Characterisation of stress protein LysU. Enzymic synthesis of diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) analogues by LysU

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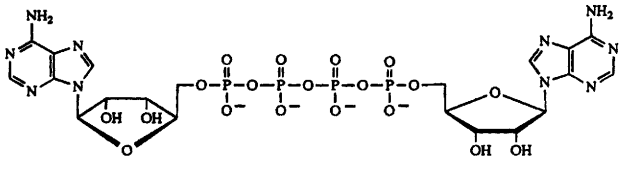
The stress protein LysU (lysyl tRNA synthetase) has been purified from a recombinant strain of *Escherichia coli* expressing the plasmid pXLys5, and kinetically characterised. Preparative syntheses of analogues of the biologically important molecule diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) are then achieved in good yield by enzyme catalysis, using purified LysU.

Introduction

All cells of all organisms synthesize small families of highly conserved proteins, known as stress proteins,[1] which protect cells against adverse affects of the environment. We are currently studying [2–6] the structure, function and chemistry of a number of stress proteins. LysU is one such stress protein from the bacterium *Escherichia coli* (*E. coli*). Whilst the majority of characterised stress proteins are known to be molecular chaperones, which assist protein folding and unfolding *in vivo*, LysU is a stress-inducible enzyme which is an isozyme of a constitutively expressed *E. coli* lysyl tRNA synthetase enzyme (LysS).[7] The genes coding for LysU and LysS (namely *lysU* and *lysS* respectively) are well separated[8] on the *E. coli* chromosome and are closely regulated such that *lysS* is expressed only under normal growth conditions and *lysU* under conditions of cell stress including heat, acidosis, anaerobosis, entry to stationary phase and metabolite stress.[9] The normal function of lysyl tRNA synthetase is to catalyse the synthesis of lysyl tRNA but the reason why two such enzymes are required, of which one is stress-inducible, is mysterious. Recently, differences in the relative lysine affinities of the two isozymes have been proposed[10] to explain the existence of the two isozymes but another explanation may be found in the ability of both isozymes, in particular LysU, to act as efficient catalysts for the formation of the dinucleotide diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) **1** from adenosine 5′-triphosphate (ATP) **2**.[10–12] A plausible mechanism for this conversion is shown in Scheme 1. This mechanism, which presumes the formation of a lysyl adenylate intermediate **3** prior to formation of Ap_4A **1**, was first suggested by Zamecnik *et al.*[13]



1 Ap_4A

Ap_4A **1** and other closely related dinucleoside polyphosphates are molecules ubiquitous in Nature. Ap_4A **1** is thought to function in cellular responses to cell proliferation and environmental stresses in prokaryotes and lower eukaryotes, as well as to play a role in extracellular signalling in higher eukaryotes.[14,15] Originally, Ap_4A **1** was thought to function as a 'signal nucleotide' acting to induce the synthesis of stress proteins.[16] However, recent evidence suggests that Ap_4A **1** may instead negatively modulate cellular stress responses by binding directly to stress proteins and/or by modulating the translation of stress protein genes.[14,17] In any event, the biological importance of Ap_4A **1** is self-evident, implying that analogues could be potentially useful therapeutic compounds. Indeed some Ap_4A analogues have already been evaluated as antithrombotic agents.[18] In recognition of this potential utility, the syntheses of some Ap_4A analogues have been reported using both chemical[19] and enzymic methods.[20,21] However, the chemical procedures were multi-step and often low yielding owing to formation of by-products, whilst enzymic syntheses were performed without purification or characterisation of the products.[20] Recently, we communicated[22] the efficient synthesis of a number of Ap_4A analogues by an enzymic procedure involving LysU. Using this procedure, Ap_4A analogues were obtained in good to excellent yield and in a homogeneous form. This paper further details the utility of this method and also describes the enzymology of the recombinant LysU used in the syntheses. Furthermore, our investigations into the mechanism of the LysU-catalysed synthesis of Ap_4A **1** and the Ap_4A analogues are also discussed.

Discussion

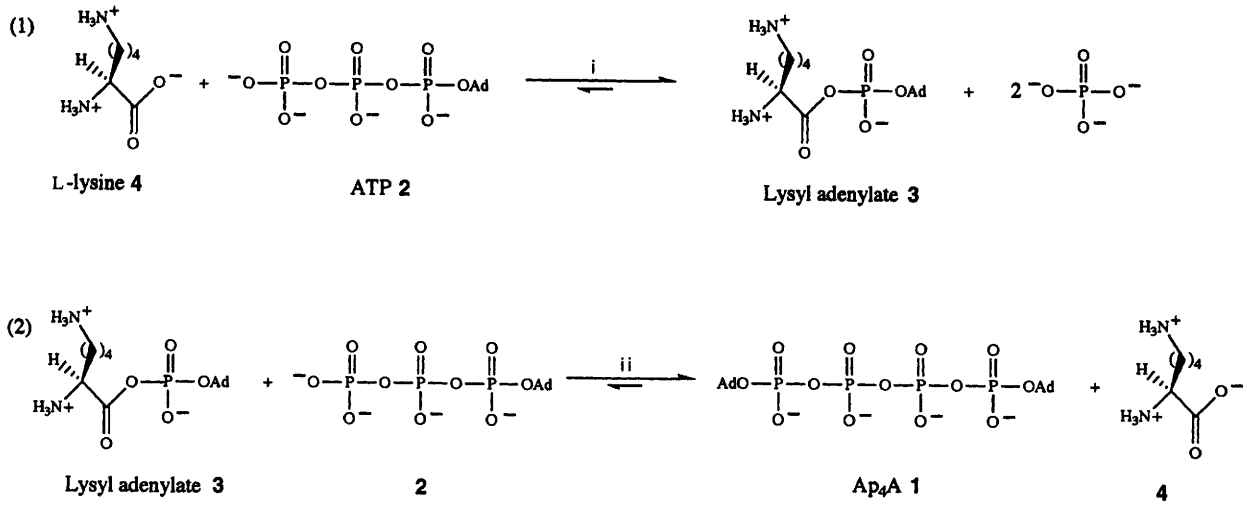
Purification of recombinant LysU

LysU was purified from a strain of *Escherichia coli* (TG2) expressing plasmid pXLys5.[12] The recombinant enzyme was purified to homogeneity [as determined by sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (PAGE)] with a yield of ~250 mg of protein per litre of cell culture. This represents a high level of protein expression which agrees with the 44-fold overexpression of LysU obtained by Brevet *et al.*[12] Such high levels of expression rule out significant contamination by the constitutively expressed synthetase LysS,[7] and since the overproduction of proteins can elicit the heat shock response[23] constitutive expression of LysS is probably suppressed further in favour of stress-induced LysU.

LysU has been reported to be unstable in dilute solution after purification.[11] Our results corroborate this finding. However at concentrations of 2–3 mg ml^{-1} with 20% (v/v) glycerol, recombinant LysU was found to be stable and showed no significant drop in specific activity (182 mol of ATP transformed min^{-1} per mol of LysU at 37 °C) following long-term storage at −20 °C.

Kinetic characterisation of recombinant LysU

A radioactive assay, adapted from the procedure of Charlier and Sanchez,[11] was used to determine the temperature and pH optima of LysU-catalysed Ap_4A **1** synthesis. The results (Fig. 1) show that Ap_4A **1** formation was maximal at pH 8 and at a



Scheme 1 *Reagents:* i, LysU, pyrophosphatase, $MgCl_2$, KCl; ii, LysU, $ZnCl_2$. Ad = adenosine

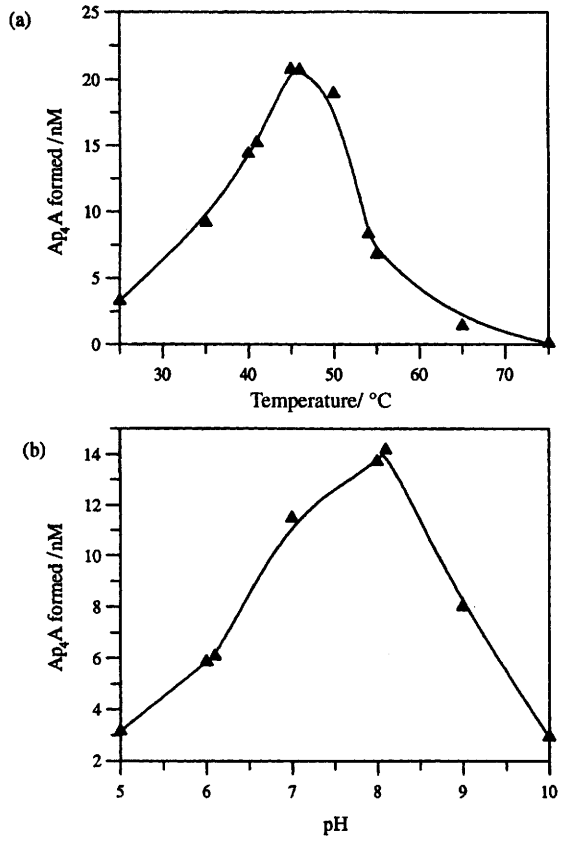


Fig. 1 Effect of temperature (°C) and pH on formation of Ap_4A by LysU. (a) The amount of Ap_4A formed in standard LysU radioactive-assays was determined at the indicated temperatures as described in the text; (b) the amount of Ap_4A formed in standard LysU radioactive-assays was determined at the indicated pH values.

temperature of 45 °C. LysU was found to be thermostable and showed no substantial loss in specific activity even after at least one hour of incubation at 45 °C. The thermostability of LysU has been observed elsewhere [10,11,24] and is in accord with the role of LysU as a heat shock protein.

Kinetic constants (k_{cat} and K_M) for LysU-catalysed conversion of ATP **2** to Ap_4A **1** were determined using ^1H NMR spectroscopy, by the adaptation of a previously published method of Plateau and Blanquet.[25] Individual reaction mixtures were made up in 5 mm NMR tubes and the conversion of ATP **2** to Ap_4A **1** followed over a period of 30 min. The formation of Ap_4A **1** was identified by the upfield shift of the 2-H and 8-H singlets of adenine by 0.15 ppm (see Fig. 2). Values of k_{cat} and K_M were evaluated for ATP **2**, L-lysine **4**, and Zn^{2+} as well as with Cd^{2+} and Co^{2+} in place of Zn^{2+} (Table 1). These kinetic measurements were made in the non-chelating HEPES {2-[*N*′-(2-hydroxyethyl)piperazin-*N*′-yl]-ethanesulfonic acid} buffer, in order to minimise distortions to the kinetic data resulting from buffer–metal ion chelation. Mildly chelating TRIS [tris(hydroxymethyl)methylamine] buffer could also be used, with little apparent distortion (results not given). The saturated rate constant (k_{cat}) of 9.9 s^{-1}, obtained for ATP **2** at 37 °C (Table 1), is higher than that previously reported [25] for LysS under similar conditions although lower than a value recently reported [10] for LysU using a bioluminescence assay. Rather unexpectedly, the k_{cat} value increased only slightly at 45 °C. However, the K_M value was halved on increasing the temperature, thereby increasing the specificity constant of the enzyme (k_{cat}/K_M) by a factor of ~2. We have no explanation for this observation at this time. Of considerable interest is the apparent sensitivity of formation of Ap_4A **1** to the Zn^{2+} concentration. Zn^{2+} stimulation of synthesis of Ap_4A **1** was found to obey typical saturation kinetics, with half-maximal metal ion stimulation (K_M) at 36 μM. In the absence of Zn^{2+} ions, enzyme-catalysed Ap_4A **1** synthesis was unmeasurable. If kinetics were measured in the presence of the chelating agent EDTA (ethylenediaminetetra-acetic acid) (100 μM) then sigmoidal kinetics were observed (results not shown) with a corresponding increase of K_M to 85 μM. Finally, metal ions Cd^{2+} (Group IIb) and Co^{2+} (Group VIII) were also found to act as reasonable surrogates of Zn^{2+} albeit stimulating Ap_4A **1** synthesis less efficiently.

These data suggest that LysU is an efficient catalyst of formation of Ap_4A **1** at both 37 °C (normal growth temperature) and 45 °C (sub-lethal heat shock temperature). The stimulatory affect of Zn^{2+} and other ions on the rate of formation of Ap_4A **1** is marked. Presumably, both Cd^{2+} and Co^{2+} are promoting Ap_4A **1** synthesis by a similar mechanism. Based on this presumption, we have attempted to determine the nature of the Zn^{2+} ion-binding site on the protein as a first step towards understanding the role of Zn^{2+}. It is not possible to observe Zn^{2+} spectroscopically, therefore in the past Cd^{2+} or Co^{2+} ions have been used in place of Zn^{2+}, so that ^{113}Cd NMR [26] and Co^{2+} UV–VIS [27] spectroscopy could be used to provide indirect information about Zn^{2+} binding site(s). However, in the case of LysU these ions bound to the protein only weakly, with the result that no useful spectroscopic data could be obtained.

Metal ion-induced aggregation of recombinant LysU

In addition to kinetic effects, Zn^{2+} was found to bring about aggregation of LysU (in presence of 10 mM $MgCl_2$) [Fig. 3(a)].

Table 1 Kinetic data for the LysU-catalysed formation of Ap_4A. Kinetic data determined by ^1H NMR spectroscopy as described in text

Substrate/ cofactor	Temperature (T/°C)	k_{cat}/s^{-1}	K_M/mM	k_{cat}/K_M
ATP[a]	37	9.9	6.0	1.7
ATP[a]	45	11.9	3.56	3.3
L-Lysine[b]	45	12.2	0.48	
$ZnCl_2$[c]	45	11.6	0.036	
$CdCl_2$[c]	45	3.2	0.286	
$CoCl_2$[c]	45	1.4	1.25	

[a] L-Lysine was 2 mM and $ZnCl_2$ 150 μM. [b] ATP was 5 mM and $ZnCl_2$ 150 μM. [c] ATP was 5 mM and L-lysine 2 mM.

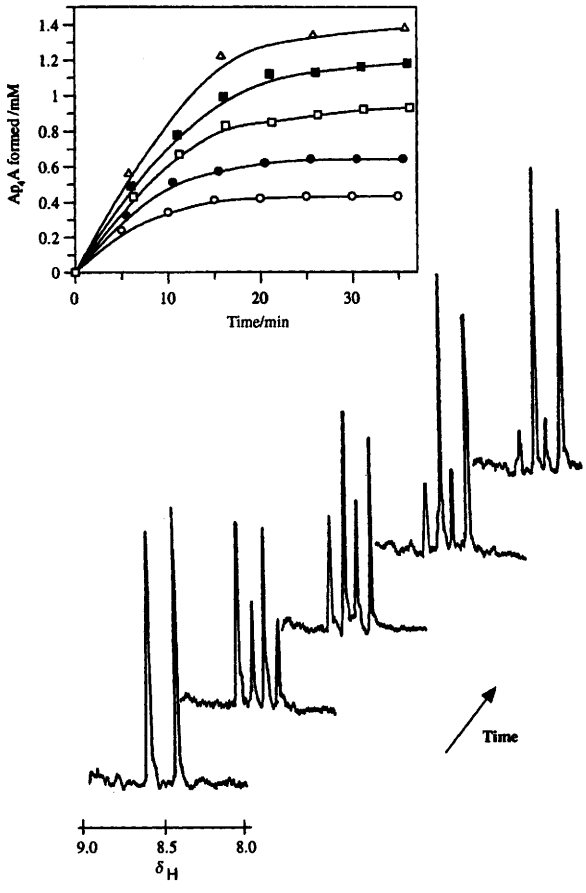


Fig. 2 Determination of kinetic constants k_{cat} and K_M for formation of Ap_4A by LysU. The figure shows a typical sequence of ^1H NMR spectra obtained during the LysU-catalysed conversion of ATP **2** to Ap_4A **1** in a 5 mm NMR tube, as described in the text. The amount of Ap_4A **1** formed with time was evaluated from the increase in the integrated intensity of Ap_4A NMR signals with time. The inset illustrates the increase in the rate of formation of Ap_4A **1** given increased initial ATP **2** concentrations. Initial ATP concentrations were 1 mM ATP (○), 1.5 mM ATP (●), 2 mM ATP (□), 2.5 mM ATP (■) and 3 mM ATP (△). The illustrated rate data were obtained with fixed concentrations of 2 mM L-lysine and 150 μM $ZnCl_2$. Kinetic constants were determined from initial rates of Ap_4A formation.

Aggregation was detected by increases in absorbance at 400 nm, caused by increased light scattering as aggregates formed. Essentially no aggregation was observed below a Zn^{2+} concentration of 20 μM. However, the state of aggregation was found to increase dramatically as the Zn^{2+} concentration was increased from 20 to 25 μM. Thereafter, aggregation effects were found to decline in a non-linear manner. Similar effects were observed when Zn^{2+} was replaced by the other Group IIb metal ions (Cd^{2+} and Hg^{2+}) or by Co^{2+}, although higher concentrations were needed to bring about aggregation, especially in the case of Co^{2+} [Fig. 3(b)]. *E. coli* phenylalanyl tRNA synthetase has previously been found to undergo similar

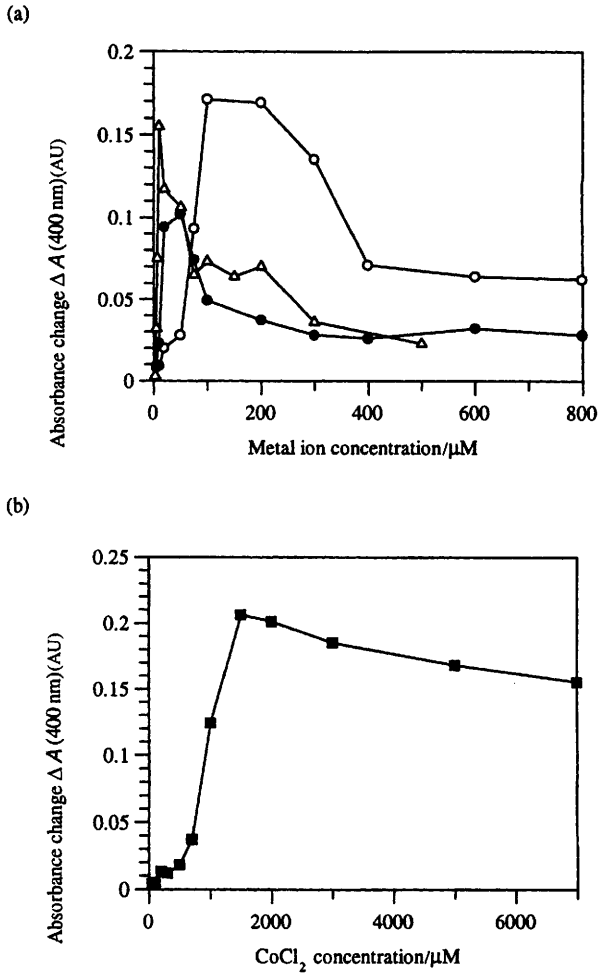
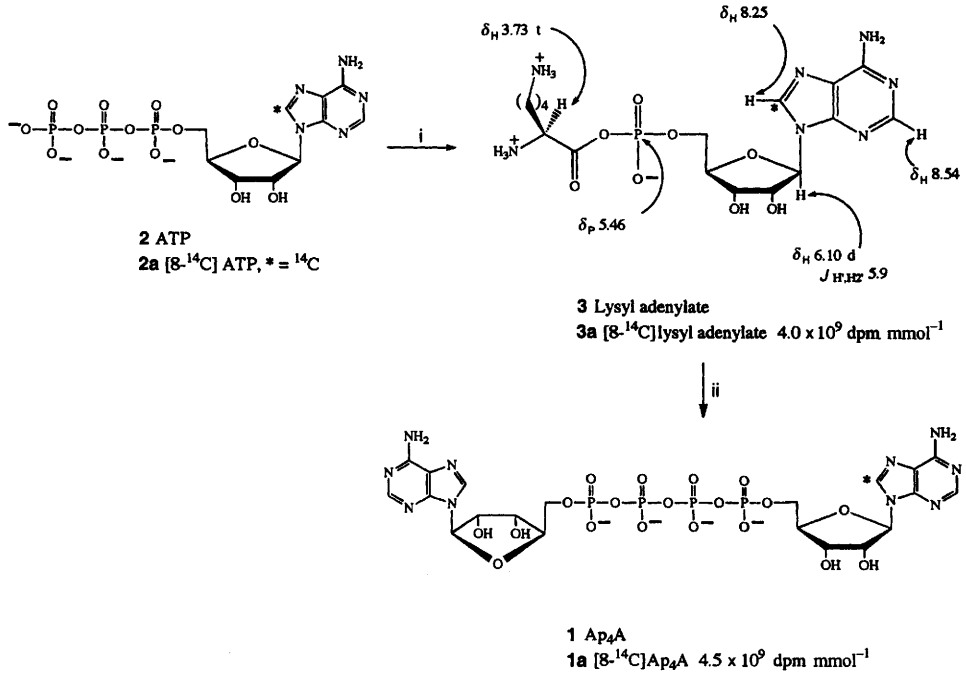


Fig. 3 Aggregation of LysU induced by heavy metal ions. Aggregation mixtures containing $ZnCl_2$ (△), $HgCl_2$ (●), $CdCl_2$ (○) or $CoCl_2$ (■) were prepared as described in the text. Following the addition of LysU to the mixtures, enzyme aggregation was detected by a change in absorbance at 400 nm and plotted as a function of metal ion concentration.

Group IIb metal ion-induced aggregation.[28] Intriguingly, it was found that aggregation of LysU in the presence of either Zn^{2+} (25 μM), Cd^{2+} (100 μM) or Co^{2+} (1500 μM) was suppressed by the addition of L-lysine **4** (2 mM) and ATP **2** (3 mM). Perhaps this suppression results from the simultaneous coordination of the heavy metal ions by substrate(s) and protein. What significance this effect has in accounting for the role of heavy metal ions (especially Zn^{2+}) in the stimulation of Ap_4A synthesis remains to be seen.

Mechanism of formation of Ap_4A

The proposed mechanism of synthesis of Ap_4A **1** (Scheme 1) is a two-stage process involving the formation of an intermediate lysyl adenylate **3**. If this mechanism were valid, then L-lysine **4** would be essential for the first step to take place, and Zn^{2+} for the second step. In fact, the kinetic data (Table 1) support the requirement for both Zn^{2+} and L-lysine **4**, so providing evidence for both steps of the mechanism (Scheme 1). However, additional evidence was sought by attempting to isolate the proposed intermediate **3**. A reaction mixture was prepared containing LysU, inorganic pyrophosphatase, ATP **2**, L-lysine **4**, $MgCl_2$ and KCl (but *without* Zn^{2+}) and the reaction was monitored by TLC. After 2.5 h, a product was formed (Scheme 2) which was isolated by reversed-phase HPLC and characterised by ^1H and ^{31}P NMR spectroscopy. This product was identified as lysyl adenylate **3**, thereby confirming the first step of the mechanism (Scheme 1). The second step was then demonstrated by adding the isolated intermediate **3** to a reconstituted reaction mixture comprising



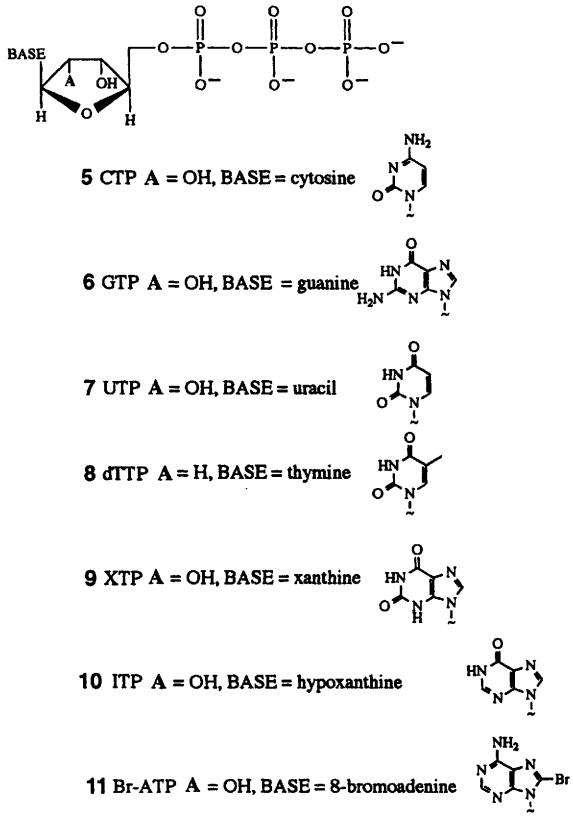
Scheme 2 *Reagents:* i, LysU, pyrophosphatase, $MgCl_2$, KCl, L-lysine; ii, LysU, ATP, $MgCl_2$, KCl, $ZnCl_2$, L-lysine

LysU, ATP **2**, L-lysine **4**, $MgCl_2$, KCl and $ZnCl_2$ (but without inorganic pyrophosphatase). Ap_4A **1** was the only product isolated (Scheme 2). A final piece of evidence for the mechanism (Scheme 1) was provided by repeating the above experiment (Scheme 2) with [8-^{14}C]ATP **2a**. A radioactively labelled intermediate **3a** was then isolated and counted before being added to a reconstituted reaction mixture containing unlabelled ATP **2**. After purification, the radioactive Ap_4A **1a** produced was counted and shown to have a similar specific radioactivity to the isolated intermediate **3a** thereby demonstrating direct, onward conversion of the intermediate **3** to Ap_4A **1** (Scheme 2).

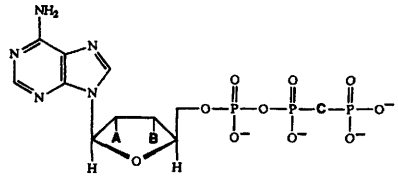
Enzymic syntheses of Ap_4A analogues

In previous reports concerning the isozyme LysS,[25] mixtures of ATP **2** and a nucleoside 5′-triphosphate (NTP, where N is a nucleoside other than adenosine) were apparently transformed by the enzyme into a mixture of Ap_4A and an analogue of the form Ap_4N (adenosine[nucleoside] 5′,5‴-P^1,P^4-tetraphosphate), provided that appropriate quantities of $ZnCl_2$ were present. Therefore, we tested whether recombinant LysU could be used to synthesize Ap_4A analogues.

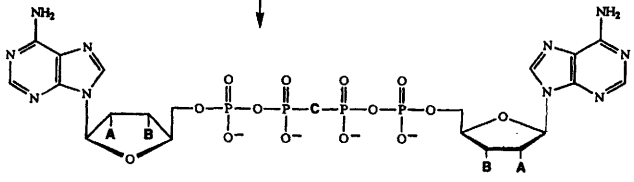
Initially, cytidine 5′-triphosphate (CTP) **5**, guanosine 5′-triphosphate (GTP) **6**, uridine 5′-triphosphate (UTP) **7**, 2′-deoxythymidine 5′-triphosphate (dTTP) **8**, xanthosine 5′-triphosphate (XTP) **9**, inosine 5′-triphosphate (ITP) **10**, 8-bromoadenosine 5′-triphosphate (Br-ATP) **11**, 2′-deoxyadenosine 5′-triphosphate (dATP) **12**, 3′-deoxyadenosine 5′-triphosphate (3′-dATP) **13** and adenine arabinofuranoside 5′-triphosphate (Ara-ATP) **18** were tested as alternative substrates to ATP **2**. Out of these, only dATP **12** and 3′-dATP **13** were transformed by the enzyme, giving di-2′-deoxyadenosine 5′,5‴-P^1,P^4-tetraphosphate (dAp_4dA) **15** and di-3′-deoxyadenosine 5′,5‴-P^1,P^4-tetraphosphate (3′-dAp_4dA) **16** in 74 and 47% yield, respectively (Scheme 3). Subsequently, adenosine 5′-[α,β-methylene]triphosphate ($ApCH_2pp$) **19**, adenosine 5′-[β,γ-methylene]triphosphate ($AppCH_2p$) **20**, adenosine 5′-[β,γ-imido]triphosphate (AppNHp) **14**, adenosine (S) 5′-[β-thio]triphosphate (β-thioATP) **21** and adenosine (S) 5′-[α-thio]triphosphate (α-thioATP) **22** were tested. In this case, only AppNHp **14** was transformed (Scheme 3), to give diadenosine 5′,5‴-P^1,P^4-[β,γ-imido]tetraphosphate (AppNHppA) **17** in 61% yield. These results suggested that formation of the intermediate [Step (1), Scheme 1] was a very specific process, possible only with ATP **2** or other closely related nucleotides such as dATP **12**, 3′-dATP **13** and AppNHp **14**. Therefore, syntheses of Ap_4A analogues would only be possible if the second step [Step (2), Scheme 1] were much less specific and able to tolerate a wide range of second nucleotide substrates in place of ATP **2**.



To test this, a *competitive procedure* was adopted in which both ATP **2** and a second nucleotide substrate were present in the reaction mixture. If the second step were non-specific, we anticipated that the ATP **2** would be consumed first in forming the adenylate intermediate **3**, which would then react in turn with the second nucleotide substrate to produce an Ap_4A

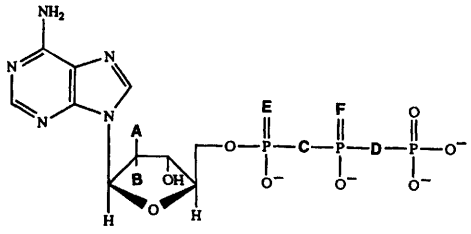


12 dATP **A** = H, **B** = OH, **C** = O
13 3′dATP **A** = OH, **B** = H, **C** = O
14 AppNHp **A** = **B** = OH, **C** = NH



15 dAp_4dA **A** = H, **B** = OH, **C** = O
16 $3'dAp_4dA$ **A** = OH, **B** = H, **C** = O
17 AppNHppA **A** = **B** = OH, **C** = NH

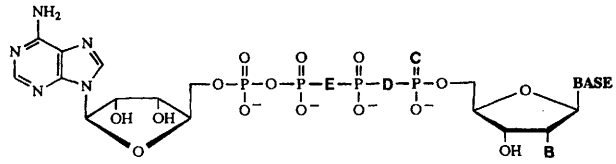
Scheme 3



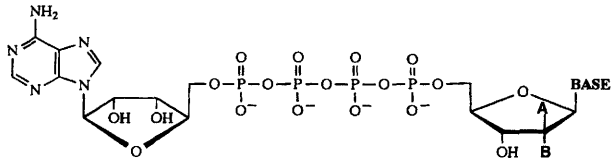
18 Ara-ATP **A** = OH, **B** = H, **C** = **D** = **E** = **F** = O
19 $ApCH_2pp$ **A** = H, **B** = OH, **C** = CH_2, **D** = **E** = **F** = O
20 $AppCH_2p$ **A** = H, **B** = OH, **D** = CH_2, **C** = **E** = **F** = O
21 β-thioATP **A** = H, **B** = OH, **F** = S, **C** = **D** = **E** = O
22 α-thioATP **A** = H, **B** = OH, **E** = S, **C** = **D** = **F** = O

analogue. Indeed, this appeared to be the case and several Ap_4A analogues were successfully synthesized in this manner (**17**, **23**–**29**). However, significant amounts of Ap_4A **1** by-product were often formed. In addition, some second nucleotides behaved not as substrates but rather as enzyme inhibitors. For example, both inosine 5′-triphosphate (ITP) **10** and cytidine 5′-triphosphate (CTP) **5** were found to act as mixed inhibitors when kinetic inhibition studies were carried out (results not shown). As a result, a *sequential procedure* was developed in order to avoid both of these problems. This procedure was based upon the observation that adenylate **3** could be preformed in the absence of Zn^{2+} and subsequently trapped by the addition of a second nucleotide substrate followed by Zn^{2+}. Using this procedure, several more Ap_4A analogues (**30**–**35**) were synthesized successfully. The synthesis of more complex Ap_4A analogues was investigated using 5-fluoro-2′-deoxyuridine 5′-triphosphate (5-fluoro-dUTP) **36**, uridine 5′-[β,γ-methylene]triphosphate ($UppCH_2p$) **37**, guanosine 5′-[β,γ-methylene]triphosphate ($GppCH_2p$) **38**, uridine 5′-[β,γ-imido]triphosphate (UppNHp) **39** and guanosine 5′-[β,γ-imido]triphosphate (GppNHp) **40** as second nucleotide substrates. Analogues **41**–**45** were formed from these substrates, using the sequential procedure, in moderate to low yields, which suggested that these second substrates were binding only weakly to LysU. Furthermore, the (S) α-thio derivatives of nucleotides **37**–**40** were not found to be substrates for LysU and so no analogues could be synthesized from these.

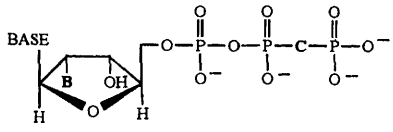
All the Ap_4A analogues, described above, were purified to homogeneity and characterised by TLC, NMR spectroscopy (^1H and ^{31}P) and negative-ion mode fast atom bombardment mass spectrometry (negative-ion FAB MS). To obtain reproducible mass spectra, special conditions were developed which we have reported elsewhere.[29] However, the conditions

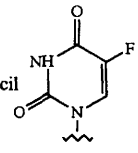


23 Ap_4G BASE = guanine
24 Ap_4X BASE = xanthine
25 Ap_4U BASE = uracil
26 Ap_4dT **B** = H, BASE = thymine
27 $AppCH_2pA$ **D** = CH_2, BASE = adenine
28 $AppCH_2ppA$ **E** = CH_2, BASE = adenine
29 $[\alpha S]Appp_sA$ **C** = S, BASE = adenine
Unless otherwise stated, **B** = OH, **C** = **D** = **E** = O



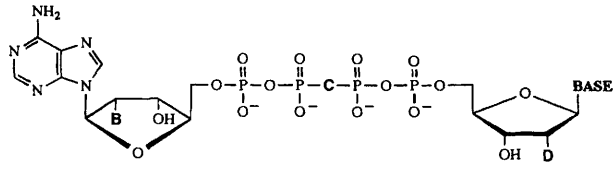
30 Ap_4I BASE = hypoxanthine
31 Ap_4BrA BASE = 8-bromoadenine
32 Ap_4C BASE = cytosine
33 Ap_4dA **B** = H, BASE = adenine
34 $Ap_4[Ara\text{-}A]$ **A** = OH, **B** = H, BASE = adenine
35 Ap_4 (no second nucleoside)
Unless otherwise stated, **A** = H, **B** = OH





36 5-fluoro-dUTP **B** = H, **C** = O, BASE = 5-fluorouracil

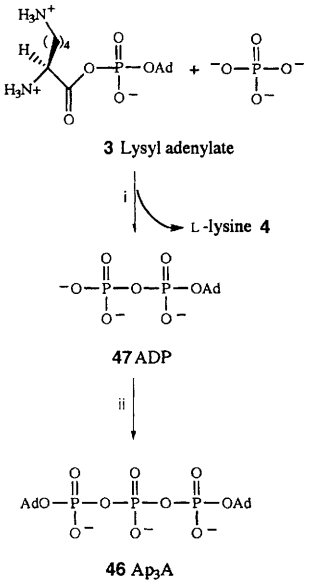
37 $UppCH_2p$ **B** = OH, **C** = CH_2, BASE = uracil
38 $GppCH_2p$ **B** = OH, **C** = CH_2, BASE = guanine
39 UppNHp **B** = OH, **C** = NH, BASE = uracil
40 GppNHp **B** = OH, **C** = NH, BASE = guanine



41 $Ap_4d[5\text{-fluoro-U}]$ **D** = H, BASE = 5-fluorouracil
42 $AppCH_2ppU$ **C** = CH_2, BASE = uracil
43 dAppNHppU **B** = H, **C** = NH, BASE = uracil
44 $AppCH_2ppG$ **C** = CH_2, BASE = guanine
45 AppNHppG **C** = NH, BASE = guanine
Unless otherwise stated, **B** = **D** = OH, **C** = O

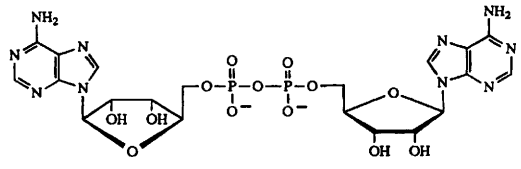
which were developed proved only adequate for low-resolution analysis, and usually poor mass-signal persistence and low signal-to-noise ratio prevented access to higher-resolution information. Almost certainly this limitation resulted from the extremely polar character of Ap_4A **1** and analogues; thus far we have not been able to improve the mass spectral analysis further. Our experience duplicates that of other workers who have chemically synthesized Ap_4A analogues since they too appear unable to report high-resolution mass spectra data for Ap_4A **1** or for any analogues.[19,30]

An intriguing side-reaction was observed when synthesis of diadenosine 5′,5‴-P^1,P^4-(S)-[β-thio]tetraphosphate ([βS]-$Appp_spA$) was attempted using a combination of ATP **2** and β-thioATP **21**. Using the competitive procedure, only Ap_4A **1** was formed, probably because β-thioATP **21** was unable to bind into the enzyme's reactive site. By contrast, diadenosine 5′,5‴-P^1,P^3-triphosphate (Ap_3A) **46** was formed when the sequential procedure was used. The formation of



Scheme 4 *Reagents:* i, Zn^{2+}; ii, Zn^{2+}, **3**. Ad = adenosine.

Ap_3A **46** was thought to be caused (Scheme 4) by the slow nucleophilic attack of free phosphate (contributed from the phosphate buffer in which LysU was stored prior to use) on the lysyl adenylate intermediate **3** to form adenosine 5′-diphosphate (ADP) **47** which was then able to react with further intermediate to form Ap_3A **46**. This suggestion was supported by the apparent ease with which Ap_3A **46** was synthesized from ATP **2** and second nucleotide ADP **47**, using the sequential procedure. Unfortunately, LysU was unable to catalyse the formation of Ap_3A analogues in spite of persistent attempts. Moreover, diadenosine 5′,5‴-P^1,P^2-diphosphate (Ap_2A) **48** and analogues could not be formed by LysU catalysis either.



48 Ap_2A

The X-ray crystal structure of LysU

Our recently published X-ray crystal structure [31,32] of LysU can help us explain the limitations of LysU as a catalyst for formation of Ap_4A analogues. The structure of LysU bears close correspondence to the crystal structure of yeast aspartyl tRNA synthetase (AspRS) complexed to ATP and its cognate transfer RNA ($tRNA^{Asp}$).[33] In the ATP-binding region, the similarity of the two structures can be used to visualise the ATP-binding site in LysU. As a result, we can identify (Fig. 4) amino acids responsible for binding the first ATP **2** molecule, which reacts with L-lysine **4** to form the reactive adenylate **3**. Paradoxically, there seem to be no amino acid residues which clearly complement adenosine functional groups, although there are two arginine residues, Arg262 and Arg480, which clearly complement the α- and γ-phosphate of ATP, respectively, thereby assisting the alignment of the α-phosphate with respect to L-lysine (Fig. 4). Nevertheless, both adenine and ribose are tightly constrained within a narrow binding pocket which probably explains the rigorous specificity of the first, intermediate-forming step.

Unfortunately, the X-ray crystal structure of LysU has not allowed us to identify either a clear Zn^{2+}-binding site or a binding site for the second nucleotide substrate. However, the X-ray crystal structure of the Hg-heavy atom derivative of LysU shows phenyl–Hg bound to a histidine residue (His382) near the active site for adenylate formation. This histidine residue could be part of a Zn^{2+} binding site on the basis that Hg is a Group IIb metal, like Zn, and Hg^{2+} is able to promote weak formation of Ap_4A (results not shown). Quite possibly, there is no defined binding site for the second nucleotide substrate. Instead, Zn^{2+} could be acting simply to chelate this substrate to the enzyme active site so that it might react with the adenylate intermediate **3** possibly with the assistance of Zn^{2+} electrophilic catalysis. In this event, no binding site for a second nucleotide substrate would be required. Such a suggestion would be in keeping with the observed suppression of Zn^{2+}-induced aggregation of LysU by ATP **2** and L-lysine **4** and the general difficulties experienced in determining the nature of the Zn^{2+} binding site using ^{113}Cd NMR and Co^{2+} visible spectroscopy. Certainly, whilst the enzyme's active-site is very specific for formation of lysyl adenylate **3**, the lack of specificity for the second nucleotide substrate suggests that LysU probably only recognises simple features such as the phosphate chain. Evidence for this suggestion is provided by the ready synthesis of adenosine 5′-tetraphosphate (Ap_4) **35** using the sequential procedure where the second 'nucleotide substrate' was tripolyphosphate (p_3).

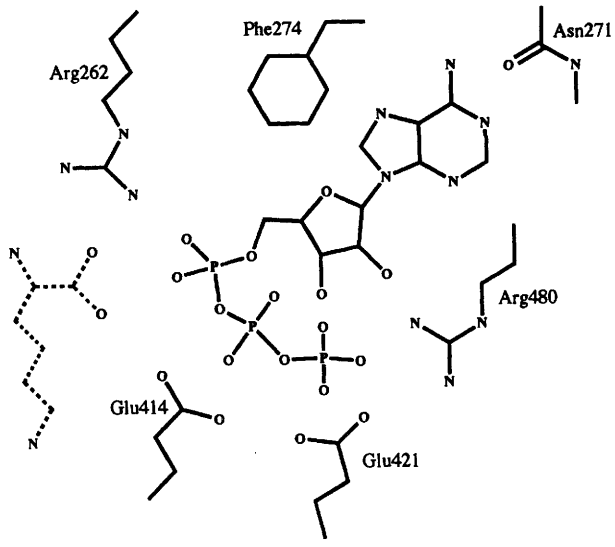


Fig. 4 Schematic of LysU active site derived from the X-ray crystal structure. Schematic to show the predicted structure of the first molecule of ATP **2** bound to the LysU active site. This was predicted [32] on the basis of the known structure of ATP bound to the yeast AspRS:$tRNA^{Asp}$ complex.[33] The similarity of the two active sites in this region was such that the atomic coordinates of the ATP molecule were simply 'pasted' into the LysU structure without any adjustment. The adenosine ring is held in a sandwich between the benzene ring of Phe 274 (conserved) and the guanidinium group of Arg 480 (conserved). The ATP-phosphate backbone assumes a bent conformation which is stabilised by conserved, charged residues Arg 262, Arg 480, Glu 414 and Glu 421. The amino acid substrate L-lysine is shown dotted and the α-phosphate group of the ATP is correctly positioned for nucleophilic attack of the L-lysine carboxylate group.

Conclusions

Here we have described the purification and characterisation of a recombinant form of the stress protein LysU. This protein has been used successfully to synthesize analogues of the biologically important molecule Ap_4A **1**. The results from these syntheses clearly show that although there are limitations on the range of Ap_4A analogues which can be synthesized by LysU, a reasonable range of assymetric Ap_4A analogues may still be produced. With the X-ray crystal structure of LysU now solved, it should now be possible to commence protein engineering of LysU so as to improve the synthetic versatility of this enzyme further.

Experimental

General

Inorganic pyrophosphatase (200 units ml^{-1}), alkaline phosphatase (1500 units mg^{-1}) and adenosine 5′-[β-thio]diphosphate were purchased from Boehringer-Mannheim (Diagnostics and Biochemicals) UK, Lewes, East Sussex, UK. All other chemicals, unless otherwise stated, were obtained from either Sigma Chemical Co., Poole, Dorset, UK or from Aldrich Chemical Co. Ltd., Gillingham, Dorset, UK. All other enzymes used were purchased from Sigma Chemical Co., Poole, Dorset, UK. The triethylammonium hydrogen carbonate buffer and phosphoenolpyruvate were from Fluka Chemika-BioChemika, Gillingham, Dorset, UK. Radioactive [8-^{14}C]ATP (50 μCi ml^{-1}) was obtained from Amersham International, Aylesbury, Bucks., UK. DEAE-cellulose filters DE-81 were purchased from Whatman, Maidstone, Kent, UK. Bactotryptone and Bactoyeast extract were from Difco Laboratories, East Molsey, Surrey, UK. Purification media S300 and Q-Sepharose were obtained from Pharmacia LKB Biotechnology, Milton Keynes, Bucks., UK. All reagents used were of AnalaR grade or the nearest equivalent. Deionised distilled Milli-Q water was used throughout.

General enzymology

pH-values for buffer solutions were adjusted at room temperature, irrespective of the temperature at which they were subsequently used. SDS/PAGE was carried out using 15% acrylamide gels according to standard procedures.[34a] Gels were stained with Coomassie-blue. Protein concentration measurements were determined by A_{280} measurements, using an $A^{0.1\%}$ value of 0.61 calculated from the ratio of A_{280} and A_{205} of a standard solution of the protein.[34b] A monomer relative molecular mass of 57 800 Da was used for the LysU homodimer.[7b] UV–VIS absorption measurements were carried out on a Pharmacia LKB Ultrospec III at room temperature. ^{1}H NMR enzyme kinetic studies were performed on a Bruker WM 250 (250 MHz) NMR spectrometer. For each kinetic experiment, an acquisition time of 2.72 s and a sweep width of 3012 Hz were used. Spectra were recorded after every 64 FIDs. Radioactivity was measured by liquid scintillation counting on a Kontron Analytical Betamatic 1 liquid scintillation counter. Liquid scintillation was performed using a 'home-made' scintillation fluid of the following composition: naphthalene, 6% (w/v), 2,5-diphenyloxazole, 0.4% (w/v), MeOH, 10% (v/v), ethane-1,2-diol, 2% (v/v) all in 1,4-dioxane.

General synthesis

TLC was performed using 1,4-dioxane–water–NH_3 (v/v/v, 6:3:1) and silica gel 60 F254 precoated (0.2 mm thick) on aluminium sheets. Preparative TLC (PLC) was performed with the same solid phase, which was eluted with the same solvent system. Products were extracted from silica over a period of 24 h with water and then freed of residual solid phase by filtration and chromatography on a column of Q-sepharose (1.5 cm × 10 cm) eluted with an increasing gradient of triethylammonium hydrogen carbonate (from 15 to 600 mM). Preparative HPLC was performed on a Pharmacia LKB Biotechnology fast-protein liquid chromatography (FPLC) system using a reversed-phase PepRPC HR 10/10 column pre-equilibrated with 15 mM triethylammonium hydrogen carbonate buffer and eluted with a shallow gradient of acetonitrile (0–9% v/v) in the same buffer. The sodium salts of pyrophosphoric acid analogues were converted to their acid forms, prior to reaction, by passage through a Dowex-50W X8 (H^+ form) column. Routine ^{31}P NMR spectra were recorded in 80% D_2O solutions either on a JEOL FX90Q or a Bruker WM 250 NMR spectrometer using 80% phosphoric acid as an external reference. Broad-band proton decoupling was employed unless otherwise stated. Routine ^{1}H NMR spectra were recorded on either a JEOL GSX 270 or a Bruker WM 250 NMR spectrometer, using tetramethylsilane as an external reference. Chemical shifts are on the δ-scale and are quoted in ppm, whilst coupling constants J are quoted in Hz. Negative ion fast atom bombardment mass spectra were obtained on a KRATOS MS890 mass spectrometer connected to a KRATOS DS90 data system. For all spectra the accelerating voltage was 4 kV, resolution was set to 1000, and the target was bombarded at 7–8 kV with xenon atoms. The special sample preparation required is recorded elsewhere.[29]

Transformation and growth of *E. coli*

E. coli strain TG2[*supE hsd*Δ5 *thi* Δ(*lac-proAB*), Δ(*srl-recA*)306::Tn*10*(*tet*^{r}) F′(*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] was transformed using standard protocols[34a] with plasmid pXLys5.[12] Strain TG2/pXLys5 was grown at 37 °C in 2YT medium containing ampicillin (100 mg l^{-1}). A 10 ml portion of an overnight shake culture was used to innoculate 2 l of growth medium and the suspension was shaken at 200 rev. min^{-1}. After 16 h, cells were collected by centrifugation (10 800 ***g***; 4 °C; 20 min) and, if not used immediately, were stored at −20 °C. A typical 2 l growth gave 18 g wet weight of cells.

Purification of LysU

All solutions used during the purification of the enzyme contained the following additions, unless otherwise stated: 2 mM β-mercaptoethanol, 2 mM EDTA, 0.6 mM phenylmethanesulfonyl fluoride (previously dissolved in ethanol, 5 ml l^{-1} of final solution), 0.6 mM benzamidine, aprotinin (1 mg l^{-1}) and pepstatin (1 mg l^{-1}). Operations were performed at 0–4 °C, except for the FPLC column steps, which were carried out at ambient temperature (~20 °C), although fractions from these columns were collected on ice. Both the S300 and Q-Sepharose purification steps below were performed with columns attached to a Pharmacia LKB Biotechnology FPLC system and the eluate was continuously monitored at 280 nm. The purity of LysU at each stage of the purification process was assessed by standard enzyme assay and SDS/PAGE.

Washed cells (thawed if previously frozen) were resuspended in 50 mM sodium phosphate buffer, pH 7.5 (2 ml g^{-1} wet mass of cells). After the addition of DNAse (0.1 mg ml^{-1}) and 2 mM $MgCl_2$, cells were lysed by sonication and the cell debris removed by centrifugation (26 000 ***g***, 30 min). A 0.2 volume of 6.2% (w/v) streptomycin sulfate solution was added dropwise to the stirred supernatant. After being stirred for a further 15 min, the precipitate was removed by centrifugation (26 000 ***g***, 30 min). The supernatant was then fractionated with $(NH_4)_2SO_4$, using three stages of saturation from 0–10, 10–30 and 30–45%. Protein precipitating in the latter two fractions was collected by centrifugation (26 000 ***g***, 30 min) and then dissolved in a minimum volume of 50 mM sodium phosphate buffer, pH 7.5, containing 20% (v/v) glycerol. The clear solution was fractionated on a column (5.0 cm × 60 cm) of S300 equilibrated with the last mentioned buffer and fractions (20 ml) were collected at a flow rate of 10 ml min^{-1}. Fractions containing LysU were pooled and applied to a Q-Sepharose column (5.0 cm × 12 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.5. The column was eluted at 10 ml min^{-1} with a linear gradient of 0–0.5 M NaCl in the same buffer. LysU eluted at a NaCl concentration ~0.3 M (60% of gradient) (occasionally, it was necessary to repeat the final Q-Sepharose step employing a longer gradient). 20% Glycerol (v/v) was added to the combined fractions and the protein was stored frozen at −20 °C. Prior to use, LysU was defrosted and dialysed against 20 mM HEPES, pH 7.8, 2 mM β-mercaptoethanol (for enzymology studies) and against 50 mM sodium phosphate, 2 mM β-mercaptoethanol (for synthetic studies).

Radioactive assay of LysU

LysU assays were performed by modification of the Ap_4A synthesis assay of Charlier and Sanchez.[11] A standard assay mixture (5 ml) was prepared containing 100 mM Hepes, 5 mM $MgCl_2$, 5 mM ATP, 160 μM $ZnCl_2$ and 2 mM L-lysine. For each assay, 50 μl aliquots of the standard assay mixture were combined with undialysed inorganic pyrophosphatase (2 μl), [8-^{14}C]ATP (2.5 μl) and an appropriate aliquot of LysU. Each reaction mixture was incubated at 37 °C for 10 min, then boiled for a further 2 min. After the mixture had cooled on ice, undialysed alkaline phosphatase (2 μl) was added and the reaction mixture was incubated at 37 °C for 40 min in order to destroy unreacted ATP. A standard aliquot (30 μl) of final reaction mixture was then diluted into 0.025 M aq. NH_4HCO_3 (1 ml) and then applied, under water-pump pressure, to three-ply pre-wet DE-81 filters (1 cm diameter) mounted on a millipore vacuum filtration manifold (1 cm diameter). The filters were washed with 0.025 M aq. NH_4HCO_3 (3 × 3 ml), dried, and then placed in scintillation fluid (5 ml). After 10 min of continuous shaking on a moving-table shaker, radioactive counting was performed. A blank without enzyme was always run under identical conditions.

Determination of pH and temperature dependence of formation of Ap_4A

The pH and temperature dependence of LysU-catalysed Ap_4A formation was determined by performing the standard radioactive assay described above under a variety of different conditions of pH and temperature, respectively. 660 nM LysU (homodimer) was used in all the assays. pH conditions different from pH 7–8 were established by substituting for 100 mM HEPES with other appropriate buffer salts at the same concentration. Sodium acetate was used for pH 4 and 5, BISTRIS for pH 6 and ethanolamine–HCl for pH 9 and 10.

Determination of k_{cat} and K_M values for Ap_4A formation by LysU

Initial rates for the LysU-catalysed formation of Ap_4A as a function of ATP, L-lysine, $ZnCl_2$, $CdCl_2$ and $CoCl_2$ concentration were determined by ^{1}H NMR spectroscopy at 37 or 45 °C by adapting the method of Plateau and Blanquet.[25] Reaction mixtures (each 0.5 ml) were prepared containing 20 mM HEPES, pH 7.8, 150 mM KCl, 5 mM $MgCl_2$, inorganic pyrophosphatase (0.05 mg ml^{-1}), 20% D_2O (v/v) together with different concentrations of ATP, L-lysine, $ZnCl_2$, $CdCl_2$ and $CoCl_2$ as appropriate for each kinetic study (see Table 1). Each reaction mixture was transferred to a 5 mm NMR tube, where 400 nM LysU (final homodimer concentration) was added. Spectra were recorded every 5 min (or 64 scans) for 40 min. The formation of Ap_4A was followed by the upfield shift of adenine protons 2- and 8-H upon conversion of ATP to Ap_4A. The increase in integrated intensities of the Ap_4A-proton signals in successive spectra provided a measure of the increase of Ap_4A concentration with time. Initial rates of formation of Ap_4A were calculated for each set of initial reaction conditions and values of k_{cat} and K_M were determined by Lineweaver–Burk plot, for each substrate/cofactor.

Investigation of metal-induced protein aggregation

Protein aggregation was measured by absorbance at 400 nm. Aggregation mixtures (each 1 ml) were prepared containing 10 mM $MgCl_2$ and appropriate concentrations of $ZnCl_2$ (0–400 μM) in 20 mM HEPES, pH 7.8. The absorbance of each mixture was measured and then LysU added, to give a final concentration of 440 nM LysU (homodimer concentration). After 30 min incubation at 37 °C, the final absorbance was determined. These experiments were repeated using $CdCl_2$, $CoCl_2$ and $HgCl_2$ in place of $ZnCl_2$.

Proof of mechanism

LysU (0.14 mg, 1.2 nmol) and inorganic pyrophosphatase (14 units) were added to a solution containing adenosine-5′-triphosphate **2** (3 mg, 5.5 μmol, 2.5 mM), L-lysine **4** (0.6 mg, 4 μmol), magnesium chloride (10 mM), potassium chloride (150 mM) in TRIS buffer (20 mM; pH 8; 1.7 ml). The solution was then incubated at 37 °C and the reaction monitored by TLC. After 2.5 h, the solution was filtered and the product was purified by preparative reversed-phase HPLC, eluting at 0.4% acetonitrile. The fractions containing the product were combined to give a freeze-dried solid which was characterised as lysyl adenylate **3** by NMR spectroscopy; δ_H(250 MHz; D_2O) 8.54 (1 H, s, 2-H-Ad), 8.25 (1 H, s, 8-H-Ad), 6.10 (1 H, d, $J_{H1',H2'}$ 5.9, H-1′-Ad) and 3.73 (1 H, t, α-H L-lysine); δ_P(D_2O; pH 7) 5.46 (s, P^{α}, 1 P). Lysyl adenylate **3** was then added to a reconstituted reaction mixture at 37 °C which contained LysU (0.14 mg), adenosine-5′-triphosphate **2** (3 mg, 5.5 μmol, 2.5 mM), L-lysine **4** (0.6 mg, 4 μmol), magnesium chloride (10 mM), potassium chloride (150 mM) and zinc chloride (160 μM) in TRIS buffer (20 mM; pH 8; 1.7 ml). When the reaction was complete, as judged by TLC, the solution was filtered and the new product was collected by reversed-phase HPLC eluting at 3.5% acetonitrile. The fractions containing the product were combined to give a freeze-dried solid which was characterised as Ap_4A **1** (6.4 mg, 78%); δ_H(250 MHz; D_2O) 8.51 (1 H, s, 2-H-Ad), 8.23 (1 H, s, 8-H-Ad), 6.17 (1 H, t, $J_{H1',H2'}$ 6.7, H-1′-Ad); δ_P(D_2O; pH 7) −8.01 (2 P, br d, $^2J_{\alpha\beta}$ 17, P^{α}, $P^{\alpha'}$), −18.43 (2 P, br t, $^2J_{\alpha\beta}$ 17, P^{β}, $P^{\beta'}$,); *m/z* (FAB) 857 ([MNa − H]$^-$, 100%). This complete experiment was repeated with [8-^{14}C]ATP **2a** and the radioactive lysyl adenylate **3a** isolated and counted before onward conversion to radioactive Ap_4A **1a**.

Diadenosine 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate, Ap_4A 1

LysU (0.34 mg, 3 nmol) and inorganic pyrophosphatase (34 units) were added to a solution containing adenosine 5′-triphosphate **2** (8 mg, 15 μmol; 4.3 mM), L-lysine **4** (1.2 mg, 8 μmol; 2.4 mM), zinc chloride (160 μM), magnesium chloride (10 mM) and potassium chloride (150 mM) in TRIS buffer (20 mM; pH 8; 3.4 ml). The solution was incubated at 37 °C and the reaction monitored by TLC until complete (2.5 h). The reaction mixture was filtered (0.2 μm sterile filter), and purified by reversed-phase HPLC with the product eluting at 3.5% acetonitrile. Combined fractions were freeze-dried to give Ap_4A **1** as a solid (8.6 mg, 74%) (Found: [MNa − H]$^-$, 857. $C_{20}H_{26}N_{10}NaO_{19}P_4$ requires 857); δ_H(250 MHz; D_2O) 8.50 (1 H, s, 2-H-Ad), 8.21 (1 H, s, 8-H-Ad) and 6.16 (1 H, t, $J_{H1',H2'}$ 6.7, H-1′-Ad); δ_P(D_2O; pH 7) −7.99 (2 P, br d, $^2J_{\alpha\beta}$ 17, P^{α}, $P^{\alpha'}$) and −18.63 (2 P, br t, $^2J_{\alpha\beta}$ 17, P^{β}, $P^{\beta'}$); *m/z* (FAB) 857 ([MNa − H]$^-$, 100%).

Di-2′-deoxyadenosine 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate, dAp_4dA 15

As for Ap_4A **1**, the desired product **15** was prepared from 2′-deoxyadenosine 5′-triphosphate **12** (20 mg, 37 μmol) as a freeze-dried solid (22 mg, 74%) (Found: [MNa_2 − H]$^-$, 847. $C_{20}H_{25}N_{10}Na_2O_{17}P_4$ requires 847); δ_H(250 MHz; D_2O) 8.31 (1 H, s, 2-H-dAd), 8.05 (1 H, s, 8-H-dAd) and 6.31 (1 H, t, $J_{H1',H2'}$ 6.7, 1′-H-dAd); δ_P(D_2O; pH 7) −11.27 (2 P, br d, $^2J_{\alpha\beta}$ 18, P^{α}, $P^{\alpha'}$) and −22.15 (2 P, br t, $^2J_{\alpha\beta}$ 18, P^{β}, $P^{\beta'}$); *m/z* (FAB) 847 ([MNa_2 − H]$^-$, 100%) and 825 ([MNa − H]$^-$).

Di-3′-deoxyadenosine 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate, 3′-dAp_4dA 16

As for Ap_4A **1**, the desired product **16** was synthesized from 3′-deoxyadenosine 5′-triphosphate **13** (2 mg, 4 μmol) as a freeze-dried solid (0.7 mg, 47%) (Found: [MNa − H]$^-$, 825. $C_{20}H_{26}N_{10}NaO_{17}P_4$ requires 825); δ_H(250 MHz; D_2O) 8.34 (1 H, s, 2-H-dAd), 8.07 (1 H, s, 8-H-dAd) and 6.19 (1 H, t, $J_{H1',H2'}$ 6.6, 1′-H-dAd); δ_P(D_2O; pH 7) −11.51 (2 P, br d, $^2J_{\alpha\beta}$ 18, P^{α}, $P^{\alpha'}$) and −20.98 (2 P, br t, $^2J_{\alpha\beta}$ 18, P^{β}, $P^{\beta'}$); *m/z* (FAB) 825 ([MNa − H]$^-$, 100%).

Diadenosine 5′,5‴-*P*^{1},*P*^{4}-[β,γ-imido]tetraphosphate, AppNHppA 17

As for Ap_4A **1**, the desired product **17** was made from

adenosine 5′-[β,γ-imido]triphosphate **14** (8 mg, 15 μmol) as a freeze-dried solid (4 mg, 61%) (Found: [MNa − H]$^-$, 856. $C_{20}H_{27}N_{11}NaO_{18}P_4$ requires 856); δ_H(250 MHz; D_2O) 8.59 (1 H, s, 2-H-Ad), 8.38 (1 H, s, 8-H-Ad) and 6.08 (1 H, d, $J_{H1',H2'}$ 5.9, 1′-H-Ad); δ_P(D_2O; pH 7) −6.67 (2 P, br t, $^2J_{\alpha\beta}$ 13.2, P$^\alpha$, P$^{\alpha'}$) and −7.61 (2 P, d, $^2J_{\alpha\beta}$ 14, P$^\alpha$, P$^{\alpha'}$); *m/z* (FAB) 856 ([MNa − H]$^-$, 100%).

Product **17** was also prepared (6 mg, 62%) in a similar way to adenosine(guanosine) 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate Ap_4G **23** (below), using adenosine 5′-[β,γ-imido]triphosphate **14** (9 mg, 16 μmol) in place of guanosine 5′-triphosphate **6**.

Adenosine(guanosine) 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate, Ap_4G 23

LysU (0.25 mg, 2 nmol) and inorganic pyrophosphatase (60 units) were added to a solution containing adenosine 5′-triphosphate **2** (13 mg, 23 μmol; 2.5 mM), guanosine 5′-triphosphate **6** (25 mg, 42 μmol; 5 mM), L-lysine **4** (3 mg, 20 μmol), zinc chloride (160 μM), magnesium chloride (10 mM) and potassium chloride (150 mM) in TRIS buffer (20 mM; pH 8; 7.5 ml). The solution was incubated at 37 °C and the reaction monitored by TLC until complete (24 h). The reaction mixture was filtered (0.2 μm sterile filter), and purified by reversed-phase HPLC with the product eluting at 5.5% acetonitrile. Combined fractions were freeze-dried to give Ap_4G **23** as a solid (11 mg, 52%) (Found: [M − H]$^-$, 851. $C_{20}H_{27}N_{10}O_{20}P_4$ requires 851); δ_H(250 MHz; D_2O) 8.46 (1 H, s, 2-H-Ad), 8.18 (1 H, s, 8-H-Ad), 8.00 (1 H, s, 8-H-Gua), 5.84 (1 H, d, $J_{H1',H2'}$ 6.0, 1′-H-Ad) and 5.65 (1 H, d, $J_{H1',H2'}$ 6.2, 1′-H-Gua); δ_P(D_2O; pH 7) −8.55 (2 P, br d, $^2J_{\alpha\beta}$ 17, P$^\alpha$, P$^{\alpha'}$) and −19.21 (2 P, br t, $^2J_{\alpha\beta}$ 17, P$^\beta$, P$^{\beta'}$); *m/z* (FAB) 851 ([M − H]$^-$, 100%).

Adenosine(xanthosine) 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate, Ap_4X 24

As for Ap_4G **23**, the desired product **24** was made using xanthosine 5′-triphosphate **9** (25 mg, 45 μmol; 5 mM) in place of guanosine 5′-triphosphate **6** and was obtained as a freeze-dried solid (13 mg, 66%) (Found: [M − H]$^-$, 852. $C_{20}H_{26}N_9O_{21}P_4$ requires 852); δ_H(250 MHz; D_2O) 8.41 (1 H, s, 2-H-Ad), 8.14 (1 H, s, 8-H-Ad), 7.88 (1 H, s, 8-H-Xan), 5.79 (1 H, d, $J_{H1',H2'}$ 5.8, 1′-H-Ad) and 5.61 (1 H, d, $J_{H1',H2'}$ 5.9, 1′-H-Xan); δ_P(D_2O; pH 7) −8.70 (2 P, br d, $^2J_{\alpha\beta}$ 18, P$^\alpha$, P$^{\alpha'}$) and −20.1 (2 P, br t, $^2J_{\alpha\beta}$ 18, P$^\beta$, P$^{\beta'}$); *m/z* (FAB) 852 ([M − H]$^-$, 100%).

Ap_4X **24** was also prepared (7 mg, 74%) in a similar way to adenosine(inosine) 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate, Ap_4I **30** (below) using xanthosine 5′-triphosphate **9** (12.5 mg, 22 μmol) for inosine 5′-triphosphate **10**.

Adenosine(uridine) 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate, Ap_4U 25

As for Ap_4G **23**, the desired product **25** was made using uridine 5′-triphosphate **7** (25 mg, 42 μmol; 5 mM) in place of guanosine 5′-triphosphate **6** and obtained as a freeze-dried solid (10 mg, 50%) (Found: [M − H]$^-$, 812. $C_{19}H_{26}N_7O_{21}P_4$ requires 812); δ_H(250 MHz; D_2O) 8.46 (1 H, s, 2-H-Ad), 8.19 (1 H, s, 8-H-Ad), 7.99 (1 H, d, $J_{H6,H5}$ 6.3, 6-H-Ur), 6.21 (1 H, d, $J_{H5,H6}$ 6.3, 5-H-Ur), 5.99 (1 H, d, $J_{H1',H2'}$ 5.9, 1′-H-Ur) and 5.89 (1 H, d, $J_{H1',H2'}$ 5.9, 1′-H-Ad); δ_P(D_2O; pH 7) −8.63 (2 P, br d, $^2J_{\alpha\beta}$ 17, P$^\alpha$, P$^{\alpha'}$) and −20.09 (2 P, br t, $J_{\alpha\beta}$ 17, P$^\beta$, P$^{\beta'}$); *m/z* (FAB) 812 ([M − H]$^-$, 100%) and 834 (60, [MNa − H]$^-$).

Adenosine(2′-deoxythymidine) 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate, Ap_4dT 26

As for Ap_4G **23**, the desired product **26** was prepared using 2′-deoxythymidine 5′-triphosphate **8** (25 mg, 40 μmol; 5 mM) in place of guanosine 5′-triphosphate **6** and obtained as a freeze-dried solid (14.5 mg, 73%) (Found: [MNa$_3$ − H]$^-$, 876. $C_{20}H_{25}N_7Na_3O_{20}P_4$ requires 876); δ_H(250 MHz; D_2O) 8.42 (1 H, s, 2-H-Ad), 8.13 (1 H, s, 8-H-Ad), 7.43 (1 H, s, 6-H-Thy), 6.22 (1 H, t, $J_{H1',H2'}$ 6.6, 1′-H-Thy) and 5.81 (1 H, d, $J_{H1',H2'}$ 5.8, 1′-H-Ad); δ_P(D_2O; pH 7) −7.69 (2 P, br d, $^2J_{\alpha\beta}$ 18, P$^\alpha$, P$^{\alpha'}$) and −18.39 (2 P, br t, $^2J_{\alpha\beta}$ 18, P$^\beta$, P$^{\beta'}$); *m/z* (FAB) 876 ([MNa$_3$ − H]$^-$, 100%), 854 ([MNa$_2$ − H]$^-$) and 832 ([MNa − H]$^-$).

Diadenosine 5′,5‴-*P*^{1},*P*^{4}-[α,β-methylene]tetraphosphate, $AppCH_2pA$ 27

As for Ap_4G **23**, product **27** was made using adenosine 5′-[α,β-methylene]triphosphate **19** (10 mg, 18 μmol; 5 mM) *in lieu* of guanosine 5′-triphosphate **6** and obtained as a freeze-dried solid (5 mg, 59%) (lit.,[30] 22%) (Found: [MNa − H]$^-$, 855. $C_{21}H_{28}N_{10}NaO_{18}P_4$ requires 855); δ_H(250 MHz; D_2O) 8.45 (1 H, s, 2-H-Ad), 8.41 [1 H, s, 2-H-Ad(CH$_2$)], 8.09 (1 H, s, 8-H-Ad), 8.08 [1 H, s, 8-H-Ad(CH$_2$)] and 6.05 (2 H, dd, $J_{H1',H2'}$ 5.9, 1′-H-Ad); δ_P(D_2O; pH 7) 19.67 (1 P, d, $^2J_{\alpha\beta}$ 23, P$^\beta$), 12.90 (1 P, d, $^2J_{\alpha\beta}$ 23, P$^\alpha$), −8.35 (1 P, d, $^2J_{\alpha\beta}$ 23.5, P$^{\alpha'}$) and −17.75 (1 P, t, $^2J_{\alpha\beta}$ 23, P$^{\beta'}$); *m/z* (FAB) 855 ([MNa − H]$^-$, 100%) and 833 ([M − H]$^-$).

Diadenosine 5′,5‴-*P*^{1},*P*^{4}-[β,γ-methylene]tetraphosphate, $AppCH_2ppA$ 28

As for Ap_4G **23**, the product **28** was made using adenosine 5′-[β,γ-methylene]triphosphate **20** (22 mg, 40 μmol; 5 mM) in place of guanosine 5′-triphosphate **6** and obtained as a freeze-dried solid (10 mg, 59%) (lit.,[19] 35%) (Found: [MNa − H]$^-$, 855. $C_{21}H_{28}N_{10}NaO_{18}P_4$ requires 855); δ_H(250 MHz; D_2O) 8.41 (1 H, s, 2-H-Ad), 8.16 (1 H, s, 8-H-Ad) and 6.10 (1 H, d, $J_{H1',H2'}$ 5.9, 1′-H-Ad); δ_P(D_2O; pH 7) 11.49 (2 P, br, $^2J_{\alpha\beta}$ 23.5, P$^\beta$, P$^{\beta'}$) and −7.99 (2 P, d, $^2J_{\alpha\beta}$ 23.5, P$^\alpha$, P$^{\alpha'}$); *m/z* (FAB) 855 ([MNa − H]$^-$, 100%) and 833 ([M − H]$^-$).

Diadenosine 5′,5‴-*P*^{1},*P*^{4}-(S)-[α-thio]tetraphosphate, $Appppp_SA$ 29

As for Ap_4G **23**, the desired product **29** was prepared using adenosine 5′-[α-thio]triphosphate **22** (30 mg, 53 μmol; 5 mM), prepared by standard methods,[35,36] *in lieu* of guanosine 5′-triphosphate **6** and obtained as a freeze-dried solid (11 mg, 46%) (Found: [MNa$_2$ − H]$^-$, 895. $C_{20}H_{25}N_{10}Na_2O_{18}P_4S$ requires 895); δ_H(250 MHz; D_2O) 8.59 [1 H, s, 2-H-Ad(S)], 8.41 (1 H, s, 2-H-Ad), 8.19 [2 H, s, 8-H-Ad, 8-H-Ad(S)] and 6.04 [2 H, d, $J_{H1',H2'}$ 6.0, 1′-H-Ad, 1′-H-Ad(S)]; δ_P(D_2O; pH 7) 45.82 [1 P, d, $^2J_{\alpha\beta}$ 26.3, P$^\alpha$(S)], −8.66 (1 P, d, $^2J_{\alpha\beta}$ 18.3, P$^{\alpha'}$), −20.55 (1 P, dd, $^2J_{\alpha\beta}$ 17.3, P$^{\beta'}$) and −21.66 (1 P, dd, $^2J_{\alpha\beta}$ 8.9, P$^\beta$); *m/z* (FAB) 895 ([MNa$_2$ − H]$^-$, 100%) and 873 ([MNa − H]$^-$).

Inhibition by cytidine 5′-triphosphate and inosine 5′-triphosphate

The mode of inhibition and inhibition constants were determined by means of the ^{1}H NMR assay described above. In these experiments, the L-lysine **4**, zinc chloride and magnesium chloride concentrations were fixed at 2 mM, 160 μM and 10 mM, respectively, and the enzyme concentration at 400 nM (homodimer concentration). The adenosine 5′-triphosphate **2** concentrations were varied between 1 and 7.5 mM, whilst concentrations of the inhibitory nucleotides cytidine 5′-triphosphate **5** and inosine 5′-triphosphate **10** were varied between 0.75 and 3 mM. For each inhibitor, Lineweaver–Burk plots were used to represent the kinetic data obtained at each fixed concentration of inhibitor, thereby demonstrating mixed inhibition by both nucleotides. The gradients of each set of Lineweaver–Burk plots were then plotted as a function of the respective inhibitor concentration to determine K_I whilst $K_{I'}$ was evaluated by plotting $1/V_{max}$ as a function of inhibitor concentration. Cytidine 5′-triphosphate **5** was found to inhibit LysU with a K_I of 3.10 mM and a $K_{I'}$ of 3.39 mM; Inosine 5′-triphosphate **10** was found to inhibit with a K_I of 4.28 mM and a $K_{I'}$ of 5.69 mM.

Adenosine(inosine) 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate, Ap_4I 30

LysU (0.23 mg, 2 nmol) and inorganic pyrophosphatase (23 units) were added to a solution containing adenosine 5′-triphosphate **2** (5 mg, 9 μmol; 2.5 mM), L-lysine **4** (1.1 mg, 7.5

μmol), magnesium chloride (10 mM) and potassium chloride (150 mM) in TRIS buffer (20 mM; pH 8; 2.75 ml). This solution was incubated at 37 °C and the reaction monitored by TLC until formation of the lysyl adenylate **3** was complete (2.5 h). Inosine 5′-triphosphate **10** (10 mg, 18 μmol; 5 mM) followed by zinc chloride (160 μM) were then added and the reaction was left to incubate at 37 °C until completion. The mixture was filtered (0.2 μm sterile filter unit), and purified by reversed-phase HPLC with product eluting at 0.3% acetonitrile product. Fractions were combined and freeze-dried to give Ap_4I **30** as a solid (5.5 mg, 70%) (Found: $[M - H]^-$, 836. $C_{20}H_{26}N_9O_{20}P_4$ requires 836); δ_H(250 MHz; D_2O) 8.46 (1 H, s, 2-H-Ad), 8.30 (1 H, s, 2-H-I), 8.17 (1 H, s, 8-H-Ad), 8.09 (1 H, s, 8-H-I), 6.07 (1 H, d, $J_{H1',H2'}$ 5.9, 1′-H-Ad) and 6.00 (1 H, d, $J_{H1',H2'}$ 6.3, 1′-H-I); δ_P(D_2O; pH 7) −8.88 (2 P, br d, $^2J_{\alpha\beta}$ 17, P^{α}, $P^{\alpha'}$) and −20.18 (2 P, br t, $^2J_{\alpha\beta}$ 17, P^{β}, $P^{\beta'}$); *m/z* (FAB) 858 ($[MNa - H]^-$, 60%) and 836 (100, $[M - H]^-$).

Adenosine(8-bromoadenosine) 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate, Ap_4BrA 31

As for Ap_4I **30**, the desired product **31** was made using 8-bromoadenosine 5′-triphosphate **11** (5 mg, 8 μmol; 5 mM) *in lieu* of inosine 5′-triphosphate **10** and obtained as a freeze-dried solid (2.5 mg, 62%) (Found: $[M - H]^-$, 914. $C_{20}H_{26}BrN_{10}O_{19}P_4$ requires 914); δ_H(250 MHz; D_2O) 8.45 (1 H, s, 2-H-Ad), 8.15 (1 H, s, 8-H-Ad), 8.06 (1 H, s, 2-H-8-Br-Ad), 6.03 (1 H, d, $J_{H1',H2'}$ 5.9, 1′-H-Ad) and 5.95 (1 H, d, $J_{H1',H2'}$ 6.3, 1′-H-8-Br-Ad); δ_P(D_2O; pH 7) −8.68 (2 P, br d, $^2J_{\alpha\beta}$ 18, P^{α}, $P^{\alpha'}$) and −20.05 (2 P, br t, $^2J_{\alpha\beta}$ 18, P^{β}, $P^{\beta'}$); *m/z* (FAB) 914 ($[M - H]^-$, 100%).

Adenosine(cytidine) 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate, Ap_4C 32

As for Ap_4I **30**, product **32** was synthesized using cytidine 5′-triphosphate **5** (15 mg, 28 μmol; 6 mM) in place of inosine 5′-triphosphate **10** and obtained as a freeze-dried solid (4 mg, 63%) (Found: $[MNa_2 - H]^-$, 855. $C_{19}H_{25}N_8Na_2O_{20}P_4$ requires 855); δ_H(250 MHz; D_2O) 8.53 (1 H, s, 2-H-Ad), 8.24 (1 H, s, 8-H-Ad), 7.83 (1 H, s, $J_{H5,H6}$ 7.6, 6-H-Cyt), 6.11 (1 H, d, $J_{H1',H2'}$ 6.4, 1′-H-Cyt) and 5.94 (2 H, dd, $J_{H5,H6}$ 8, 5-H-Cyt, $J_{H1',H2'}$ 5.9, 1′-H-Ad); δ_P(D_2O; pH 7) −8.69 (2 P, d, $^2J_{\alpha\beta}$ 12.2, P^{α}, $P^{\alpha'}$) and −20.40 (2 P, br t, $^2J_{\alpha\beta}$ 7.5, P^{β}, $P^{\beta'}$); *m/z* (FAB) 855 ($[MNa_2 - H]^-$, 100%) and 833 ($[MNa - H]^-$).

Adenosine(2′-deoxyadenosine) 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate, Ap_4dA 33

As for Ap_4I **30**, the desired product **33** was prepared using 2′-deoxyadenosine 5′-triphosphate **12** (10 mg, 19 μmol; 3 mM) instead of inosine 5′-triphosphate **10** and obtained as a freeze-dried solid (10 mg, 73%) (Found: $[M - H]^-$, 819. $C_{20}H_{27}N_{10}O_{18}P_4$ requires 819); δ_H(250 MHz; D_2O) 8.41 (1 H, s, 2-H-Ad), 8.38 (1 H, s, 2-H-d-Ad), 8.19 (1 H, s, 8-H-Ad), 8.17 (1 H, s, 8-H-d-Ad), 6.38 (1 H, t, $J_{H1',H2'}$ 6.1, 1′-H-d-Ad) and 6.05 (1 H, d, $J_{H1',H2'}$ 6.1, 1′-H-Ad); δ_P(D_2O; pH 7) −8.46 (2 P, br d, $^2J_{\alpha\beta}$ 17, P^{α}, $P^{\alpha'}$) and −20.26 (2 P, br t, $^2J_{\alpha\beta}$ 17, P^{β}, $P^{\beta'}$); *m/z* (FAB) 819 ($[M - H]^-$, 100%).

Adenosine(adenine arabinofuranoside) 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate, Ap_4[Ara-A] 34

As for Ap_4I **30**, the desired product **34** was made using adenosine arabinofuranoside 5′-triphosphate **18** (20 mg, 36 μmol; 5 mM), prepared by standard procedures,[37,38] *in lieu* of inosine 5′-triphosphate **10** and obtained as a freeze-dried solid (9 mg, 52%) (Found: $[MNa - H]^-$, 857. $C_{20}H_{26}N_{10}NaO_{19}P_4$ requires 857); δ_H(250 MHz; D_2O) 8.53 (1 H, s, 2-H-Ad), 8.50 (1 H, s, 2-H-Ara-Ad), 8.26 (1 H, s, 8-H-Ad), 8.21 (1 H, s, 8-H-Ara-Ad), 6.43 (1 H, d, $J_{H1',H2'}$ 6.1, 1′-H-Ara-Ad) and 6.12 (1 H, d, $J_{H1',H2'}$ 6.3, 1′-H-Ad); δ_P(D_2O; pH 7) −3.38 (2 P, br d, $^2J_{\alpha\beta}$ 18, P^{α}, $P^{\alpha'}$) and −7.62 (2 P, br t, $^2J_{\alpha\beta}$ 18, P^{β}, $P^{\beta'}$); *m/z* (FAB) 857 ($[MNa - H]^-$, 100%).

Adenosine 5′-tetraphosphate, Ap_4 35

As for Ap_4I **30**, product **35** was prepared using sodium tripolyphosphate (13 mg, 35 μmol; 5 mM) in place of inosine 5′-triphosphate **10** and obtained as a freeze-dried solid (5.5 mg, 52%) (Found: $[M - H]^-$, 586. $C_{10}H_{16}N_5O_{16}P_4$ requires 586); δ_H(250 MHz; D_2O) 8.62 (1 H, s, 2-H-Ad), 8.44 (1 H, s, 8-H-Ad) and 6.15 (1 H, d, $J_{H1',H2'}$ 6.1, 1′-H-Ad); δ_P(D_2O; pH 7) −3.63 (1 P, d, $^2J_{\gamma\delta}$ 15.5, P^{δ}), −9.56 (1 P, d, $^2J_{\alpha\beta}$ 14.5, P^{α}), −16.98 (1 P, t, $^2J_{\alpha\beta}$ 14.5, P^{β}) and −19.36 (1 P, m, $^2J_{\gamma\delta}$, 15.5, P^{γ}); *m/z* (FAB) 586 ($[M - H]^-$, 100%).

Adenosine(5-fluoro-2′-deoxyuridine) 5′,5‴-*P*^{1},*P*^{4}-tetraphosphate, Ap_4d[5-fluoro-U] 41

As for Ap_4I **30**, product **41** was prepared using 5-fluoro-2′-deoxyuridine 5′-triphosphate **36** (12 mg, 23 μmol; 5 mM), synthesized by standard methods,[37,38] in place of inosine 5′-triphosphate **10** and obtained as a freeze-dried solid (5.5 mg, 55%) (Found: $[MNa - H]^-$, 836. $C_{19}H_{24}FN_7NaO_{20}P_4$ requires 836); δ_H(250 MHz; D_2O) 8.54 (1 H, s, 2-H-Ad), 8.23 (1 H, s, 8-H-Ad), 7.75 (1 H, d, $J_{5,6}$ 6.3, 6-H-fluoro-dU), 6.25 (1 H, t, $J_{H1',H2'}$ 6.2, 1′-H-fluoro-dU) and 6.13 (1 H, d, $J_{H1',H2'}$ 6.3, 1′-H-Ad); δ_P(D_2O; pH 7) −8.53 (2 P, br d, $^2J_{\alpha\beta}$ 17, P^{α}, $P^{\alpha'}$) and −19.27 (2 P, br t, $^2J_{\alpha\beta}$ 17, P^{β}, $P^{\beta'}$); *m/z* (FAB) 858 ($[MNa_2 - H]^-$, 60%), 836 (100, $[MNa - H]^-$) and 814 ($[M - H]^-$).

Adenosine(uridine) 5′,5‴-*P*^{1},*P*^{4}-[β,γ-methylene]tetraphosphate, $AppCH_2ppU$ 42

As for Ap_4I **30**, the desired product **42** was prepared using uridine 5′-[β,γ-methylene]triphosphate **37** (30 mg, 60 μmol; 5 mM), synthesized by a standard procedure,[38] in place of inosine 5′-triphosphate **10** and obtained as a freeze-dried solid (10 mg, 40%) (Found: $[M - H]^-$, 810. $C_{20}H_{28}N_7O_{20}P_4$ requires 810); δ_H(250 MHz; D_2O) 8.60 (1 H, s, 2-H-Ad), 8.39 (1 H, s, 8-H-Ad), 7.98 (1 H, d, $J_{H5,H6}$ 6.2, 6-H-Ur), 6.17 (1 H, d, $J_{H1',H2'}$ 5.9, 1′-H-Ur) and 5.99 (2 H, dd, $J_{H1',H2'}$ 6.3, 1′-H-Ad, $J_{H5,H6}$ 5.8, 5-H-Ur); δ_P(D_2O; pH 7) 12.05 (2 P, br, $^2J_{\alpha\beta}$ 20.7, P^{β}, $P^{\beta'}$) and −7.87 (2 P, d, $^2J_{\alpha\beta}$ 22.5, P^{α}, $P^{\alpha'}$); *m/z* (FAB) 810 ([M − H]$^-$, 100%).

2′-Deoxyadenosine(uridine) 5′,5‴-*P*^{1},*P*^{4}-[β,γ-imido]tetraphosphate, dAppNHppU 43

As for Ap_4I **30**, product **43** was made from reactions of 2′-deoxyadenosine 5′-triphosphate **12** (11 mg, 20 μmol; 2.5 mM) with uridine 5′-[β,γ-imido]triphosphate **39** (20 mg, 40 μmol; 5 mM), synthesized by a standard procedure,[38] and obtained as a freeze-dried solid (6.5 mg, 38%) (Found: $[M - H]^-$, 795. $C_{19}H_{27}N_8O_{19}P_4$ requires 795); δ_H(250 MHz; D_2O) 8.49 (1 H, s, 2-H-Ad), 8.21 (1 H, s, 8-H-Ad), 7.93 (1 H, d, $J_{H5,H6}$ 6.4, 6-H-Ur), 6.49 (1 H, t, $J_{H1',H2'}$ 6.6, 1′-H-Ad) and 5.91 (2 H, dd, $J_{H1',H2'}$ 5.9, 1′-H-d-Ur, $J_{H5,H6}$ 6.3, 5-H-Ur); δ_P(D_2O; pH 7) −3.82 (2 P, br, $^2J_{\alpha\beta}$ 15, P^{β}, $P^{\beta'}$) and −8.21 (2 P, d, $^2J_{\alpha\beta}$ 16, P^{α}, $P^{\alpha'}$); *m/z* (FAB) 795 ($[M - H]^-$, 100%).

Adenosine(guanosine) 5′,5‴-*P*^{1},*P*^{4}-[β,γ-methylene]tetraphosphate, $AppCH_2ppG$ 44

As for Ap_4I **30**, the desired product **44** was made using guanosine 5′-[β,γ-methylene]triphosphate **38** (5.5 mg, 10 μmol; 5 mM), synthesized by a standard procedure,[38] in place of inosine 5′-triphosphate **10** and obtained as a freeze-dried solid (2 mg, 35%) (Found: $[M - H]^-$, 849. $C_{21}H_{29}N_{10}O_{19}P_4$ requires 849); δ_H(250 MHz; D_2O) 8.51 (1 H, s, 2-H-Ad), 8.23 (1 H, s, 8-H-Ad), 8.15 (1 H, s, 8-H-Gua), 6.09 (1 H, d, $J_{H1',H2'}$ 5.9, 1′-H-Ad) and 5.88 (1 H, d, $J_{H1',H2'}$ 6.1, 1′-H-Gua); δ_P(D_2O; pH 7) 11.99 (2 P, br, $^2J_{\alpha\beta}$ 21.1, P^{β}, $P^{\beta'}$) and −7.95 (2 P, d, $^2J_{\alpha\beta}$ 22.5, P^{α}, $P^{\alpha'}$); *m/z* (FAB) 849 ($[M - H]^-$, 100%).

Adenosine(guanosine) 5′,5‴-*P*^{1},*P*^{4}-[β,γ-imido]tetraphosphate, $AppNHppG$ 45

As for Ap_4I **30**, product **45** was synthesized using guanosine

5′-[β,γ-imido]triphosphate **40** (19 mg, 34 μmol; 5 mM), synthesized by a standard procedure,[38] *in lieu* of inosine 5′-triphosphate **10** and obtained as a freeze-dried solid (5 mg, 31%) (Found: $[M - H]^-$, 850. $C_{20}H_{28}N_{11}O_{19}P_4$ requires 850); δ_H(250 MHz; D_2O) 8.55 (1 H, s, 2-H-Ad), 8.25 (1 H, s, 8-H-Ad), 8.16 (1 H, d, 8-H-Gua), 6.13 (1 H, d, $J_{H1',H2'}$ 6.1, 1′-H-Ad) and 5.93 (1 H, d, $J_{H1',H2'}$ 5.9, 1′-H-Gua); $\delta_P(D_2O$; pH 7) − 6.60 (2 P, br, $^2J_{\alpha\beta}$ 21, P^{β}, $P^{\beta'}$) and −7.31 (2 P, d, $^2J_{\alpha\beta}$ 23, P^{α}, $P^{\alpha'}$); *m/z* (FAB) 850 ($[M - H]^-$, 100%).

Diadenosine 5′,5‴-*P*^{1},*P*^{3}-triphosphate, Ap_3A 46

As for Ap_4I **30**, triphosphate **46** was prepared using adenosine 5′-diphosphate **47** (8 mg, 17 μmol; 5 mM) in place of inosine 5′-triphosphate **10** and obtained as a freeze-dried solid (2 mg, 31%) (Found: $[M - H]^-$, 755. $C_{20}H_{26}N_{10}O_{16}P_3$ requires 755); δ_H(250 MHz; D_2O) 8.57 (1 H, s, 2-H-Ad), 8.37 (1 H, s, 8-H-Ad) and 6.15 (1 H, d, $J_{H1',H2'}$ 6.1, 1′-H-Ad); $\delta_P(D_2O$; pH 7) −8.60 (2 P, d, $^2J_{\alpha\beta}$ 18.5, P^{α}, $P^{\alpha'}$) and −19.90 (1 P, t, $^2J_{\alpha\beta}$ 18.1, P^{β}); *m/z* (FAB) 755 ($[M - H]^-$, 100%).

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