Purification and some properties of an IMP-specific 5'-nucleotidase from yeast

Roichi ITOH

The National Institute of Health and Nutrition, 1-23-1, Toyama, Shinjuku-ku, Tokyo 162, Japan

An IMP-hydrolysing enzyme was purified to homogeneity from yeast extract. It was a soluble protein with an apparent molecular mass of 220 kDa, with a subunit molecular mass of 55 kDa. It was highly specific for IMP, and there was virtually no detectable activity with the other purine and pyrimidine nucleotides tested, including AMP and dIMP. The enzyme had a pH optimum of 6.0–6.5. Its activity was absolutely dependent on bivalent metal salts: Mg^{2+} was most potent, followed by Co^{2+} and Mn^{2+} . The

velocity/substrate-concentration plot of the enzyme was slightly sigmoidal ($h = 1.7$) and the $s_{0.5}$ was 0.4 mM. ATP stimulated the enzyme by decreasing both h and $s_{0.5}$. Diadenosine tetraphosphate stimulated the enzyme as effectively as ATP. Although the properties of the enzyme are similar to those of the IMP/GMP 5'-nucleotidase identified in various animals [Itoh (1993) Comp. Biochem. Physiol. 105B, 13–19], the substrate specificity of the former was much more strict than the latter.

INTRODUCTION

The intracellular degradation of AMP in eukaryotes may occur through two different pathways: either by deamination by AMP deaminase, followed by dephosphorylation of IMP by nucleotidases, or by dephosphorylation by nucleotidases, followed by deamination of adenosine by adenosine deaminase. It has been suggested that the conversion of AMP into inosine in animals is initiated mainly through deamination of AMP to IMP [1–4]. The dephosphorylation of IMP in this pathway is proposed to be catalysed by IMP/GMP 5'-nucleotidase [5] or by a 5'-nucleotidase of the c-N-II type designated by Zimmermann [6], which has about a 30–70-fold preference for IMP over AMP, based on the ratio of $V_{max}/s_{0.5}$ [5].

Merkler et al. [7] demonstrated that, in cell-free extracts of the yeast *Saccharomyces cerevisiae*, degradation of [^{14}C]AMP was accompanied by the production of an almost equivalent amount of [^{14}C]IMP and a small but significant amount of [^{14}C]adenosine and [^{14}C]inosine. The transient increase in adenosine that occurs during the time course of AMP degradation suggests that inosine production occurs mainly through dephosphorylation of AMP by nucleotidases in yeast extracts. In accordance with this, two 5'-nucleotidases, which have a preference for AMP, have been characterized in *Saccharomyces oviformis*, which is now considered to be a variant race of *S. cerevisiae* [8,9].

There is, however, the possibility that inosine is, at least in part, formed through dephosphorylation of the IMP produced by the AMP deaminase reaction in yeast as well as in animals. To confirm the presence of a yeast 5'-nucleotidase with a preference for IMP, which presumably participates in IMP dephosphorylation in the yeast, we purified IMP-hydrolysing activity from extracts of *S. cerevisiae*. We obtained a 5'-nucleotidase as a homogeneous protein and defined its catalytic and molecular properties. The enzyme was highly specific for IMP and barely hydrolysed the other 5'-nucleotides tested, including AMP and dIMP.

EXPERIMENTAL

Materials and reagents

Baker's yeast (*S. cerevisiae*) was purchased from Oriental Yeast Co. (Tokyo, Japan). The ion-exchange resin, DEAE-Toyopearl 650M, and the medium for size-exclusion column chromatography, Toyopearl HW-55F, were products of Tosoh Corporation (Tokyo, Japan). Protein standards used to calibrate the size-exclusion columns were from Pharmacia LKB (Uppsala, Sweden). Purine nucleoside phosphorylase (20 units/ml; from calf spleen) and xanthine oxidase (20 units/ml; from cow milk) were purchased from Boehringer (Mannheim, Germany). IMP, dIMP, Ap_3A , Ap_4A , Ap_5A , 2,3-DPG (defined in title-page footnote) and ribose 5-phosphate were products of Sigma (St. Louis, MO, U.S.A.). AMP, ADP, ATP, GMP, GTP, 2'(3')-IMP (i.e. mixture of the two isomers) XMP, UMP, UTP, CMP, CTP, IDP and ITP were from Yamasa Shoyu Co. (Choshi, Japan). All other chemicals were of reagent grade or of the highest quality available.

Electrophoresis equipment and supplies were from TEF Corp. (Hotaka-machi, Nagano, Japan). All h.p.l.c. apparatus and columns were from Waters (Milford, MA, U.S.A.).

Enzyme assay

The enzyme 5'-nucleotidase was assayed as the formation of P_i or inosine at 37 °C. The standard reaction mixture contained 100 mM imidazole/HCl buffer (pH 6.5), 25 mM $MgCl_2$, 0.1 % BSA, 10 mM IMP and the enzyme. One unit of enzyme activity corresponded to the hydrolysis of 1 μ mol of IMP/min. The usual concentration of the enzyme in the reaction mixture was 0.015–0.025 unit/ml.

Assay 1

5'-Nucleotidase was assayed colorimetrically by measuring P_i liberated from IMP as described previously [10].

Abbreviations used: Ap_3A , diadenosine 5',5'''- P^1,P^3 -triphosphate; Ap_4A , diadenosine 5',5'''- P^1,P^4 -tetraphosphate; Ap_5A , diadenosine 5',5'''- P^1,P^5 -pentaphosphate; $p[CH_2]pA$, adenosine 5'-[$\alpha\beta$ -methylene]diphosphate; 2,3-DPG, 2,3-bisphosphoglycerate.

Assay 2

The inosine formed was converted enzymically into uric acid and determined spectrophotometrically. The reaction was terminated by heating the reaction mixture (0.5 ml) at 100 °C for 2 min. After centrifugation at 15000 *g* for 10 min, 0.39 ml of 20 mM sodium phosphate (pH 7.4), 0.1 unit of xanthine oxidase in 5 μ l and 0.1 unit of purine nucleoside phosphorylase in 5 μ l were added to 0.4 ml of the supernatant. Immediately after the addition of purine nucleoside phosphorylase, the amount of uric acid produced was determined by monitoring the increase in A_{300} .

Assay 3

Nucleotides and nucleosides were separated by h.p.l.c., and changes in their concentrations were determined spectrophotometrically. The reaction was terminated as described for Assay 2. After centrifugation at 15000 *g* for 10 min, 1 μ l of the supernatant was injected into an h.p.l.c. system equipped with a PCSS Guard-Pak C-18 followed by a column of μ Bondasphere C-18 (3.9 mm $\times$ 15 cm). Samples were eluted isocratically with 25% acetonitrile in 10 mM KH_2PO_4 /2 mM tetrabutylammonium (pH 5.0) at a flow rate of 1.5 ml/min. The concentration of nucleotides and nucleosides was determined by spectrophotometry at 254 nm.

Determination of protein

Protein precipitated with 5% (w/v) trichloroacetic acid and dissolved in 0.1 M NaOH, containing 2% (w/v) Na_2CO_3 , 0.01% (w/v) CuSO_4 and 0.02% (w/v) potassium tartrate, was determined by using the Folin phenol reagent as described by Lowry et al. [11]. Crystalline BSA was used as the standard.

Gel electrophoresis

PAGE in the presence of 0.1% (w/v) SDS was performed as described by Laemmli [12] in 4–20% (w/v) gradient polyacrylamide slab gels. The pre-stained protein markers were: myosin (200 kDa), β -galactosidase (116.3 kDa), phosphorylase *b* (rabbit muscle; 97.4 kDa), BSA (66.3 kDa), glutamate dehydrogenase (bovine liver; 55.4 kDa), lactate dehydrogenase (pig muscle; 36.5 kDa), carbonic anhydrase (bovine erythrocytes; 31.0 kDa), trypsin inhibitor (soybean; 21.5 kDa), lysozyme (chicken egg-white; 14.4 kDa) and aprotinin (bovine lung; 6.0 kDa). The proteins were revealed with Coomassie Brilliant Blue G-250.

Estimation of molecular mass by gel filtration

The enzyme was gel-filtered on a column of Toyopearl HW-55F (16 mm $\times$ 85 cm) equilibrated with 50 mM Tris/HCl (pH 7.4) containing 1 mM EDTA, 10 mM 2-mercaptoethanol and 0.4 M NaCl. The column was eluted at a rate of 18 ml/h, and 2.0 ml fractions were collected. The apparent molecular mass was estimated by comparing the elution profile with those of the following standards: ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa) and ovalbumin (43 kDa).

Isolation of yeast 5'-nucleotidase

Extraction

Baker's yeast (*S. cerevisiae*) was suspended in 5 vol. of Tris/HCl buffer (pH 7.4) containing 1 mM EDTA and 10 mM 2-mercaptoethanol (Buffer A). The yeast was disrupted in a

continuous-flow Impandex Dyano-Mill containing 0.5–0.7 mm glass beads. The resulting solution was centrifuged to remove cellular debris.

(NH_4) $_2\text{SO}_4$ fractionation

Solid (NH_4) $_2\text{SO}_4$ was slowly added to 1.2 litres of the extract to give 35% saturation. After 30 min, the precipitate was removed by centrifugation at 17500 *g* for 30 min. More (NH_4) $_2\text{SO}_4$ was added to the supernatant to give 50% saturation, and after 30 min the solution was centrifuged. The resulting precipitate was dissolved in Buffer A and dialysed against 20 vol. of the same buffer for 3 h, and then again for 15 h.

DEAE-Toyopearl chromatography

The dialysed solution was centrifuged at 17500 *g* for 30 min. The supernatant (71.4 ml) was applied to a column (25 mm $\times$ 38 cm) containing DEAE-Toyopearl, previously equilibrated with Buffer A. The column was washed with 300 ml of Buffer A, and the enzyme was eluted with a linear gradient of 0–0.4 M NaCl in the same buffer. The volume of the limiting buffer was 500 ml; 10 ml fractions were collected at a flow rate of 120 ml/h. The active fractions (0.24–0.27 M NaCl) were pooled and concentrated by (NH_4) $_2\text{SO}_4$ fractionation as described above. The fraction precipitated between 35 and 50% saturation was dissolved in a minimal amount of Buffer A.

First Toyopearl HW-55 chromatography

The fractions obtained from the previous step were applied to a column of Toyopearl HW-55F (16 mm $\times$ 85 cm) equilibrated with Buffer A containing 0.4 M NaCl. The column was eluted with the same buffer, containing 0.4 M NaCl, at 18 ml/h, and 2 ml fractions were collected. The fractions with high activity were pooled.

Second Toyopearl HW-55 chromatography

A sample of the pooled fractions was applied to the column that was used for the first Toyopearl HW-55 chromatography, and the enzyme was eluted under exactly the same conditions as described above. The fractions with high activity were pooled and stored at –20 °C until use.

RESULTS

Isolation of yeast 5'-nucleotidase

Typical results of the isolation of the yeast 5'-nucleotidase are given in Table 1. The activity for β -glycerophosphate was removed by the chromatography on DEAE-Toyopearl. Elution profiles of the first and second Toyopearl HW-55 chromatographies are shown in Figures 1(a) and 1(b) respectively. The activity was eluted from the first column as a single peak just after Blue Dextran and was followed by a broad shoulder. When the fractions with the highest activity were pooled and applied to the second Toyopearl HW-55 column, the activity was eluted as a single peak at a similar position to that of thyroglobulin, followed by a shoulder. As indicated in Figure 2, each fraction from the second Toyopearl HW-55 chromatography migrated as a single polypeptide band on SDS/PAGE, and the staining intensity of each band paralleled the enzyme activity. The position of the band corresponded to that of glutamate dehydrogenase from bovine liver, of which the subunit molecular mass is 55.4 kDa.

Table 1 Isolation of yeast 5'-nucleotidase

The enzyme activity was determined after each isolation step by Assay 1; —, not determined.

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)	β -Glycerophosphate:IMP*
Crude extract	1200	9032	510.2	0.056	1	100	1.41
(NH ₄) ₂ SO ₄ fractionation	714	2285	401.8	0.176	3.1	78.8	0.41
DEAE-Toyopearl (concentrated)	1	33.3	234.3	7.04	125.7	45.9	0.00
First Toyopearl HW-55	10	2.1	132.8	63.2	1128.6	26.0	—
Second Toyopearl HW-55†	14	0.047	4.8	102.5	1830.4	0.9	—

* The ratio of β -glycerophosphate-hydrolysing activity to IMP-hydrolysing activity.

† For this, 10% of the pooled fractions from the first Toyopearl HW-55 chromatography were applied to the second.

Figure 1 Elution profile of yeast 5'-nucleotidase from the first (a), the second (b) and the third (c) Toyopearl HW-55 chromatography

The 5'-nucleotidase activity (■) was determined by Assay 1. The activity was determined by using 1, 10 and 50 μ l portions of fractions from the first, second and third chromatography respectively. Protein (●) was estimated as A_{280} . The arrows indicate the elution positions of Blue Dextran (1) and standard proteins: (2) thyroglobulin; (3) ferritin; (4) catalase; (5) aldolase; (6) BSA; (7) ovalbumin. A 1 ml portion of concentrated DEAE-Toyopearl fraction (200 units/ml) was applied to the first Toyopearl HW-55 column chromatography; 1 ml of the fraction 39 obtained from the first chromatography was applied to the second; 1 ml of the fraction 47 obtained from the second chromatography was applied to the third. The same column and the same conditions were used for each elution.

Figure 2 SDS/PAGE of fractions obtained by the second Toyopearl HW-55 chromatography

Electrophoresis was performed with 4–20% gradient polyacrylamide resolving gels as described in the Experimental section. Fraction numbers of the samples eluted from the second Toyopearl HW-55 chromatography are indicated on the lower side of the gel. On the left side, the molecular mass of each marker protein is indicated.

Figure 3 Effect of bivalent metal salts on the activity of yeast 5'-nucleotidase

The reaction mixture contained 100 mM imidazole/HCl buffer (pH 6.5), 10 mM IMP and various metal salts at the concentrations indicated: ●, MgCl_2 ; ○, CoCl_2 ; ■, MnCl_2 . The enzyme activity was determined by Assay 1.

Table 2 Substrate specificity of yeast 5'-nucleotidase

The reaction mixture contained 10 mM substrate. The reaction velocity was determined by Assay 1. The data are expressed as a percentage of the activity with IMP.

Substrate	Relative activity (%)
IMP	100
dIMP	0.73
AMP	1.06
GMP	0.63
XMP	0.00
UMP	0.00
CMP	0.00
dTMP	2.72
2'(3')-IMP	0.24
D-Ribose 5-phosphate	1.07
Phenyl phosphate	0.73
β -Glycerophosphate	0.40

Figure 4 Effect of ATP or Ap_4A on the yeast 5'-nucleotidase

(a) The reaction mixture contained 100 mM imidazole/HCl buffer (pH 6.5), 25 mM MgCl_2 , and various concentrations of IMP with (●) or without (○) 2.5 mM ATP. A Hill plot of the data is presented in the inset. (b) The reaction mixture contained 100 mM imidazole/HCl (pH 6.5), 25 mM MgCl_2 and 0.15 mM IMP with ATP (●) or Ap_4A (■) at the concentrations indicated. The ratio of reaction velocities in the presence of an effector (v) to those in its absence (v_0) is indicated on the ordinate.

Estimation of the molecular mass of the native enzyme

A fraction from the second Toyopearl HW-55 chromatography, which was eluted at the position corresponding to thyroglobulin, was applied to the same column that was used in the first and second Toyopearl HW-55 separations. The activity was eluted as a single symmetrical peak with no shoulder, at the position corresponding to an apparent molecular mass of 220 kDa (Figure 1c).

Some catalytic properties of the enzyme

pH optimum

The pH optimum measured with 100 mM imidazole/HCl buffer or 100 mM sodium acetate buffer in the presence of 25 mM MgCl_2 was 6.0–6.5.

Bivalent metal requirement

The yeast 5'-nucleotidase required bivalent cations for activity (Figure 3). CoCl_2 and MnCl_2 were substituted for MgCl_2 ,

Table 3 Effects of various phosphoesters on the activity of yeast 5'-nucleotidase

Each reaction mixture contained 0.15 mM substrate and various phosphoesters at a concentration of 2.5 mM. The reaction velocity was determined by Assay 2. The data are expressed as the percentage of the activity without an effector.

Effector	Relative activity (%)
None	100
AMP	101
ADP	121
ATP	243
GTP	75
UTP	139
CTP	86
Cyclic AMP	130
p[CH ₂] ₂ pA	113
Ap ₃ A	200
Ap ₄ A	283
Ap ₅ A	252
2,3-DPG	114

resulting in about 50 and 10% of the activity with MgCl₂ respectively. There was no activity with NiCl₂ or CaCl₂.

Substrate specificity

As indicated in Table 2, the enzyme was highly specific for IMP. There was virtually undetectable activity with the 5'-nucleoside monophosphates of other purines and pyrimidines. The enzyme barely hydrolysed dIMP or 2'(3')-IMP. The enzyme was almost inactive with D-ribose 5-phosphate, phenyl phosphate or β -glycerophosphate. Activity with IDP, ITP, ADP or ATP was determined by Assay 3. There was no detectable activity with these compounds (results not shown).

Kinetic characteristics and stimulation by ATP and Ap₄A

As shown in Figure 4(a), the velocity/substrate-concentration plot of the enzyme was slightly sigmoidal. ATP at a concentration of 2.5 mM stimulated the enzyme by decreasing h from 1.7 to 1.2 and $s_{0.5}$ from 0.4 to 0.3 mM without changing V . In Table 3, the effects of various phosphoesters on the activity of the enzyme are presented. Ap₃A, Ap₄A and Ap₅A also stimulated the enzyme. Figure 4(b) shows the effect of the concentrations of ATP and Ap₄A on the activity. P_i up to a concentration of 10 mM showed no inhibitory effect (results not shown).

DISCUSSION

IMP-hydrolysing activity in yeast extracts was purified about 1800-fold to apparent homogeneity as determined by SDS/PAGE.

Highly concentrated enzyme (about 200 units/ml) was eluted from Toyopearl HW-55 between the exclusion volume and the position of thyroglobulin, followed by a broad shoulder. The presence of the shoulder implied that the enzyme, which aggregates at a high concentration, dissociates by dilution during chromatography. In fact, when the enzyme diluted to a concentration of about 10 units/ml was applied to size-exclusion chromatography, it was eluted at the position corresponding to thyroglobulin with a molecular mass of 669 kDa, although it was

still followed by a shoulder. Due to the behaviour of the enzyme, it was isolated by performing size-exclusion chromatography twice: the first was performed with a highly concentrated preparation and the second with a diluted sample.

The homogeneous enzyme eluted from the second size-exclusion chromatography was again applied to the same column at a concentration of 0.025 mg/ml (0.4 unit/ml). The activity was eluted as a symmetrical peak with no shoulder at the position corresponding to a molecular mass of 220 kDa. The concentration of the enzyme recovered in the eluted fractions was less than 0.03 unit/ml, which is equivalent to that used in the standard reaction conditions. A tetrameric structure of the active enzyme at this concentration was suggested, since the subunit molecular mass estimated by SDS/PAGE was about 55 kDa.

Takei [8,9] isolated two 5'-nucleotidases from *Saccharomyces* and reported their properties. These two enzymes hydrolyse various purine and pyrimidine 5'-nucleoside monophosphates, of which AMP seems to be hydrolysed most effectively. These two enzymes are active without bivalent metal salts, but are stimulated by Co²⁺ and Ni²⁺. Mg²⁺ has almost no effect on the activity. One of these two enzymes possesses nucleotide pyrophosphatase activity for hydrolysis of NAD⁺, FAD, ATP or ITP.

In contrast with these 5'-nucleotidases, the enzyme described here had some unique properties. Firstly, it had a high preference for IMP as a substrate and little activity with AMP. Secondly, its activity was absolutely dependent on the presence of bivalent metal salts, of which Mg²⁺ was the most effective. The enzyme was inactive with ATP, ADP, ITP or IDP.

The *Saccharomyces* 5'-nucleotidase described here rather has some features in common with the IMP/GMP 5'-nucleotidase of animals [5,6,13]. Besides its preference for IMP and absolute dependence on bivalent metal salts, the enzyme was soluble, had a pH optimum of 6.0–6.5, and was stimulated by ATP or Ap₄A. These catalytic properties, as well as its molecular mass, are close to those reported for most IMP/GMP 5'-nucleotidases from various sources of mammals.

However, there are some differences in the regulatory characteristics between *Saccharomyces* 5'-nucleotidase and IMP/GMP 5'-nucleotidase [13]. Firstly, the effect of Ap₄A on the activity of the former was much weaker than that of the latter. Secondly, p[CH₂]₂pA and 2,3-DPG potentially stimulated the latter, whereas they barely stimulated the former. Thirdly, P_i is a potent inhibitor of the latter, whereas it had no effect on the former.

These two enzymes also differed with respect to substrate specificity. Both enzymes prefer IMP as a substrate over other 5'-nucleoside monophosphates. IMP/GMP 5'-nucleotidase purified from animal sources, however, shows significant activities with many 5'-nucleoside monophosphates other than IMP, although, based on the ratio of $V/s_{0.5}$, the preference for AMP or pyrimidine nucleotides is much lower than that for 6-hydroxypurine nucleotides such as IMP, dIMP, GMP or dGMP [5]. In contrast, the yeast enzyme had much more strict specificity, and in fact hydrolysed only IMP. Even the activity with dIMP was less than 1% of that with IMP.

Whether the enzyme belongs to the same category as the IMP/GMP 5'-nucleotidases of animals remains to be elucidated.

Most 5'-nucleotidases previously obtained from various sources were shown to have substrate specificity for a broad spectrum of purine and pyrimidine 5'-nucleoside monophosphates, although they display significant differences in preference for nucleotides [6]. Even 5'-nucleotidases, which have been referred to as AMP-specific 5'-nucleotidase, show activity with IMP and GMP of approximately one-third to one-half

of that with AMP [14,15]. However, prokaryotic and plant 5'-nucleotidases which are highly specific for only one nucleotide have been reported; after infection of *Bacillus subtilis* SB19 with phage SP5C, a new enzyme arises, which cleaves dTMP, but does not hydrolyse other naturally occurring nucleoside 5'-monophosphates [16]. The membrane-bound 5'-nucleotidase characterized in peanut cotyledons is highly specific for AMP and does not hydrolyse other nucleotides [17].

This is the first report to provide evidence for the existence of a 5'-nucleotidase that is highly specific for IMP in eukaryotes.

In yeast, the AMP deaminase reaction has been suggested to be the main pathway of AMP catabolism [7]. Further degradation of IMP produced by the reaction to inosine is possibly catalysed by the IMP-specific 5'-nucleotidase described in the present paper. The activity of this enzyme may be depressed by the decrease in intracellular ATP. This suggests that, under the de-energized condition, dephosphorylation of IMP to diffusible inosine by this 5'-nucleotidase is decelerated. This probably serves to maintain the level of intracellular IMP as a precursor of adenine and guanine nucleotides.

REFERENCES

- 1 Lomax, C. A. and Henderson, J. F. (1973) *Cancer Res.* **33**, 2825–2829
- 2 Van den Berghe, G., Bronfman, M., Vanneste, R. and Hers, H.-G. (1977) *Biochem. J.* **162**, 601–609
- 3 Van den Berghe, G., Van Pottersberghe, G. and Hers, H.-G. (1977) *Biochem. J.* **162**, 610–616
- 4 Van den Berghe, G., Bontemps, F. and Hers, H.-G. (1980) *Biochem. J.* **188**, 913–920
- 5 Itoh, R. (1993) *Comp. Biochem. Physiol.* **105B**, 13–19
- 6 Zimmermann, H. (1992) *Biochem. J.* **285**, 345–365
- 7 Merkler, D. J., Wali, A. S., Taylor, J. and Schramm, V. L. (1989) *J. Biol. Chem.* **264**, 21422–21430
- 8 Takei, S. (1966) *Agric. Biol. Chem.* **30**, 1215–1200
- 9 Takei, S. (1967) *Agric. Biol. Chem.* **31**, 1251–1255
- 10 Itoh, R. (1981) *Biochim. Biophys. Acta* **657**, 402–410
- 11 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 12 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 13 Itoh, R. and Yamada, K. (1990) *Int. J. Biochem.* **22**, 231–238
- 14 Skladanowski, A. C. and Newby, A. C. (1990) *Biochem. J.* **268**, 117–122
- 15 Darvish, A. and Metting, P. J. (1993) *Am. J. Physiol.* **264**, H1528–H1534
- 16 Aposhian, H. V. and Tremblay, G. Y. (1966) *J. Biol. Chem.* **241**, 5095–5101
- 17 Sharma, C. B., Mittal, R. and Tanner, W. (1986) *Biochim. Biophys. Acta* **884**, 567–577