than the inhibiting effect on the dephosphorylation of IMP and GMP, both at pH 7.4 and 10.4. The inhibition of the dephosphorylation of all three purine mononucleotides was less at pH 7.4 than at 10.4, for the liver enzyme, while the opposite was the case with regard to the intestinal enzyme. The inhibiting effect on alkaline phosphatase from *E. coli* was less than 10%, both at pH 7.4 and 10.4. There was no difference between the inhibition of the three purine mononucleotides with regard to this enzyme.

Fig. 3 shows how the dephosphorylation of AMP, IMP and GMP by means of alkaline phosphatases is inhibited by different concentrations of *p*-nitrophenol phosphate at pH 7.4 and 10.4. The inhibition was similar for each of the three enzymes. No qualitative or pronounced quantitative difference appears to be present between the three enzymes.

Discussion

AMP, IMP and GMP differ from each other in the purine component, while the ribose phosphate parts are identical. AMP is substituted with an amino group in the 6-position, while both IMP and GMP have a hydroxy group in this

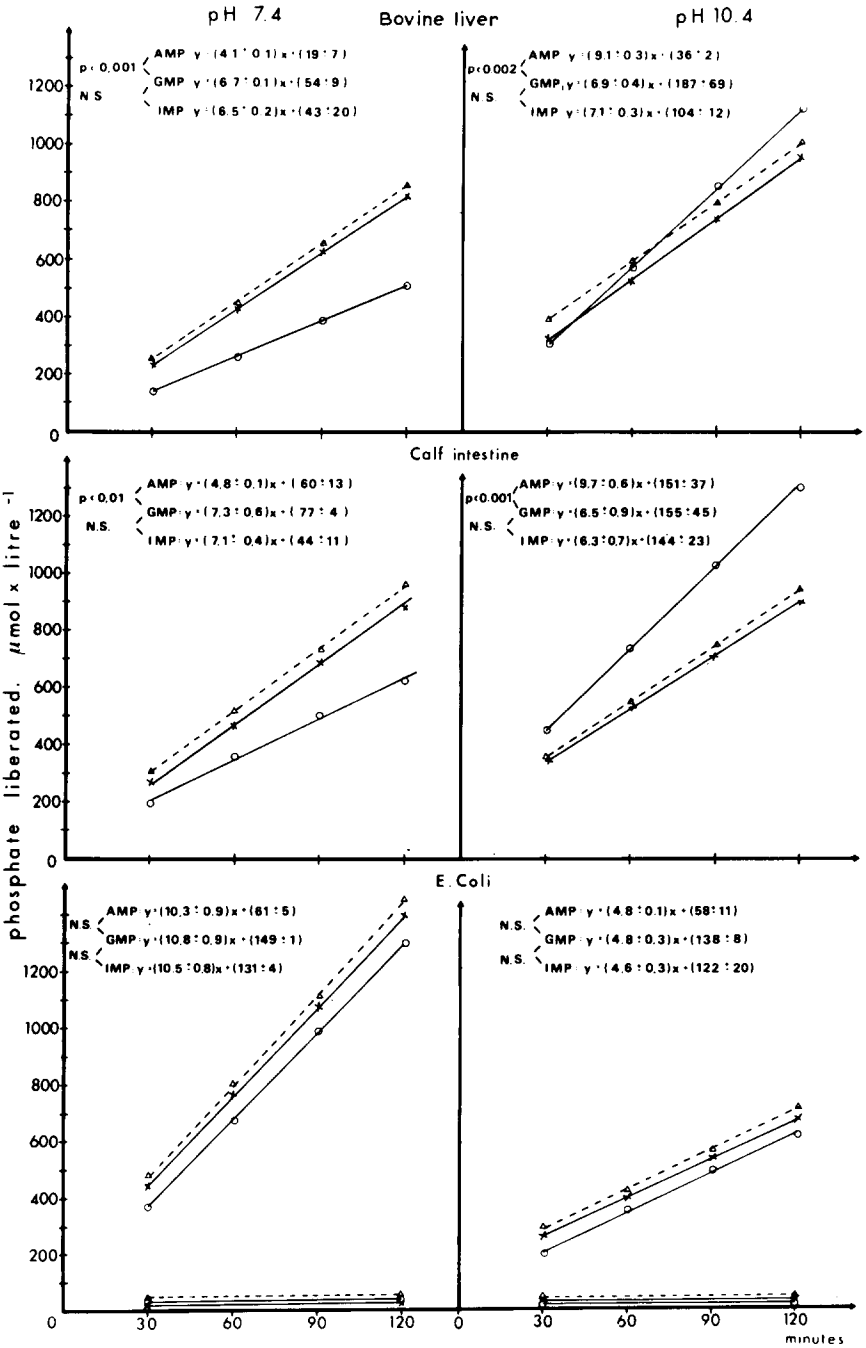

Fig. 1. Dephosphorylation of purine mononucleotides by alkaline phosphatases. Dephosphorylation at pH 7.4 (left) and 10.4 (right) at 37° C of the purine mononucleotides, AMP (○———○), IMP (X———X) and GMP (△- - - - -△) during incubation of varying duration with three different alkaline phosphatases. The concentration of the enzymes was 200 units/l at pH 7.4 and 50 at 10.4. Each of the 18 lines are mean lines for four determinations. The correlation of coefficient for a linear relationship was 0.98—1.00. The equation for the line is stated as the coefficient of slope ± 2 S.E. and intercept ± 2 S.E. The coefficient of slope = the amount of phosphate liberated/min. The lines in the lower part of the figure show the amount of phosphate liberated after incubation of 0 and 120 min duration without the addition of an enzyme. The *P* values for the comparison of the coefficients of slope are shown in the figure. N.S., non-significant difference at the 5% level.

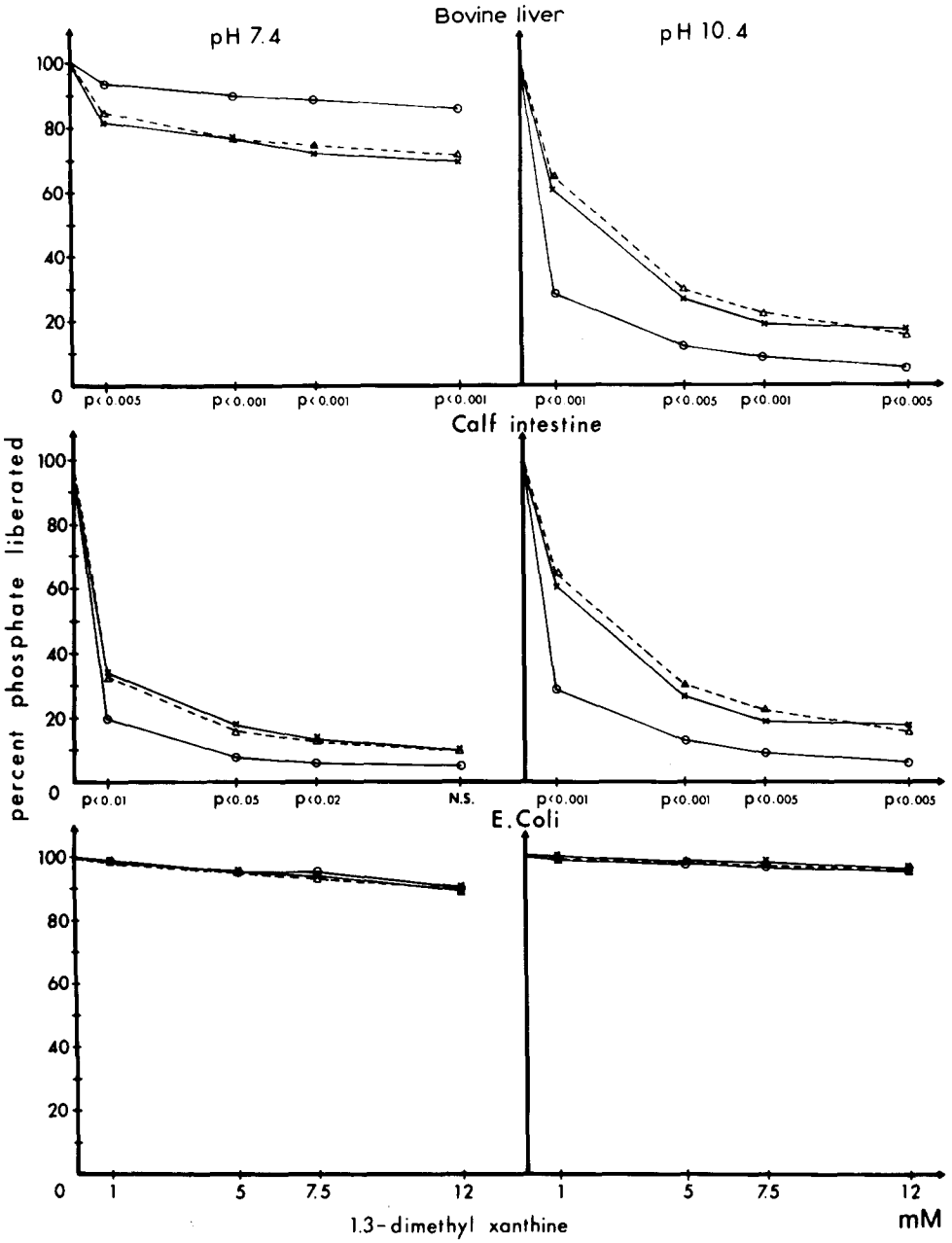

Fig. 2. The inhibiting effect of 1,3-dimethyl xanthine on the dephosphorylation of purine mononucleotides by alkaline phosphatases. The inhibiting effect of 1,3-dimethyl xanthine on the dephosphorylation of purine mononucleotides, AMP (○———○), IMP (X———X) and GMP (△- - - - -△) by alkaline phosphatase at pH 7.4 (left) and 10.4 (right). The formed amount of free phosphate without the addition of the inhibitor is put to 100%. All the incubations were of 90 min at 37°C. The enzyme concentrations employed were 200 units/l at pH 7.4 and 50 units/l at 10.4. S.E. varied from ±0 to ±6% for four analyses. In no case was it possible to demonstrate a statistically significant difference at the 5% level, between the inhibiting effect on the dephosphorylation of IMP and GMP. The values for the comparison between the inhibiting effect on the dephosphorylation of AMP and GMP are shown in the figure. N.S., non-significant difference at the 5% level.

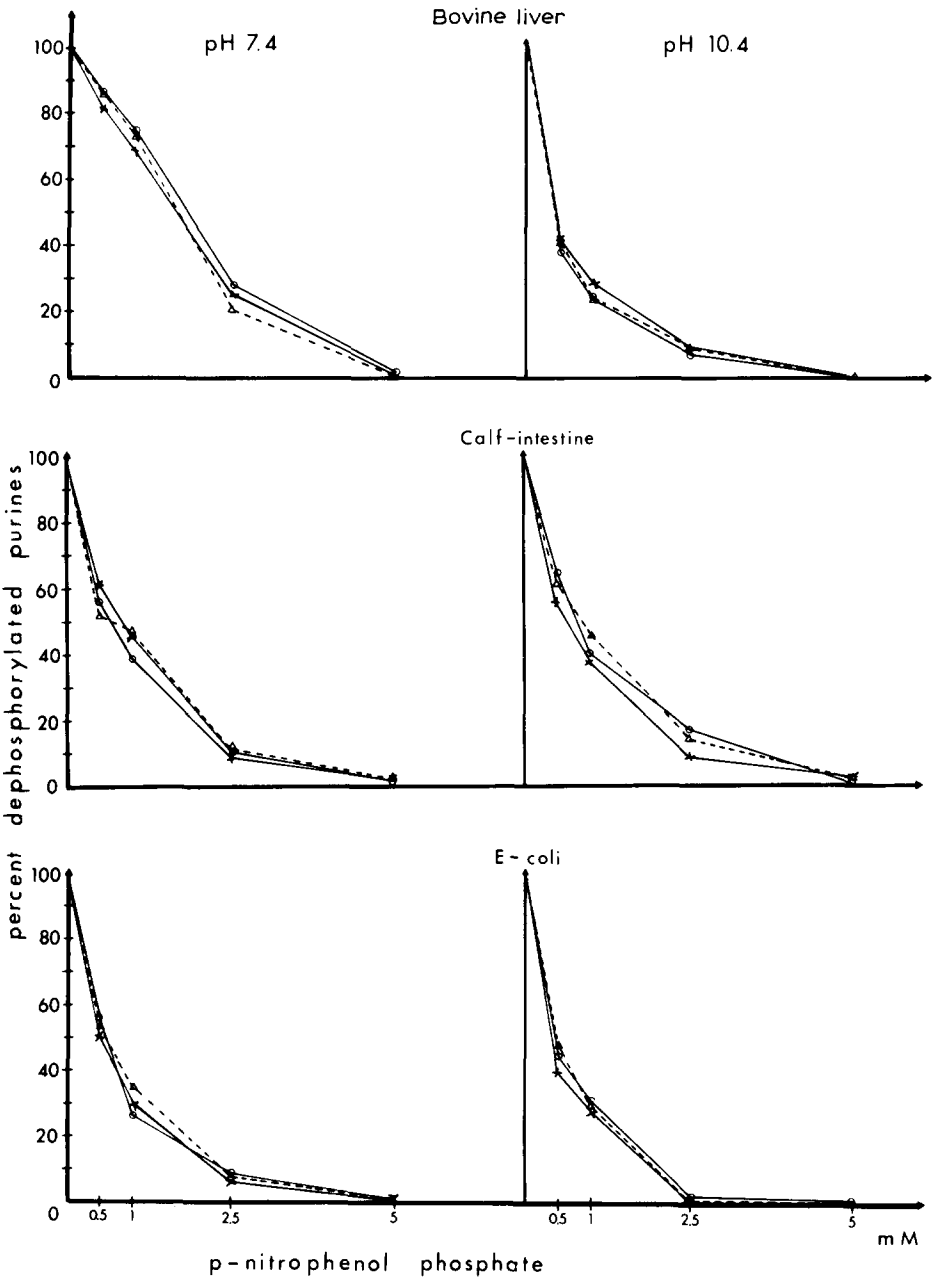

Fig. 3. The inhibiting effect of *p*-nitrophenol phosphate on the dephosphorylation of purine mononucleotides. The inhibiting effect of *p*-nitrophenol phosphate on the dephosphorylation of AMP (○———○), IMP (X———X) and GMP (△- - - - -△) by alkaline phosphatases at pH 7.4 (left) and 10.4 (right). The amount of formed dephosphorylated purines, without the addition of *p*-nitrophenol phosphate was put to 100%. All the incubations were of 45 min at 37° C. The enzyme concentrations were 200 units/l at pH 7.4 and 50 units/l at 10.4. All the values are mean of four determinations. 2 S.E. varied from ±0 to ±3%. In no case was it possible to demonstrate a statistical difference at the 5% level between the inhibiting effect on the dephosphorylation of the three purine mononucleotides.

position. GMP in addition, is substituted with an amino group in the 2-position. These differences do not appear to influence the activity of alkaline phosphatase from *E. coli*, but play a significant role for the enzymes from liver and intestine both with regard to substrate specificity and patterns of inhibition with 1,3-dimethyl xanthine (see Figs. 1 and 2). The different substitution in the 6-position of the purine component seems to be the only possible explanation. The differences in the purine component were, however, not of any significance when the dephosphorylation was inhibited by *p*-nitrophenol phosphate (see Fig. 3). The composition and the mode of action of the esteratic site of the three alkaline phosphatases used is well-studied. All three enzymes contain a so-called 'active' serine group and phosphomonoesters as well as pyrophosphates are split after binding of the phosphate group to the active serine group during the formation of intermediary phosphorylserine enzyme compounds [4—6,9].

The pronounced substrate-independent inhibition with *p*-nitrophenol phosphate suggests that the active serine group is responsible also for the dephosphorylation of the three purine mononucleotides. It seems, however, unlikely that the same phosphate-binding serine groups can be responsible for the substrate specificity and for the substrate-dependent inhibition with 1,3-dimethyl xanthine.

Our findings that the purine loss from ischaemic kidneys can be inhibited by 1,3-dimethyl xanthine [1] seems well explained by the fact that this purine analogue inhibits the dephosphorylation of purine mononucleotides by alkaline phosphatases. Adenosine is a powerful vasodilator, e.g. in the heart, skeletal muscles and brain [10—12], but oddly enough a powerful vasoconstrictor in the kidneys [13]. Alkaline phosphatase may therefore play an important role in the regulation of the regional blood flow, via adenosine formation (= AMP dephosphorylation). It has been found that 1,3-dimethyl xanthine has the opposite effect on the regional blood flow to that of adenosine [13,14]. A likely explanation is that the endogenous adenosine formation is decreased by the inhibiting effect of 1,3-dimethyl xanthine on alkaline phosphatase. The demonstrated specificity at physiological pH for the two mammalian enzymes studied and their localization to the membrane structure suggests a role in the regulation of the intracellular content of purine nucleotides, as only dephosphorylated purine compounds appear to be able to pass the cell membrane [1,15,16].

Acknowledgement

This investigation was supported by the Danish Medical Council.

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