A soluble 5'-nucleotidase in rat kidney

Stimulation by decavanadate

Michel LE HIR

Departement Forschung, Kantonsspital, CH-4031 Basel, Switzerland

A soluble 5'-nucleotidase was identified in rat kidney and partially purified. Compared with 5'-IMP, 5'-AMP was a poor substrate. The affinity for 5'-IMP was very low ($s_{0.5} > 1$ mM) in the absence of an activator, and it was much increased ($s_{0.5} = 0.1$ mM) by 2,3-bisphosphoglycerate (2,3-DPG). ATP and bisadenosyl tetraphosphate were further activators. The pH optimum was 6.3. Those properties suggest that the renal soluble 5'-nucleotidase is identical with the 'high- K_m ' 5'-nucleotidase purified previously from liver, heart and erythrocytes. Decavanadate (100 nM) increased the rate of hydrolysis of 1 mM-5'-IMP 16-fold. The effect was specific for the decameric form of vanadate, since it was not reproduced by either decavanadate-free orthovanadate or pervanadate. Half-maximal activation was obtained at 1.4 nM-decavanadate. Decavanadate increased the affinity of the soluble 5'-nucleotidase for 5'-IMP. The effects of 2,3-DPG and of vanadate were not additive. Thus decavanadate probably influences the soluble 5'-nucleotidase in the same way as 2,3-DPG, but with a much higher potency.

INTRODUCTION

Adenosine exerts through specific membrane receptors physiological effects in a variety of tissues. In the kidney it affects glomerular filtration, tubular transport and the release of renin (Spielman & Thompson, 1982).

In order to understand the regulatory role of adenosine, it is important to know the pathways for its production. Adenosine is produced mainly by the hydrolysis of 5'-AMP by 5'-nucleotidase. The membrane-bound ecto-5'-nucleotidase has been known for many years. In addition, two types of soluble 5'-nucleotidases have been described in mammals. Spychala *et al.* (1988) proposed to call them 'high- K_m ' and 'low- K_m ' 5'-nucleotidase. In the absence of specific activators, the first shows $s_{0.5}$ values in the millimolar range, and the second one in the micromolar range. The occurrence of a renal ecto-5'-nucleotidase has been demonstrated in previous studies (Le Hir *et al.*, 1989; Dawson *et al.*, 1989). Likewise we described a soluble 'low- K_m ' 5'-nucleotidase in the rat kidney (Le Hir & Dubach, 1988). However, we found recently that it corresponds to solubilized ecto-5'-nucleotidase (Piec & Le Hir, 1991). A 'high- K_m ' 5'-nucleotidase has been detected in several tissues (Van den Berghe *et al.*, 1977; Itoh, 1981; Bontemps *et al.*, 1988; Spychala *et al.*, 1988; Truong *et al.*, 1988). It is characterized by its higher affinity for 5'-IMP than for 5'-AMP, and is probably located in the cytosol (Yokota *et al.*, 1988). Although data of Bontemps *et al.* (1989) are suggestive of its existence in the kidney, this point has not been established definitively. In the present study we attempted to purify a soluble 'high- K_m ' 5'-nucleotidase from renal homogenates. The purification protocol included steps devised by other investigators for the purification of the soluble 5'-nucleotidase from heart, erythrocytes and liver. It appeared that a soluble 'high- K_m ' 5'-nucleotidase exists in the rat kidney, and that it is very similar to the enzyme purified from other sources.

The repertoire of substances useful for the experimental manipulation of the activity of the soluble 5'-nucleotidase is poor. As vanadate is a potent inhibitor of various phosphohydrolases, we tested its effect on the renal soluble 5'-nucleotidase. Decavanadate, an oligomeric form of orthovanadate, proved to be an extremely potent activator.

MATERIALS AND METHODS

Materials

Orthovanadate (Na_3VO_4), the nucleotides and 2,3-DPG were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phosphocellulose was a product of Whatman (Maidstone, Kent, U.K.) and Blue Sepharose CL 6B of Pharmacia (Uppsala, Sweden). $[8\text{-}^{14}\text{C}]5'$ -IMP was obtained from Amersham International (Amersham, Bucks., U.K.).

Vanadate solutions

Decavanadate-free solutions were obtained by dissolving orthovanadate at a concentration of 50 mM in distilled water. Final dilutions of the assay solution were not followed by an adjustment of the pH, in order to avoid the formation of decavanadate (Kadota *et al.*, 1987a). The alkalinity of vanadate was not disturbing, because of its very low final concentrations in the assay medium (micromolar concentrations). Decavanadate solutions were prepared by titration of 50 mM-orthovanadate to pH 2 (Varga *et al.*, 1985). This yields a stock solution of 5 mM-decavanadate. Pervanadate was produced as described by Kadota *et al.* (1987b).

Purification of soluble 5'-nucleotidase

Male Wistar rats (200–300 g body wt.) were decapitated and the kidneys excised. The preparation of high-speed supernatants of homogenates, the $(\text{NH}_4)_2\text{SO}_4$ precipitation and the phosphocellulose chromatography with NaCl-gradient elution were performed as described for the purification of soluble 5'-nucleotidase from rat heart by Truong *et al.* (1988), starting with 20–30 g of whole kidneys. The column was 1 cm $\times$ 8 cm, the gradient volume 200 ml and the flow rate 15 ml/h. The single peak with 5'-IMP phosphohydrolase activity was eluted at a concentration of 500 mM-NaCl from the phosphocellulose column. The collected fractions (about 10 ml) were passed at a flow rate of 10 ml/h through a Blue Sepharose column (1 cm $\times$ 2 cm) equilibrated with 50 mM-Hepes/NaOH, pH 7.0, containing 1 mM-EDTA and 10 mM-mercaptoethanol. The column was washed with 20 ml of the same buffer containing 500 mM-NaCl, and the 5'-IMP phosphohydrolase was eluted by increasing the concentration of

Abbreviation used: 2,3-DPG, 2,3-bisphosphoglycerate.

Table 1. Purification of soluble 5'-nucleotidase from 30 g of rat kidney

Activities were measured in the presence of 100 nM-decavanadate. The results are representative of three experiments. One unit represents 1 $\mu\text{mol/min per mg}$.

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Supernatant	75	35.22	1170	0.0300	1	100
(NH ₄) ₂ SO ₄ precipitate	13	5.94	463.7	0.0128	0.43	16.9
Residue of dialysis	15	5.12	441.5	0.0116	0.39	14.5
Phosphocellulose pool	22	3.66	14.12	0.259	8.63	10.4
Blue Sepharose	5.8	2.38	0.95	2.50	83.3	6.8
Low-ionic-strength precipitation	0.5	1.98	0.50	3.97	132	5.6

NaCl in one step to 2 M (Bontemps *et al.*, 1988). As in the protocol of Itoh (1981) for the liver soluble 5'-nucleotidase, the last step was a low-ionic-strength precipitation. The active fractions eluted from the Blue Sepharose column were pooled and dialysed twice against 200 ml of 20 mM-Tris/HCl, pH 8.0, containing 10 mM-mercaptoethanol for a total of 20 h at 4 °C. The dialysis residue was left for 3 days at 4 °C and was then centrifuged at 10000 *g* for 15 min. The pellet was dissolved in 0.5 ml of 50 mM-Mops/NaOH buffer, pH 6.8, supplemented with 500 mM-NaCl.

Assay of proteins

The Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, U.S.A.) was used, with BSA as the standard.

Assays of 5'-nucleotidase

The standard incubation medium consisted of 1 mM-5'-IMP, 10 mM-MgCl₂ and 1 mM-EDTA in 50 mM-Mops/NaOH buffer, pH 6.5. The reaction was started by addition of the enzyme (final protein concentration between 0.12 and 0.60 $\mu\text{g/ml}$). Incubation proceeded for between 15 and 120 min at 37 °C. It was stopped by addition of an equal volume of 0.2 M-H₂SO₄. The liberated P_i was assayed as described by Baykov *et al.* (1988). In experiments where the effect of P_i or phosphoesters was tested, [8-¹⁴C]5'-IMP was added in the standard incubation medium at a final radioactivity of 100 $\mu\text{Ci/l}$. The activity of 5'-nucleotidase was assayed by measuring the production of labelled inosine as described by Truong *et al.* (1988).

RESULTS

Table 1 shows the purification factors and recoveries at the successive steps of one purification of the soluble 5'-nucleotidase from rat kidneys. The activities in Table 1 were measured under conditions of full activation of the enzyme, obtained by the addition of 100 nM-decavanadate to the standard assay medium. In the standard assay medium, i.e. in the absence of decavanadate, the activity of the enzyme preparation used for all experiments shown in the Figures was 0.188 $\mu\text{mol/min per mg}$ with 1 mM-5'-IMP and 0.0094 $\mu\text{mol/min per mg}$ with 1 mM-5'-AMP. No activity was detectable with 1 mM-3'-AMP (< 0.001 $\mu\text{mol/min per mg}$) in the standard assay medium, or with 1 mM-5'-IMP if MgCl₂ was omitted. The optimum pH with 1 mM-5'-IMP was about 6.3 (Fig. 1).

Addition to the assay medium of 1 mM-P_i and 0.1 mM-adenosine 5'-[α,β -methylene]diphosphate resulted respectively in 88 % and 11 % decrease in activity. Compared with the standard medium, 1 mM-ATP, 0.1 mM-P_i, P⁴-bis-(5'-adenosyl)tetraphosphate and 1 mM-2,3-DPG provoked 6.3-fold, 9.1-fold and

Fig. 1. Influence of the pH on the activity of the soluble 5'-nucleotidase

The assay medium consisted of 1 mM-5'-IMP, 1 mM-EDTA, 10 mM-MgCl₂ and 50 mM-Mes (▲) or -Mops (●) titrated with NaOH to the indicated pH values, without decavanadate (top) or with 100 nM-decavanadate (bottom). The production of P_i was measured.

16-fold increases of the activity respectively. In the presence of 1 mM-2,3-DPG the concentration of 5'-IMP yielding half-maximal activity was close to 0.1 mM, the lowest concentration tested, whereas it was higher than 1 mM in the absence of activator (Fig. 2). Addition of 500 mM-NaCl to the standard assay medium did not influence the activity. However, when the assay medium described by Itoh (1981) was used, containing 50 mM-MgCl₂ and 3 mM-5'-IMP in 100 mM-imidazole/HCl at pH 6.5, the activity with 500 mM-NaCl was 694 % of the control without NaCl.

Decavanadate was an activator of the soluble 5'-nucleotidase. With 1 mM-5'-IMP and 1 mM-5'-AMP the activity was respectively 16-fold and 76-fold higher in the presence of 100 nM-decavanadate than in the control. Half-maximal activity was obtained at 1.4 nM-decavanadate with 1 mM-5'-IMP as substrate (Fig. 3). Neither decavanadate-free orthovanadate nor per-

Fig. 2. Influence of the concentration of 5'-IMP on the activity of the soluble 5'-nucleotidase

The standard assay medium (see the Materials and methods section) was used without supplement (▲), with 1 mM-2,3-DPG (■) or with 100 nM-decavanadate (●). The production of P_i was measured.

Fig. 3. Influence of the concentration of decavanadate on the activity of the soluble 5'-nucleotidase

The standard assay medium (see the Materials and methods section) was supplemented with various concentrations of decavanadate. The production of P_i was measured.

vanadate up to 10 μ M had any effect on the hydrolysis of 5'-IMP. The affinity of the soluble 5'-nucleotidase for 5'-IMP and the maximal rate of hydrolysis of 5'-IMP were identical with 1 mM-2,3-DPG and 1 μ M-decavanadate (Fig. 2). In the presence of 1 μ M-decavanadate, 2,3-DPG had no influence on the activity (Fig. 4). The pH-dependence of the rate of hydrolysis of 5'-IMP was somewhat modified by decavanadate (Fig. 1). The slope of decrease in activity at pH values above the optimum was steeper in the absence than in the presence of decavanadate. In other words, stimulation by decavanadate was more pronounced at higher pH values. The substrate specificity was not modified by vanadate. At the substrate concentration of 1 mM in the presence of 100 nM-decavanadate, the activity towards 5'-AMP was 25%

Fig. 4. Influence of the concentration of 2,3-DPG on the activity of the soluble 5'-nucleotidase

The standard assay medium (see the Materials and methods section) was supplemented with various concentrations of 2,3-DPG, without decavanadate (●) or with 100 nM-decavanadate (▲). The production of [8- 14 C]inosine from 5'-[8- 14 C]IMP was measured.

of that measured with 5'-IMP, and no activity could be detected with 3'-AMP.

DISCUSSION

The aim of the present study was the characterization of a soluble 5'-nucleotidase activity in the rat kidney. Data of Bontemps *et al.* (1989) are indicative of the existence in the supernatant of renal homogenates of an enzyme similar to the soluble 5'-nucleotidase purified from liver, heart and erythrocytes. To establish this unequivocally, it was necessary to study the properties of the purified enzyme. The first steps of purification corresponded to the protocol used for the purification of the soluble 5'-nucleotidase from heart (Truong *et al.*, 1988). Further steps, namely Blue Sepharose affinity chromatography and low-ionic-strength precipitation, were adapted from protocols used for the purification of the soluble 5'-nucleotidase from erythrocytes (Bontemps *et al.*, 1988) and from liver (Itoh, 1981).

The overall purification factor indicated in Table 1 does not represent the actual purification of the soluble 'high- K_m ' 5'-nucleotidase described in the present paper, because the starting material contains other enzymes that hydrolyse 5'-mononucleotides. We found in supernatants of kidney homogenates a 'low- K_m ' 5'-nucleotidase (Le Hir & Dubach, 1988) that corresponds to a product of solubilization of the ecto-5'-nucleotidase (Piec & Le Hir, 1991), and a tartrate-inhibitable acid phosphatase active with mononucleotides (M. Le Hir, unpublished work). The soluble 5'-nucleotidase purified from rat kidney was devoid of non-specific phosphatase activity, as evidenced by the lack of activity towards 3'-AMP and the absence of activity towards 5'-IMP if $MgCl_2$ was omitted from the assay medium. The absolute requirement for $MgCl_2$ indicates additionally that the solubilized ecto-5'-nucleotidase has been eliminated during purification.

The enzyme purified from rat kidney displayed typical properties of the soluble 5'-nucleotidase purified from liver (Naito & Tsushima, 1976; Van den Berghe *et al.*, 1977; Itoh, 1981), heart (Truong *et al.*, 1988), placenta (Spychala *et al.*, 1988) and erythrocytes (Bontemps *et al.*, 1988), namely the preference for 5'-IMP over 5'-AMP as substrate, the requirement for $MgCl_2$, the low affinity for the substrate in the absence of activators, the susceptibility to stimulation by 2,3-DPG, ATP and P^1, P^4 -bis-(5'-

adenosyl)tetraphosphate as well as to inhibition by P_i and the localization of the optimal pH in the vicinity of 6.5. Moreover, the $s_{0.5}$ of 0.1 mM for the soluble 5'-nucleotidase from rat kidney in the presence of decavanadate was in the range (0.04–0.2 mM) of the values determined for the soluble 5'-nucleotidases from other sources in the presence of activators (Itoh *et al.*, 1978, 1986; Bontemps *et al.*, 1988; Spychala *et al.*, 1988; Truong *et al.*, 1988). The very potent inhibitor of ecto-5'-nucleotidase, adenosine 5'-[$\alpha\beta$ -methylene]diphosphate, with an apparent K_i value of 5 nM calculated for the renal enzyme (Le Hir *et al.*, 1989), had little effect at a concentration of 0.1 mM on the soluble 5'-nucleotidase described in the present paper. This enzyme was activated by ATP, which is an inhibitor of the ecto-enzyme (Le Hir *et al.*, 1989).

Thus the kidney contains a soluble 5'-nucleotidase which is very similar to the soluble 5'-nucleotidase found in other tissues and very different from the ecto-5'-nucleotidase. The issue of a possible production of adenosine by this type of soluble 5'-nucleotidase is still controversial, because the ecto-5'-nucleotidase and the AMP-specific soluble 5'-nucleotidase found in heart (Skladanowski & Newby, 1990) are further possible sources of the nucleoside. To assess the role of each of these enzymes, it will be important to find substances which can be used experimentally to modify the activity of the enzyme in living cells (Skladanowski *et al.*, 1989). Likewise, a more profound knowledge of the kinetic properties of the soluble 5'-nucleotidase might be important. In this respect decavanadate might constitute an interesting tool.

Vanadate influences the activity of a number of phosphohydrolases. Its effect on the soluble 5'-nucleotidase is somewhat atypical for three reasons. Firstly, in the case of the soluble 5'-nucleotidase, the active species is decavanadate. Until now decavanadate has not been identified as the effective form of vanadate with other enzymes, except for kinases (Boyd *et al.*, 1985). Secondly, vanadate activates the soluble 5'-nucleotidase in the nanomolar range, whereas most described effects of vanadate require micromolar or millimolar concentrations (literature cited by Nechay *et al.*, 1986). Thirdly, in its decameric form, vanadate stimulates the soluble 5'-nucleotidase, whereas it is mostly known as an inhibitor of various phosphohydrolases (reviewed by Nechay *et al.*, 1986). The known inhibitory effects of vanadate on phosphohydrolases can be explained by structural analogies with P_i or with phosphoesters (Coan *et al.*, 1986). For the soluble 5'-nucleotidase, considering our finding that the stimulatory effects of decavanadate and of 2,3-DPG are identical and not additive, it is likely that decavanadate reacts with the binding site of 2,3-

DPG. This site probably binds ATP also, because the stimulatory effects of ATP and 2,3-DPG are not additive at saturating concentrations (Bontemps *et al.*, 1988).

This study was supported by the Swiss National Foundation, grant 32-25353.88. The skilful technical assistance of Mrs. T.-M. Baer is gratefully acknowledged.

REFERENCES

- Baykov, A. A., Evtushenko, O. A. & Avaeva, S. M. (1988) *Anal. Biochem.* **171**, 266–270
- Bontemps, F., Van den Berghe, G. & Hers, H.-G. (1988) *Biochem. J.* **250**, 687–696
- Bontemps, F., Vincent, M. F., Van den Berghe, F., Vanwaeg, G. & Van den Berghe, G. (1989) *Biochim. Biophys. Acta* **997**, 131–134
- Boyd, D. W., Kustin, K. & Niwa, M. (1985) *Biochim. Biophys. Acta* **827**, 472–475
- Coan, C., Scales, D. J. & Murphy, A. J. (1986) *J. Biol. Chem.* **261**, 10394–10403
- Dawson, T., Gandhi, R., Le Hir, M. & Kaissling, B. (1989) *J. Histochem. Cytochem.* **37**, 39–47
- Itoh, R. (1981) *Biochim. Biophys. Acta* **657**, 402–410
- Itoh, R., Usami, C., Nishino, T. & Tsushima, K. (1978) *Biochim. Biophys. Acta* **526**, 154–162
- Itoh, R., Oka, J. & Ozasa, H. (1986) *Biochem. J.* **235**, 847–851
- Kadota, S., Fantus, I. G., Deragon, G., Guyda, H. J. & Posner, B. I. (1987a) *J. Biol. Chem.* **262**, 8252–8256
- Kadota, S., Fantus, I. G., Deragon, G., Guyda, H. J., Hersh, B. & Posner, B. I. (1987b) *Biochem. Biophys. Res. Commun.* **147**, 259–266
- Le Hir, M. & Dubach, U. C. (1988) *Am. J. Physiol.* **254**, F191–F195
- Le Hir, M., Gandhi, R. & Dubach, U. C. (1989) *Enzyme* **41**, 87–93
- Naito, Y. & Tsushima, K. (1976) *Biochim. Biophys. Acta* **438**, 159–168
- Nechay, B. R., Nanninga, L. B., Nechay, P. S. E., Post, R. L., Grantham, J. J., Macara, I. G., Kubena, L. F., Phillips, T. D. & Nielsen, F. H. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45**, 123–132
- Piec, G. & Le Hir, M. (1991) *Biochem. J.* **273**, 409–413
- Skladanowski, A. C. & Newby, A. C. (1990) *Biochem. J.* **268**, 117–122
- Skladanowski, A. C., Sala, G. B. & Newby, A. C. (1989) *Biochem. J.* **262**, 203–208
- Spielman, W. S. & Thompson, C. I. (1982) *Am. J. Physiol.* **242**, F423–F435
- Spychala, J., Madrid-Marina, V. & Fox, I. H. (1988) *J. Biol. Chem.* **263**, 18759–18766
- Truong, V. L., Collinson, A. R. & Lowenstein, J. M. (1988) *Biochem. J.* **253**, 117–121
- Van den Berghe, G., Van Pottelsberghe, C. & Hers, H.-G. (1977) *Biochem. J.* **162**, 611–616
- Varga, S., Csermely, P. & Martonosi, A. (1985) *Eur. J. Biochem.* **148**, 119–126
- Yokota, S., Oka, J., Ozasa, H. & Itoh, R. (1988) *J. Histochem. Cytochem.* **36**, 983–989