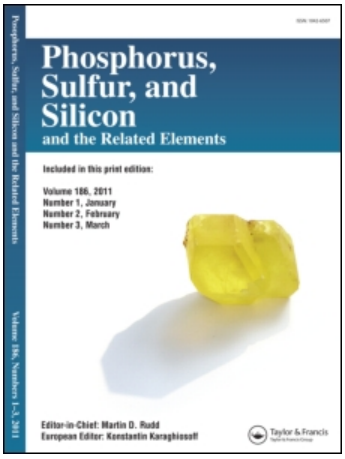

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Studies on Bis(5'-Adenosyl)-P^1, P^4-Tetraphosphate, Appppa, and Some Synthetic Isosteric and Isopolar Phosphonate Analogues Using the Specific Appppaases from *Artemia* and Lupin

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STUDIES ON BIS(5'-ADENOSYL)-P^1,P^4-TETRAPHOSPHATE, AppppA, and SOME SYNTHETIC ISOSTERIC AND ISOPOLAR PHOSPHONATE ANALOGUES USING THE SPECIFIC AppppAases from *Artemia* and LUPIN

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We have recently reported the synthesis and separation of three diastereoisomers 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* (1), while Guranowski *et al.*(2) 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.

We have now synthesised a wider range of phosphonate analogues of AppppA which have CHF, CF_2, CCl_2, and CH_2CH_2 bridges in the tetrapolyphosphate chain. These afford analogues that are on the one hand isosteric or non-isosteric, and on the other hand are polar or non-isopolar (3). This range of compounds has been studied as modified substrates for the AppppAase from *Artemia salina* and from lupin. The results provide unique mechanistic insights into the nature of the enzyme-catalysed mechanism of hydrolysis of such polyphosphate chains. Unexpectedly, they demonstrate that these enzymes can manifest a changed regioselectivity by adapting their reactive site to switch attack from the regular phosphate to a flanking phosphate when they are challenged by a phosphonate residue that is resistant to normal hydrolytic cleavage

(4,5).

Cleavage of AppppA by this specific *asymmetric* Ap_4A hydrolase in $^{18}OH_2$ yields exclusively ^{18}O-AMP and ^{16}O-ATP as products, indicating attack at P-α. In order to probe further the active site of this enzyme, five β,β'-substrates having carbon bridges were prepared and used as substrates for this *Artemia* enzyme. Their order of reactivity relative to AppppA decreases from $AppCF_2ppA$ (V_{rel}=0.7) > CHF > CCl_2 > CHCl > CH_2 (V_{rel}=0.025). By contrast, $AppCH_2ppA$ was the most effective competitive inhibitor of this enzyme with a K_i eight-fold lower than the K_m for AppppA. The non-isosteric analogues having as β,β'-bridge either CH_2CH_2 or (*E*)-CH=CH- show a further fall in the value of V_{rel} and a substantial loss of inhibitory activity.

As anticipated, the corresponding $\alpha\beta,\alpha'\beta'$-disubstituted compounds strongly resist cleavage by this enzyme and are also less effective as inhibitors, paticularly those with bulkier substituents, suggesting poor binding recognition by the enzyme. However, ApCHFppCHFpA was slowly cleaved (V_{rel}=0.03) in a symmetrical fashion to give ApCHFp as the exclusive product. This shows that, under certain conditions, attack at P-β must be possible and indicates some significant degree of flexibility in the active site for this specific *Artemia* enzyme, on the part of either the substrate or the protein. One interpretation of this anomalous cleavage is that the enzyme can operate in a "frameshift" mode when cleavage at the regular site is frustrated by chemical modification (4).

For the range of AppXppA analogues, the rate of cleavage by the *Artemia* hydrolase shows a direct relation to the fourth dissociation constant of the corresponding methylene bisphosphonate $(O_3PXPO_3H)^{3-}$ The Bronsted relationship has a slope of 0.50 (correlation coefficient

0.98) and it clearly establishes that there is a significant degree of breaking of the P^1-O(P^2) bond in the transition state for the reaction. It follows that the enzyme-catalysed reaction is manifesting **rate-determining breakdown** following attack of water at P^1 with some 50% of the oxyanion charge being 'loaded' onto the bridge oxygen in the transition state. It thus provides a rare example of the identification of the rate-determining step in an enzyme-catalysed phosphate transfer process. Somewhat suprisingly, there is an effective compensation between enhanced V_{rel} and K_i which leads to a significant degree of uniformity in the relative specificity (V/K) over a fourty-fold range for V_{max} (4).

The *asymmetrical* specific hydrolase from lupin shows a similar pattern of cleavage for the P^2,P^3-bridged analogues (CF_2 > CCl_2 > O > CHBr > CH_2 > CH_2CH_2). However, for this enzyme, the range of V_{rel} was not matched by significant changes in K_i which varied by a factor of only seven for this group of substrates (5).

In order further to probe the active site for the lupin enzyme, cleavage reactions were carried out on AppppA and AppppppA in the presence of water which was 50% enriched with 18-oxygen. The nucleotide products were isolated by FPLC, and their 18-oxygen content was determined by negative ion FAB MS in a glycerol matrix containing *p*-toluenesulphonic acid. The Lupin AppppA hydrolase cleaves Ap_4A to give ATP and ^{18}O-AMP while it cleaves Ap_5A to give ATP and ^{18}O-ADP. In view of the fact that this enzyme cleaves Ap_4A with inversion of configuration (6), these data show that the active site of this lupin hydrolase effectively measures to the fourth phosphate distant from the bound adenosine residue and cleaves the inward located bridged oxygen (Figure 1). Similar studies are in progress using Ap_4A hydrolase from

E.coli and the lupin Ap_3A hydrolase.

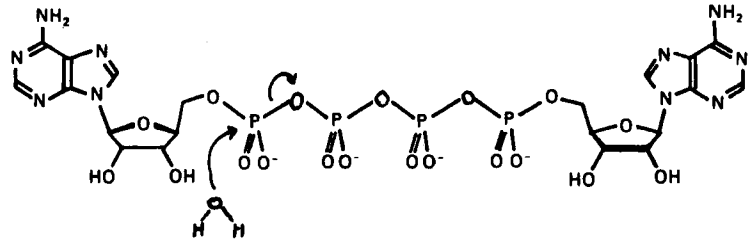

Figure 1. Mode of action of the *asymmetrical* specific lupin Ap_4A hydrolase.

Clearly, further mechanistic studies are called for investigation of this fascinating class of hydrolases and, in particular, attempts must be made to identify the mode of anomalous cleavage of analogues such as ApCHFppCHGpA by the *Artemia* enzyme (4).

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