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Synthesis of bisphosphonate derivatives of ATP by T4 RNA ligase

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Dedicated to Gabriela Sillero, for her inspiration

Abstract T4 RNA ligase catalyzes the synthesis of ATP β,γ-bisphosphonate analogues, using the following substrates with the relative velocity rates indicated between brackets: methylenebisphosphonate (pCH_2p) (100), clodronate ($pCCl_2p$) (52), and etidronate ($pC(OH)(CH_3)p$) (4). The presence of pyrophosphatase about doubled the rate of these syntheses. Pamidronate ($pC(OH)(CH_2–CH_2–NH_2)p$), and alendronate ($pC(OH)(CH_2–CH_2–CH_2–NH_2)p$) were not substrates of the reaction. Clodronate displaced the AMP moiety of the complex E-AMP in a concentration dependent manner. The K_m values and the rate of synthesis (k_{cat}) determined for the bisphosphonates as substrates of the reaction were, respectively: methylenebisphosphonate, 0.26 ± 0.05 mM (0.28 ± 0.05 s^{-1}); clodronate, 0.54 ± 0.14 mM (0.29 ± 0.05 s^{-1}); and etidronate, 4.3 ± 0.5 mM (0.028 ± 0.013 s^{-1}). In the presence of GTP, and ATP or $AppCCl_2p$ the relative rate of synthesis of adenosine 5′,5‴-P^1,P^4-tetraphosphoguanosine (Ap_4G) was around 100% and 33%, respectively; the methylenebisphosphonate derivative of ATP ($AppCH_2p$) was a very poor substrate for the synthesis of Ap_4G. To our knowledge this report describes, for the first time, the synthesis of ATP β,γ-bisphosphonate analogues by an enzyme different to the classically considered aminoacyl-tRNA synthetases.

Keywords: Osteoporosis; Bisphosphonates; Ligases; Dinucleoside polyphosphates; Methylenebisphosphonate; Clodronate; Etidronate

1. Introduction

Bisphosphonates are analogs of pyrophosphate in which the oxygen bridge between the two phosphates is replaced by a methylene group (–CH_2–) [1]. This structure allows a great number of possible variations, mostly by changing the two lateral chains on the carbon atom. As happens with the amino acids (which also have different structures on the common basis of an α-amino acid) each bisphosphonate, depending on the nature of the attached moieties can be classified by several criteria and has its own physicochemical and biological properties [2].

Bisphosphonates were employed to prevent scaling in water installations due to their ability to inhibit calcium carbonate precipitation [1]. Their use in the treatment of osteoporosis is based both on empirical clinical grounds and on experimental approaches. Being the osteoporosis an insidious disease, the beneficial effects of bisphosphonates are not rapid and require prolonged periods of medication and statistical and systematic studies to decide on the worthiness of each treatment. It is known that bisphosphonates bind to the hydroxyapatite bone mineral surfaces and deposit on the remodeling zones of the bone where due to an acidic local medium are absorbed by osteoclasts, diminishing their viability [3]. Their deleterious effects are in part due to their ability to generate bisphosphonate derivatives of ATP [4–8] and to their inhibitory effect on the mevalonate pathway and on the synthesis of cholesterol [9–13]. However, many unknowns still prevail on their molecular mechanism of action.

The work presented below stems from the following two points:

1. Based on previous studies with aminoacyl-tRNA synthetases and on the noxious effects of bisphosphonates in *Dictyostelium discoideum* it is thought that bisphosphonates are incorporated into analogs of ATP by the back reaction catalyzed aminoacyl-tRNA synthetases [4–8].

$$E + ATP + Aa \leftrightarrow E - Aa - AMP + PPi \quad (a)$$

$$E - Aa - AMP + pCH_2p \leftrightarrow E + Aa + AppCH_2p \quad (b)$$

2. In 1990 [14] we suggested that the transfer of AMP to other compounds with terminal phosphates catalyzed by the aminoacyl-tRNA synthetases was but a particular case of a more general type of reactions that could be carried out by the AMP-forming ligases and by some transferases. This proved to be the case for a number of such enzymes [15]. In line with this, the results presented below show that T4 RNA ligase uses very effectively some bisphosphonates as

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Abbreviations: Aa, amino acid; Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; Ap_4G, adenosine 5′,5‴-P^1,P^4-tetraphosphoguanosine; $AppCCl_2p$, adenosine 5′-(β,γ-dichloromethylenetriphosphate); $AppCH_2p$, adenosine 5′-(β,γ-methylenetriphosphate); $AppC(R_1)(R_2)p$, adenosine 5--triphosphate derivative of any bisphosphonate; $AppC(R_1)(R_2)ppA$, diadenosine 5′,5‴-P^1,P^4-tetraphosphate derivative of any bisphosphonate; P_3, tripolyphosphate; $pC(OH)(CH_2–CH_2–NH_2)p$, pamidronate or 3-amino-1-hydroxpropylidene-1,1-bisphosphonate; $pC(OH)(CH_2–CH_2–CH_2–NH_2)p$, alendronate or 4-amino-1-hydroxybutylidene-1,1-bisphosphonate; $pC(OH)(CH_3)p$ (HEBP), etidronate or ethane-1-hydroxy-1,1-bisphosphonate or hydroxyethylidenebisphosphonate; $pCCl_2p$ (Cl_2MBP), clodronate or methane-1-dichloro-1,1-bisphosphonate or dichloromethylenebisphosphonate; pCH_2p (MBP) or methylenebisphosphonate; $pC(R_1)(R_2)p$, any bisphosphonate

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analogs of pyrophosphate (PPi) and synthesizes potentially toxic derivatives of ATP. In our view this finding may offer a more general perspective on the effect of bisphosphonates at the cellular level.

2. Materials and methods

2.1. Materials

Bisphosphonates used: methylenebisphosphonate, pCH_2p (MBP) (Cat. No. 274291) and etidronate, $pC(OH)(CH_3)p$ (HEBP) (Cat. No. P5248) were from Sigma; clodronate, $pCCl_2p$ (Cl_2MBP) (Cat. No. 233183), pamidronate, $pC(OH)(CH_2–CH_2–NH_2)p$, (Cat. No. 506600) and alendronate, $pC(OH)(CH_2–CH_2–CH_2–NH_2)p$ (Cat No: 126855) were from Calbiochem. Sodium tripolyphosphate, P_3 (Cat. No. 5633) was from Sigma. [α-^{32}P] ATP (3000 Ci/mmol) was from Amersham. Shrimp alkaline phosphatase (EC 3.1.3.1) was from Roche Applied Science. T4 RNA ligase was from the following sources: Roche Applied Science (1449478, lot No. 85123921), BioLabs (M0204S, lot No. 34; Promega (M105A, lot No. 21004402) with specific activities of 3500, 12000, and 5500 U/mg, respectively. One unit (U) is the enzyme activity required to catalyze the formation of 1 nmol [5′-^{32}P]$rA_{12–18}$ from [α-^{32}P] ATP into a phosphatase-resistant form in 30 min at 37 °C. Electrophoresis on polyacrylamide gels, in the presence of SDS showed a unique band with an apparent molecular mass of 43.5 kDa. The preparations from BioLabs and Promega (but not from Roche) contained an ATPase activity that, in the course of the reaction, produced ADP in appreciable amounts (see Fig. 4, lane 2). 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 Agilent Technologies.

2.2. T4 RNA ligase–AMP complex formation

The reaction mixture (0.02 ml) contained 50 mM Tris/HCl (pH 7.4), 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 10 μM (0.1 μCi) [α-^{32}P] ATP, 0.02 U of desalted pyrophosphatase and clodronate, as indicated. The formation of the E-AMP complex was initiated by addition of 2 U of T4 RNA ligase (Roche). After 15 min incubation, reactions were stopped with 6.5 μl of concentrated SDS sample buffer (0.25 M Tris/HCl (pH 6.8), 8% SDS, 40% glycerol, 240 mM dithiothreitol, 0.005% bromophenol blue). The mixtures were heated at 90 °C for 3 min and 6-μl aliquots loaded onto a 12% denaturing polyacrylamide gel.

2.3. Synthesis of bisphosphonates derivatives of ATP

In the course of these experiments, we were compelled to modify the assay of T4 RNA ligase. Initially the enzyme used was from Roche; when most of the experiments of this work had been performed, Roche discontinued to sell this ligase, and we were forced to get the enzyme from other sources: BioLabs or Promega. The main results obtained with those preparations were similar to that obtained with the enzyme from Roche, except for the presence of a strong ATPase activity (transforming ATP to ADP), that interfered in the assays when low concentrations of [α-^{32}P] ATP were used. After several unsuccessful attempts to inhibit this ATPase activity (with fluoride) we found that the addition of an ATP regenerating system (pyruvate kinase and phosphoenolpyruvate) to the reaction mixtures was adequate to overcome this difficulty. Accordingly, with the enzyme from Roche, the reaction mixtures contained 50 mM Tris/HCl (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$, 1 U/ml of desalted pyrophosphatase, T4 RNA ligase, ATP and bisphosphonates as indicated. The assays performed with enzyme from BioLabs or from Promega were additionally supplemented with 1 mM phosphoenolpyruvate and 0.2 U pyruvate kinase. When required the T4 RNA ligase was diluted in 50 mM Tris/HCl (pH 7.4), 10 mM DTT and 0.05% bovine serum albumin. A molecular mass of 43.5 kDa was considered to calculate the concentration of the enzyme. After incubation at 30 °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 (0.2 μCi). Aliquots of 1.5-μl of the reaction mixtures were spotted on silica gel plates and developed for 90 min in dioxane:ammonium hydroxide:water (6:1:6 or 6:1:7, by volume, as indicated). Radioactivity was measured by autoradiography and/or with an InstantImager.

HPLC. The reaction mixtures (0.05 ml) contained 0.3 mM ATP. After the indicated times of incubation, 15-μl aliquots were transferred into 0.09 ml of water and kept at 95 °C for 1.5 min.

Aliquots of 0.05 ml were analyzed by HPLC as previously described [16].

3. Results and discussion

3.1. Formation of the E-AMP complex and its reaction with clodronate ($pCCl_2p$)

Formation of an E-AMP complex, migrating on SDS/PAGE in a position corresponding to a molecular weight of around 45 kDa, was observed when T4 RNA ligase (Roche) was incubated with [α-^{32}P] ATP (Fig. 1). When clodronate was added to the reaction mixtures, the AMP moiety of the complex was displaced by this bisphosphonate, in a concentration dependent manner, similarly as occurred with P_3 [16].

3.2. Relative efficiency of methylenebisphosphonate, clodronate, etidronate, tripolyphosphate, pamidronate, or alendronate as substrates of the reaction. Influence of pyrophosphatase

Tripolyphosphate, clodronate, methylenebisphosphonate, or etidronate were added to a final concentration of 1 mM to reaction mixtures containing 0.02 mM [α-^{32}P] ATP. Samples were taken from the reaction mixtures at different times of incubation and subjected to TLC. This experiment was repeated twice with the enzyme from Roche and twice with the enzyme from BioLabs. The results obtained were similar, in spite of the contaminant ATPase activity present in the enzyme from BioLabs. One representative experiment (using the last enzyme) is depicted in Fig. 2. The relative rate of velocities obtained for methylenebisphosphonate, clodronate, etidronate, and tripolyphosphate in the synthesis of the corre-

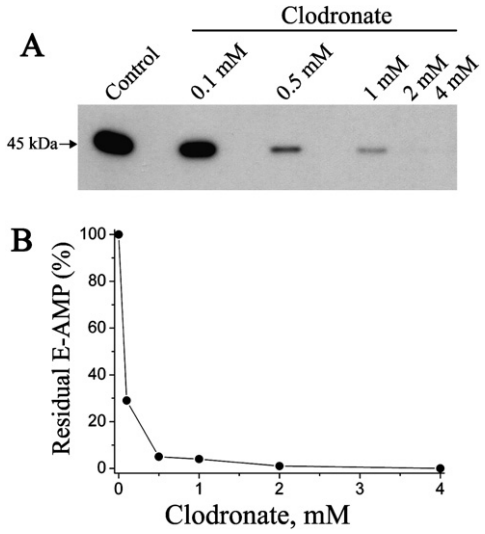

Fig. 1. Displacement of the AMP moiety of the T4 RNA ligase-adenylyl complex by clodronate. (A) The enzyme was incubated with [α-^{32}P] ATP in the absence or presence of increasing concentrations of clodronate as indicated, and subjected to SDS–PAGE as described in Section 2. The relevant portion of the autoradiogram is shown. The size (in kDa) of coelectrophoresed marker proteins are indicated by the arrow at the left. (B) The residual (%) adenylylated enzyme (E-AMP) in the presence of increasing concentrations of clodronate was quantified in an InstantImager.

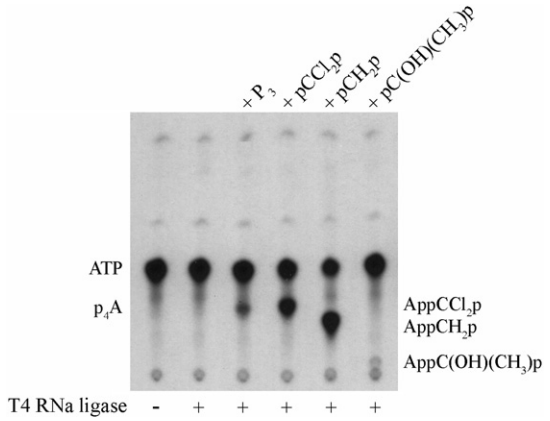

Fig. 2. Tripolyphosphate (P_3), clodronate ($pCCl_2p$), methylenebisphosphonate (pCH_2p), and etidronate ($pC(OH)(CH_3)p$) as substrates of the reaction. The reaction mixtures contained 0.02 mM [α-^{32}P] ATP, 1 mM bisphosphonates or P_3 as indicated, and 0.04 U of enzyme from BioLabs; other components as in Material and methods. At different times of incubation (3, 6, 12, and 20 min) aliquots were spotted on TLC plates, developed with dioxane:ammonium hydroxide:water (6:1:7) and subjected to autoradiography. The figure shows the result obtained after 12 min incubation.

sponding ATP derivatives were: 100, 52, 4, and 10, respectively. In our hands, pamidronate and alendronate were not substrates of the reaction (result not shown).

The influence of the addition of pyrophosphatase was assayed in reaction mixtures containing methylenebisphosphonate or clodronate: in both cases the ratio of the velocities obtained in the presence and absence of pyrophosphatase was around 2 (result not shown).

3.3. K_m values determination for clodronate, methylenebisphosphonate, and etidronate

The assays to calculate the K_m values for these bisphosphonates in the reaction

$$E + ATP + pC(R_1)(R_2)p \rightarrow AppC(R_1)(R_2)p + E + PPi$$

were carried out in the presence of a fixed concentration of [α-^{32}P] ATP (0.02 mM) or 0.3 mM non-radioactive ATP, using the TLC or HPLC methods, respectively. Other conditions as described in Section 2. The range of bisphosphonate concentrations used in the assay, the K_m values and the rate of synthesis (k_{cat}) determined were, respectively: clodronate, range: 0.1–1.5 mM; 0.54 ± 0.14 mM (0.29 ± 0.05 s^{-1}); methylenebisphosphonate, range: 0.1–1.5 mM; 0.26 ± 0.05 mM (0.28 ± 0.05 s^{-1}); etidronate, range 1–6 mM; 4.3 ± 0.5 mM (0.028 ± 0.013 s^{-1}). For comparison, the K_m and k_{cat} values calculated for P_3 in the synthesis of p_4A were: 1.26 ± 0.09 mM and 0.23 ± 0.04 s^{-1}, respectively [16]. The above values were determined by Lineweaver–Burk analysis and represent the mean and standard deviation of at least four determinations.

3.4. Characterization of $AppCCl_2p$

Although we have assumed $AppCCl_2p$ as the expected product to be synthesized in the presence of ATP, clodronate, and T4 RNA ligase, the validation of such structure was confirmed by treatment with alkaline phosphatase and snake venom phosphodiesterase. As shown in Fig. 3, the presumptive $AppCCl_2p$ was resistant to phosphatase, and yielded adenosine

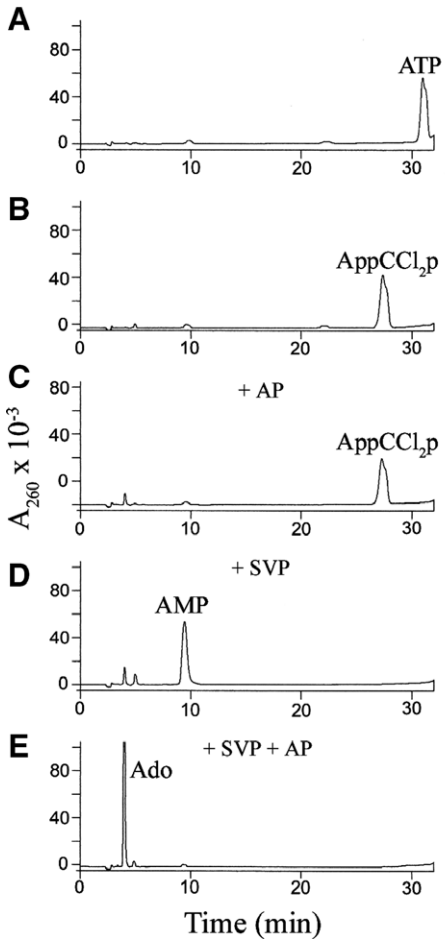

Fig. 3. Characterization of $AppCCl_2p$. A reaction mixture (0.12 ml) containing 15 U T4 RNA ligase (from Roche), 0.3 mM ATP and 4 mM clodronate was incubated until complete transformation of ATP into $AppCCl_2p$ and an aliquot analyzed by HPLC as described (B). The rest of the reaction mixture was treated with 2 μl (2U) of shrimp alkaline phosphatase (AP) for 1 h, and an aliquot analyzed by HPLC (C). The added phosphatase was denatured by heating at 90 °C for 6 min and thereafter the reaction mixture was treated with snake venom phosphodiesterase (SVP) (D), and treated again with AP (E). The content of ATP of the reaction mixture before addition of T4 RNA ligase is shown in (A).

as the only UV absorbing product after consecutive treatment with phosphodiesterase and phosphatase.

3.5. Bisphosphonate, and/or their ATP derivatives, as substrates or inhibitors of some of the reactions catalyzed by T4 RNA ligase

In these experiments the following reactions were considered:

$$E + ATP \leftrightarrow E - AMP + PPi \quad (c)$$

$$E - AMP + GTP \rightarrow Ap_4G + E \quad (d)$$

$$E + ATP + pC(R_1)(R_2)p \rightarrow AppC(R_1)(R_2)p + E + PPi \quad (e)$$

$$E + AppC(R_1)(R_2)p + GTP \rightarrow Ap_4G + pC(R_1)(R_2)p + E \quad (f)$$

$$E + AppC(R_1)(R_2)p + AppC(R_1)(R_2)p \rightarrow AppC(R_1)(R_2)ppA + pC(R_1)(R_2)p + E \quad (g)$$

$$E + AppC(R_1)(R_2)p + PPi \rightarrow ATP + pC(R_1)(R_2)p + E \quad (h)$$

where $pC(R_1)(R_2)p$ is any bisphosphonate; $AppC(R_1)(R_2)p$ and $AppC(R_1)(R_2)ppA$ are the corresponding ATP or Ap_4A derivatives.

It was previously known that in the presence of ATP and GTP the T4 RNA ligase catalyzed the synthesis of Ap_4G in two steps: formation of the E-AMP complex and transfer of the AMP moiety of the complex to GTP (reactions (c) and (d)), respectively [16]. As shown above, methylenebisphosphonate and clodronate (but not alendronate or pamidronate) reacted with ATP to synthesize the corresponding derivatives, $AppCH_2p$ and $AppCCl_2p$, respectively (reaction (e)). We wondered whether these derivatives, once formed in vitro: (i) could behave as ATP to generate the E-AMP complex and (ii) could serve as substrates for the synthesis of Ap_4G (reaction (f)) or even $AppC(R_1)(R_2)ppA$ (reaction (g)).

For that purpose the following experiment was set up (Fig. 4A–D). In A, the enzyme was incubated in the presence of ATP, a regenerative ATP system and the bisphosphonate indicated (Fig. 4A, lanes 4–7). One control without enzyme (Fig. 4A, lane 1) and two control reactions (with no added bisphosphonate) were carried out in parallel, in the absence (Fig. 4A, lane 2) or in the presence of the ATP regenerating system (Fig. 4, lane 3). After 30 min incubation, the following results were obtained (Fig. 4A): (i) the ATP regenerating system was very effective (compare the amount of ADP in Fig. 4A, lanes 2 and 3); (ii) complete conversion of ATP into the corresponding ATP bisphosphonate derivative were obtained in the presence of methylenebisphosphonate and clodronate (Fig. 4A, lanes 4 and 5) but not in the presence of alendronate or pamidronate (Fig. 4A, lanes 6 and 7) where, as in the absence of bisphosphonates (Fig. 4A, lane 3), similar small amounts of AMP and Ap_4A were formed in both cases, showing that alendronate or pamidronate were neither substrates of the enzyme nor inhibitors of the synthesis of Ap_4A (Fig. 4A, compare lanes 3, with lanes 6 and 7).

After 30 min incubation, and in order to promote the potential synthesis of Ap_4G the reaction mixtures were supplemented with GTP to a final concentration of 2 mM and further incubated for 15, 30, and 60 min (Fig. 4B–D, respectively). As expected [16], an important transformation of ATP into Ap_4G (reaction (d)) was obtained in the absence of bisphosphonates and in the presence of an ATP regenerating system (Fig. 4B–D, lanes 3). The synthesis of Ap_4G was evident after 15 min incubation (Fig. 4B, lane 3), with practically complete transformation of ATP into Ap_4G + AMP after 60 min incubation (Fig. 4D, lane 3). The methylene derivative of ATP remained almost unaltered even after 60 min incubation with GTP (Fig. 4B–D, lanes 4), showing that this ATP analogue ($AppCH_2p$) was hardly substrate for the synthesis of Ap_4G. In contrast, the clodronate derivative of ATP ($AppCCl_2p$) (Fig. 4B–D, lanes 5), synthesized Ap_4G, in the presence of GTP (reaction (f)), at a rate of 2.6 nmoles/mg/min, about 33% of that obtained in the presence of ATP (i.e., in the absence of bisphosphonate) (compare Fig. 4B–D, lanes 3 and 5). The presence of alendronate or palmidronate in the reaction mixture did not alter appreciably the rate of synthesis of Ap_4G (compare in Fig. 4B–D, lane 3 with lanes 6 and 7), showing that they were not inhibitors of that synthesis.

In a similar experiment, T4 RNA ligase was incubated for 30 min in the presence of radioactive ATP, clodronate, or methylenebisphosphonate until complete transformation of ATP into the corresponding bisphosphonate derivatives, $AppCCl_2p$ or $AppCH_2p$ (results not shown). Thereafter the reaction mixtures were supplemented with 2 mM PPi (final concentration) and further incubated for 15, 30, 60, and 120 min. The results were similar to those shown in Fig. 4: in the presence of PPi, the bisphosphonate derivatives $AppCCl_2p$ and $AppCH_2p$ synthesized ATP (reaction (h)) at a rate of 4.0 and 0.6 nmoles/mg/min, respectively (results not shown).

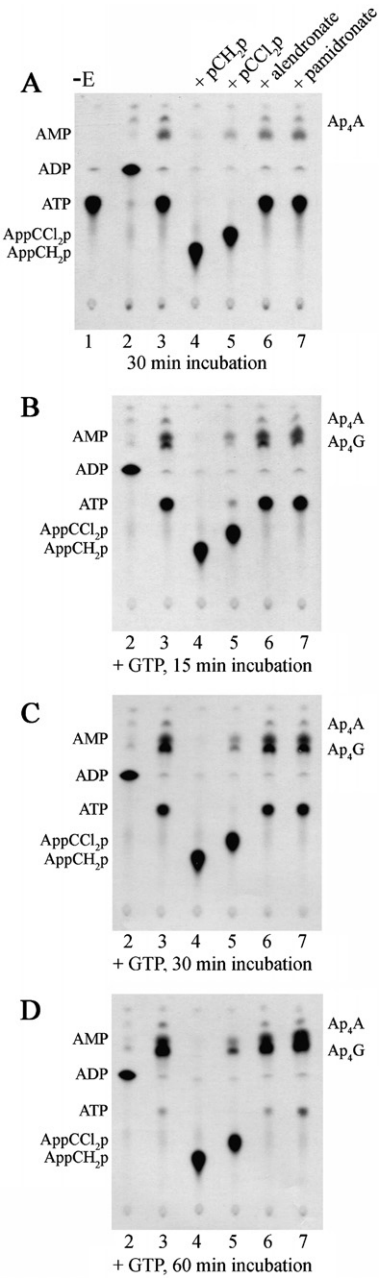

Fig. 4. Synthesis of ATP β,γ-bisphosphonate analogues and Ap_4G catalyzed by T4 RNA ligase. (A) The enzyme was incubated for 30 min in the presence of radioactive ATP and the bisphosphonates indicated in the upper part of the figure (lanes 4–7); a control without enzyme (lane 1) and controls without bisphosphonates (lanes 2 and 3) were carried out in parallel; all the reaction mixtures (except that in lane 2) contained the ATP regenerating system. The illustrations in B–D were obtained after incubation of the reaction mixtures shown in A (lanes 2–7) with 2 mM GTP for 15, 30, and 60 min, respectively. The nature of the radioactive spots are indicated at both sides of the figure. Analysis of the reaction mixtures were performed by TLC and developed with dioxane:ammonium hydroxide:water (6:1:6) as indicated in Section 2.

3.6. Concluding remarks

The aim of this work was to examine whether the synthesis of bisphosphonate derivatives of ATP could be carried out by ligases other than the well-established aminoacyl-tRNA synthetases. The results here presented show that T4 RNA ligase uses methylenebisphosphonate, clodronate and, to a much lesser extent, etidronate, but not alendronate nor pamidronate as substrates for the synthesis of the corresponding ATP derivatives. This finding seems to support the view that some non-nitrogen-bisphosphonates are incorporated into toxic bisphosphonates derivatives of ATP. This conclusion was reached with the use of aminoacyl-tRNA synthetases [3,5–7,11,17] and is here confirmed with T4 RNA ligase. In contrast, nitrogen-containing bisphosphonates, do not seem to incorporate into ATP derivatives but do interfere with the mevalonate pathway [3,9,11,13,18] by inhibiting farnesylpyrophosphate synthase [12,18]. The reason for the different behavior of bisphosphonates, depending on the occurrence of a nitrogen atom in its molecule, is currently unknown.

Since some aminoacyl-tRNA synthetases catalyze the synthesis of bisphosphonate derivatives of ATP, the possibility that the syntheses here reported were due to an aminoacyl-tRNA synthetase present as a contaminant in the commercial preparations of T4 RNA ligase, was contemplated. This possibility was ruled out, since cytidine 3′,5′-bisphosphate, a specific inhibitor of T4 RNA ligase [16,19] strongly inhibited the synthesis of the ATP derivative of clodronate. In addition the following amino acids: asparagine, aspartate, glycine, histidine, lysine, phenylalanine, or serine, substrates of the Type II subclass of aminoacyl-tRNA synthetases [3] did not stimulated, but rather inhibited, the synthesis of the ATP clodronate derivative catalyzed by T4 RNA ligase (results not shown).

In our view and related with bisphosphonates, the conclusions reached with a certain enzyme, cannot be extrapolated to other enzymatic systems: methylenebisphosphonate was a good substrate for the synthesis of both its ATP (reaction (e)) and its Ap_4A derivatives (reaction (g)) in the aminoacyl-tRNA synthetases system [17]. However, with T4 RNA ligase, methylenebisphosphonate is a good acceptor of AMP (reaction (e)) but its ATP derivative was a bad acceptor of GTP (reaction (f)) and of PPi (reaction (h)). Based on that, we think that the knowledge of the biochemical bases of the mechanism of action of bisphosphonates is still rather scanty and could depend on the enzymatic system considered. We are at present engaged in a systematic study of the efficiency of other ligases to use different bisphosphonates for the synthesis of ATP derivatives.

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