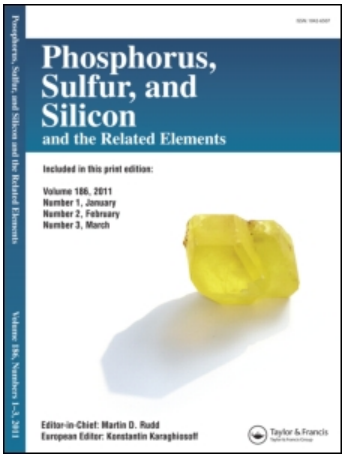

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Highly Potent Bisphosphonate Ligands for Phosphoglycerate Kinase and Protein Binding Studies

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Several novel α,ω-bisphosphonate analogs (**1**) of 1,3-bisphospho-D-glyceric acid, 1,3-BPG (**2**) have been made and their binding to phosphoglycerate kinase, PGK (EC 2.7.2.3) evaluated. Non-scissile methanephosphonic acids replace the two phosphate monoesters of 1,3-BPG. Fluorine substitution in the α-methylene groups of the phosphonic acid analogs (**1**) markedly improves their binding to PGK as determined by NMR analysis. The best ligands bind some 50–100 times more strongly than does 3-phospho-D-glyceric acid and show a requirement for pK_a3 to be below 6.0 while the presence of a β-carbonyl group (**1**, Y = CO) seems to be of secondary importance. One analog, 1,1,5,5-tetrafluoropentanebisphosphonic acid, has been linked to adenosine 5'-phosphate and 5'-diphosphate to generate bisubstrate analogs (**3**) whose affinity for PGK and an X-ray crystal structure of a binary complex with PGK will be discussed.

Keywords: Phosphonates; PGK; binding studies; bisubstrate; p*K*a difluoromethylenephosphonates

INTRODUCTION

Phosphoglycerate kinase (PGK) is a key glycolytic enzyme which catalyses the interconversion of 1,3-*bis*-phosphoglycerate (1,3-BPG, (**2**)) and adenosine diphosphate with 3-phosphoglycerate (3-PGA) and adenosine triphosphate. The enzyme is an attractive drug design target since the bloodstream form of *trypanosoma brucei*, the causative agent of African sleeping sickness, relies on glycolysis as its sole source of energy. [1] The recent crystal structure of the enzyme from this trypanosome[2] demonstrates that the enzyme is highly flexible and undergoes a dramatic conformational change in response to substrate binding as first proposed by Banks [3]. For several years we have synthesized a range of bisphosphonate analogues of (**2**) with a general structure (**1**) and reported their binding potential to PGK.[4,5] Here we report the synthesis of novel bisphosphonate analogues (**3**) of the transition state for the reaction catalyzed by PGK their binding potential to the enzyme and show how one analogue (**12**) when crystallized with PGK induces a hitherto unknown conformation of the enzyme.

The transition state of PGK has been shown to proceed *via* an "in-line" mechanism, with direct phosphoryl transfer between substrates.[6] Thus any bisubstrate inhibitor of the enzyme 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. Figure 1 depicts bisphosphonate and bisubstrate ligands for PGK. Analogues **4**,**5** and **7** were selected as typical weak and medium affinity ligands for condensation with AMP and ADP for two main reasons. First, because of

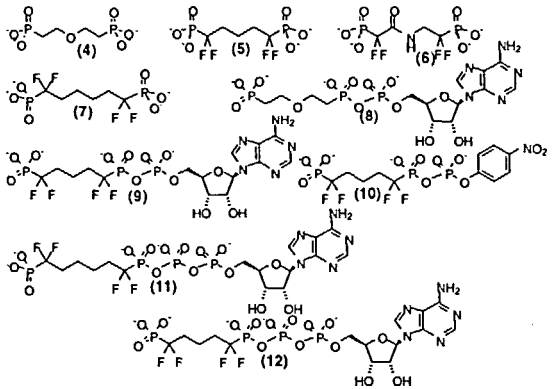

Figure 1 Bisphosphonate and bisubstrate ligands for PGK

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. By contrast, analogue **6** could form two condensation products with AMP, and the problems inherent in a regioselective synthesis have not yet been resolved. Moreover, any enhancement of its affinity for PGK (K_d 4 μM) through conjugation to AMP/ADP would lead to an off scale result.

The reaction of the tris(tributylammonium) salts of the phosphonates **4** and **5** with either AMP morpholidate[7] or *p*-nitrobenzyl phosphoromorpholidate[8] in pyridine gave the adenosine monophosphate (AMP) analogues **8** and **9** and the *p*-nitrobenzyl analogue **10**, respectively. The lower reactivity of the difluoromethylenephosphonates necessitated reaction times of 6 d, whilst the reaction to give **4** required only 4 d. Diphenyl phosphorochloridate (2 equiv) was the activating agent for **7**, and **11** was isolated in 3% yield after ion exchange chromatography and extensive purification by reversed phase HPLC. The condensation of ADP morpholidate[9] and **5** gave the desired compound **12** in 17% yield in pure form following ion exchange chromatography.

Table 1 presents the binding constants determined by NMR for 1,3BPG analogues (**4-12**). All the bisubstrate analogues bind PGK more potently than the bisphosphonates from which they are derived.

TABLE 1 Dissociation constants for bisphosphonate and bisubstrate ligands of PGK

Structure	K_d / μM (s.d.)	Average Δδmax / ppb (s.d)	# determinations	pK_a3,4 (statistically corrected)
(4)	5000 (880)	115 (40)	2	6.90, 7.72
(5)	140 (20)	62 (30)	2	5.09, 5.27
(6)	4 (2)	58 (18)	3	4.02, 4.88
(7)	1000 (260)	52 (13)	3	4.95, 4.95
(8)	120 (52)	19 (n.d.)	2	n.d.
(9)	6 (3)	21 (n.d.)	3	n.d.
(10)	13	90	1	n.d.
(11)	1	35	1	n.d.
(12)	1 (1)	35 (n.d.)	3	n.d.

Analogue **(12)** was successfully crystallized with *T. brucei* PGK[10] and Figure 2 shows a comparison of the two crystal structure forms of the enzyme. Figure 2A represents the PGK • ADP • 3PGA complex in which the enzyme adopts a 'closed' conformation where the substrates are aligned for phosphoryl transfer. Figure 2B represents the PGK • **(12)** structure. It shows that the enzyme adopts an intermediate state when binding **(12)**,

between the 'open' and 'closed' forms of the enzyme. In the 2B structure, helix 13 (purple in Figure 2) adopts a unique position, not seen in any of the previous PGK structures,[10] differing in position by up to 2.3 Å. Prior to this observation it was thought each domain moved about the 'hinge points' at either end of helix 7 with no intradomain motion; however, this crystal structure reveals that helix 13 moves independently within the C-terminal domain, switching between its two known conformations as substrates bind. The synthesis of stable bisubstrate analogues of PGK has afforded an altogether new view of the mechanism of domain closure of this enzyme, and has provided new insight into how synthetic chemistry may aid enzyme mechanism elucidation.

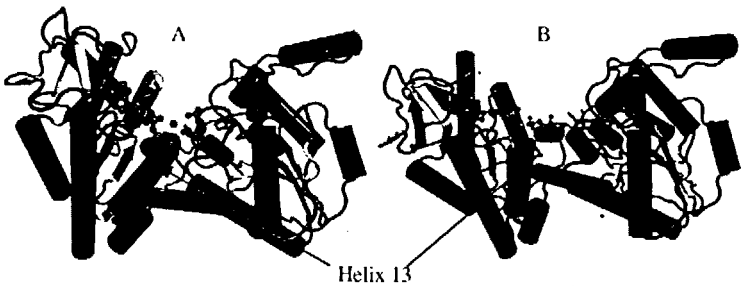

FIGURE 2 *T. brucei* PGK complexed with (A) ADP and 3PGA and (B) $AppCF_2(CH_2)_3CF_2p$. (**12**). Figure prepared using VMD[11] and Pov-ray.[12]

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