Synthesis of (di)nucleoside polyphosphates by the ubiquitin activating enzyme E1

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Abstract **Previous work from this laboratory had shown that ligases may catalyze the synthesis of (di)nucleoside polyphosphates. Here, we show that one of the enzymes of the proteasome system (E1 or the ubiquitin (Ub) activating enzyme, EC 6.3.2.19) catalyzes very effectively ($k_{cat} = 0.29 \pm 0.05\ s^{-1}$) the transfer of AMP from the E–AMP–ubiquitin complex to tripolyphosphate or tetrapolyphosphate with formation of adenosine tetra- or pentaphosphate (p_4A or p_5A), respectively. Whereas the concomitant formation of AMP is stimulated by the presence of dithiothreitol in a concentration dependent manner, the synthesis of p_4A is only slightly inhibited by this compound. Previous treatment of the enzyme (E1) with iodoacetamide inhibited only partially the synthesis of p_4A. p_4A can substitute for ATP as substrate of the reaction to generate the ubiquityl adenylate complex. A small amount of diadenosine pentaphosphate (Ap_5A) was also synthesized in the presence of p_4A.**

Keywords: Diadenosine pentaphosphate; (Di)nucleoside polyphosphates; Ligases; Proteasome; Tetrapolyphosphate; Tripolyphosphate

1. Introduction

Based on previous results obtained by others on aminoacyl t-RNA synthetases [1], we suggested that all the enzymes classified as AMP-forming ligases could be considered as potentially able to catalyze the synthesis of dinucleoside polyphosphates [2]. This proved to be the case for a variety of enzymes, including luciferase, acetyl-CoA synthetase, acyl-CoA synthetase, T4 RNA ligase, T4 DNA ligase, and others (for a review see [3]).

Ligases catalyze the synthesis of diadenosine tetraphosphate (Ap_4A) and other (di)nucleoside polyphosphates through the general reactions shown in Eqs. (a) and (b) below:

(a) $E + X + ATP \leftrightarrow E–X–AMP + PPi$

(b) $E–X–AMP + ATP \rightarrow Ap_4A + X + E$

As reaction (a) is reversible, the synthesis of Ap_4A is greatly favored in the presence of pyrophosphatase (PPase). In our experience, reaction (b) is rather unspecific and the AMP residue of the E–X–AMP complex (or E–AMP in some cases [3]) may react with the terminal phosphate of almost any molecule containing an intact terminal P–P–P, such as tripolyphosphate (P_3), tetrapolyphosphate (P_4), pentapolyphosphate (P_5) ATP, GTP, adenosine 5′-tetraphosphate (p_4A), guanosine 5′-tetraphosphate (p_4G), etc. In the case of luciferase, the moiety of AMP from E–luciferin–AMP can even be transferred to polyphosphates as long as linear-chain polyphosphates with an average chain length of 15 ± 3 (P_{15}) giving rise to a family of compounds as bizarre as $p_{16}A$, $p_{20}A$, $Ap_{15}A$, $Ap_{16}A$, etc. [4].

Following this reasoning, we have paid attention to the ubiquitin (Ub) system of degrading proteins, a complex metabolic pathway thoroughly explored by the groups of Ciechanover, Hershko, Rose and others [5–7]. The knowledge developed by these authors on this system is the cornerstone of the work shown below.

Two main processes can be considered in the proteasome system (i) ubiquitination of proteins and (ii) the proteolytic cleave of the tagged proteins by the 26 S proteasome complex with liberation of Ub (Fig. 1). In the first process, Ub is activated by the following enzymes: E1 (Ub activating enzyme), E2s (Ub carrier proteins) and E3s (Ub–protein ligases) [8]. Finally, the ubiquitinated protein is degraded in the proteasome with liberation of peptides and regeneration of Ub (Fig. 1).

Here we shall center our attention on E1. This enzyme is a homodimer composed of two subunits of 105 kDa [9]. The three reactions catalyzed by this enzyme, represented diagrammatically in Fig. 1 [10,11], can be summarized in the following global equation:

$$E + 2Ub + 2ATP \leftrightarrow \text{Ub–S–E}_{\text{AMP–Ub}} + AMP + 2PPi$$

In order to simplify the presentation of this work, two "functional regions" (regions in subsequent lines), A–U (acronym of AMP–ubiquitin) and U (from ubiquitin) are considered in E1 (Fig. 1). The synthesis of ubiquityl adenylate (AMP–Ub) and the firm, but non-covalent, attachment of this complex to region A–U of the enzyme take place with liberation of PPi (Fig. 1, step 1). In step 2, the Ub moiety of the $E_{AMP–Ub}$ complex is transferred from the region A–U to a sulfhydryl residue in the area U of E1, with liberation of AMP. In step 3, similar to step 1, an AMP–Ub complex is attached to region A–U of the enzyme. In the presence of E2, the Ub moiety on area U is transferred to E2 and the region U of E1 (unoccupied now by Ub) is

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Abbreviations: Ap_4A, adenosine (5′) tetraphospho (5′) adenosine or diadenosine tetraphosphate; Ap_4N, adenosine (5′) tetraphospho (5′) nucleoside; BSA, bovine serum albumin; DTT, dithiothreitol; Ub, ubiquitin; AMP–Ub, ubiquityl adenylate; IAA, iodoacetamide; NTP, nucleoside triphosphate; PPase, pyrophosphatase; P_3, tripolyphosphate; P_4, tetrapolyphosphate; P_5, pentapolyphosphate; P_{15}, linear-chain polyphosphates with an average chain length of 15 ± 3; p_4A, adenosine 5′-tetraphosphate; p_5A, adenosine 5′-pentaphosphate; p_4G, guanosine 5′-tetraphosphate

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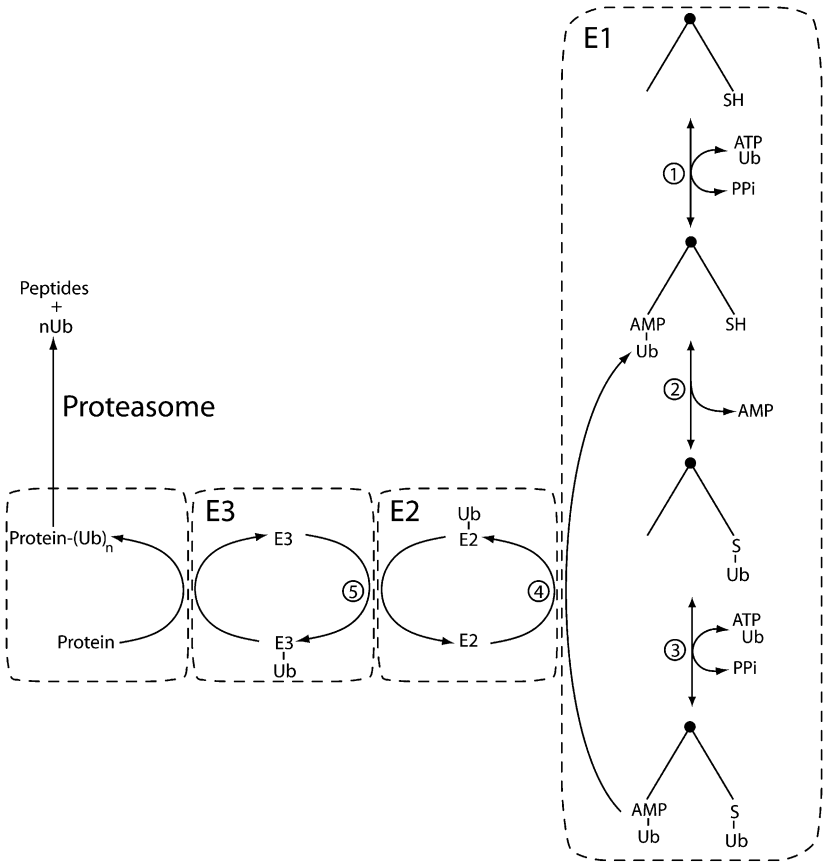

Fig. 1. Ubiquitin system and potential degradation of proteins in the proteasome. Three interconnected pathways can be considered in this process: E1 or ubiquitin activating enzyme, E2 or ubiquitin carrier proteins and E3 or ubiquitin protein ligases. Whereas only one type of E1 is known, many species of E2 and multiple families of E3 have been described. The (poly)ubiquitinated protein is degraded in the proteasome. Here, E1 is represented with the form of an angle to facilitate the location of the two functional regions: for the formation of E$_{AMP-Ub}$ (right arm or A–U region) and for the coupling of ubiquitin to a –SH residue of the enzyme (left arm or U region). For details of steps 1–5, see text.

ready to start a new cycle. The protein to be ubiquitinated and (potentially) digested by the proteasome is tagged through additional steps 4, 5 and 6 (Fig. 1).

The Enzyme Commission assigns the number EC 6.3.2.19 to the reaction catalyzed by E1 or to the total process of ubiquitination of the target protein catalyzed by E1, E2 and E3

$$\text{ATP} + \text{ubiquitin} + \text{ubiquitin–protein ligase} = \text{AMP} + \text{protein N–ubiquitin} + \text{PPi}$$

In our view, E1 is a special type of ligase as it catalyzes: (i) the first step of a ligase, i.e.: formation of the AMP–Ub complex and (ii) the binding of the activated Ub to a –SH group in a Cys residue of E1, in a thiolester linkage: in this case, the proper enzyme E1 serves as the other substrate of the reaction affording the –SH residue to which the carboxyl end of Ub is joined [11,12] (Fig. 1).

Based on previous results with other ligases we considered that the enzyme E1 could also be a candidate to catalyze the synthesis of (di)nucleoside polyphosphates. Experiments showing that E1 catalyzes the synthesis of p_4A and adenosine 5′-pentaphosphate (p_5A), and to a small extent the synthesis of a dinucleotide such as Ap_5A, are described below.

2. Materials and methods

2.1. Materials

Ub activating enzyme (E1) (110 kDa) (Ref. U1758; lot numbers 064K2742; 025K2733); Ub (U6253); sodium tripolyphosphate and hexaammonium tetrapolyphosphate were from Sigma. Yeast inorganic PPase (EC 3.6.1.1) was from Roche Molecular Biochemicals. [α-^{32}P] ATP (3000 Ci/mmol) was from Amersham. TLC silica-gel fluorescent plates were from Merck. Radioactively labeled nucleotides were quantified with the help of an InstantImager (Packard Instrument Co.). HPLC was carried out in a Hewlett Packard chromatograph (model 1090) with a diode array detector. The Hypersil ODS column (4.6 × 100 mm) was from Hewlett Packard.

2.2. Synthesis of nucleoside polyphosphates by Ub activating enzyme (E1)

Unless otherwise indicated the reaction mixtures contained 50 mM Tris/HCl (pH 7.5), 9 mM $MgCl_2$, 0.05 μg PPase, 6 μM Ub, 0.05% bovine serum albumin (BSA), ATP, dithiothreitol (DTT), polyphosphates and E1, as indicated. When required the enzyme (E1) was diluted in 0.1 M Tris/HCl, pH 7.5/0.5% BSA. A molecular mass of 110 kDa was considered to calculate the concentration of E1. After incubation at 37 °C the reaction mixtures were analyzed by one of the following methods.

TLC. The reaction mixtures (0.02 ml) contained 0.02 mM [α-^{32}P] ATP (0.4 μCi). Aliquots of 1.5 μl of the reaction were spotted on silica gel plates and developed for 2 h in dioxane:ammonium hydroxide:water (6:1:6 by volume). Radioactivity was measured by autoradiography and/or with an InstantImager.

HPLC. The reaction mixtures (0.02 ml) contained: 0.3 mM ATP, 58 μM Ub, 20 mM DTT, and 0.8 mM P_3, P_4 or p_4A, as indicated, and 4.5 pmol of E1. After incubation at 37 °C for 16 h the mixtures were transferred into 0.180 ml of water and kept at 95 °C for 1.5 min. Aliquots of 0.05 ml were analyzed by HPLC as described in [13].

3. Results and discussion

3.1. Synthesis of p_4A by the Ub activating enzyme E1

Since an Ub-dependent ATP:PPi exchange had been shown to be catalyzed by E1 [8,10–12], we wondered if P_3 in the presence of PPase could also be an acceptor of the AMP moiety of the E$_{AMP-Ub}$ complex. As shown in Fig. 2, when E1 was incubated in the presence of 0.02 mM [α-^{32}P] ATP, 0.8 mM P_3, and in the absence or presence of PPase, the formation of a compound migrating slower than ATP, in the same position as a marker of p_4A, and of another one corresponding to AMP were detected (Fig. 2A). The formation of both AMP and p_4A was enzyme dependent. The presence of PPase in the reaction mixture doubled the rate of synthesis of p_4A (Fig. 2B) and decreased about 25% the rate of synthesis of AMP (Fig. 2C).

The apparent K_m values determined for ATP for the synthesis of p_4A (in the presence of 0.8 mM P_3 and 6 μM Ub), for P_3 (in the presence of 20 μM ATP and 6 μM Ub) and Ub (in the presence of 20 μM ATP and 0.8 mM P_3) were 4.9 ± 0.8 μM, 2.8 ± 0.6 mM and 2.0 ± 0.5 μM, respectively (results not shown). The rate of synthesis (k_{cat}) of p_4A in the presence of 0.8 mM P_3 was 0.29 ± 0.05 s^{-1}.

3.2. Synthesis of p_4A, p_5A and minor amounts of Ap_5A

To get a better knowledge of the reaction catalyzed by E1, the reaction mixtures were also analyzed by HPLC. From the chromatograms depicted in Fig 3, the following conclusions could be drawn: in the absence of E1, only peaks corresponding to ATP, contaminant ADP and DTT were observed (Fig. 3A); in the presence of E1 and in the absence of P_3, a small peak corresponding to AMP was observed (Fig. 3B); in the presence of P_3, a significant amount of ATP was converted into p_4A (Fig. 3C); in the absence of PPase the synthesis of p_4A was significantly reduced (Fig. 3D); the formation of both p_4A and AMP was Ub dependent (Fig. 3E); P_4 seemed to be almost as good substrate as P_3 as evidenced by the amount of p_5A formed in the presence of P_4; the concomitant synthesis of p_4A observed in this chromatogram (Fig. 3F) is due to the contaminant P_3 present in the commercial sample of P_4 [4,14]. In the presence of 0.8 mM p_4A a very small amount of Ap_5A was also synthesized (Fig. 3G).

In experiments aiming to check whether the moiety of AMP from the E$_{AMP-Ub}$ complex could be transfer to a nucleoside triphosphate (NTP) with formation of adenosine (5′) tetraphospho (5′) nucleoside (Ap_4N), reaction mixtures, containing ATP and PPase, were supplemented with either 0.8 mM GTP or UTP but the syntheses of the potential products, Ap_4G or Ap_4U, were not observed (results not shown). In the presence

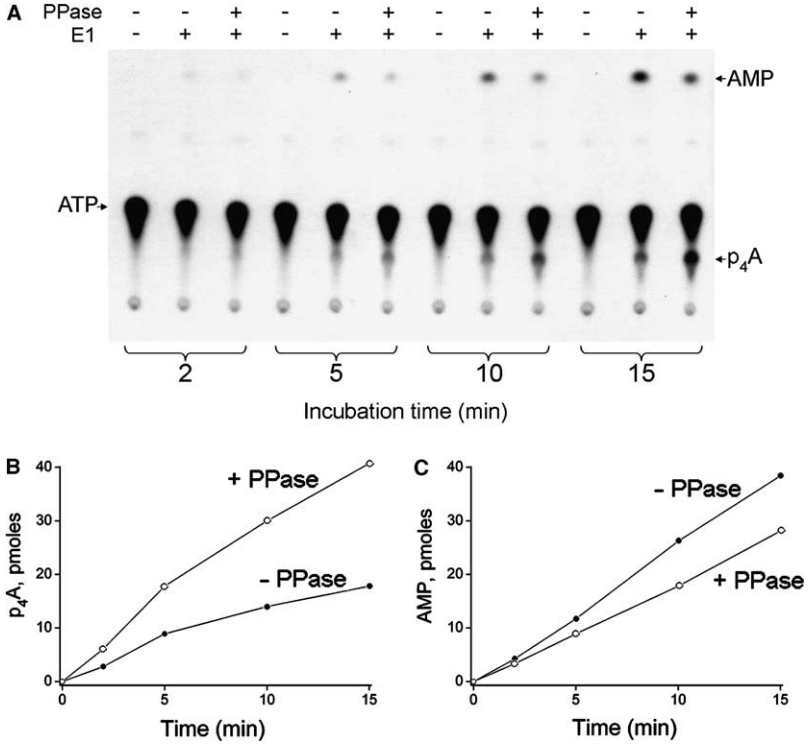

Fig. 2. Synthesis of p_4A catalyzed by the ubiquitin activating enzyme E1. The reaction mixtures contained: 0.02 mM [α-^{32}P] ATP (0.4 μCi); 6 μM Ub; 0.8 mM P_3; 20 mM DTT; E1 (1.5 pmol or 0.17 μg) and PPase (0.05 μg), when indicated. Other components as indicated in Section 2. (A) At different times of incubation, aliquots were spotted on TLC plates, developed with dioxane:ammonium hydroxide:water (6:1:6) and subjected to autoradiography. [α-^{32}P] p_4A and [α-^{32}P] AMP (pmol) formed in the absence or presence of PPase are represented in B and C, respectively.

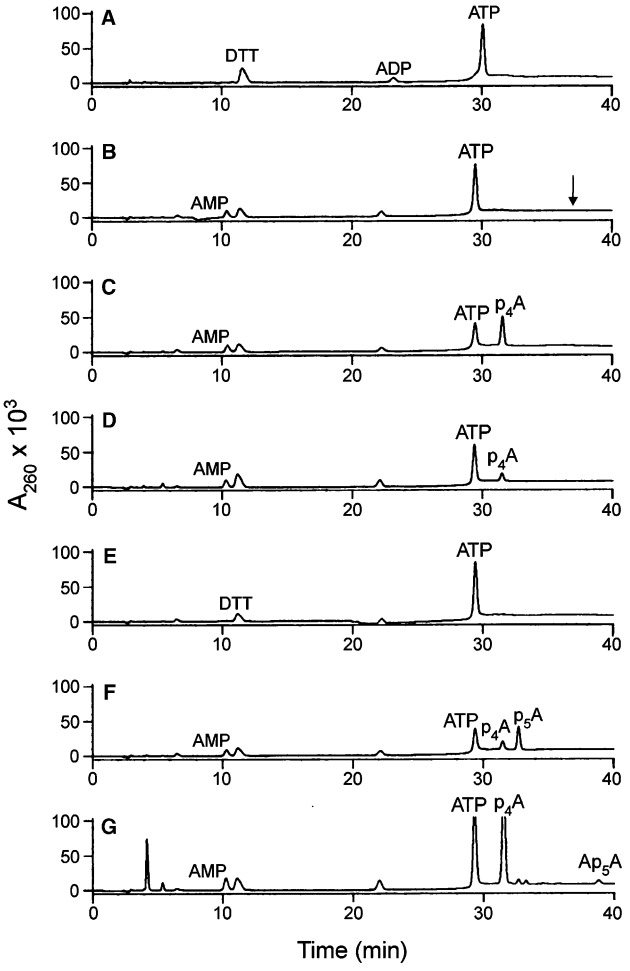

Fig. 3. Synthesis of p_4A, p_5A and Ap_5A catalyzed by the ubiquitin activating enzyme E1. The reaction mixtures contained 0.8 mM P_3 (C, D, E); 0.8 mM P_4 (F); or 0.8 mM p_4A (G). Other components as indicated in Section 2. Controls were carried out in the absence of E1 (A), Ub (E) or PPase (D). Aliquots were analyzed by HPLC as described [13]. The arrow in part B of the figure indicates the elution time of a marker of Ap_4A.

of PPase, the transfer of AMP from the E_{AMP}–Ub complex to ATP to form Ap_4A, was never observed.

3.3. Influence of DTT and iodoacetamide on the rate of synthesis of p_4A and AMP

These experiments were performed based on previous findings [8,15] showing that the transfer of Ub from the E_{AMP}–Ub complex in region A–U to the region U of E1 was inhibited by iodoacetamide (IAA) and by DTT in different ways: IAA reacts with the cysteine residue of region U, and DTT reacts with the activated Ub in the E_{AMP}–Ub complex, hindering its reaction with the –SH residue in the region U.

As shown in Fig. 4, in the absence of DTT the amount of AMP synthesized is very low and the AMP moiety of the complex E_{AMP}–Ub reacts mainly with P_3 to form p_4A (Fig. 4, lanes 2–3). As the amount of DTT increases, the rate of formation of AMP also increases in a concentration dependent manner due to the cleavage of the enzyme-bound Ub adenylate by DTT [15] (Fig. 4). In the presence of 20 mM DTT, while the hydrolysis of ATP to AMP increased from 0.2 to 4.1 pmol/min/pmol

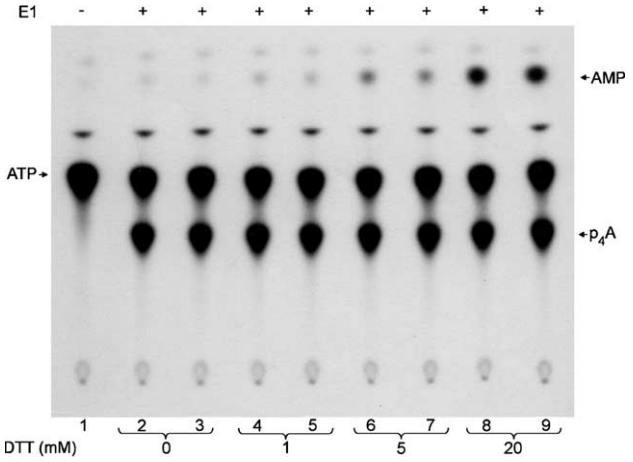

Fig. 4. Influence of DTT on the synthesis of p_4A and AMP. Reaction mixtures (in duplicate) contained: 0.02 mM [α-^{32}P] ATP (0.4 μCi); 6 μM Ub; 0.8 mM P_3; 0.05 μg PPase; 0; 1; 5 and 20 mM DTT as indicated, and 1 pmol of E1. After 10 min of incubation, aliquots were spotted on TLC plates, developed with dioxane:ammonium hydroxide:water (6:1:6) and subjected to autoradiography.

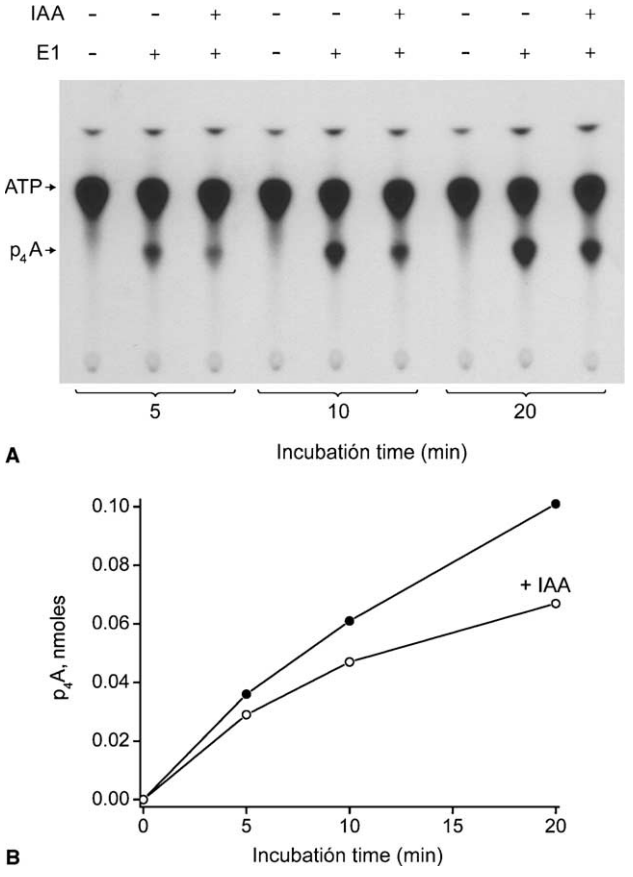

Fig. 5. Synthesis of p_4A. Influence of the pre-treatment of E1 with IAA. E1 at a final concentration of 0.75 nmol/ml was incubated in 50 mM Tris–HCl, pH 7.5, and 1 mg/ml BSA for 30 min at 37 °C in the absence or presence of 0.5 mM IAA. (A) Aliquots containing 1.5 pmol of E1 were used to determine its capacity to synthesize p_4A as in Fig. 4, but omitting DTT in the assay. (B) pmoles of p_4A synthesized.

of E1, the synthesis of p_4A decreased about 8% from an initial value of 14.5 pmol/min/pmol of E1 (Fig. 4, lanes 8–9).

The effect of IAA had been previously tested [15] and found that there was a selective derivatization of the thiol site required for Ub thiolester formation and that the derivatized enzyme still retained 86% of the ATP:PPi exchange activity. In our working conditions when E1 was pre-treated with 0.5 mM IAA for 30 min at 37 °C as described in [15], but omitting DTT, the synthesis of p_4A decreased by $32 \pm 5\%$ ($n = 4$) (Fig. 5A and B), which points to step 1 (Fig. 1) as the main source of p_4A, although the possibility that the complex Ub–S–E$_{AMP}$–Ub, as well as a IAA derivatized complex, could be able to react with P_3 cannot be ruled out.

3.4. Linearity with the amount of enzyme: p_4A as substrate of the reaction

Different amounts of enzyme (0.5; 1; 2 and 4 pmol of E1) were added to reaction mixtures containing 0.8 mM P_3 and aliquots spotted on TLC plates after 5, 10, 20, 40 120 min and 22 h of incubation (Fig. 6A). The linearity with the amount of E1 in the first 5 min of the reaction is shown in Fig. 6B. In the presence of 4 pmol of E1 and after 20 min incubation, the ATP had been completely transformed into p_4A. Further incubation of this mixture converted gradually the p_4A into AMP (Fig. 6C). The same conversion occurred at the other enzyme concentrations although at lower rates. From these results, it seems as p_4A could substitute ATP in establishing the following equilibrium:

$$E + Ub + p_4A \leftrightarrow E_{AMP}\text{–}Ub + P_3$$

and that the complex E$_{AMP}$–Ub, due to the presence of DTT in the reaction mixture, is slowly degraded liberating AMP [15].

Further evidence that p_4A is substrate of the reaction was obtained incubating the enzyme E1 (for 6 h) in the presence of 0.25 mM p_4A and 2 mM PPi as in Fig. 3, but in the absence of PPase and DTT: in this conditions a 80% of the p_4A was transformed into ATP (results not shown).

3.5. Concluding remarks

The work presented above is based on previous results obtained by others on the Ub system [5–7] and in our previous work [3] on the catalytic properties of the enzymes ligases.

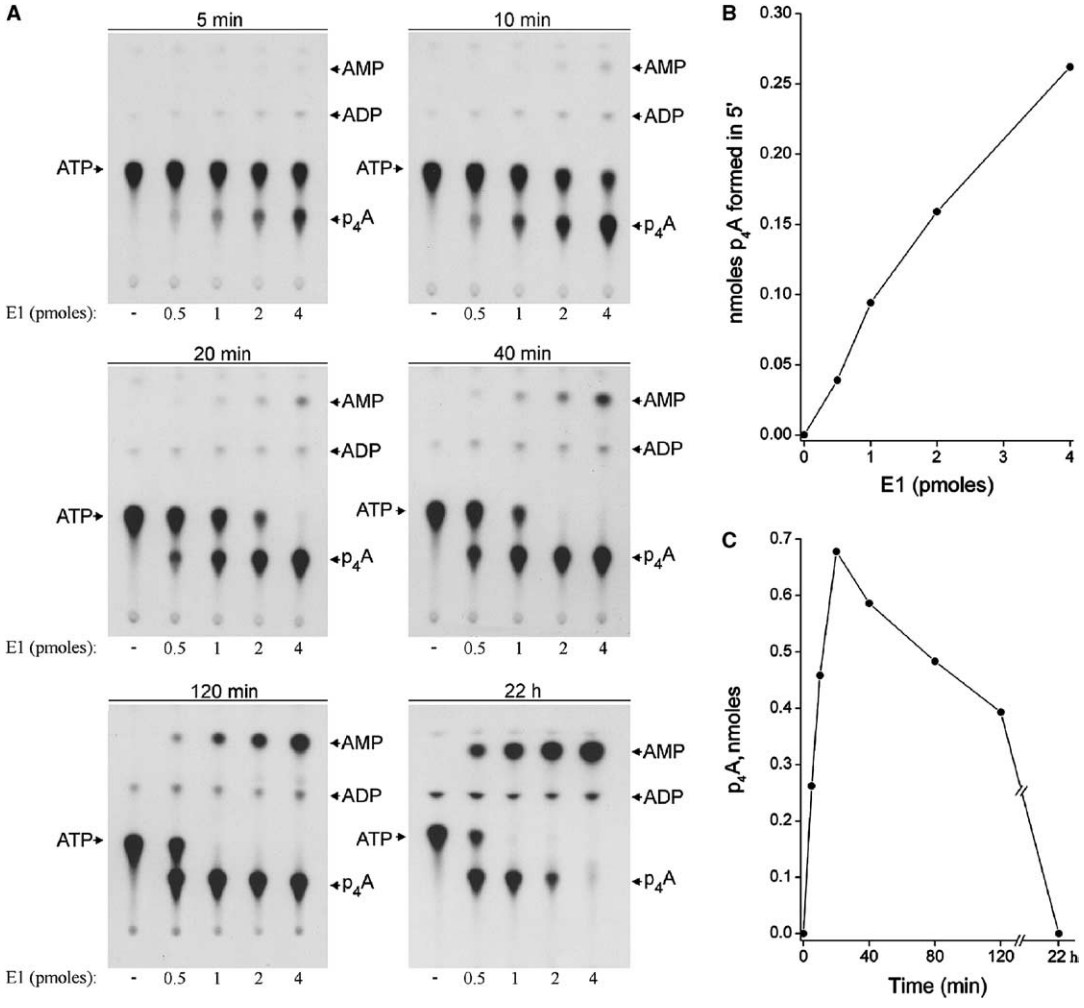

Fig. 6. Linearity with the amount of enzyme. The reaction mixtures contained: 0.04 mM [α-^{32}P] ATP (0.4 μCi); 12 μM Ub; 0.8 mM P_3; 9 mM $MgCl_2$; 0.05 μg PPase, 2 mM DTT, 0.05% BSA and E1 as indicated. (A) At different times of incubation, aliquots were spotted on TLC plates, developed with dioxane:ammonium hydroxide:water (6:1:6) and subjected to autoradiography. (B) pmoles of p_4A synthesized after 5 min incubation. (C) Synthesis and degradation of p_4A, along time of incubation, in the presence of 4 pmol E1.

The main contribution presented here is to show that P_3 (and P_4) in the presence of PPase (reaction (d)), replaces PPi in the reverse of reaction (c):

(c) E + Ub + ATP ↔ E~AMP~–Ub + PPi

(d) E~AMP~–Ub + P_3 ↔ E + p_4A + Ub

The possibility of reaction (d) to take place was implicit (but it was not tested) in [11]. Haas et al. stated that "inorganic phosphate, arsenate, methyl phosphate and triphosphate, but not NTPs, can serve as alternate substrates in place of PPi in the reverse of Ub adenylate formation". However, they did not explore whether E1 did synthesize the expected derivatives.

The E~AMP~–Ub complex is the center of a small metabolic crossroads that may follow different routes (Fig. 7) depending on the conditions of the assay mixture. In the presence of ATP and Ub, and in the absence of PPase, the reaction reaches a stationary phase in which 2 mol of Ub and 2 mol of ATP are used for the formation of the complexes E~AMP~–Ub and Ub–S–E~AMP~–Ub with liberation of 1 mol of AMP (see steps 1–3 in Fig. 1). In the presence of P_3 and PPase the AMP moiety from the E~AMP~–Ub complex is transferred to P_3 with formation of p_4A (Fig. 7). Pre-incubation of the enzyme with IAA, that prevents formation of the second complex, inhibited the synthesis of p_4A by only 32% in comparison to a non-treated enzyme. Based on this result, the synthesis of p_4A seems to proceed mainly through the E~AMP~–Ub complex but some synthesis from the Ub–S–E~AMP~–Ub and IAA–S–E~AMP~–Ub complex could also occur (Fig. 7). In the presence of DTT the synthesis of p_4A is slightly inhibited, due to the catalytic cleavage of the enzyme-bound Ub-adenylate by DTT, with synthesis of DTT–Ub and subsequent liberation of AMP from the E~AMP~–Ub and Ub–S–E~AMP~–Ub complexes. Most probably, once AMP is liberated from the complex is not apt for the synthesis of p_4A.

Inorganic polyphosphates are probably present in every cell [17–19] and are particularly abundant in yeast extracts where

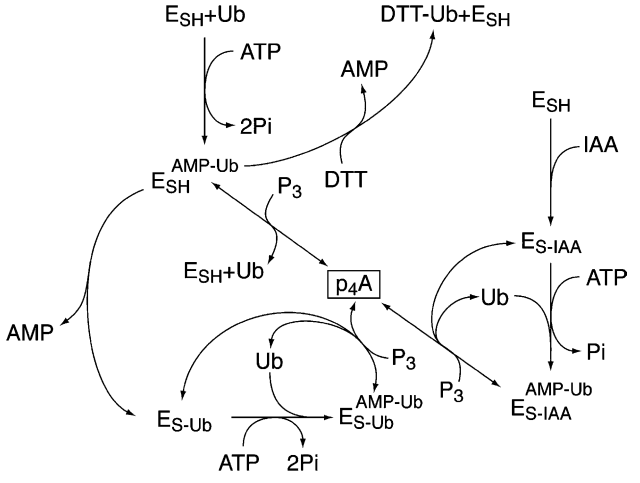

Fig. 7. Overview of possible reactions catalyzed by the ubiquitin activating enzyme E1, in the presence of PPase and with participation of ATP, Ub, P_3, p_4A, DTT and IAA. Synthesis of p_4A can be obtained by transfer of AMP from the E1 complexes, indicated in the figure, to P_3. In the presence of DTT the rate AMP formation is accelerated in a concentration dependent manner and the synthesis of p_4A results slightly inhibited. IAA reacting with one critical SH-group in E1, inhibits the formation of the Ub–S–E~AMP~–Ub complex, but not the ATP:PPi exchange [15]. The synthesis of p_4A with the IAA treated E1 decreased by about 32%. For more details see text.

P_3 and P_4 have been described at mM concentrations [17]. As previously pointed out [16], the potential effects carried out by polyphosphates (here P_3 and P_4) can be considered under two different aspects: either due their presence at high concentration and frequently condensed in organelles or due to their occurrence at low concentration in different cellular compartments. Although their role in the last case is not clear, there is a common concern about the possibility of polyphosphates playing more general and universal functions in biology [14,17–20]. Being this so, these specific effects would entail the action of polyphosphates at (sub) μM concentration in animal cells [19]. In the case of E1, it is difficult for us to envisage the physiological role of P_3, considering its high apparent K_m value prevailing in the reactions here described and the expected low concentrations of P_3 present in the cytosol of animal cells. Nevertheless, E1 could be contemplated as one of the AMP-forming ligases able to catalyze the synthesis of p_4A from ATP and P_3 [3]. The occurrence of p_4A has been described in chromaffin granules of the adrenal medulla [21], in skeletal and cardiac muscle [22,23], and in yeast [24]. p_4A has been involved in yeast sporulation [24] and as a modulator of cardiac vascular tone [23]. p_4A could modulate also indirectly the levels of Ap_4A and Ap_4N (nucleotides whose physiological role seems to be elusive [25,26]), by inhibiting (K_i, nM) the activity of the (*asymmetrical*) dinucleoside tetraphosphatase (EC 3.6.1.17) [27]. In another context, the effect of P_3 could also be contemplated from a pharmacological point of view. In this sense, experiments are being carried out in this laboratory to explore the possible effect of P_3 on the degradation of proteins, using the reticulocyte system.

Finally, it could be recall that, in our hands, no observable synthesis of Ap_4A (from ATP) or Ap_4N (from ATP + UTP or GTP) was obtained with E1. As shown previously by others NTPs cannot serve as alternative substrates in place of PPi in the reverse of Ub adenylate formation [11]. This property excludes E1 as an enzyme participating in the rise of Ap_4N after metabolic stress [28]. It seems as if this active site of E1 could accommodate P_3 or P_4, but not the bulky residue of a nucleoside attached to one of their ends. The enzyme specificity of E1 (compared with that of other ligases) could be better understood with a deeper knowledge of the crystallographic structures and the topography of the active areas of these enzymes.

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