Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 2603–2608

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SYNTHESIS AND BINDING OF STABLE BISUBSTRATE LIGANDS FOR PHOSPHOGLYCERATE KINASE

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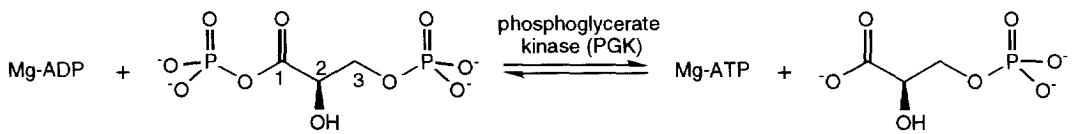
Received 12 June 1998; accepted 12 August 1998

Abstract. Stable bisubstrate ligands of phosphoglycerate kinase (PGK) have been synthesised with AMP or ADP conjugated to hydrolytically-stable, symmetrical analogues of 1,3-*bis*phosphoglycerate and their binding to yeast PGK evaluated. Their K_ds decrease with net negative charge, with a penta-anionic analogue **7** showing highest affinity - in accordance with its approximation to the transition state for the reaction catalysed by PGK.

Introduction

Phosphoglycerate kinase (PGK) is a key glycolytic enzyme which catalyses the interconversion of 1,3-*bis*-phosphoglycerate (1,3-BPG) and adenosine diphosphate with 3-phosphoglycerate (3-PGA) and adenosine triphosphate (Scheme 1).[1]

Scheme 1 The interconversion of 1,3-BPG and 3-PGA catalysed by phosphoglycerate kinase (PGK)



1,3-Bisphosphoglycerate (1,3-BPG) 3-Phosphoglycerate (3-PGA)

PGK has been crystallised from a variety of sources[2-6] and exists as a bilobal enzyme in which the binding of the two substrates is known to occur within the two distinct domains. 3-PGA binds to the *N*-terminal domain at a locus comprised of several basic amino acids (the 'basic patch') whilst the nucleotide is bound within the other lobe at the *C*-terminal domain. Since the separation of these two domains is too large (12-15 Å) for direct phosphoryl transfer, it was envisaged that upon binding of the two substrates to this 'open' form of the enzyme, a 'hinge bending' mechanism might allow the reaction to take place *via* the 'closed' form of the enzyme.[7] Recently, a partially 'closed' form of the enzyme has been characterised by x-ray crystallography of *T. brucei* PGK in ternary complex with Mg-ADP and 3-PGA. This revealed a 32° hinge-bending relative to the 'open' form of the horse muscle enzyme[7] in which the two substrates are brought close together in the absence of water to enable phosphoryl transfer to occur. Such "closed" conformations have been seen in crystal structures

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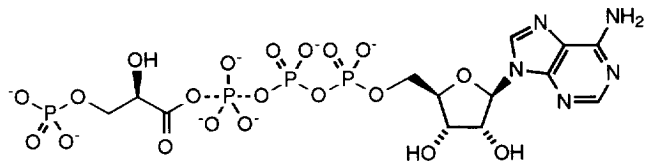
PII: S0960-894X(98)00466-1

for adenylate kinase [EC 2.7.4.3] in a binary complex with 5',5'''-diadenosyl pentaphosphate,[8] for thymidylate kinase [EC 2.7.4.9] complexed with 5'-adenosyl 5'''-thymidyl pentaphosphate,[9] and for cytidylate kinase [EC 2.7.4.14] complexed with 5'-adenosyl 5'''-uridyl pentaphosphate.[10]

The stereochemistry of the phosphoryl transfer reaction for PGK has been shown to proceed *via* an "in-line" mechanism[11] and the transition state for the reaction with magnesium co-ordination is likely to be as illustrated (Figure 1). Details of the binding of the substrates and the transition state for trypanosomal PGK have emerged from x-ray studies with Arg39, Gly376, and Gly399 identified as substrate phosphate ligands and with Lys219 as a ligand for the transition state only.[12]

Figure 1 Proposed pentacoordinate transition state involved in PGK-catalysed phosphoryl transfer

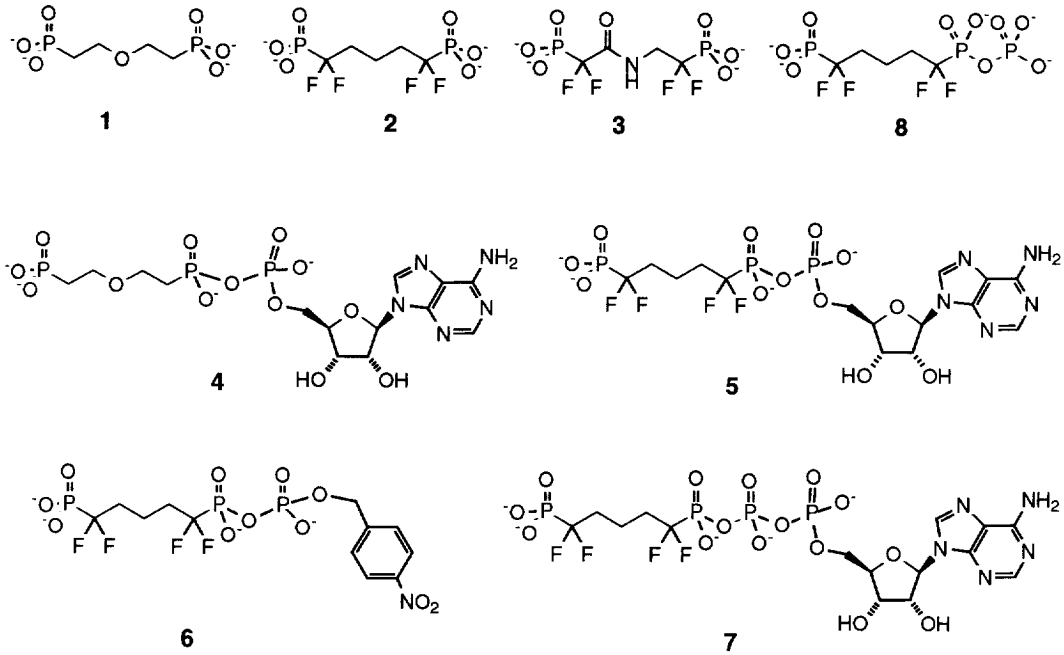


We have for some years pursued the rational design of bisubstrate inhibitors of PGK incorporating several structural features envisaged to be present within the transition state[11] (Figure 1). Ideal analogues should have five negative charges in the active site in addition to two on the glycerate 3-phosphate ester. They should be stable to hydrolysis in the normal reaction by incorporation of non-hydrolysable phosphonate linkages and these should have significant electronegative character to support the apical disposition of the glycerate moiety relative to the trigonal bipyramidal phosphorus in the transition state.

By comparison with the pentaphosphate bisubstrate ligands successfully used with adenylate, thymidylate, and uridylate kinases, a polyphosphate chain linking adenosine and 3-phosphoglycerate would be too unstable to spontaneous hydrolysis for use as a bisubstrate analogue. We therefore developed chemical syntheses and determined binding characteristics for several non-hydrolysable bisphosphonate analogues of 1,3-BPG[13] with a view to linking suitable candidates to adenosine nucleotides. From this study it was clear that a chain of five atoms between the two phosphoryl groups generally gives medium to high affinity binding to PGK. For the simple pentane 1,5-bisphosphonic acid derivatives, the presence of an α-difluoromethylene moiety dramatically increases binding while the introduction of a carbonyl function in the chain provides a further advantage. We concluded that the increased charge resulting from enhanced acidity (pK_{a2}) of difluoromethylenephosphonic acids would be needed to optimise coulombic interactions with "basic patch" residues around the glycerate 3-phosphate binding site.

Analogues **1** and **2** were selected as typical weak and medium affinity ligands for condensation with AMP and ADP for two main reasons. First, because of symmetry there is no ambiguity in their mode of linkage to the nucleotide. Secondly, affinity for PGK enhanced by some two orders of magnitude as a result of nucleotide binding would remain "on scale" for the NMR assay used.[14] By contrast, analogue **3** could form two condensation products with AMP and the problems inherent in a regioselective synthesis have not yet been resolved (Figure 2). Moreover, any enhancement of its affinity for PGK (K_d 3 μM) through conjugation to AMP/ADP would lead to an off scale result. We present here details of the chemical synthesis and PGK binding of analogues **4** - **6** and of a novel bisubstrate ligand **7** in which **2** is linked to ADP.

Figure 2 1,3-Bisphosphoglycerate analogues and their bisubstrate ligands of PGK



Results and Discussion

The reaction of the tris(tributylammonium) salts of the phosphonates **1** and **2** with either AMP morpholidate[15] or *p*-nitrobenzyl phosphoromorpholidate[16] in pyridine gave the adenosine monophosphate (AMP) analogues **4** and **5** and the *p*-nitrobenzyl analogue **6**, respectively. The lower reactivity of the difluoromethylenephosphonates necessitated reaction times of 6 d, whilst the reaction to give **4** required only 4 d. The analogues were converted into their sodium salts by anion exchange chromatography: isolated yields and NMR data are displayed in Table 1. Products were further characterised by negative ion ESMS and were shown to be pure by ion-exchange HPLC.

In view of the reduced nucleophilic reactivity of the *bis*-difluoromethylenephosphonate analogue **2**, an alternative synthetic route to the ADP-bisubstrate ligand **7** was explored, involving activation of the phosphonate **2** prior to condensation with AMP. However, with diphenyl phosphorochloridate (2 equiv) as the activating agent for **2**, analogue **7** was isolated in only 3% yield after ion exchange chromatography and extensive purification by reversed phase HPLC. A large number of side products appeared to arise from activation of bisphosphonate **2** at both ends. By contrast, the condensation of ADP morpholidate[17] and **2**, gave the desired compound **7** in 17% yield in pure form following ion exchange chromatography. A second synthetic route involved deprotection of the phosphonophosphate anhydride **6** by catalytic hydrogenation to afford the bisphosphonophosphate species **8**. It was envisaged that **8** would react at the phosphate centre (rather than at the difluoromethylenephosphonate moiety) with AMP morpholidate and so would generate the bisubstrate ligand **7**. A similar reaction with ADP morpholidate would then give the corresponding ATP analogue. In the

event, such reaction with AMP morpholidate at the phosphate terminus was not achieved and mixtures of products in which the nucleotidic component was attached to either and/or both termini were obtained.

Table 1 Experimental data for bisubstrate ligands of PGK

Compound	Yield	δ_P (ppm)	δ_F (ppm)	$^2J_{PP}$ / Hz	$^2J_{PF}$ / Hz
4	35 %	P^1 -10.79 P^2 +14.52 P^3 +20.74		P^1P^2 26.1	
5	30 %	P^1 -11.51 P^2 -4.12 P^3 +5.82	-114.82 -115.23	P^1P^2 30.6	P^2 102.6 P^3 90.8
6	45 %	P^1 -11.52 P^2 -4.45 P^3 +5.58	-115.00 -115.03	P^1P^2 30.8	P^2 102.8 P^3 93.4
7	17 %	P^1 -10.92 P^2 -22.74 P^3 -3.33 P^4 +6.14	-115.13 -115.20	P^1P^2 18.7 P^2P^3 28.9	P^3 103.0 P^4 90.3

K_d values for binding the bisubstrate ligands to PGK and those of the corresponding 1,3-BPG derivatives were determined by ^{1}H NMR as described previously (Table 2).[13,14] The K_d values of the AMP bisubstrate ligands **4** and **5** were both very much reduced relative to the corresponding 1,3-BPG analogues **1** and **2**. In particular, the *bis*-difluoromethylenephosphonate analogue attains higher binding affinity than the methylenephosphonate when linked to AMP. The fact that the *p*-nitrobenzyl ester **6** displays an approximate ten-fold enhancement in binding to PGK relative to the parent 1,3-BPG **2** suggests that the greater affinity of these analogues for the enzyme results predominantly from the multiplicity and placing of anionic charge in the binding site while it is noteworthy that *p*-nitrobenzyl ester binds only two-fold weaker than the corresponding adenosyl ester **5**. These results strongly suggest that the affinity of the bisubstrate analogues for PGK is dominated by coulombic interactions of the phosphoryl moieties. It is particularly promising that the bisubstrate ligand **7** constructed on an ADP moiety exhibits the lowest K_d value achieved.[14] This accords with its increased charge and the potential binding of the adenylate portion of the molecule within the nucleotide-binding domain of PGK. These results clearly demonstrate the superiority of binding of bisubstrate analogues to PGK over affinities for either isolated adenosine nucleotides or for the 1,3-BPG analogues alone.

The complex of the AMP analogue **5** with *T. brucei* PGK has recently been characterised in two different crystal forms[18] with the phosphonate chain being fully extended and the adenylate portion bound within the C-terminal domain of the protein in an isomorphous manner to that observed in the ternary complex.[12] In both structures, position-5 of the phosphonate occupies a similar location within the N-terminal domain to that occupied by 1,3-BPG. The two structures provide novel intermediate 'closed' structures of the ternary complex in that they display hinge bending motions within both the C- and N-domains and a dislocation of the catalytic element helix-13 within the C-terminal domain. In the ternary complex of PGK with ADP and 3PGA,[12] hinge bending within both domains occurs with helix-13 in its original position. It is to be expected that analogue **7**

Table 2 Dissociation constants (K_d) for 1,3-BPG analogues and 1,3-BPG-bisubstrate ligands.

Compound	K_d (SD) / μM	$\Delta\delta_{max}$ / ppb	No. of Histidines used
3-PGA	109 (10)	299	3
1	4,999 (833)[a]	115	2[b]
2	140 (21)[a]	62	2[b]
4	120 (52)	19	2[c]
5	6 (3)	21	3
6	13	90	1[d]
7	≤ 1 (1)	35	3

All data were the mean of three independent determinations. [a] Data from reference 13. [b] His62 did not shift to give data of high enough quality for analysis. [c] His167 did not shift to give data of high enough quality for analysis. [d] His62 did not shift to give data of high enough quality for analysis and signal of His170 was obscured by ring protons of **6**.

would more closely resemble a 'true' bisubstrate ligand in that it contains both ADP and 1,3-BPG moieties. Crystallisation experiments with the analogue **7** and *T. brucei* PGK are in progress.

Nonetheless, the affinities of Up_5A for UMP/CMP kinase (K_d 3 nM)[19] and that of Ap_5A for adenylate kinase (K_i 40 nM)[20] are much higher than that achieved here for **7** ($K_d \leq 1$ μM). This would appear to relate in part to the limited analogy of **2** with 1,3-BPG, which might be remedied in a condensation product of ADP with **3**, and in part to the better coulombic status of Up_5A and Ap_5A (5 minus), which have only one negative charge less than the transition state (6 minus) for the nucleotide kinase process. By contrast, **7** has two negative charges fewer than the corresponding transition state for the PGK reaction. That highlights a key dilemma: in order to gain better charge approximation one has to introduce an additional phosphate residue in the chain with consequent loss of steric compatibility. Alternative solutions to this problem, including the use of supercharged nucleotide analogues,[21] are under investigation.

Experimental.

Ion exchange HPLC was performed using a SAX 4.6 x 250 mm column with a linear gradient of 0.1 - 1 M potassium phosphate pH 6.6 in 20 min and a flow rate of 1 mL min^{-1}. NMR data were obtained for ^{31}P at 101.26 MHz and ^{19}F at 235.36 MHz.

AMP and p-*nitrobenzyl phosphate bisubstrate ligands:* AMP morpholidate or *p*-nitrobenzyl phosphoromorpholidate (1 mmol) was reacted with the *tris*(tributylammonium) salt of phosphonate **1** or **2** (1.4 mmol) in dry pyridine (10 mL) for 4 and 6 d respectively. The crude products were purified on DEAE Sephadex with a linear gradient of 0.05-0.45 M triethylammonium bicarbonate solution (2.5 L each) and converted into their sodium salts. Analogues **4** and **5** eluted between 0.3 and 0.37 M TEAB, whilst **6** eluted between 0.32-0.45 M TEAB. Yields and NMR data are presented in Table 1. Ion exchange HPLC elution times were 10.4 min for both **4** and **5** and 10.3 min for **6**.

ADP bisubstrate analogue 7: ADP morpholidate (1 mmol) was reacted with the *tris*(tributylammonium) salt of

phosphonate **2** in dry pyridine (5 mL) for 6 d and purified on DEAE-Sephadex with a linear gradient of 0.025-0.6 M TEAB (2.5 L each). The *product* eluted between 0.54 and 0.57 M TEAB. Yield and NMR data is presented in Table 1; HPLC elution time was 13.9 min.

K_d values were measured using ^{1}H nmr chemical shift data (with a Bruker AMX-500) for His62, His167 (upfield shifts), and His170 (downfield shift) based on the work of Boyle *et al.*[22] and analysed by a programme provided by Prof. C. A. Hunter (Sheffield University).[13]

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