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Research Overview

Recent Progress in the Study of the Intracellular Functions of Diadenosine Polyphosphates

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ABSTRACT Recent developments in the effort to understand the metabolism and function of the intracellular dinucleoside polyphosphates were described by nine speakers from some of the world's leading laboratories in this field in a workshop at the Purines 2000 International Symposium on Nucleosides and Nucleotides held in Madrid in July, 2000. Topics were wide-ranging and included phenotypic analyses of yeast mutants defective in enzymes of dinucleoside polyphosphate degradation, virally encoded catabolic enzymes, the structure and function of the Fhit tumor suppressor and Fhit-nitrilase fusion proteins and the relationship of Fhit to human diadenosine triphosphate hydrolase, site-directed mutagenesis of diadenosine tetraphosphate hydrolase, novel nucleotide analogs for studying hydrolase function, the synthesis of dinucleoside polyphosphates by ligases, and the possible roles of diadenosine tri- and tetraphosphates in insulin function and of diadenosine tetraphosphate in the heat-shock response of *Escherichia coli*. The results presented and the ensuing discussions showed that, while considerable progress is being made in the field, it still has the capacity to tease and frustrate and produce the unexpected result. Drug Dev. Res. 52:249–259, 2001.

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INTRODUCTION

The functions of the intracellular, mainly cytosolic, diadenosine 5′, 5‴-P^1, P^n polyphosphates (Ap_nA, n = 3–6) are still not clear despite many years of investigation. Their levels respond to various metabolic stresses, they bind with relatively high affinity and specificity to a number of intracellular proteins, and they have been implicated in a variety of processes, including the regulation of DNA replication and repair, but none of these possibilities is supported by unequivocal evidence [McLennan, 2000]. Several new developments were reported at a workshop devoted to the intracellular dinucleoside polyphosphates organized by Antonio Sillero (Universidad Autonoma de Madrid) and Sandy McLennan (University of Liverpool) at the Purines 2000 International Symposium on Nucleosides and Nucleotides held in Madrid in July, 2000, which encourage the view that the functions of the Ap_nAs are finally nearing clarification, although it was also clear that there are still some unexpected obstacles in the way.

Enzymes of Ap_nA Catabolism

For example, recent research in Larry Barnes' laboratory (University of Texas Health Science Center, San Antonio) focusing on the deletion and overexpression of enzymes of Ap_nA metabolism and subsequent measurement of Ap_nA levels has yielded some surprising results.

Schizosaccharomyces pombe Aps1 is one of three enzymes, including *Saccharomyces cerevisiae* Ddp1p (YOR163w) and *Homo sapiens* DIPP, that will hydrolyze both Ap_nA and diphosphorylated inositol polyphosphates (DIPs) in vitro [Safrany et al., 1999]. Aps1, Ddp1p, and DIPP all contain the Nudt sequence motif (also called the MutT motif or nudix box) [Yang et al., 1999]. Aps1 prefers Ap_6A and Ap_5A over all other Ap_nAs and will degrade both bisdiphosphoinositol tetrakisphophate ($[PP]_2$-$InsP_4$) and diphosphoinositol pentakisphosphate ([PP]-$InsP_5$) [Ingram et al., 1999; Safrany et al., 1999]. Which of these substrates is degraded in vivo by Aps1 is unknown. Ap_5A and Ap_6A exist in higher eukaryotes and are proposed to function as both intracellular and extracellular signaling molecules. Extracts of *S. pombe* can synthesize DIPs in vitro [Ongusaha et al., 1998] but the function of these compounds in *S. pombe* is unknown.

To investigate further the in vivo function of Aps1, the *aps1* gene was disrupted and overexpressed in *S. pombe* and the endogenous levels of Ap_6A and Ap_5A measured using boronate and anion-exchange chromatographies. Ap_5A was present in wild-type yeast at about 5–10% the level of Ap_4A, while Ap_6A was undetectable. Disruption of *aps1* resulted in a significant decrease in Ap_6A hydrolase activity, no detectable change in intracellular Ap_5A concentration, and no detectable change in morphology or growth rate in rich and minimal media. When *aps1* and its promoter region were cloned into pUR18, a multicopy plasmid, and transformed into *S. pombe*, transformants displayed slow growth in minimal medium and showed a subtle change in morphology. The cells were slightly enlarged (both width and length) throughout the growth phase and did not shorten upon entering stationary phase. Such transformants exhibited a 2-fold increase in Ap_6A hydrolase activity in vitro. To intensify the change in phenotype, *aps1* was expressed using an integrated expression cassette which drives *aps1* expression via the cytomegalovirus promoter. These cells displayed almost no growth in minimal medium and microcolonies on plates contained multiseptate cells. When *aps1* was expressed at an intermediate level via pREP3X, a plasmid with a thiamine-repressible promoter, the growth rate was three times slower than wild-type and the cells exhibited a "round-bottom flask" morphology. Such transformants exhibited a 5-fold increase in Ap_6A hydrolase activity in vitro. To verify that enzyme activity was required for the change in phenotype, a mutated form of the enzyme, E93Q, which has no detectable Ap_6A hydrolase activity in vitro, was also expressed. *Schizosaccharomyces pombe* transformed with pREP3X*aps1*E93Q displayed no changes in growth rate or morphology. The levels of Ap_5A in the pUR18*aps1* and pREP3X*aps1* overexpression strains were, paradoxically, 3 and 50 times, respectively, the Ap_5A level in wild-type strain. Ap_6A was undetectable in the disruption and overexpression strains. These data indicate that: 1.) Ap_5A and Ap_6A probably are not in vivo substrates for Aps1; 2.) the enzymatic activity of Aps1 is required for the changes in phenotype in the overexpression strains; and 3.) either Aps1 or one of its likely DIP substrates may be involved in fission yeast morphogenesis and normal progression through septation and cytokinesis. Although Aps1 was initially characterized as an Ap_nA hydrolase [Ingram et al., 1999], previous results [Safrany et al., 1999] and the data presented here suggest that the DIPs are the in vivo substrates.

Results similar to the above were also obtained in Sandy McLennan's laboratory (University of Liverpool) with the budding yeast *S. cerevisiae*. Cell extracts were made from log and stationary phase wild-type and null mutants in the *DDP1* gene (encoding the Ap_6A/DIP hydrolase). After exhaustive alkaline phosphatase treatment of the extracts, ion-exchange chromatography was used to separate the remaining dinucleotides into the families Ap_4N (where N = A, G, C, or U), Ap_5N and $Ap_{\geq 6}N$ (presumed to contain putative Ap_6N and possible higher-order polyphosphates) on the basis of the known chromatographic properties of authentic standards.

Four- to eight-fold increases in Ap_5N and $Ap_{\geq 6}N$ were observed in stationary phase wild-type cells com-

pared to log phase cells, but only a slight increase in Ap_4N. Similar increases in Ap_5N and $Ap_{\geq 6}N$ were seen in the *DDP1* null mutant as it entered stationary phase; however, the differences in levels between the wild-type and null mutant in both phases were at most 2-fold. The greatest difference found was in the amount of Ap_4N nucleotides when comparing stationary phase wild type (4.9 ± 0.6 pmol/mg protein) and *DDP1* null mutant (18.5 ± 2.8 pmol/mg protein) cells. The corresponding values for log phase cells were 3.5 ± 0.7 and 7.0 ± 0.6 pmol/mg protein, respectively. Thus, despite the fact that Ap_4A is barely used by Ddp1p as a substrate in vitro [Cartwright and McLennan, 1999], the greatest effect of disrupting the *DDP1* gene on Ap_nN pools is on Ap_4N. Ap_5N and $Ap_{\geq 6}N$ show only slight increases.

Corresponding measurements of DIP concentrations have been made in collaboration with Steve Safrany (University of Dundee) and show a 4–8–fold higher level of a [PP]-$InsP_5$ isomer (but no change in $[PP]_2$-$InsP_4$) in the *DDP1* null mutant compared to wild-type (both log and stationary phase). Therefore, as with *S. pombe*, there is as yet no strong evidence that the Ddp1p is responsible for metabolizing the higher-order Ap_nA compounds in *S. cerevisiae* but may be of more relevance to DIP metabolism and function.

Like the Ap_6A/DIP hydrolases, the more widely studied (asymmetrical) dinucleoside tetraphosphate hydrolases (EC 3.6.1.17), commonly referred to as Ap_4A hydrolases, are also members of the nudix hydrolase family and possess the Nudt motif [Maksel et al., 1998; Cartwright et al., 1999]. Andrzej Guranowski (Agricultural University of Poznan) reported on site-directed mutagenesis studies of the enzyme from the narrow-leafed lupin *Lupinus angustifolius* recently carried out in collaboration with Ken Gayler's group at the Department of Biochemistry and Molecular Biology, University of Melbourne, as part of their effort to determine the importance to catalysis of particular amino acid residues acids within this motif and in a short region towards the carboxyl terminus.

The sequence encoding the first 39 amino acids was removed from the cloned Ap_4A hydrolase cDNA [Maksel et al., 1998] in order to generate a product equivalent to the native (18.5 kDa) protein (residues previously designated M40 to L199 are now 1–160). In addition to K_m and k_{cat}, the mutants were characterized by two other measurable parameters—the positional preference of cleavage of a model substrate, 2′-deoxyadenylated Ap_4A, (2′-pdA)Ap_4A (produced by the action of 2′,5′-oligoadenylate synthetase on Ap_4A), and the susceptibility to fluoride inhibition. The former parameter was chosen, first because it was recently demonstrated that human Ap_4A hydrolase easily hydrolyzes 2′-adenylated- and 2′-deoxyadenylated Ap_4A [Guranowski et al., 2000], second because the plant counterpart exhibited the same property, and finally because the preference of cleavage of (2′-pdA)Ap_4A by the human enzyme to the product pairs ATP + (2′-pdA)AMP (80%) rather than to (2′-pdA)ATP + AMP (20%), differed from the preference observed with the lupin wild-type Ap_4A hydrolase. (These measurements were achieved using the enzymically synthesized model substrate, (2′-pdA)[^{3}H]Ap_4A, and a thin-layer chromatography system which separates [^{3}H]ATP from (2′-pdA)[^{3}H]ATP [Guranowski et al., 2000]).

Some mutants had an altered cleavage pattern for (2′-pdA)Ap_4A. Whereas the wild-type enzyme hydrolyzed this model compound to ATP + (2′-pdA]AMP (60%) and to (2′-pdA)ATP + AMP (40%), mutants R54Q, E58D, and E125Q hydrolyzed this substrate to the same product pairs in a 70:30 ratio, while E58Q did so in a 55:45 ratio. These results showed that the mutations produced subtle changes in binding of the substrate analog, although they changed neither the overall structure of the enzyme, as detected by UV circular dichroism, nor the pattern of Ap_4A cleavage from asymmetrical to symmetrical.

The other parameter was chosen because high sensitivity to fluoride is a feature very typical of (asymmetrical) Ap_4A hydrolases [Guranowski, 1990; Maksel et al., 1998; Cartwright et al., 1999] and it was expected that a comparison of different mutants would reveal amino acid(s) potentially responsible for such strong inhibition of catalysis by this anion—the IC_{50} values are 2–3 μM for the plant enzymes [Guranowski, 1990; Maksel et al., 1998]. The E55Q mutant, which had a 20-fold lower K_m for Ap_4A and a 10,000-fold lower k_{cat} than the wild-type enzyme, was insensitive to fluoride. Mutant R54Q retained residual susceptibility to fluoride with an IC_{50} of 1 mM. Mutants E58D and E58Q retained moderate, and E59D almost full, sensitivity to fluoride with IC_{50} values of 50, 20, and 6 μM, respectively. The same values for the mutants generated in another part of the enzyme protein, E122Q and E125Q, were 4 and 250 μM, respectively. These data suggest that it is arginine 54, properly oriented to glutamate 55 and to glutamate 125, that interacts with fluoride. These structural–functional studies are of great importance and hopefully will soon be extended to the animal Ap_4A hydrolases, particularly in view of the possibility highlighted by McLennan (University of Liverpool) that the plant and animal Ap_4A hydrolases may be more distantly related within the nudix family than might be expected from their very similar properties. A phylogenetic analysis of the genes for all known nudix asymmetrical Ap_4A hydrolases shows that the animal enzymes cluster with those from thermophilic bacteria and are in fact more closely related to the Ap_6A/DIP hydrolases than to the plant Ap_4A hydrolases, which cluster with another group of prokaryotic enzymes, those from Gram-negative invasive pathogens. Thus, the plant

and animal Ap_4A hydrolases may have acquired a common substrate specificity within the nudix family by convergent evolution.

A new member of the nudix hydrolase family with specificity for dinucleoside and nucleoside polyphosphates and which clusters with the animal Ap_4A hydrolases was also described by McLennan. African swine fever virus (ASFV) is a double-stranded DNA virus with a single nudix hydrolase gene (g5R or D250). This gene was cloned and expressed in *Escherichia coli* and shown to encode an enzyme that degrades a number of (di)nucleoside polyphosphates. Good substrates (with relative percentage degradation at 200 μM in brackets) were adenosine 5′-pentaphosphate (100), adenosine 5′-tetraphosphate (92), GTP (87), Gp_4G (76), Gp_3G (46), ATP (71), Ap_5A (41), Ap_4A (29), and Ap_3A (13). In the case of Gp_4G, for example, hydrolysis was mainly symmetrical with GDP as the major product. However, some asymmetrical hydrolysis to GTP and GMP was also evident. No interconversion of products was observed. Vaccinia virus, another double-stranded DNA virus, has two nudix hydrolase genes, one of which (D10) selectively inhibits cap-dependent translation when overexpressed [Shors et al., 1999]. This suggested that D10 protein might hydrolyze cap structures. However, when the ASFV g5R protein was tested with 7-methylguanine-containing dinucleotides such as 7-methylguanosine(5′)tetraphospho(5′)guanosine ($m7Gp_4G$), the activity was greatly reduced to the point that cap analog $m7Gp_3A$ was inactive as a substrate. Thus, one possible function of the ASFV g5R protein may be to eliminate free guanine dinucleotides such as Gp_4G, which may be produced by viral guanylyltransferases, to prevent them from interfering with cap-dependent reactions. Since vaccinia D10 differs considerably from g5R and lacks a conserved tyrosine residue found in all Ap_4A hydrolases, D10 may have a different function from g5R.

FHIT Ap_3A Hydrolase and Nucleotide Analogs

Charles Brenner (Thomas Jefferson University, Philadelphia) reported on one of the most exciting developments in the field of intracellular Ap_nA function—the recent demonstration that the product of the human *FHIT* tumor suppressor gene, Fhit, has Ap_3A hydrolase activity. Such an activity, which cleaves Ap_3A into ADP and AMP, was first purified from rat liver by Sillero et al. [1977], but largely ignored until the *FHIT* gene was positionally cloned from FRA3B, the most fragile site in the human genome, by Huebner and co-workers [Ohta et al., 1996] and shown to be an Ap_3A hydrolase by Barnes et al. [1996]. Fhit is the ortholog of the *S. pombe* Ap_4A hydrolase [Huang et al., 1995] and thus it is considered that Fhit-related enzymes are a branch of the histidine triad superfamily of dimeric nucleotide-binding proteins that hydrolyze a range of dinucleoside polyphosphates [Brenner et al., 1999], generating an AMP product plus the other mononucleotide. The structure of Fhit explains how these enzymes accomplish this specificity: in Fhit bound to nonhydrolyzable Ap_3A, one nucleoside is tightly bound to each protomer while the other nucleoside moiety is more solvent-exposed [Pace et al., 1998]. Genetic, biochemical, and crystallographic data support the notion that the active signaling form of Fhit is bound to Ap_nA substrates [Pace et al., 1998]. Thus, more slowly cleaved dinucleoside polyphosphates might actually promote the biological activity of Fhit.

Human and murine genetics have demonstrated that loss of Fhit is associated with cancer in many tissues and that re-expression of Fhit induces programmed cell death [Huebner et al., 1999; Fong et al., 2000]. If Fhit function depends on complexing with Ap_nA, then the cellular systems that generate intracellular polyphosphates ought to be the upstream regulators of Fhit activity. Novel fluorescent and fluorigenic Fhit substrates, ApppBODIPY and GpppBODIPY, have been used to test whether rare Ap_nA would be able to bind Fhit in the face of high cellular concentrations of ATP. Indeed, Fhit binds the Ap_nA's 100-fold better than ATP and 10-fold better than pyrophosphate, potentially indicating that Ap_nA's might displace pyrophosphate in a postulated "ground-state" of Fhit [Draganescu et al., 2000].

The downstream effectors that might transmit Fhit-substrate complex formation to pro-apoptotic machinery are being hotly pursued. One fascinating molecule to emerge is Nit, a member of the nitrilase superfamily, that is fused to Fhit in flies and worms and coordinately expressed from separate genes in vertebrates and fungi [Pekarsky et al., 1998]. Whether Nit is a Fhit effector is not known. Nonetheless, the existence of the NitFhit as a fusion protein in invertebrates, the coordinated expression of Nit and Fhit in mouse, and the fact that the Nit branch of the nitrilase superfamily and the Fhit branch of the histidine triad superfamily are found in the same forms of life [Pace et al., 2000] satisfy Eisenberg's criteria for likely functional significance of NitFhit as a "Rosetta Stone" protein fusion event [Marcotte et al., 1999]. NitFhit turns out to be a tetramer in which a central Nit domain binds two Fhit dimers, presenting them on the opposite poles of a 200 kDa complex, now defined at 2.8 Å resolution. Curiously, the C-terminal elements of Nit polypeptides are extended out of the Nit tetramer and are in intimate contact with Fhit dimer domains [Pace et al., 2000]. While the substrates of Nit proteins remain to be identified and the nature of regulation by Fhit is still unknown, the community of "weird-nucleotide" fanciers appears to be ready to embrace Nit substrates and products if they lead to the normal cell and cancer cell biology of dinucleoside polyphosphates.

An important aspect of understanding Fhit function is to assimilate the albeit limited data from much earlier studies of Ap_3A hydrolases into the story. In particular, are Fhit and the previously described mammalian Ap_3A hydrolase the same enzyme? Pedro Rotllán and co-workers (University of La Laguna) have addressed this problem. The enzyme isolated from various rat tissues appears to be a monomeric protein of ~32 kDa. The human Fhit protein, also an Ap_3A hydrolase, is a homodimer of two 16.8 kDa [Barnes et al., 1996], suggesting that the previously known Ap_3A hydrolase and Fhit could be the same protein. While human Fhit protein expressed in *E. coli* is readily available [Barnes et al., 1996; Pawelczyk et al., 2000], Ap_3A hydrolase has not yet been isolated directly from human cells.

Rotllán and co-workers have undertaken the purification of human Ap_3A hydrolase and compared its properties with those of the human Fhit protein [Asensio et al., 2000]. Di-ethenoadenosine triphosphate, ε-(Ap_3A), a fluorogenic Ap_3A analog, was used for fluorimetric measurement of reaction rates. Human Ap_3A hydrolase was purified from platelet, leukocyte, and placental cytosolic fractions using standard procedures: ammonium sulfate fractionation, gel-filtration (Sephacryl S-200 HR), and ion-exchange chromatography (DEAE-Sephacel). Specific activity in the cytosol of blood cells was very low, about 0.1 mU/mg protein. Nonspecific phosphodiesterase activity was absent from platelet and leukocyte extracts but clearly detected in placenta. Partially purified Ap_3A hydrolase after the ion-exchange chromatography step was used for biochemical studies.

Both the human Ap_3A hydrolase and Fhit proteins had K_m values close to 2 μM for ε-(Ap_3A), exhibited similar pH optima of 7.0–7.2, and were similarly activated by Mg^{2+}, Ca^{2+}, and Mn^{2+} and inhibited by Zn^{2+}. Suramin, the most potent inhibitor of rat brain Ap_3A hydrolase [Rotllán et al., 1998], displayed a competitive pattern of inhibition for human Ap_3A hydrolase but a rather mixed pattern for Fhit, while K_i values for both enzymes were about 25 nM. Fluoride, heparin, and heparin disaccharides were not inhibitors. The histidine-specific reagent diethyl pyrocarbonate rapidly inactivated both human Ap_3A hydrolase and Fhit. Thus, kinetically, both hydrolases are barely distinguishable.

Fhit antibodies differentially inhibited the enzymic activity of Fhit and Ap_3A hydrolase, Fhit protein being the more sensitive. Purified human Ap_3A hydrolase eluted during gel filtration as a single peak with an apparent molecular mass of ~32 kDa. However, Fhit protein eluted as a broad peak, suggesting the existence of several enzymically active Fhit oligomers in addition to the homodimer. This point was confirmed by native PAGE and immunodetection with Fhit antibodies. SDS/PAGE, however, proved that the Fhit monomer is a 17 kDa polypeptide.

Attempts to achieve further purification of human Ap_3A hydrolase were performed using affinity chromatography on N^6-AMP-agarose. The Ap_3A hydrolase activity present in the cytosolic fractions of human platelets and leukocytes and rat brain was adsorbed and specifically eluted as a single peak by 100 μM Ap_3A. This procedure yielded highly purified preparations but the Ap_3A hydrolase proved to be extremely unstable at this stage. Electrophoresis of these affinity-purified fractions revealed at least three bands, probably indicating the presence of several Ap_3A-binding proteins in addition to Ap_3A hydrolase.

A near homogeneous preparation was obtained from the human leukocyte cytosolic fraction after ammonium sulfate fractionation, ion-exchange chromatography, and a final affinity chromatography step. After native PAGE the final preparation showed a single Coomassie blue-stained band, coincident with the band detected after immunoblotting using a Fhit antibody, and with the enzymic activity peak detected after extraction and assaying Ap_3A hydrolase activity in parallel sliced lanes. SDS/PAGE revealed an intense silver-stained band corresponding to a polypeptide of about 17 kDa.

Ap_3A hydrolase-containing fractions at several stages of purification were subjected to Western blotting analysis. Results indicated that Ap_3A hydrolase activity and immunoreactivity against Fhit antibodies copurified. After SDS/PAGE and immunoblotting of purified human Ap_3A hydrolase (gel filtration, ion-exchange, and affinity chromatography fractions) and rat brain Ap_3A hydrolase (affinity chromatography), only bands close to 17 kDa, the size of Fhit monomers, were detected. However, after native PAGE and immunoblotting, evident differences of mobility between the bands detected in human and rat fractions and Fhit were observed. Isoelectric focusing followed by immunoblotting allowed the detection of bands with pI values of about 6.3 for platelet and leukocyte Ap_3A hydrolase and around 6.5 for Fhit. Human Ap_3A hydrolase is therefore a slightly more acidic protein than Fhit.

Taken together, these results indicate that human Ap_3A hydrolase and human Fhit protein are closely related proteins kinetically and immunochemically. They present some slight structural differences accounting for some distinctions in their observed behavior. Their unequal pI values suggest minor posttranslational or purification-generated differences involving charged amino acids.

The areas of extra- and intracellular Ap_nA function and metabolism come together in the work of Mike Blackburn (University of Sheffield). The design and preparation of successful agonists/antagonists for nucleotide receptors has to meet three requirements. First, the analog has to have high affinity for the receptor protein. Second, it has to resist hydrolysis by specific and

nonspecific nucleotide phosphatases and pyrophosphohydrolases. Third, it has to possess good bioavailability. Over the last 15 years the work of Blackburn and collaborators on Ap_nA analogs has primarily focused on the rational design of the first two of these features, with particular attention to nucleotide analog stability to eukaryotic and prokaryotic degradative enzymes. Many of these enzymes show high specificities with K_m values in the micromolar range for both natural and synthetic substrates.

Two devices have been prominent in delivering resistance of analogs to hydrolysis in vitro and in vivo. The first employed a range of carbon bridges to replace the P-O-P pyrophosphate by an alkylenebisphosphonate, $P\text{-}(C)_n\text{-}P$. The virtue of this construct is the stability of the C-P(O) bond to enzymic hydrolysis and its close stereochemical relationship to the parent O-P(O) entity. Its disadvantage is the inevitable reduction of electronegativity resulting from the exchange of an oxygen ligand for a carbon ligand at phosphorus. This deficiency was corrected in two ways to restore isoelectronic character to the phosphonate moieties: the use of the CF_2 group and the insertion of an acetylenic linkage adjacent to phosphorus, as in $P(O)CF_2P(O)$ and P(O)-C≡C-P(O) bisphosphonates, respectively [Blackburn et al., 1984]. X-ray analysis of nucleotides containing the former show that they are isosteric and isopolar with the parent pyrophosphate linkage. By contrast, the acetylenic bridge is strongly electronegative for phosphorus but is clearly nonisosteric. In particular, it means that an analog of ATP such as AdoP-O-P-C≡C-P will not bind magnesium between the β- and γ-phosphoryl groups. The single insertion of CX_2 bisphosphonates into Ap_nA analogs can maintain nucleotide binding in the micromolar range. However, the insertion of two such moieties, as in αβ:α′β′-disubstituted phosphonate analogs of Ap_4A, has generally resulted in weakened affinity for the enzyme [McLennan et al., 1989].

It has therefore proved desirable to introduce a second feature to endow analogs with resistance to hydrolysis: the substitution of sulfur for oxygen as a nonbridging phosphorus ligand. This device, introduced for nucleotides in the 1960s by Fritz Eckstein, can be employed readily in complementation with alkylenebisphosphonates. It has to be noted that the introduction of each P(S) moiety creates a new stereochemical center in the nucleotide analog, for which chirality can be controlled either by stereospecific synthesis or by chromatographic separation of diastereoisomers. Some early results showed a preference for one or the other chirality at the P(S) center for resistance to specific enzyme cleavage, while good overall affinity for the protein was retained [Blackburn et al., 1990a].

In designing analogs of Ap_3A resistant to eukaryotic enzyme hydrolysis, both of these features were introduced into species such as (P^1,P^2-methylene-P^3-thio)-diadenosine triphosphate, $AdoPCH_2POP_SAdo$ [Blackburn et al., 1990b]. This has a useful affinity for the Fhit protein with a K_d of 40 μM and effectively resists hydrolysis. X-ray crystal structures of two diastereoisomers of this analog complexed to wild-type and to an active-site mutant of Fhit show one adenosine bound in a deep cleft and with opposite stereospecificities for the stereochemistry of a P(S) ligand in the P^1 and P^3 sites [Pace et al., 1998]. This explains the general observation that one adenosine is recognized with high specificity while there is ample scope for modification of the second adenosine either to other nucleosides or to non-nucleoside ligands. Some indication of weakened binding of thiophosphate relative to phosphate analogs was also observed for Fhit, although the reverse is the case for the P2T receptor.

Studies were next directed to the question of the contribution of anionic charge on nucleotide binding to Fhit by the synthesis of "supercharged" analogs of pyrophosphoric acid—specifically, methylenetrisphosphonic acid, $HC(PO_3H_2)_3$, and its halo-derivatives. The corresponding supercharged ATP analog, $AdoPOPC(P)_2$, has increased anionic charge (≥5 –ve) relative to ATP (4 –ve). Nucleotides incorporating such trisphosphonates show differential competitive inhibition between two specific hydrolases: the human Fhit protein and the Ap_3A hydrolase from yellow lupin seeds [Liu et al., 1999]. The adenylated polyphosph(on)ates are not hydrolyzed by these enzymes; however, they strongly and competitively inhibit Fhit with K_i values similar to K_m for Ap_3A (1.9 μM), while they are significantly less effective as inhibitors of the lupin enzyme. The competitive inhibition of Fhit shows a rather good LFE (linear free energy) relationship to anionic charge for six adenosine phosphonate analogs, requiring at least one adenosine moiety for binding and with the best inhibitor having six negative charges. However, the apparently beneficial effect of additional negative charge will certainly have adverse effects on bioavailability and makes a search for non-ionic bioisosteres of phosphates a key feature of future work. By contrast, the use of a very sensitive fluorescence-based assay [Draganescu et al., 2000] for nucleotide binding to Fhit has revealed that incorporation of sulfur at the phosphoryl centers adjacent to the adenosine moiety can promote protein affinity by up to two orders of magnitude and provide ligands with nanomolar affinities for Fhit. Clearly, the stereochemical preferences for such modification will be the focus of future work.

Assays with two recombinant asymmetrical Ap_4A hydrolases (lupin and human) has shown that none of the above nucleotide analogs acts as a substrate, while they are poor to moderately effective inhibitors of these en-

zymes. For example, the derivative with two adenylates and one adenosine did not inhibit the human enzyme and was a poor inhibitor of the lupin counterpart (I_{50} = 650 μM). Only the three-adenylated compound moderately and competitively inhibited both enzymes with I_{50} values (estimated at 50 μM Ap_4A) of 80 μM and 40 μM for the human and lupin enzymes, respectively.

These results raise the question of improved intracellular availability of nucleotide analogs. The inherent liability to ready hydrolysis and alkylating potential of fully esterified polyphosphate species (e.g., tetraethyl pyrophosphate) disfavors the pursuit of fully esterified analogs of Ap_nAs as delivery vehicles and pro-ligands for receptor proteins in vivo. Moreover, the potential benefit of thiophosphates for cell availability remains largely unexplored. Consequently, a search for non-ionic bioisosteres of phosphate will dominate the next phase of work on nucleotide ligand design and synthesis. A few leads are available, as from the design of stable mimics for anionic intermediates in the hydrolysis of esters by catalytic antibodies of peptides by aspartyl proteases. In particular, the potential of tetrahedral moieties such as secondary hydroxyl functions (CH(OH) as in statin) and fluoroketones (as $CF_2(OH)_2$ covalent hydrates) are two promising candidates for further exploration.

Synthesis of Ap_nAs

The ability of a variety of ligases to synthesize dinucleoside polyphosphates has been investigated in detail in recent years by Antonio Sillero's group. The following enzymes belonging to this group were shown to catalyze the synthesis of nucleoside polyphosphates (p_nN) and dinucleoside polyphosphates (Np_nN): aminoacyl-tRNA synthetases; firefly luciferase (EC 1.13.12.); acetyl-CoA synthetase (EC 6.2.1.1); acyl-CoA synthetase (EC 6.2.1.8); DNA ligase (AMP forming) (EC 6.5.1.1); RNA ligase (EC 6.5.1.3) [see Sillero and Sillero, 2000 for a review].

T4 RNA ligase catalyzes the joining of RNA molecules via the following reversible steps:

1.) E + ATP ↔ E-AMP + PPi (Enzyme adenylylation)
2.) E-AMP + 5′-P-RNA ↔ E-AppRNA (RNA adenylylation)
3.) E-AppRNA + 3′-OH-*RNA* ↔ RNA-p-*RNA* + AMP + E (RNA joining)

The 5′-P and the 3′-OH ends correspond to that of the RNA *donor* and RNA *acceptor*, respectively. T4 RNA ligase also transfers the adenylyl moiety of the E-AMP complex to a nucleoside triphosphate (NTP) giving rise to a dinucleoside tetraphosphate [Atencia et al., 1999]:

4.) E-AMP + NTP → Ap_4N + E

Because a dinucleoside polyphosphate resembles the 3′-OH end of an RNA, it could conceivably behave as an RNA acceptor analog in reaction 3. This possibility was explored using cytidine-3′,5′-bisphosphate (pCp) as an RNA donor in reaction 2 and several Np_nN as RNA acceptors in reaction 3.

Reaction mixtures were set up containing ATP, pCp, Np_nN and T4 RNA ligase and analyzed by HPLC at different times of incubation. In this way, it could be demonstrated that the 5′-adenylated cytidine 5′,3′-bisphosphate (AppCp) synthesized from ATP and pCp in reaction 5 is used as a substrate to transfer the pCp residue to the 3′-OH end of a dinucleoside polyphosphate (reaction 6) and to a lesser extent to both 3′-OH ends (reaction 7):

5.) E-AMP + pCp ↔ E-AppCp
6.) E-AppCp + Np_nN → Np_nNp_nCp + E + AMP
7.) E-AppCp + Np_nNpCp → $pCpNp_nNpCp$ + E + AMP

Using pCp and Gp_4G as substrates, in the presence of ATP, formation of Gp_4GpCp and $pCpGp_4GpCp$ was obtained in a ratio of 10:1, respectively. Reactions 6 and 7 can also be followed indirectly by measuring the formation of AMP. The Gp_4GpCp synthesized was isolated and characterized by treatment with snake venom phosphodiesterase and alkaline phosphatase and analysis (chromatographic position and UV spectra) of the reaction products by HPLC. The apparent K_m values measured for Gp_4G and Ap_2Cp were around 4 and 0.4 mM, respectively. In the presence of 0.5 mM ATP and 0.5 mM pCp, the relative efficiencies of the following Np_nN as acceptors of pCp from Ap_2Cp are indicated in parentheses: Gp_4G (100); Gp_5G (101); Ap_4G (47); Ap_4A (39). Gp_2G, Gp_3G and Xp_4X were not substrates for the reaction [Atencia et al., 2000]. Thus, T4 RNA ligase: 1.) discriminates between Np_nN containing the same number of internal phosphates (Gp_4G, Ap_4G, Ap_4A, and Xp_4X) and between homodinucleotides containing chains of internal phosphates of differing lengths (Gp_2G, Gp_3G, Gp_4G, and Gp_5G); 2.) requires a minimum of four internal phosphates; and 3.) prefers guanine to adenine or xanthine dinucleotides. The nucleotide specificity of T4 RNA ligase is different from that exhibited by two enzymes that specifically cleave dinucleoside polyphosphates: Ap_3A hydrolase and Ap_4A hydrolase [Guranowski and Sillero, 1992].

Apart from its utility for dinucleotide modification in vitro, it is difficult to grasp the possible physiological meaning of the reactions described here. Very little is known about the function of the enzyme itself in vivo. The only phenotype attributed to mutations in T4 gene 63, coding for T4 RNA ligase, is the failure of the noncovalent attachment of the tail fibers, leading to

fiberless T4 particles. In the process of T4 phage morphogenesis, the enzyme could recognize certain motifs of the phage proteins which resemble the structures of Gp_4G and Gp_5G and which have been revealed by chance using this experimental approach.

Ap_nA and Insulin Function

Insulin secretion from pancreatic β-cells is essential in glucose homeostasis. This hormone is released by exocytosis in response to the presence of glucose in plasma. Glucose enters the cell, generating metabolites that selectively inhibit ATP-sensitive potassium (K_{ATP}) channels, depolarizing the cell and triggering insulin secretion. K_{ATP} channels are involved in a variety of signaling pathways that transduce cellular metabolic events into membrane potential changes. Due to their potential clinical implications, K_{ATP} has generated most interest in two particular processes: glucose-induced insulin secretion of pancreatic β-cells and ischemia-associated action potential in heart muscle. Although the defining property of K_{ATP} channels is their inhibition by intracellular ATP, it is now widely accepted that other intracellular modulators of K_{ATP} channels may also be involved in the regulation of insulin secretion in response to glucose stimulation. Particularly, the role of several nucleotides in K_{ATP} channel regulation, namely ADP, GTP, GDP, or UTP, has been extensively analyzed.

Recent studies have demonstrated that Ap_nA modulate the gating of K_{ATP} channels in isolated membrane patches of ventricular cells [Jovanovic and Terzic, 1995]. Given the similarities found in the physiological behavior of K_{ATP} channel activity in cardiac and β-cells, it seems feasible that Ap_nA could also be pharmacologically active on β-cells. Current work from Bernat Soria's laboratory (Miguel Hernández University) was outlined by Juan Manuel Rovira. These studies are directed toward determining the effects of Ap_nA on pancreatic K_{ATP} channels and then analyzing their suitability as potential mediators in β-cell signaling.

To investigate the effect of Ap_nA on pancreatic K_{ATP} channels, the patch-clamp technique was used. Ap_4A was applied at micromolar concentrations to the intracellular side of β-cell membrane patches and found to provoke a rapid and fully reversible decrease of K_{ATP} channel activity. The effect was comparable to that observed for ATP, both in time-course and effective concentration range. The estimated half-maximal inhibition (18 μM) for Ap_4A closely resembled that found for ATP channel inhibition (17 μM). These results demonstrated that Ap_4A acts as an effective inhibitor of β-cell K_{ATP} channel activity, as expected from results previously obtained in cardiac muscle [Ripoll et al., 1996]. This first observation led Soria and co-workers to investigate the potential significance of Ap_nA as a modulator of β-cell function. As there were no published data on the existence or concentrations of Ap_nA in pancreatic β-cells, measurements of Ap_3A and Ap_4A were first made using high-performance liquid chromatography. The concentrations of both nucleotides were estimated under two different sets of conditions: in nonstimulated cells, i.e., cells incubated in basal glucose (3 mM), and in cells incubated in high glucose (22 mM). The increase in glucose augmented Ap_4A and Ap_3A levels from submicromolar to tens of micromolar. Stimulatory glucose concentrations were found to increase cytosolic Ap_nA to concentrations sufficient to block K_{ATP} channels. Furthermore, other nutrients such as leucine and 2-ketoisocaproate raised Ap_4A and Ap_3A as efficiently as glucose, while non-nutrient secretagogues, such as high extracellular potassium or tolbutamide, had no effect. In conclusion, β-cell K_{ATP} channels can be effectively blocked by Ap_nA at concentrations induced by nutrient (glucose, amino acids) stimulation [Martin et al., 1998]. However, unlike ATP, Ap_4A does not restore K_{ATP} channel activity after rundown [Ripoll et al., 1996; Martin et al., 1998].

In the light of these results, Soria's group proposed Ap_nA as a new K_{ATP} channel regulator in pancreatic β-cells, and so provide a physiological meaning to the existence of two metabolic pathways in the early steps that lead from glucose sensing to insulin secretion that converge in K_{ATP} channel regulation. These data also highlight the fact that the list of messengers derived from the metabolism of glucose may still be incomplete.

Ap_nA In Prokaryotic Systems

Although most current investigations of Ap_nA involve eukaryotic systems, it must not be forgotten that many of the early studies describing their response to metabolic stresses were carried out in prokaryotes and some recent studies on the possible involvement of Ap_4A in the heat-shock response in *E. coli*, in particular the binding of Ap_4A to the molecular chaperone GroEL and the posttranslational modification of the LysU lysyl-tRNA synthetase, were described by Julian Tanner from Andrew Miller's lab (Imperial College, London). Ap_4A is present in submicromolar concentrations in prokaryotes, but is induced to much higher levels (>100 μM) under a variety of stress conditions [Lee et al., 1983]. Ap_4A may act as a signal molecule whose function is to regulate or modify stress responses on the basis that it binds to several *E. coli* heat-shock proteins, including molecular chaperones GroEL, DnaK, and ClpB that assist protein folding/refolding in vivo [Johnstone and Farr, 1991]. This view is supported by experiments showing that Ap_4A overproduction causes cells to be oversensitive to heat shock [Farr et al., 1989]. Aminoacyl-tRNA synthetases are a major source of Ap_4A in cells and in *E. coli* the heat-inducible lysyl-tRNA synthetase (LysU) is the most ef-

fective Ap_4A synthetase—80% of total Ap_4A synthesis in *E. coli* cell extracts has been ascribed to lysyl-tRNA synthetase catalysis [Charlier and Sanchez, 1987].

Escherichia coli lysyl-tRNA synthetase (heat-inducible LysU and constitutively expressed LysS) can be resolved into several peaks of enzymic activity during the last stage of purification by hydroxyapatite chromatography [Charlier and Sanchez, 1987]. Recombinant LysU was further resolved in the Miller laboratory into two main peaks of activity by hydroxyapatite chromatography. Using anti-phosphothreonine Western blots and Ames malachite green assays, it was shown that the first peak eluting at 70 mM potassium phosphate corresponded with unphosphorylated LysU, while that eluting at 100 mM potassium phosphate corresponded with LysU phosphorylated on threonine. Under normal growth conditions (37°C), approximately 30% of the recombinant LysU was found to be phosphorylated. This result is consistent with some of the earliest studies on the lysyl-tRNA synthetase of *E. coli*, which suggested that the enzyme was phosphorylated, but at less than 1 mole of phosphate per mole of enzyme [Stern and Peterkofsky, 1969]. An analysis of phosphorylated and unphosphorylated LysU by circular dichroism (CD) spectroscopy revealed only very slight changes in tertiary structure as a result of phosphorylation. However, phosphorylation did induce some changes in the kinetics of Ap_4A synthesis. The overall rate of Ap_4A synthesis was slightly increased as a consequence of phosphorylation (134 ± 10 mol min^{-1} for unphosphorylated LysU, 150 ± 12 mol min^{-1} for phosphorylated LysU). However, the specificity constant k_{cat}/K_m for lysine of phosphorylated LysU (290 $sec^{-1}.mol^{-1}$) was found to be over three times that of the unphosphorylated enzyme (80 $sec^{-1}mol^{-1}$), suggesting that phosphorylation had significantly increased the velocity of aminoacylation. Phosphorylated LysU also appeared to be significantly more thermostable than the unphosphorylated enzyme.

In order to characterize a molecular role for Ap4A in the heat-shock response, the interaction between Ap4A and the molecular chaperone GroEL was also studied. Ap_4A was found to bind tightly to GroEL ($K_d < 10\ \mu M$), both in the presence and absence of ADP, according to the results of a CD binding assay [Holler, 1984]. Using isothermal calorimetry, Ap_4A was found not to inhibit the binding of ADP to GroEL. Moreover, no hydrolysis of Ap_4A could be detected as a result of binding to GroEL. The potential significance of this tight binding interaction was investigated using two model protein refolding assay systems. At low temperatures (<30°C), Ap_4A was found to have no effect on the refolding of unfolded malate dehydrogenase (MDH) mediated by GroEL (and co-chaperone GroES). However, at higher heat-shock temperatures (>40°C), Ap_4A was found to promote the release of over five times more refolded MDH from GroEL than was released in the absence Ap_4A. This observation was corroborated by differential scanning calorimetry studies that showed Ap_4A to significantly destabilize a complex formed between GroEL and unfolded α-lactalbumin, giving rise to a two-step unfolding transition consistent with differing interactions between *cis* and *trans* rings of GroEL. Taken together, these results suggest that Ap_4A is able to bind to GroEL at a site distinct from the well-established ADP/ATP binding site. The functional purpose of this allosteric binding interaction then appears to be destabilization of the high-temperature protective complex formed between an unfolded protein substrate and GroEL under heat-shock conditions [Llorca et al., 1998], forcing the release of refolded protein back into solution.

In conclusion, phosphorylation of LysU resulted in some significant changes to the rate and thermal persistence of Ap_4A synthesis catalyzed by LysU. Therefore, phosphorylation of LysU may represent an important way to control the synthesis Ap_4A in *E. coli* under stress conditions. There may also be a link between Ap_4A, the *E. coli* hsp70 DnaK, and the aminoacyl-tRNA synthetases. DnaK has been shown to participate in the phosphorylation of the glutaminyl-tRNA synthetase and threonyl-tRNA synthetase of *E. coli* [Wada et al., 1986]. Therefore, DnaK may well be the source of LysU phosphorylation as well, a possibility that is under active investigation. The effects of Ap_4A on GroEL point towards a cellular role in which Ap_4A assists the return of cells to normal growth conditions following stress, and promotes release of molecular chaperone-bound cellular proteins under prolonged stress to maintain cellular viability. These effects could also account for the heat-sensitivity of Ap_4A hydrolase-deficient *apaH* mutants, which have elevated intracellular levels of Ap_4A [Farr et al., 1989].

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