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Recognition of $\beta\beta'$-Substituted and $\alpha\beta,\alpha'\beta'$-Disubstituted Phosphonate Analogues of Bis(5′-adenosyl) Tetraphosphate by the Bis(5′-nucleosidyl)-tetraphosphate Pyrophosphohydrolases from *Artemia* Embryos and *Escherichia coli*†

Alexander G. McLennan,*,‡ Graham E. Taylor,§,∥ Mark Prescott,‡ and G. Michael Blackburn§

Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K., and Department of Chemistry, University of Sheffield, Sheffield S3 7HF, U.K.

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ABSTRACT: A total of 13 phosphonate analogues of bis(5′-adenosyl) tetraphosphate (AppppA) have been tested as substrates and inhibitors of the asymmetrically cleaving bis(5′-nucleosidyl) tetraphosphatase (NppppNase) from *Artemia* and the symmetrically cleaving NppppNase from *Escherichia coli*. With the *Artemia* enzyme, the substrate efficiency of $\beta\beta'$-substituted compounds decreased with decreasing substituent electronegativity (O > CF_2 > CHF > CCl_2 > CHCl > CH_2) such that AppCF_2ppA and AppCH_2ppA were hydrolyzed at 70% and 2.5% of the rate of AppppA, respectively. These compounds were competitive inhibitors of this enzyme with K_i values that generally also decreased with electronegativity from 12 μM for AppCF_2ppA to 0.4 μM for AppCH_2ppA (K_m for AppppA = 33 μM). AppCH=CHppA and AppCH_2CH_2ppA were neither effective substrates nor inhibitors of the *Artemia* enzyme. $\alpha\beta,\alpha'\beta'$-Disubstituted analogues were generally less effective inhibitors with K_i values ranging from 23 μM (AppCH_2ppCH_2pA) to > 1.5 mM (AppCH_2CH_2ppCH_2CH_2pA). However, they displayed a low and unexpected rate of *symmetrical* cleavage by the *Artemia* enzyme: e.g., ApCHFppCHFpA yielded ApCHFp at 3% of the rate of AppppA breakdown. Both sets of analogues were also competitive inhibitors of the *E. coli* NppppNase with K_i values ranging from 7 μM (AppCH_2ppA) to 250 μM (AppCH_2CH_2ppCH_2CH_2pA) (K_m for AppppA = 28 μM). The only $\alpha\beta,\alpha'\beta'$-disubstituted analogue to be hydrolyzed by the *E. coli* enzyme was ApCF_2ppCF_2pA at 0.2% of the rate of AppppA; however, several of the $\beta\beta'$-substituted compounds showed a limited degree of *asymmetrical* cleavage. These results are interpreted in terms of a "frameshift" model for substrate binding in which the oligophosphate moiety of the substrate can position itself in the active site of either enzyme with either P^α or P^β adjacent to the attacking nucleophile depending on the electronegativity of the phosphonate substituent.

In 1963, the first bis(5′-nucleosidyl) oligophosphate, bis(5′-guanosyl) tetraphosphate (GppppG),[1] was isolated from encysted embryos of the brine shrimp *Artemia*, where it serves as a purine ring store for the developing organism (Finamore & Warner, 1963; Warner, 1980). Since then, several other nucleotides of general structure Np_nN' have been detected in a wide variety of prokaryotes and eukaryotes, but their functions remain to be clearly established. Interest has focused mainly on the adenine-containing members of this family and in particular bis(5′-adenosyl) tetraphosphate (AppppA). This nucleotide was originally implicated in the initiation of DNA replication [reviewed in Zamecnik (1983), McLennan and Prescott (1984), Baril et al. (1985), and Grummt (1988)]; however, evidence to the contrary also exists (Bambara et al., 1985; Plateau et al., 1987), and this has led to the search for alternative functions.

For example, AppppA and a number of other adenylated nucleotides including ApppA, AppppG, ApppGpp, and ApppG accumulate rapidly in bacteria when they are subjected to metabolic stresses such as heat shock, oxidation, and organic solvents (Lee et al., 1983a,b; Bochner et al., 1984; VanBogelen et al., 1987; Balodimos et al., 1988); however, with the possible exception of the *oxy* R-controlled oxidation stress regulon of

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* Address correspondence to this author.
‡ University of Liverpool.
§ University of Sheffield.
∥ Present address: Fisons plc, Loughborough, U.K.

[1] Abbreviations: GppppG, bis(5′-guanosyl) tetraphosphate; AppppA, bis(5′-adenosyl) tetraphosphate; other bis(5′-nucleosidyl) oligophosphates are abbreviated in a similar fashion; NppppNase, bis(5′-nucleosidyl)-tetraphosphate pyrophosphohydrolase; AppCH_2ppA, diadenosine 5′,5‴-P^1,P^4-(P^2,P^3-methylene)tetraphosphate; AppCH_2p, adenosine 5′-(β,γ-methylene)triphosphate; AppCH_2ppCH_2pA, diadenosine 5′,5‴-P^1,P^4-(P^1,P^2-methylene-P^3,P^4-methylene)tetraphosphate; AppCH_2p, adenosine 5′-(α,β-methylene)diphosphate; other phosphonate analogues of AppppA, ATP, and ADP are abbreviated in a similar fashion.

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Escherichia coli (VanBogelen et al., 1987), there is no clear evidence that they are acting as "alarmones" to initiate the subsequent stress responses as originally proposed by Varshavsky (1983a,b). Furthermore, in eukaryotes the degree of Np_nN' accumulation is low and does not correlate with the kinetics of stress protein induction. Rather, the degrees of stress required to produce significant increases in these nucleotides tend to be lethal (Miller & McLennan, 1986; Guêdon et al., 1986; Baltzinger et al., 1986). More recent experiments that demonstrate an accumulation of AppppA in mammalian cells upon exposure to DNA-damaging agents and conditions that produce single-strand DNA breaks still provide no real clues as to the function of this nucleotide (Baker & Ames, 1988; Gilson et al., 1988). Finally, extracellular roles for AppppA and ApppA in blood platelet function, vasoconstriction, and vasodilation have been suggested (Flodgaard & Klenow, 1982; Busse et al., 1988).

The adenylated bis(5′-nucleosidyl) oligophosphates are probably synthesized in vivo by aminoacyl-tRNA synthetases (Goerlich et al., 1982; Blanquet et al., 1983), though in some cases the enzyme AppppA phosphorylase may also be responsible. This activity has been isolated from yeast (Guranowski & Blanquet, 1985; Brevet et al., 1987) and *Euglena gracilis* (Guranowski et al., 1988) and is a candidate enzyme in the synthesis of some of the nonadenylated compounds such as GppppG and GppppC, which have recently been detected in yeast and *E. coli* (Coste et al., 1987).

AppppA phosphorylase also catalyzes the phosphorolysis of NppppN to NTP and NDP. However, the major intracellular catabolic activity in other cells appears to be NppppN pyrophosphohydrolase (NppppNase) of which two types exist. Cells from higher organisms such as rat (Cameselle et al., 1982, 1984), mouse (Höhn et al., 1982; Moreno et al., 1982), human (Ogilvie & Antl, 1983), lupin (Jakubowski & Guranowski, 1983), and *Artemia* (Warner & Finamore, 1965; Vallejo et al., 1974; Prescott et al., 1989) contain an *asymmetrical* NppppNase that cleaves one of the P^{α}–O–P^{β} linkages to produce equimolar amounts of NTP and NMP, while *Physarum polycephalum* (Barnes & Culver, 1982; Garrison et al., 1982) and *E. coli* and other prokaryotes (Guranowski et al., 1983; Plateau et al., 1985) possess a *symmetrical* NppppNase that hydrolyzes a P^{β}–O–$P^{\beta'}$ linkage, yielding 2 mol of NDP/mol of NppppN. Specific bis(5′-nucleosidyl) triphosphatases have also been found in *E. coli* (Hurtado et al., 1987), rat tissues (Sillero et al., 1977; Costas et al., 1984, 1986), and *Artemia* (M. Prescott, A. D. Milne, and A. G. McLennan, unpublished experiments).

The existence of these synthetic and degradative pathways would seem to imply a definite biological role for AppppA and other members of this family. A powerful approach toward the clarification of these functions involves the application of structural analogues of these compounds to systems representing varying degrees of subcellular organization from the microinjected cell to the isolated enzyme. In the case of AppppA, rapid destruction of added nucleotide in cells and cell extracts has limited the use of the parent nucleotide itself in such experiments (A. G. McLennan, unpublished experiments). Nonhydrolyzable analogues would therefore be of particular value. We have recently reported the synthesis and separation of the three diastereomers of diadenosine 5′,5‴-P^1,P^4-(P^1,P^4-dithio-P^2,P^3-methylene)tetraphosphate ($Ap_spCH_2pp_sA$) and demonstrated their resistance to hydrolysis by and competitive inhibition of the asymmetrical NppppNase from *Artemia* (Blackburn et al., 1987a), while Guranowski et al. (1987) have studied the ability of four phosphonate analogues of AppppA to act as substrates and inhibitors of the lupin and *E. coli* NppppNases, lupin phosphodiesterase I, and the yeast AppppA phosphorylase.

In addition to their use in evaluating the various roles proposed for AppppA in vivo, analogues are also important tools in the analysis of those factors that are responsible for substrate recognition and catalysis by such enzymes. Here we present a series of 13 $\beta\beta'$-substituted and $\alpha\beta,\alpha'\beta'$-disubstituted phosphonate analogues of AppppA and their interactions as substrates and inhibitors with the asymmetrical NppppNase from *Artemia* and the symmetrical NppppNase from *E. coli*.

Experimental Procedures

Chemicals. [^{3}H]AppppA (4.3 Ci/mmol) was purchased from Amersham International. Unlabeled AppppA, calf intestine alkaline phosphatase, and rabbit muscle pyruvate kinase were from Boehringer. Adenosine, AMP, ADP, and ATP were from Sigma. Phosphonate analogues of AppppA, ATP, and ADP were synthesized by procedures that will be published in detail elsewhere. Briefly, $\alpha\beta$-substituted ADP analogues were first prepared by the reaction of the tris(tetra-*n*-butylammonium) salt of the appropriate substituted bisphosphonate with 5′-*O*-tosyl-2′,3′-isopropylideneadenosine (Davisson et al., 1987). After removal of excess bisphosphonate by precipitation, the products were purified by anion-exchange chromatography on DEAE-Sephadex with yields of 50–70%. The ADP analogues were then self-condensed by using dicyclohexylcarbodiimide to form the $\alpha\beta,\alpha'\beta'$-disubstituted AppppA derivatives (yield = 40%). $\beta\beta'$-Substituted AppppA analogues were prepared by the reaction of the appropriate substituted bisphosphonate with AMP–morpholidate in pyridine. The $\beta\gamma$-substituted ATP that slowly formed at room temperature during 3 weeks was converted into the $\beta\beta'$-substituted AppppA by heating at 60 °C for 24 h and the product purified with a final yield of 40–50% by anion-exchange chromatography. For those analogues where diastereomers exist, these were not resolved.

Enzymes. Asymmetrical NppppN pyrophosphohydrolase from *Artemia* embryos was purified to homogeneity (Prescott et al., 1989). Briefly, an extract prepared from 250 g of dry cysts was chromatographed successively on Q–Sepharose, Ultrogel AcA44, Mono Q, AppppA–Sepharose, and Bio-Gel HPHT. The final fraction (2.7% yield) gave a single polypeptide of M_r 17 600 on a silver-stained SDS–polyacrylamide gel with a K_m for AppppA of 33 ± 2 μM and k_{cat} = 12.7 s^{-1} (37 °C).

The symmetrical NppppNase was purified from *E. coli* MC4100 by the following procedure (all operations were carried out at 4 °C unless otherwise stated):

(*a*) *Step 1*: *Crude Extract.* Twenty-eight grams wet weight of frozen cells (previously grown at 37 °C in Oxoid 2 nutrient broth to an optical density of 1.5 at 450 nm) were suspended in 25–30 mL of 20 mM potassium phosphate buffer, pH 8.0, 0.1 mM EDTA, and 0.1 mM dithiothreitol and disrupted by two passages through a French pressure cell at 40 MPa. Homogenization buffer was then added to a final volume of 75 mL and the extract centrifuged at 150000*g* for 1 h.

(*b*) *Step 2*: *Ammonium Sulfate Precipitation.* The supernatant from step 1 (65 mL) was brought to 60% saturation by the addition of solid ammonium sulfate over a 30-min period and the precipitated protein collected by centrifugation at 20000*g* for 30 min. The pellet was dissolved in Q–Sepharose running buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, and 0.1 mM dithiothreitol) to a final volume of 19 mL and then dialyzed overnight against 100 vol of the same buffer.

Table I: HPLC Retention Times (RT) of Nucleotides and Nucleotide Analogues[a]

compound	RT (min)	compound	RT (min)	compound	RT (min)	compound	RT (min)
AppppA	10.54	ATP	14.24			ADP	8.40
$AppCF_2ppA$	10.06	$AppCF_2p$	14.27	$ApCF_2ppCF_2pA$	10.13	$ApCF_2p$	8.91
$AppCCl_2ppA$	9.97	$AppCCl_2p$	13.02	$ApCCl_2ppCCl_2pA$	9.60	$ApCCl_2p$	7.94
AppCHFppA	10.07	AppCHFp	11.49	ApCHFppCHFpA	9.81	ApCHFp	6.68
AppCHClppA	10.21	AppCHClp	10.70	ApCHClppCHClpA	10.00	ApCHClp	6.20
$AppCH_2ppA$	9.50	$AppCH_2p$	7.82	$ApCH_2ppCH_2pA$	8.81	$ApCH_2p$	5.25
AppCH=CHppA	8.36	AppCH=CHp	8.36				
$AppCH_2CH_2ppA$	7.55	$AppCH_2CH_2p$	6.21	$ApCH_2CH_2ppCH_2CH_2pA$	6.45	$ApCH_2CH_2p$	4.32
AMP	4.50	adenosine	2.65				

[a] See footnote 1 for an explanation of the abbreviations.

(*c*) *Step 3*: *Q–Sepharose Chromatography*. The solution from step 2 was applied to a column of Q–Sepharose (2.6 × 10 cm) equilibrated in running buffer at a flow rate of 50 mL/h. After the elution of unbound protein, the column was developed with a 2 × 500 mL linear gradient of 0–1 M NaCl in running buffer. Ten-milliliter fractions were collected and the active fractions pooled (37 mL) and concentrated by the addition of ammonium sulfate to 100% saturation and centrifugation (20000*g*, 30 min). The pellet was dissolved in AcA44 running buffer [50 mM potassium phosphate buffer, pH 7.5, 0.1 mM EDTA, and 0.1 mM dithiothreitol (final volume = 4.5 mL)].

(*d*) *Step 4*: *Ultrogel AcA44 Chromatography*. The sample from step 3 was applied at 8 mL/h to a column of Ultrogel AcA44 (1.6 × 95 cm) equilibrated in running buffer. Two-milliliter fractions were collected and the active fractions pooled (20 mL) and dialyzed against 100 vol of Bio-Gel HPHT running buffer (10 mM potassium phosphate buffer, pH 6.8, and 10 μM $CaCl_2$).

(*e*) *Step 5*: *High-Performance Hydroxylapatite Chromatography*. The enzyme was purified to final homogeneity by chromatography at room temperature on Bio-Gel HPHT (Bio-Rad). The sample from step 4 was applied at 0.8 mL/h, and after elution of the unbound protein, the column was developed with a 2 × 12 mL gradient of 10–350 mM potassium phosphate buffer, pH 6.8, containing 10 μM $CaCl_2$. Fractions (0.5 mL) were collected and the active fractions pooled, adjusted to 50% glycerol, 0.1 mg/mL bovine serum albumin, and 0.1 mM dithiothreitol, and stored in aliquots at −70 °C. The final preparation had a K_m for AppppA of 28 ± 3 μM and k_{cat} = 180 s^{-1} (30 °C) and was judged to be homogeneous by SDS–polyacrylamide gel electrophoresis and silver staining (Laemmli, 1970; Wray et al., 1981) with a single band of protein at M_r 30000 (data not shown). These properties are very similar to those reported previously (Guranowski et al., 1983; Plateau et al., 1985).

Assay of Column Fractions for E. coli NppppNase. NppppNase activity was determined with a luminescence assay exactly as described for the *Artemia* enzyme (Prescott et al., 1989) but with the inclusion of 100 μM $CoCl_2$, 2 mM phosphoenolpyruvate, and 20 μg/mL pyruvate kinase.

Determination of Inhibition Constants. Incubation mixtures (50 μL) contained 20 mM bicine–KOH, pH 8.4, 2 mM magnesium acetate, 5 units of alkaline phosphatase, 10 or 50 μM [^{3}H]AppppA (200 mCi/mmol), either 1 ng of homogeneous *Artemia* NppppNase or 0.1 ng of homogeneous *E. coli* NppppNase plus 100 μM $CoCl_2$, and various concentrations of analogues. After 10 min at 30 °C (*E. coli*) or 37 °C (*Artemia*), assays were chilled on ice, and 300 μL of a 25% (v/v) suspension of DEAE-Sephacel in 10 mM Tris-HCl, pH 7.5, was then added followed by centrifugation at 16000*g* for 5 min. A total of 200 μL of the supernatants was added to 4 mL of Optiphase MP scintillant (LKB) and the radioactivity in the [^{3}H]adenosine released by alkaline phosphatase from the ^{3}H-labeled mononucleotide products determined. K_i values were calculated according to the method of Cheng and Prusoff (1973).

Determination of Degradation Rates. Analogues (400 μM) were incubated at 37 °C with *Artemia* NppppNase (1 ng–0.75 μg) or at 30 °C with *E. coli* NppppNase (0.1–60 ng) for various times between 5 and 60 min at 37 °C in 20 mM bicine–KOH, pH 8.4, 2 mM magnesium acetate, and 5 units of alkaline phosphatase (and 100 μM $CoCl_2$ for the *E. coli* enzyme) in a volume of 50 μL. Ten microliters of 0.3 M ammonium phosphate, pH 5.2, was added and 50 μL of the mixture injected on to a 4.6 × 250 mm Partisil 10-SAX column. The column was developed at 1 mL/min with a 20-min gradient of 5–60% buffer B, where buffer A was 50 mM ammonium phosphate, pH 5.2, and buffer B was 1 M ammonium phosphate, pH 5.7. Areas of UV-absorbing peaks (254 nm) were integrated by using a 1040 data capture unit and ChromMac software (Drew Scientific, London) and were then adjusted to compensate for the hyperchromicity of the products. Initial degradation rates were determined from these data. Retention times of the phosphonate analogues of AppppA used in this study and of their potential hydrolysis products are shown in Table I.

Results

Phosphonate Analogues of AppppA as Substrates and Inhibitors of the Artemia NppppNase. Hydrolysis of AppppA by an asymmetrically cleaving enzyme such as that from *Artemia* embryos can conceivably proceed by attack at either P^α or P^β. We have recently shown by mass spectrometric analysis of the cleavage products of a reaction performed in $H_2{}^{18}O$ that attack is normally at P^α, although the nature of the attacking nucleophile remains as yet unknown (McLennan et al., 1989). Therefore, substitution of O in the P^β–O–$P^{\beta'}$ bridge by a methylene group to give $AppCH_2ppA$ (compound **1e** in Table II) might be expected to yield a product still sensitive to hydrolysis. However, Guranowski et al. (1987) have previously shown that **1e** is highly resistant to hydrolysis by the lupin NppppNase, and our results with the *Artemia* enzyme confirm this (Table II). **1e** is hydrolyzed at only 2.5% of the rate of AppppA compared to 6% with the lupin NppppNase. It is known that isosteric phosphonate analogues of nucleotides containing a P–CH_2–P bridge tend strongly to resist enzymic hydrolysis owing to the reduced electrophilicity of the phosphorus atoms, in this case P^β and $P^{\beta'}$ (Scheit, 1980). Therefore, since it appears that the electrophilicity of P^α is unlikely to be reduced sufficiently to prevent nucleophilic attack by transmission of the effect through the bridging oxygen, the reduced rate of cleavage must be attributed to the diminished leaving-group ability of the methylenephosphonate anion.

Replacement of the hydrogen atoms of a phosphonate CH_2

Table II: Efficiency of Phosphonate Analogues of AppppA as Substrates and Inhibitors of the *Artemia* NppppNase[a]

	analogue	V_{rel}	K_i (μM)	rel spec
	AppppA	1.0	33[b]	1.0
1a	$AppCF_2ppA$	0.70	12	1.9
1b	AppCHFppA	0.20	3.5	1.9
1c	$AppCCl_2ppA$	0.18	23	0.26
1d	AppCHClppA	0.11	2.3	1.6
1e	$AppCH_2ppA$	0.025	0.4	1.8
1f	AppCH=CHppA	0.012	120	3.4×10^{-3}
1g	$AppCH_2CH_2ppA$	0.003	120	8.2×10^{-4}
2a	$ApCF_2ppCF_2pA$	0.002	300	2.2×10^{-4}
2b	ApCHFppCHFpA	0.030	50	0.02
2c	$ApCCl_2ppCCl_2pA$	0.001	1200	1.3×10^{-4}
2d	ApCHClppCHClpA	0.003	40	2.6×10^{-3}
2e	$ApCH_2ppCH_2pA$	0.004	23	5.8×10^{-3}
2g	$ApCH_2CH_2ppCH_2CH_2pA$	0.004	>1500	$<8 \times 10^{-5}$

[a] Assays were performed as described under Experimental Procedures. Alkaline phosphatase was included in the incubations to keep conditions the same as for the inhibition assays. Rates were determined by integration of the product peaks (adenosine and β,γ-ATP analogue for the $\beta\beta'$-substituted compounds and the α,β-ADP analogue for the $\alpha\beta,\alpha'\beta'$-disubstituted compounds). The identity of products was also confirmed in the absence of alkaline phosphatase. [b] K_m for Ap_4A.

group by more electronegative halogen atoms can in many cases improve the substrate efficiency of such compounds (Blackburn et al., 1986, 1987b). We therefore prepared a series of $\beta\beta'$-substituted compounds of progressively increasing substituent electronegativity (**1a–1d**, Table II). When tested with the *Artemia* NppppNase, their efficiencies as substrates increased with increasing electronegativity precisely as expected (CH_2 < CHCl < CCl_2 < CHF < CF_2 < O) (Table II). Thus, **1a** was hydrolyzed at 70% of the rate of AppppA, indicating that it is a very effective substrate. The active site of the enzyme appears to be able to accommodate the larger F and Cl atoms reasonably well in this position. Guranowski et al. (1987) reported that AppCHBrppA was hydrolyzed approximately 3-fold faster than **1e** by the lupin NppppNase. This places AppCHBrppA in the correct position between **1d** and **1e** in terms of electronegativity. Analogue **1f** in which a planar, isopolar *trans*-ethylene group had been introduced in the $\beta\beta'$ position was a very poor substrate, indicating that molecular shape is important too. The poorest substrate in this group was **1g**, which is correct in neither shape nor polarity. The correct distance between the two P^{β} atoms is obviously important. In each case, the products of hydrolysis were the corresponding β,γ-substituted ATP analogue and AMP (or adenosine when alkaline phosphatase was included).

Analogues **1a–1e** were also effective competitive inhibitors of AppppA hydrolysis by the *Artemia* enzyme with K_i values below the K_m for AppppA (Table II). With the exception of **1c** where the two large Cl atoms presumably restrict access of the analogue to the active site, the K_i values decreased with decreasing electronegativity of the substituent, possibly indicating a progressive reduction of some form of unfavorable binding interaction. The specificity constant, k_{cat}/K_m (Fersht, 1974) for AppppA was 3.8×10^5 M^{-1} s^{-1}. The values of the specificity constants of the analogues relative to that of AppppA (relative specificities) give a truer indication of the effectiveness of these compounds as substrates than do k_{cat} or K_m (K_i) individually. With the exception once more of **1c**, the values for relative specificities of **1a–1e** were very similar, between 1.6 and 1.9 (Table II). Thus, there appears to be a close inverse relationship between the tightness of binding and the susceptibility to hydrolysis. Not unexpectedly, analogues **1f** and **1g** had K_i values 4-fold higher than the K_m for AppppA

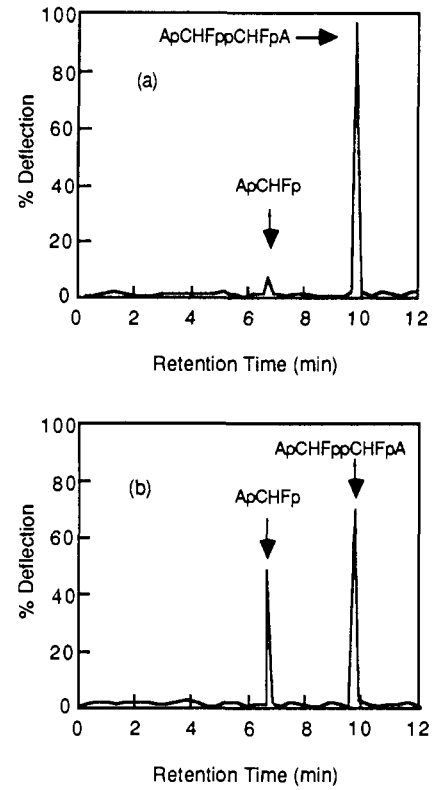

FIGURE 1: High-performance liquid chromatographic analysis of the hydrolysis of ApCHFpCHFpA by the bis(5′-nucleosidyl)-tetraphosphate pyrophosphohydrolase from *Artemia*. ApCHFppCHFpA (16.7 nmol) was incubated (a) without enzyme and (b) with 0.2 μg of *Artemia* NppppNase for 90 min, and the products were analyzed on Partisil 10-SAX as described under Experimental Procedures. The arrows indicate the positions of ApCHFpCHFpA and ApCHFp standards authenticated by negative ion FAB mass spectrometry and ^1H NMR.

due to their poor recognition, a fact that may also be influenced by alterations in the metal-binding properties of these compounds. Their very low relative specificities show them to be extremely poor substrates.

Replacement of the O atom in both P^{α}–O–P^{β} bridges by substituted methylene groups would be expected to result in compounds with a strong resistance to hydrolysis by asymmetrically cleaving enzymes. Analogue **2e** has already been shown to be totally refractory to breakdown by the lupin NppppNase even when incubated under conditions that would permit a degradation rate as low as 0.01% of that of AppppA to be observed (Guranowski et al., 1987). This compound is only poorly recognized by the enzyme as evidenced by the K_i/K_m ratio of 10. Our results with a series of $\alpha\beta,\alpha'\beta'$-disubstituted analogues confirm their resistance to degradation compared to the $\beta\beta'$-substituted compounds (**2a–2g**, Table II). However, in marked contrast to what has been reported for the lupin enzyme, a low but significant degree of breakdown was observed ranging from 0.1% for **2c** to an appreciable 3% for **2b**. As with the $\beta\beta'$-analogues, **2e** had the lowest K_i value (23 μM), and K_i increased with increasing electronegativity. As before, steric reasons probably account for the very high K_i values of 1200 and 1500 μM observed with **2c** and **2g**, respectively.

To clarify the nature of the unexpected hydrolytic reaction occurring with compounds **2a–2g**, the HPLC products were examined after prolonged incubation with the enzyme. For example, in the case of **2c** (ApCHFppCHFpA), Figure 1 clearly shows that the P–C bonds have remained intact and that cleavage has occurred symmetrically, as the sole product

Table III: Efficiency of Phosphonate Analogues of AppppA as Substrates and Inhibitors of the *E. coli* NppppNase[a]

	analogue	V_{rel}	K_i (μM)	rel spec
	AppppA	1.0	28[b]	1.0
1a	$AppCF_2ppA$	0.020	15	0.037
1b	AppCHFppA	0.008	8	0.028
1c	$AppCCl_2ppA$	0	16	0
1d	AppCHClppA	0	10	0
1e	$AppCH_2ppA$	0.040	7	0.16
1f	AppCH=CHppA	0	21	0
1g	$AppCH_2CH_2ppA$	0.003	30	2.8×10^{-3}
2a	$ApCF_2ppCF_2pA$	0.002	25	2.2×10^{-3}
2b	ApCHFppCHFpA	0	130	0
2c	$ApCCl_2ppCCl_2pA$	0	66	0
2d	ApCHClppCHClpA	0	125	0
2e	$ApCH_2ppCH_2pA$	0	15	0
2g	$ApCH_2CH_2ppCH_2CH_2pA$	0	250	0

[a] Conditions were as described for Table II. [b] K_m for Ap_4A.

of the reaction cochromatographed with authentic α,β-monofluoromethylene ADP (ApCHFp). Symmetrical cleavage of the other analogues in this group was also shown by HPLC. The fact that the monofluoromethylene analogue (**2c**) is 100 times better a substrate than the difluoromethylene analogue (**2a**) requires more than the involvement of adverse steric factors to explain the reduced reactivity of **2a**. Such a reaction necessitates attack at P^{β} rather than P^{α} but cannot be due to a contaminating symmetrical NppppNase since the enzyme preparation used was judged to be homogeneous on a silver-stained polyacrylamide gel and, during the course of our studies with *Artemia*, we have never observed an activity capable of cleaving AppppA in this manner (Prescott et al., 1989). Interestingly, a similar result was observed when the mixed stereoisomers of diadenosine 5′,5‴-P^1,P^4-(P^1,P^4-dithio)tetraphosphate, Ap_3ppp_sA, were treated with the *Artemia* enzyme. They were broken down symmetrically at 3.5% of the rate of AppppA to yield the single product adenosine 5′-(α-thio)diphosphate (Blackburn et al., 1987a). Therefore, symmetrical cleavage with attack shifted to P^{β} may be an intrinsic, alternative mode of action for the asymmetrically cleaving NppppNases. The reduced reactivity of **2a** relative to **2b** could then be attributed to opposition of the dipole of the CF_2 group to "in line" attack of a nucleophile at P^{β}.

Phosphonate Analogues as Substrates and Inhibitors of the E. coli NppppNase. Symmetrical cleavage of AppppA can only occur by attack at P^{β} or $P^{\beta'}$ with cleavage of a P^{β}–O bond in the P^{β}–O–$P^{\beta'}$ linkage. Hence, both ββ′-substituted and αβ,α′β′-disubstituted analogues would be expected to resist hydrolysis owing to the reduced electrophilicity of P^{β} and, in the first case, to the presence of the P^{β}–C–$P^{\beta'}$ bond. Guranowski et al. (1987) have shown that both compounds **1e** and **2e** are completely stable in the presence of the *E. coli* enzyme and that increasing the electronegativity of **1e** by replacement of one methylene hydrogen by bromine has no effect. Our own data with a wider range of analogues are in broad agreement, although low but significant rates of reaction were detected with certain compounds (Table III). For example, with the αβ,α′β′-disubstituted analogues (**2a–2g**), total resistance to hydrolysis was indeed observed except with the most electronegative difluoromethylene compound (**2a**). α,β-Difluoromethylene ADP was the sole product as would be predicted. All were recognized by the enzyme since they acted as competitive inhibitors with K_i values either similar to or at most 4-fold greater than the K_m for AppppA with the exception of **2g**, whose K_i of 250 μM can be attributed to its abnormal shape. Our values for the K_m for AppppA (28 μM) and the K_i for **2e** (15 μM) compare favorably with those determined by Guranowski et al. (1987) of 25 μM and 8 μM, respectively.

Compounds **1a–1g** were also recognized by the *E. coli* enzyme with K_i values equal to or below the K_m for AppppA (Table III). Interestingly **1a**, **1b**, **1e**, and **1g** were also slowly hydrolyzed in an asymmetrical fashion (details not shown), a reaction not observed by Guranowski et al. (1987) for **1e**. In our studies, the least electronegative (**1e**) was cleaved at twice the rate of the most electronegative (**1a**), suggesting that factors other than polarity are involved. Although we have not tested this directly, the asymmetrical reaction may be a consequence of the inclusion of Mg^{2+} ions in the assays in addition to the preferred Co^{2+} ions. Guranowski et al. (1987) had only Co^{2+} ions in their incubation mixtures. Asymmetrical cleavage may occur either by attack at the original P^{β} with cleavage of the P^{α}–O(P^{β}) bond or, by analogy with what has been found to occur with the *Artemia* enzyme, by a shift of attack to P^{α}. This apparent ability of both types of NppppNase to hydrolyze phosphonate substrates by attacking an alternative electrophilic center has encouraged us to suggest a model for the active site of these enzymes (see below).

Discussion

Analogues of AppppA have recently been used as aids toward the understanding of the binding-site geometry and reaction mechanisms of a number of enzymes for which AppppA is a substrate, product, or target such as NppppNase and AppppA phosphorylase (Guranowski, 1987; Guranowski et al., 1987; Blackburn et al., 1987a,b), aminoacyl-tRNA synthetases (Merkulova et al., 1986; Biryukov et al., 1987; Tarussova et al., 1987), and poly(ADP-ribose) polymerase (Suzuki et al., 1987) as well as enzymes for which it is thought to behave as a transition-state analogue. The latter include adenosine kinase and adenylate kinase (Feldhaus et al., 1975; Bone et al., 1986; M. Prescott and A. G. McLennan, unpublished results).

The results reported here with ββ′-substituted and αβ,α′β′-disubstituted phosphonate analogues of AppppA allow some further conclusions to be drawn concerning the nature of the AppppA-binding sites of the asymmetrically cleaving NppppNase from *Artemia* embryos and the symmetrically cleaving enzyme from *E. coli*, and a model is now proposed that emphasizes the similarities rather than the differences between these two enzymes. On the basis of previous data on the substrate specificity of the *Artemia* NppppNase (Prescott et al., 1989) we have suggested the existence of a site on the enzyme that recognizes and binds a pppN portion of the substrate, as shown in Figure 2. It should be noted that although the binding site is represented here by a closed pocket, it may equally well be depicted by a groove.

It seems reasonable to assume that AppppA and ββ′-modified analogues normally bind to the *Artemia* NppppNase in the fashion shown in Figure 2a. This is the normal productive binding mode for this enzyme in which the adenosine distant from the cleavage site is specifically recognized and the tetrapolyphosphate chain adopts a conformation that aligns a water molecule to attack P^{α}, most probably "in line" with the scissile P^{α}–O(P^{β}) bond. When the P^{β}–X–$P^{\beta'}$ bridging oxygen is replaced by a two-carbon component, as in **1f** and **1g**, this conformation cannot readily be attained, and these two substrate analogues show very weak binding and are cleaved very slowly. This is manifest in a reduction in relative specificity of some 3 orders of magnitude (Table II).

A more complex picture results from replacement of this oxygen by a single-carbon bridge. On the one hand, there is a progressive increase in the tightness of binding in the series

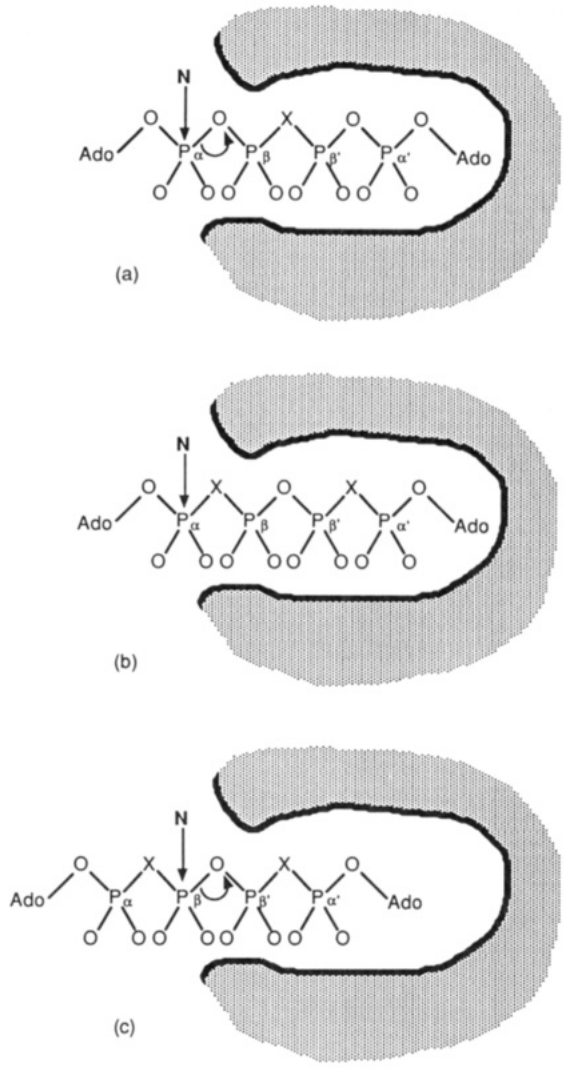

FIGURE 2: Model for the binding of AppppA and its phosphonate analogues within the active site of the bis(5′-nucleosidyl)-tetraphosphate pyrophosphohydrolase from *Artemia*. N represents the attacking nucleophile, which may be water or an amino acid side chain. Details of the model are described under Discussion. (a) Normal (productive) binding mode for AppppA (X = O) and $\beta\beta'$-substituted phosphonate analogues (X = substituted CH_2 group). (b) Normal (nonproductive) binding mode for $\alpha\beta,\alpha'\beta'$-disubstituted phosphonate analogues (X = substituted CH_2 group). (c) Frameshift (productive) binding mode for $\alpha\beta,\alpha'\beta'$-disubstituted phosphonate analogues (X = substituted CH_2 group).

$CCl_2 \leq CF_2 < CHF \leq CHCl < CH_2$, which approximately corresponds to decreasing electronegativity. This may signal a specific interaction between enzyme and the bisphosphonate oxygens, which are generally better chelating agents than pyrophosphate, or the adoption by the less electronegative bridged species of a more favorable conformation for binding. On the other hand, the rate of cleavage of the P^{α}–$O(P^{\beta})$ bond is greatest for the most electronegative CF_2 bridge. A direct comparison of V_{rel} for AppppA and its analogues **1a–c** and **1e** with the fourth dissociation constant for pyrophosphate and its PXP analogues (Blackburn et al., 1980) leads to a Brønsted coefficient of 0.5 (correlation coefficient = 0.98) (Figure 3). This suggests that there is a significant degree of P^{α}–$O(P^{\beta})$ bond breaking in the transition state for the asymmetrical enzyme-catalyzed hydrolysis and that the oxyanion generated is not particularly well stabilized in the transition state. Interestingly, compensation between the variation in V_{rel} and K_i results in a surprising degree of uniformity in the relative specificity of the *Artemia* enzyme for AppppA and four of its

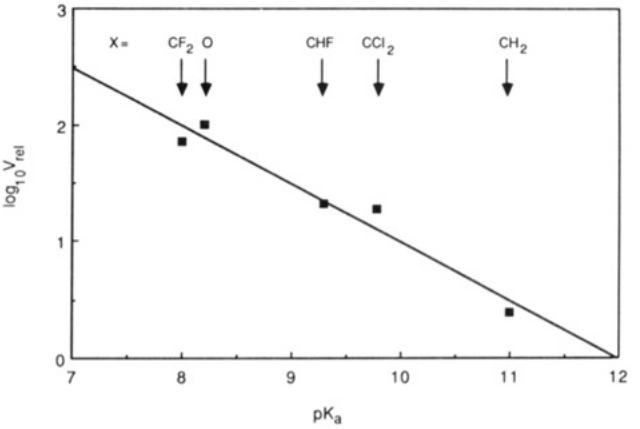

FIGURE 3: Brønsted plot of the fourth dissociation constant for pyrophosphate and the substituted methylenebisphosphonates $O_3PCXYPO_3^{4-}$ against log V_{rel} for the *Artemia* NppppNase catalyzed hydrolysis of AppppA and the corresponding analogues AppCXYppA (**1a–c** and **1e**) (Table II).

analogues over a 40-fold range of V_{max} (Table II).

In the case of the $\alpha\beta,\alpha'\beta'$-disubstituted analogues of AppppA, the tightness of binding to the *Artemia* enzyme again increases with decreasing electronegativity of the bridging carbon atom (Table II). However, the normal binding mode is necessarily nonproductive because of the stability of the P^{α}–$CXY(P^{\beta})$ bond in species **2a–e** (Figure 2b). Unexpectedly, the enzyme effects cleavage of the P^{β}–$O(P^{\beta'})$ bond and most readily for the CHF species (**2b**), which shows a relative specificity 2% of that of AppppA itself. We attribute this behavior to a frameshift mode of binding that brings the catalytic functions and the polyphosphate chain into juxtaposition for nucleophilic attack at P^{β} rather than at P^{α} (Figure 2c). It seems more reasonable to attribute the changed site of attack on phosphorus to the flexibility of the substrate than to a conformational change of the protein at the active site, though both possibilities deserve further investigation. The existence of such a frameshift mode of binding (as depicted in Figure 2c) can also be invoked to explain the established hydrolysis of ApppA, giving AMP and ADP, and of GpppG, giving GMP and GDP (relative rates 0.5% and 2% of that of AppppA) (Prescott et al., 1989). It must also be operating in the symmetrical cleavage of Ap_spppA by the *Artemia* enzyme to give ADPαS (Blackburn et al., 1987a). We also note the possibility that the frameshift mode of binding may offer an explanation for the resistance to hydrolysis of the $\beta\beta'$-substituted analogues by the *Artemia* enzyme, with the probability of the adoption of this mode, in which P^{β} would become the fruitless target for the attacking nucleophile, increasing with decreasing substituent electronegativity.

By contrast, no simple trends can be discerned in the 10-fold variation in K_i for the one-carbon bridged analogues **2a–e** with the *E. coli* enzyme (Table III). It is also surprising to observe that five of the six analogues resist cleavage totally, even though the scissile P^{β}–O–$P^{\beta'}$ bridge remains intact. Whether this signals that the P^{α}–O–P^{β} bridging oxygen is an essential binding site for the catalytic process or that the transition state for this cleavage has a metaphosphate-like character must remain a matter for speculation at this time.

What is clear is that the *E. coli* enzyme has a less stringent specificity for the oligophosphate chain length and can, for example, hydrolyze ApppG at 25% of the rate of AppppA (Plateau et al., 1985). This would be expected if the normal mode for nucleotide binding of the *E. coli* enzyme is also similar to that depicted in Figure 2c, with attack at P^{β} leading to symmetrical cleavage for AppppA. We therefore conclude

that there is no major incompatibility between the cleavage processes for the *Artemia* and *E. coli* enzymes. Rather, the major difference between these, and possibly other asymmetrical and symmetrical cleavage activities, lies in the nature of the groups that determine the register of binding of the phosphoryl anions. If this is the case, NppppNases might be profitable subjects for protein-engineering studies with the objective of interconverting the two types of activity.

The validity of our model of frameshift binding may also be tested directly by additional, designed modifications to substrate nucleotides. For example, the analogue α,β-mono(fluoromethylene) AppppA (ApCHFppA) might well satisfy the energy requirements of both binding modes and lead to the formation of four products. Alternatively, large substitutions on one or both adenine rings might force an unmodified oligophosphate chain into the frameshift binding mode. Such studies with new analogues of AppppA are continuing in our laboratories.

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NMR Assignments for the Amino-Terminal Residues of *trp* Repressor and Their Role in DNA Binding†

Cheryl H. Arrowsmith, Jannette Carey,* Lynda Treat-Clemons, and Oleg Jardetzky
Stanford Magnetic Resonance Laboratory and Department of Biochemistry, Stanford University Medical Center, Stanford, California 94305

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ABSTRACT: The *trp* repressor of *Escherichia coli* specifically binds to operator DNAs in three operons involved in tryptophan metabolism. The NMR spectra of repressor and a chymotryptic fragment lacking the six amino-terminal residues are compared. Two-dimensional *J*-correlated spectra of the two forms of the protein are superimposable except for cross-peaks that are associated with the N-terminal region. The chemical shifts and relaxation behavior of the N-terminal resonances suggest mobile "arms". Spin-echo experiments on a ternary complex of repressor with L-tryptophan and operator DNA indicate that the termini are also disordered in the complex, although removal of the arms reduces the DNA binding energy. Relaxation measurements on the armless protein show increased mobility for several residues, probably due to helix fraying in the newly exposed N-terminal region. DNA binding by the armless protein does not reduce the mobility of these residues. Thus, it appears that the arms serve to stabilize the N-terminal helix but that this structural role does not explain their contribution to the DNA binding energy. These results suggest that the promiscuous DNA binding by the arms seen in the X-ray crystal structure is found in solution as well.

Escherichia coli trp repressor regulates transcription initiation by binding to operator targets within three operons involved in tryptophan metabolism (Klig et al., 1988). The X-ray crystal structure of the protein–DNA complex shows that the repressor uses a symmetry-related pair of helix–turn–helix modules to bind in successive major grooves of the DNA (Otwinowski et al., 1988). Several other DNA binding proteins use terminal "arms" to wrap around their DNA targets [summarized by Jen-Jacobson et al. (1986)]. The amino-terminal regions of *trp* repressor are disordered in the crystal structure of the protein in both the presence and absence of DNA (Schevitz et al., 1985). We have developed methods for selective, preparative removal of the arm residues using chymotrypsin. Comparison of the two-dimensional ^{1}H NMR spectra of intact and armless repressors allows the assignment of resonances from the arm, and confirms that the fine details of the *trp* repressor structure are preserved after removal of the arms. We have used the assignments to directly assess the dynamic behavior of the arms in solution and their role in DNA binding. The identification of independently mobile regions of proteins by NMR is a well-established method (Jardetzky et al., 1978; Wade-Jardetzky et al., 1979) and has been used recently to identify mobile N-terminal residues of λ repressor (Weiss et al., 1984).

Results and Discussion

The circular dichroism spectrum of *trp* repressor is typical of a protein with high α-helix content, with strong minima at 208 and 222 nm. Upon addition of chymotrypsin, the intensity of the 222-nm minimum decreases by about 7% over the first few minutes and then decreases further in a much slower reaction. SDS gel electrophoresis and Edman analysis show that the first phase correlates with removal of the first six residues, Ala-Gln-Gln-Ser-Pro-Tyr, from the N-terminus (Carey, 1989). Using the spectral change as a real-time assay, we prepared milligram amounts of armless repressor. Chymotrypsin was removed by purification through a column of phosphocellulose; unlike intact repressor (Joachimiak et al., 1983), the armless protein elutes during the 100 mM NaCl column wash.

The aliphatic regions of the two-dimensional *J*-correlated spectra of intact and armless repressors are shown in Figure 1, along with the corresponding one-dimensional spectrum of the intact protein. *trp* repressor is a 25 000-dalton dimer (107 residues per subunit) and therefore has a relatively long rotational correlation time, which gives rise to broad resonances in the ^{1}H NMR spectrum. As a consequence, not all the

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* Address correspondence to this author at the Department of Biochemistry, Stanford University Center for Molecular and Genetic Medicine.

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