Stimulation by ATP–Mg²⁺ and inactivation by cyclic-AMP-dependent phosphorylation of a cytosolic monkey brain aminopeptidase

Sammanda RAMAMOORTHY and Aiylam S. BALASUBRAMANIAN

Department of Neurological Sciences, Christian Medical College and Hospital, Vellore 632004, India

The activity of a purified cytosolic aminopeptidase (M_r 79000) from monkey brain was stimulated about 4-fold by ATP–Mg²⁺. The stimulation was seen with either synthetic aminopeptidase substrates or natural peptides such as enkephalins. Both ATP and Mg²⁺ were required for stimulation, and ADP did not inhibit the stimulation. Non-hydrolysable analogues of ATP, deoxy-ATP and other nucleoside triphosphates stimulated to a lesser extent compared with ATP, whereas nucleoside mono- or di-phosphates were ineffective. The enzyme did not exhibit any ATPase activity. An ATPase inhibitor, orthovanadate, had no inhibitory effect on the ATP–Mg²⁺ stimulation. The aminopeptidase was not autophosphorylated by [γ -³²P]ATP and Mg²⁺, but in the presence of cyclic AMP-dependent protein kinase underwent phosphorylation on serine residue(s). Phosphorylation resulted in inactivation of the aminopeptidase activity, and also resulted in a decreased stimulation of the enzyme by ATP–Mg²⁺.

INTRODUCTION

The requirement for ATP in protein degradation has been extensively studied in the non-lysosomal proteolytic system [1,2] from several sources such as rat liver cytoplasm [3], mouse liver [4], mouse erythroleukaemia cells [5], rabbit reticulocytes [6,7], bovine adrenal-cortex mitochondria [8], rat liver mitochondria [9] and *Escherichia coli* [10–12]. Two distinct ATP-dependent protein-degradation systems are known, one the ubiquitin-dependent system [2,13] and the other the vanadate-sensitive system [9–12,14,15]. In the ubiquitin system, ATP is required for ubiquitin conjugation to the substrate, which results in enhancement of the susceptibility of cellular proteins to proteolytic attack [16,17]. An alternative hypothesis is that ubiquitin represses a proteinase inhibitor [18]. In the second mechanism ATP is directly utilized without involvement of ubiquitin. Proteinases isolated from liver mitochondria [9] and *E. coli* [10,14,19] contain a vanadate-sensitive ATPase activity that is essential for protein breakdown [9,14,15]. Inhibitors of ATPase activity inhibit the proteolytic activity also, suggesting that the proteolytic activity and ATP hydrolysis are closely coupled in these systems [15,20].

In the above studies, high- M_r proteins have been used as substrates. In one study using protease La, an ATP-dependent proteinase of *E. coli*, it was shown that, unlike large proteins, the degradation of small synthetic peptides was not coupled to ATP hydrolysis by the ATPase present in the enzyme, and also that PP_i or inorganic triphosphate could substitute for ATP in promoting degradation [11,12]. Apart from the above-mentioned ATP-dependent proteinases, there are also cytosolic proteinases stabilized by ATP against thermal denaturation [21,22].

We wished to identify an aminopeptidase that is considered to act on monkey brain neutral α -D-mannosidase [23]. During these studies we purified an aminopeptidase to homogeneity (M_r 79000) from

monkey brain cytosolic fraction [24]. The enzyme was characterized with respect to its substrate specificity, inhibitor sensitivity and requirement for thiol groups for activity [24]. The enzyme acts on synthetic peptide substrates as well as naturally occurring peptides such as enkephalins and angiotensin, resulting in the sequential cleavage of amino acids from the *N*-terminal [24]. It appears very similar in its M_r , substrate specificity and inhibition characteristics to an aminopeptidase (arylamidase) from monkey brain purified independently and studied by Hayashi & Oshima [25] and Hayashi [26]. In the present studies we show that ATP–Mg²⁺ stimulated the activity of this aminopeptidase. We also show that this aminopeptidase could undergo phosphorylation in the presence of cyclic AMP-dependent protein kinase, resulting in its inactivation.

MATERIALS AND METHODS

The peptides, amino acid *p*-nitroanilides, amino acid β -naphthylamides, dithiothreitol, 2-mercaptoethanol, sodium orthovanadate, *o*-phthalaldehyde, phospho-amino acids, some of the nucleotides, catalytic subunit of cyclic AMP-dependent protein kinase from bovine heart, protein kinase inhibitor from rabbit muscle and *E. coli* alkaline phosphatase (type III) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Adenosine 5'-[β -methylene]triphosphate, adenosine 5'-[β -imido]triphosphate, ITP and UTP were from Serva, Heidelberg, Germany. [γ -³²P]ATP was prepared by the method of Penefsky [27] as described previously [28].

Purification of aminopeptidase

The aminopeptidase was purified to homogeneity from a 12000 *g* supernatant of monkey brain homogenate by 30–60 % -satd.-(NH₄)₂SO₄ fractionation, DEAE-cellulose chromatography, lysine-Sepharose chromatography and hydroxyapatite chromatography [24]. The final purified enzyme in 20 mM-potassium phosphate

buffer, pH 7.0, contained 5 mM-dithiothreitol for stabilization.

The purified aminopeptidase gave a single protein band corresponding to enzyme activity on polyacrylamide-gel electrophoresis under non-denaturing conditions. SDS/polyacrylamide-gel electrophoresis under reducing or non-reducing conditions followed by silver staining gave a single protein band of M_r 79000 [24]. The M_r of the enzyme by gel filtration on Sephadex G-150 was ~ 90000 [24].

Enzyme assays

Aminopeptidase activity was assayed by the spectrophotometric determination of *p*-nitroaniline released by the hydrolysis of L-amino acid *p*-nitroanilides, or by fluorimetric determination of β -naphthylamine released from L-amino acid β -naphthylamides as described previously [24]. Briefly, the reaction mixture containing purified enzyme (4–5 μ g of protein), 0.5 mM substrate and other additions where indicated in 50 mM-potassium phosphate buffer, pH 8.5, in a total volume of 0.2 ml was incubated at 37 °C for 15 min and the enzyme activity determined [24]. Unless otherwise indicated, L-leucine *p*-nitroanilide was used as substrate in the assays.

Peptide-hydrolysing activity was assayed by measuring the released amino acids fluorimetrically by the *o*-phthalaldehyde method of Roth [29] as described previously [24].

ATPase activity was determined by incubating the purified enzyme (4–5 μ g) dialysed against 20 mM-Tris/HCl (pH 7.4)/2 mM-2-mercaptoethanol with 0.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ $[(4.0\text{--}5.0) \times 10^5 \text{ c.p.m./nmol}]$ and 10 mM-magnesium acetate in 0.2 ml of 50 mM-Tris/HCl, pH 8.5. Control tubes contained heat-denatured enzyme. After incubation for 15 min at 37 °C, the mixture was diluted 10-fold with ice-cold water, and a 5 μ l sample was spotted on Whatman 3MM paper, followed by high-voltage electrophoresis at 700 V for 4 h in the solvent system pyridine/acetic acid/water (1:10:189, by vol.), pH 3.5 [30]. The $[\text{P}_i\text{-}^{32}\text{P}]$ released from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was determined by autoradiography of the paper and by counting the radioactivity on the paper corresponding to the areas of ^{32}P and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Alternatively, the liberated P_i from non-radioactive ATP was measured by the method of Ames [31].

Phosphorylation of aminopeptidase

The purified aminopeptidase (20 μ g) was phosphorylated by incubating 20 units (one unit transfers 1.0 pmol of phosphate from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to hydrolysed and partially dephosphorylated casein/min, as per manufacturer's specifications) of the catalytic subunit of cyclic AMP-dependent protein kinase in 50 mM-Tris/HCl, pH 7.0, 32 mM-dithiothreitol, 10 mM-magnesium acetate and 0.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ $[(4.0\text{--}5.0) \times 10^5 \text{ c.p.m./nmol}]$ (total volume 0.1 ml) at 30 °C for various periods. The phosphorylation was arrested by adding 25 μ g of protein kinase inhibitor, and the mixture chilled and dialysed extensively at 4 °C against 20 mM-potassium phosphate buffer, pH 7.0, containing 2 mM-2-mercaptoethanol. The radioactivity incorporated into the protein and the aminopeptidase activity of the contents of the dialysis bag were determined.

Alkaline phosphatase treatment

The phosphorylated aminopeptidase was dialysed against 20 mM-Tris/HCl buffer, pH 7.0 at 4 °C. The dialysed phosphoenzyme (10 μ g) was incubated at 30 °C for the desired period with 1.5 units (one unit hydrolyses 1.0 μ mol of *p*-nitrophenyl phosphate/min at pH 10.4 and 37 °C) of *E. coli* alkaline phosphatase in an 0.1 ml reaction mixture containing 50 mM-Tris/HCl, pH 8.0, and 10 mM-magnesium acetate. The mixture was again dialysed against 20 mM-potassium phosphate buffer (pH 7.0)/2 mM-2-mercaptoethanol at 4 °C.

Other methods

SDS/polyacrylamide-gel electrophoresis by the method of Laemmli [32] and phosphoamino acid analysis [30] were done as described previously [33]. Protein was measured as described by Lowry *et al.* [34]. Radioactivity measurements of ^{32}P -labelled protein were done by fixing on phosphocellulose paper (Whatman P-81) and washing [35], followed by counting in an LKB liquid-scintillation counter [33]. Spectrophotometric measurements were made in a LKB Ultrospec instrument and fluorimetric measurements in a Hitachi 204A fluorescence spectrophotometer.

RESULTS

Effect of ATP-Mg²⁺ on the aminopeptidase

In the presence of both ATP and magnesium acetate the activity of the aminopeptidase was enhanced (Fig. 1). There was a 4-fold activation at 1 mM-ATP + 10 mM-Mg²⁺ during 15 min of reaction. In different batches of purified enzyme the activation varied from 3- to 4-fold.

Fig. 1. Effect of ATP, ADP and Mg²⁺ on the time course of hydrolysis of L-leucine *p*-nitroanilide by the brain aminopeptidase

Enzyme activity was assayed with L-leucine *p*-nitroanilide as substrate for various time periods as described in the Materials and methods section, with the following additions: ■, 10 mM-magnesium acetate; ○, 1 mM-ATP; ●, 1 mM-ATP + 10 mM-magnesium acetate; ▲, 1 mM-ADP + 10 mM-magnesium acetate; △, 1 mM-ATP + 1 mM-ADP + 10 mM-magnesium acetate; □, no addition. The percentage of substrate hydrolysed in 15 min was 16% in the absence of ATP-Mg²⁺ and 72% in its presence.

Fig. 2. ATP-Mg²⁺ activation of aminopeptidase with various amino acid *p*-nitroanilides and amino acid β -naphthylamides as substrates

Enzyme activity was assayed with various substrates (0.5 mM) in the presence of magnesium acetate (10 mM) and different concentrations of ATP as given in the Figure: Δ , L-leucine *p*-nitroanilide; $\blacktriangle$, L-alanine *p*-nitroanilide; $\bullet$, glycine *p*-nitroanilide; $\circ$, L-leucine β -naphthylamide; $\square$, L-tyrosine β -naphthylamide; $\blacksquare$, γ -N-L-glutamyl β -naphthylamide; $\times$, L-arginine β -naphthylamide. Enzyme activity at zero concentration of ATP is taken as 100 %.

No activation was observed with either ATP or Mg²⁺ alone (Fig. 1). With 1 mM-ATP and various concentrations of Mg²⁺ (0.1–20 mM), the maximal activation was observed at 10 mM-Mg²⁺ (results not shown). Other metal ions such as Ca²⁺ and Mn²⁺, at 10 mM or lower concentrations, were less effective than Mg²⁺ (results not shown). ADP (1 mM) alone or in the presence of 1 mM-ATP and Mg²⁺ did not affect the enzyme activity significantly (Fig. 1). The ATP-Mg²⁺ stimulation of aminopeptidase activity was unaffected by the presence of ADP even up to 5 mM (results not shown).

The enzyme preincubated with ATP-Mg²⁺ for 15 min at 37 °C and dialysed to remove ATP and Mg²⁺ did not show any enhanced aminopeptidase activity compared with a control enzyme treated similarly in the absence of ATP-Mg²⁺, thus ruling out irreversible changes in the enzyme molecule by ATP-Mg²⁺.

Effect of pH

The pH optimum of the enzyme tested from pH 6.0 to 9.0 in potassium phosphate buffer in the presence of 10 mM-Mg²⁺ alone was in the range pH 7.0–8.5. With 1 mM-ATP and 10 mM-Mg²⁺ the enzyme activity was increased at all pH values, with a maximum at pH 8.5. Similar results were observed when Tris/HCl buffer in the range pH 6.0–9.0 was used (results not shown).

Stimulation of the enzyme activity by ATP-Mg²⁺ with various amino acid *p*-nitroanilides and amino acid β -naphthylamides as substrates

Various amino acid *p*-nitroanilides and amino acid β -naphthylamides were used as substrates for the activation by ATP-Mg²⁺ at different concentrations (Fig. 2). The hydrolysis of all substrates was stimulated by ATP-Mg²⁺. Maximal degree of ATP activation was seen with leucine *p*-nitroanilide and alanine *p*-nitroanilide, whereas hydrolysis of glycine *p*-nitroanilide was activated to a lesser degree. With amino acid β -naphthylamides as substrates, activation by ATP was maximum with leucine β -naphthylamide as substrate, followed by tyrosine β -naphthylamide and γ -N-L-glutamyl β -naphthylamide. Hydrolysis of arginine β -naphthylamide showed the lowest activation by ATP-Mg²⁺. Since some of the substrates have to be used at concentrations below their *K_m* value [24] because of their poor solubility, a comparison of stimulation by ATP-Mg²⁺ of their hydrolysis may not be very significant.

Kinetic analysis of the activation by ATP-Mg²⁺ of the aminopeptidase indicated a 2-fold increase in the *V_{max}* (from 400 to 880 nmol/min per mg of protein), with no change in the *K_m* value (0.46–0.48 mM) of the enzyme with leucine *p*-nitroanilide as substrate.

Effect of ATP-Mg²⁺ on the aminopeptidase with peptide substrates

Since the purified aminopeptidase is known to cleave amino acids sequentially from the *N*-terminus of [methionine]enkephalin and [leucine]enkephalin [24], we examined the effect of ATP-Mg²⁺ with peptide substrates. As seen in Fig. 3, ATP at various concentrations in the presence of Mg²⁺ significantly enhanced the hydrolysis of

Fig. 3. ATP-Mg²⁺ stimulation of peptide hydrolysis by aminopeptidase

Peptide-hydrolysing activity of the aminopeptidase was assayed fluorimetrically as described in the Materials and methods section, with various peptides (0.1 mM) in 25 mM-potassium phosphate buffer, pH 8.5, containing 10 mM-magnesium acetate and different concentrations of ATP. The peptides used were [leucine]enkephalin ($\circ$), [methionine]enkephalin ($\bullet$), des-Tyr-[leucine]enkephalin ($\blacktriangle$), des-Tyr-[methionine]enkephalin (Δ) and tuftsin ($\square$). Enzyme activity at zero concentration of ATP is taken as 100 %.

Fig. 4. Effect of ATP-Mg²⁺ on thermal stability of aminopeptidase

Enzyme (5 µg) was preincubated in 50 mM-potassium phosphate buffer, pH 8.5, for the indicated time periods at 37 °C (continuous lines) or 50 °C (broken lines) in the presence (●) or the absence (○) of 1 mM-ATP and 10 mM-magnesium acetate. At the end of the preincubation period, the mixture was chilled in ice, and magnesium acetate and ATP were added to the tubes devoid of ATP-Mg²⁺ and assayed with L-leucine *p*-nitroanilide as substrate as described in the Materials and methods section. Original enzyme activity is taken as 100 %.

both enkephalins. A lesser degree of activation was seen with des-Tyr-[methionine]enkephalin, des-Tyr-[leucine]enkephalin and tuftsin as substrates. No activation by ATP-Mg²⁺ was observed with the tripeptides Tyr-Gly-Gly and Gly-Gly-Phe and the dipeptides Gly-Leu and Phe-Leu as substrates, even though these peptides could be degraded by the enzyme [24,26].

Nucleotide specificity

The effect of various concentrations of different nucleoside triphosphates (0.5–5 mM) on aminopeptidase activation was tested. Among the nucleotides tested, ATP stimulated the enzyme activity maximally. The ATP analogues adenosine 5'-[βγ-methylene]triphosphate and 5'-[βγ-imido]triphosphate, which are non-hydrolysable by ATPase [12], also activated the enzyme, indicating that the hydrolysis of the nucleotide was not obligatory for the activation of the aminopeptidase. The rate of activation by various nucleoside triphosphates at 1 mM concentration was in the order ATP (3-fold activation) > adenosine 5'-[βγ-imido]triphosphate > deoxy-ATP > UTP > ITP > adenosine 5'-[βγ-methylene]triphosphate > CTP > GTP (1.5-fold activation).

Other nucleoside phosphates such as ADP, AMP, CDP and IMP when tested at different concentrations did not stimulate the aminopeptidase significantly. Sodium pyrophosphate at 0.1–5 mM with 10 mM-Mg²⁺ did not stimulate the aminopeptidase.

Thermal stability of the enzyme in the presence and absence of ATP

The purified enzyme was preincubated at 37 °C and 50 °C with and without ATP-Mg²⁺ for various time periods, followed by assay for aminopeptidase in the presence of ATP-Mg²⁺ (Fig. 4). The loss of enzyme

Fig. 5. SDS/polyacrylamide-gel electrophoresis and [³²P]-phosphorylation of the purified aminopeptidase

(a) Lanes 1 and 2 show the purified enzyme on SDS/polyacrylamide-gel electrophoresis under reducing and non-reducing conditions respectively, stained with AgNO₃. (b) Autoradiogram of dried gel after SDS/polyacrylamide-gel electrophoresis under reducing conditions of reaction mixture used for [³²P]phosphorylation of purified aminopeptidase in the presence of [γ-³²P]ATP, Mg²⁺ and catalytic subunit of cyclic AMP-dependent protein kinase. Details for phosphorylation are given in the Materials and methods section. Lanes: 1, reaction mixture without protein kinase; 2, reaction mixture without aminopeptidase; 3, reaction mixture containing all the components. Arrow indicates the ³²P-labelled band corresponding to the aminopeptidase. (c) Autoradiogram showing the presence of [³²P]phosphoserine obtained by high-voltage paper electrophoresis of the acid hydrolysate of ³²P-labelled aminopeptidase. Details are given in the Materials and methods section.

activity by preincubation at these temperatures did not vary by more than 10 % between the ATP-protected and the control tubes (except for a 40 % difference at 50 °C for 2 min), suggesting that ATP-Mg²⁺ offered only a minor protection against thermal denaturation. Moreover, the 4-fold increase in aminopeptidase activity observed during assay at 37 °C in the presence of ATP-Mg²⁺ (Fig. 1) was far higher than the small percentage of protection offered by it against thermal denaturation at 37 °C.

Absence of ATPase activity

We tested whether any ATPase activity was present in the purified aminopeptidase by incubating the enzyme with [γ-³²P]ATP or unlabelled ATP as mentioned in the Materials and methods section. There was no evidence for the hydrolysis of ATP or release of P_i, the lower limit of detection being 0.2 % of ATP hydrolysis. Moreover, addition of sodium orthovanadate, an ATPase inhibitor, up to 5 mM did not affect the stimulation of aminopeptidase activity by ATP-Mg²⁺. From these observations, it was clear that ATPase activity was not involved in the aminopeptidase activation.

Absence of autophosphorylation of the enzyme

Incubation of the enzyme with [γ-³²P]ATP and Mg²⁺ did not result in the incorporation of radioactivity into the enzyme. This was also confirmed by SDS/

Fig. 6. Time course of phosphorylation of aminopeptidase by cyclic AMP dependent-protein kinase and the inactivation of aminopeptidase

The purified aminopeptidase (20 µg) was phosphorylated in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase, Mg²⁺ and [γ -³²P]ATP as described in the Materials and methods section for various periods. Control tubes in the absence of protein kinase were also incubated. At the end of various periods of incubation, 25 µg of protein kinase inhibitor was added to all the tubes and the mixture was dialysed against 20 mM-potassium phosphate buffer (pH 7.0)/2 mM-2-mercaptoethanol. The aminopeptidase activity was assayed with both L-leucine *p*-nitroanilide (0.5 mM) (○) and [leucine]-enkephalin (0.1 mM) (△) as substrates as described in the Materials and methods section. Samples of the phosphorylation mixture were transferred to P-81 phosphocellulose paper, and the radioactivity incorporated into the aminopeptidase was counted (●). There was only about 15% loss of the aminopeptidase activity in the control tubes after incubation for 60 min at 30 °C (results not shown). Activity of the unphosphorylated aminopeptidase is taken as 100%.

polyacrylamide-gel electrophoresis and autoradiography, where no radiolabelled protein band was observed. This observation ruled out the possibility of autophosphorylation or formation of a phosphoenzyme intermediate during ATP-Mg²⁺ stimulation.

Phosphorylation of the aminopeptidase *in vitro* by cyclic AMP-dependent protein kinase

The purified aminopeptidase could undergo phosphorylation in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase, [γ -³²P]ATP and Mg²⁺, as evidenced by SDS/polyacrylamide-gel electrophoresis and autoradiography (Fig. 5). Phospho-amino acid analysis of the ³²P-labelled enzyme showed the presence of phosphoserine (Fig. 5).

Effect of phosphorylation on aminopeptidase activity

Phosphorylation of aminopeptidase resulted in inactivation of the enzyme. Fig. 6 shows the extent of phosphorylation and the corresponding enzyme activity at various periods of phosphorylation. With increasing phosphorylation there was a marked decrease in aminopeptidase activity with both L-leucine *p*-nitroanilide and [leucine]enkephalin as substrates. Under the given conditions of phosphorylation, the maximum

Fig. 7. Time course of dephosphorylation of the phosphorylated aminopeptidase by *E. coli* alkaline phosphatase and the recovery of aminopeptidase activity

The purified aminopeptidase was phosphorylated for 1 h in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase, [γ -³²P]ATP and Mg²⁺ as described in the Materials and methods section. As a control, enzyme was also incubated in the absence of protein kinase. At the end of the incubation, both enzymes were dialysed against 20 mM-Tris/HCl, pH 7.0. The dialysed ³²P-labelled enzyme was incubated with 1.5 units of *E. coli* alkaline phosphatase as described in the Materials and methods section. After specified periods of incubation, samples of the reaction mixture were transferred to P-81 phosphocellulose paper for determining radioactivity (●) and also used for assaying aminopeptidase activity with L-leucine *p*-nitroanilide as substrate (○). The percentage recovery of the aminopeptidase activity was calculated by taking as 100% the activity of the control unphosphorylated enzyme incubated simultaneously.

incorporation of [³²P]P_i into the aminopeptidase (0.41 mol/mol of protein) was reached at 2 h and remained constant up to 4 h. About 75% of the aminopeptidase activity was lost within 1 h of phosphorylation.

When protein kinase inhibitor (25 µg) was included in the reaction mixture for phosphorylation, there was no incorporation of ³²P into the aminopeptidase, and there was only less than 5% loss of aminopeptidase activity after incubation for 60 min.

Additional evidence suggesting a correlation between protein phosphorylation and loss of aminopeptidase activity came from studies on the dephosphorylation of the [³²P]phosphorylated aminopeptidase by *E. coli* alkaline phosphatase, which is known to remove phosphate from many phosphoproteins [36]. Fig. 7 shows the time-dependent removal of phosphate from the phosphorylated aminopeptidase by alkaline phosphatase and the recovery of aminopeptidase activity. Almost 90% of the control activity was regained when most of the [³²P]phosphate was removed from the enzyme.

ATP-Mg²⁺ activation of unphosphorylated and phosphorylated aminopeptidase

The ATP-Mg²⁺ stimulation of unphosphorylated and phosphorylated aminopeptidase was compared. The percentage activation by ATP-Mg²⁺ was lower with the phosphorylated enzyme than with the unphosphorylated

Fig. 8. ATP-Mg²⁺ activation of the phosphorylated and unphosphorylated aminopeptidase

The purified aminopeptidase (10 µg) was phosphorylated by catalytic subunit of cyclic AMP-dependent protein kinase by using unlabelled ATP at 30 °C for 30 min as described in the Materials and methods section. A control aminopeptidase was subjected to the same treatment in the absence of the protein kinase. At the end of the incubation period, 25 µg of protein kinase inhibitor was added. Part of the reaction mixture was dialysed against 20 mM-potassium phosphate buffer (pH 7.0)/2 mM-2-mercaptoethanol, and another part was dialysed against 20 mM-Tris/HCl, pH 7.0. The phosphorylated enzyme dialysed against Tris/HCl buffer was incubated with 1.5 units of *E. coli* alkaline phosphatase in 20 mM-Tris/HCl, pH 8.0, containing 10 mM-magnesium acetate for 30 min at 30 °C, and again dialysed against 20 mM-potassium phosphate buffer (pH 7.0)/2 mM-2-mercaptoethanol. The activation of the control unphosphorylated aminopeptidase (○), the phosphorylated aminopeptidase (●) and the phosphorylated aminopeptidase treated with alkaline phosphatase (△) by various concentrations of ATP in the presence of 10 mM-magnesium acetate was determined with L-leucine *p*-nitroanilide as substrate as described in the Materials and methods section. Aminopeptidase activity at zero concentration of ATP is taken as 100%. (In separate experiments it was confirmed that the alkaline phosphatase did not hydrolyse ATP to any detectable extent in the presence of 50 mM-phosphate buffer, pH 8.5, used for assay of aminopeptidase.)

enzyme (Fig. 8). This lowering of the activation by ATP-Mg²⁺ could be partially reversed by alkaline phosphatase treatment of the phosphorylated enzyme (Fig. 8).

DISCUSSION

In the present studies we have shown that the monkey brain aminopeptidase can be stimulated by ATP-Mg²⁺ and inactivated by phosphorylation in the presence of cyclic AMP-dependent protein kinase. The mechanism of activation by ATP-Mg²⁺ does not involve an ATPase activity, since the aminopeptidase had no detectable ATPase activity. Moreover, orthovanadate, the powerful inhibitor of ATPase, did not inhibit the ATP-Mg²⁺ stimulation of the peptidase activity. This is in accordance with the postulated mechanism of ATP-Mg²⁺ activation of protease La from *E. coli*, with small peptides as

substrates [11,12]. Other characteristics of the aminopeptidase activation by non-hydrolysable structural analogues of ATP, the requirement for both ATP and Mg²⁺ for activation, and the lack of activation by ATP-Mg²⁺ when di- and tri-peptides were used as substrates, also closely resemble the characteristics of activation of protease La acting on small peptides [11,12]. However, one major difference observed was that, unlike protease La, the aminopeptidase activation by ATP-Mg²⁺ was not inhibited by ADP. Another difference was that, unlike protease La, the aminopeptidase could not be stimulated by PP_i. Goldberg & Waxman [12] have hypothesized in their model for protease La acting on small peptides that a reversible binding site exists in the enzyme where ATP or ADP can bind by competition. Further, it was suggested that the ATPase activity present in protease La could hydrolyse the bound ATP and allow the product ADP to remain in the same site [37]. Unlike protease La, the monkey brain aminopeptidase is devoid of ATPase activity, and even high concentrations of ADP could not inhibit its activation by ATP-Mg²⁺. By analogy with protease La, we suggest that the brain aminopeptidase is activated by ATP through a reversible binding site, except that this binding site is unaffected by ADP. The activation of the aminopeptidase by ATP-Mg²⁺ was completely reversible after removal of ATP-Mg²⁺ by dialysis, and also there was no evidence for an autophosphorylation of the enzyme by [γ -³²P]-ATP-Mg²⁺, ruling out the possibility of formation of a phosphoenzyme intermediate. We have also provided evidence to suggest that ATP-Mg²⁺ activation does not arise as a result of thermal stabilization of the aminopeptidase.

Although ATP-Mg²⁺ can stimulate the aminopeptidase, it is inactivated by phosphorylation in the presence of cyclic AMP-dependent protein kinase. The maximum incorporation of phosphate into the aminopeptidase was 0.41 mol/mol of enzyme under the conditions of the experiment described in Fig. 6. Almost 75% of the aminopeptidase activity was lost when the phosphate incorporated was 0.3 mol/mol of enzyme. One of the reasons for the loss of enzyme activity even at low extents of phosphate incorporation could be that the aminopeptidase was already in a partially phosphorylated state. The following evidence strongly suggested that the loss of enzyme activity occurs as a consequence of phosphorylation of the aminopeptidase. (a) When protein kinase inhibitor was included during phosphorylation, there was no ³²P incorporation into the aminopeptidase and there was less than 5% loss of enzyme activity. (b) Dephosphorylation of the phosphorylated aminopeptidase resulted in regaining almost 90% of the enzyme activity. (c) There was only one ³²P-labelled band corresponding to the purified aminopeptidase, as evidenced by SDS/polyacrylamide-gel electrophoresis of the reaction mixture used for phosphorylating the enzyme, excluding any other phosphorylated protein intermediate from being responsible for inactivation.

The drastic decrease in the ATP-Mg²⁺ activation of the aminopeptidase after phosphorylation suggests that phosphorylation is a mechanism not only to inactivate the enzyme but also to prevent stimulation by ATP-Mg²⁺. A common feature of all the reported ATP-Mg²⁺-dependent proteinases with associated ATPase activity is their high *M_r* (450 000–650 000) and oligomeric nature [1]. In this respect the aminopeptidase

reported here, which is devoid of ATPase and which has a native M_r of ~ 90000 [24,25], differs. To our knowledge this is the first demonstration of the activation by ATP-Mg²⁺ of an aminopeptidase from mammalian sources which can also undergo phosphorylation in the presence of cyclic AMP-dependent protein kinase. Since physiologically important peptides such as enkephalins, angiotensins and tuftsin can act as substrates for this aminopeptidase [24,26], it is conceivable that the modulation of this enzyme by ATP-Mg²⁺ and by phosphorylation could significantly alter the potency and biological half-lives of these peptides in the brain.

This work was supported by a grant from the Council of Scientific and Industrial Research, India.

REFERENCES

- Bond, J. S. & Butler, P. E. (1987) *Annu. Rev. Biochem.* **56**, 333–364
- Hershko, A. & Ciechanover, A. (1982) *Annu. Rev. Biochem.* **51**, 335–364
- DeMartino, G. N. & Goldberg, A. L. (1979) *J. Biol. Chem.* **254**, 3712–3715
- Rose, I. A., Warms, J. V. B. & Hershko, A. (1979) *J. Biol. Chem.* **254**, 8135–8138
- Waxman, L., Fagan, J. M., Tanaka, K. & Goldberg, A. L. (1985) *J. Biol. Chem.* **260**, 11994–12000
- Hough, R., Pratt, G. & Rechsteiner, M. (1986) *J. Biol. Chem.* **261**, 2400–2408
- Etlinger, J. D. & Goldberg, A. L. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 54–58
- Watabe, S. & Kimura, T. (1985) *J. Biol. Chem.* **260**, 5511–5517
- Desautels, M. & Goldberg, A. L. (1982) *J. Biol. Chem.* **257**, 11673–11679
- Charette, M. F., Henderson, G. W. & Markovitz, A. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4728–4732
- Waxman, L. & Goldberg, A. L. (1985) *J. Biol. Chem.* **260**, 12022–12028
- Goldberg, A. L. & Waxman, L. (1985) *J. Biol. Chem.* **260**, 12029–12034
- Hershko, A., Ciechanover, A., Heller, H., Haas, A. L. & Rose, I. A. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1783–1786
- Larimore, F. S., Waxman, L. & Goldberg, A. L. (1982) *J. Biol. Chem.* **257**, 4187–4195
- Waxman, L. & Goldberg, A. L. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4883–4887
- Wilkinson, K. D., Urban, M. K. & Haas, A. L. (1980) *J. Biol. Chem.* **255**, 7529–7532
- Hershko, A., Heller, H., Eytan, E., Kaklij, G. & Rose, I. A. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7021–7025
- Speiser, S. & Etlinger, J. D. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3577–3580
- Chung, C. H. & Goldberg, A. L. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4931–4935
- Chung, C. H. & Goldberg, A. L. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 795–799
- Ismail, F. & Gevers, W. (1983) *Biochim. Biophys. Acta* **742**, 399–408
- Dahlmann, B., Kuehn, L. & Reinauer, H. (1983) *FEBS Lett.* **160**, 243–247
- Mathur, R., Panneerselvam, K. & Balasubramanian, A. S. (1988) *Biochem. J.* **253**, 677–685
- Ramamoorthy, S. & Balasubramanian, A. S. (1986) *Ind. J. Biochem. Biophys.* **23**, 326–333
- Hayashi, M. & Oshima, K. (1977) *J. Biochem. (Tokyo)* **81**, 631–639
- Hayashi, M. (1978) *J. Biochem. (Tokyo)* **84**, 1363–1372
- Penefsky, H. S. (1967) *Methods Enzymol.* **10**, 702–703
- Ramamoorthy, S. & Balasubramanian, A. S. (1985) *Ind. J. Biochem. Biophys.* **22**, 321–326
- Roth, M. (1971) *Anal. Chem.* **43**, 880–882
- Hunter, T. & Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1311–1315
- Ames, B. N. (1966) *Methods Enzymol.* **8**, 115–118
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Panneerselvam, K., Ramamoorthy, S. & Balasubramanian, A. S. (1987) *Biochem. Biophys. Res. Commun.* **147**, 927–935
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Sahal, D. & Yamaguchi, Y. F. (1987) *Anal. Biochem.* **167**, 23–30
- Mellgren, R. L., Slaughter, G. R. & Thomas, A. J. (1977) *J. Biol. Chem.* **252**, 6082–6084
- Menon, A. S. & Goldberg, A. L. (1987) *J. Biol. Chem.* **262**, 14929–14934

Received 19 April 1988/20 September 1988; accepted 16 November 1988