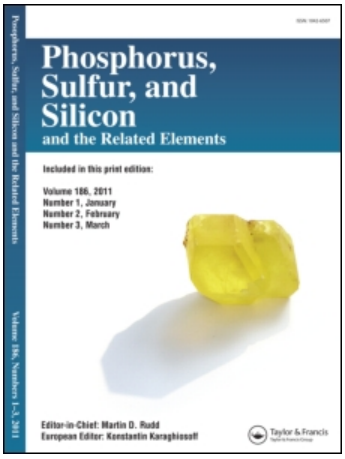

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Role of the Phosphate Moiety in the Affinity of Inhibitors for the Glycolytic Enzyme Glyceraldehydephosphate Dehydrogenase (GAPDH)

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Role of the phosphate moiety in the affinity of inhibitors for the glycolytic enzyme glyceraldehydephosphate dehydrogenase (GAPDH)

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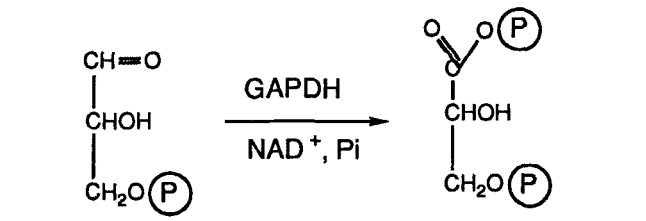
Abstract :

Kinetic inhibition studies of the title enzyme by substrate analogues bearing reactive groups, epoxyde or α-enone, indicate that the substituents on the phosphorus atom play a crucial role in the efficiency of these inhibitors. This result is accounted for by their affinity for an hydrophobic part of the protein, in close vicinity to the residue responsible for the covalent binding.

In the prospect of inhibitors for the title enzyme, part of the glucose metabolism, we came interested in molecules similar in structure to the substrate glyceraldehyde-3-phosphate that is transformed by GAPDH with NAD^+ and inorganic phosphate to 1,3- diphospho-glycerate.



glyceraldehyde phosphate

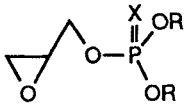
1,3 diphospho-glycerate

The inhibitors are substrate analogues bearing reactive groups such as an epoxide or α-enone, able to bind covalently to the protein (1). The aim of the study is twofold : to get a better insight on the active site and on other features for this enzyme from different sources (2), and also develop new leads with antiparasitic properties based on blocking the glucose metabolism, essential for parasites such as trypanosome and leishmania. A first approach

based on differences for glycolytic enzymes between mammal and trypanosoma brucei has already been developped (3).

To be active in vivo and reach their target, such inhibitors have to cross several membranes and therefore have to have proper lipophilic-hydrophilic balance. This requierement can be met by introducing around the phosphorus atom lipophilic groups. For this purpose the following compounds were synthesized :

p

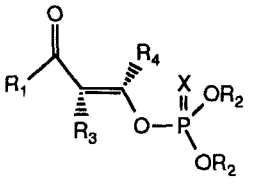


X=O

- $R_1 = H \quad R_2 = Na^+$ **1**
- $R_1 = R_2 = C_2H_5$ **2**
- $R_1 = R_2 = CH_2C_6H_5$ **3**

X=S

- $R_1 = H \quad R_2 = Na^+$ **4**
- $R_1 = R_2 = C_2H_5$ **5**
- $R_1 = OCH_3$; N iPr_2 **6**

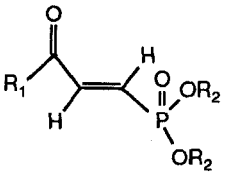


X=O

- $R_1 = CH_3$; $R_2 = CH_3$; $R_3 = H$; $R_4 = CH_3$ Z and E **7**
- $R_1 = CH_3$; $R_2 = C_6H_5$; $R_3 = H$; $R_4 = CH_3$ Z and E **8**

X=S

- same substituents as **7** **9**
- same substituents as **8** **10**



- $R_1 = R_2 = CH_3$ **11**
- $R_1 = CH_3$; R_2 =iPr **12**
- $R_1 = H$; $R_2 = H$ **13**
- $R_1 = H$; $R_2 = CH_3$ **14**
- $R_1 = H$; R_2 = iPr **15**

The compounds bear phosphate or phosphonate groups diversely substituted around the phosphorus atom. In a first study they were assayed on the isolated enzyme GAPDH of mammalian rabbit muscle. Values are given in Table I.

TABLE I : Inhibition kinetic parameters on GAPDH

	K_i (mM)	K_i/K_m	k_i (mn^{-1})	k_i/K_i ($M^{-1}\ mn^{-1}$)
1	22,22	278	0.29	$0,13.10^2$
2	1,36	19	0.69	$5,07.10^2$
3	7,82	98	1.86	$2,37.10^2$
13	3,33	42	0.33	$1,00.10^2$
14	0,06	0.75	0.73	121.10^2
15				$K_{2nd} = 116.10^2$

The best results are obtained with epoxi-phosphates ; phosphonates of the same chain length or corresponding episulfide or thiophosphoric acid are inactive (not shown). In the set of compounds bearing the α-enone group, in opposite phosphonates are the most active. The best results are obtained with epoxide compounds **1**, **2**, **3** and α-enone **13**, **14**, **15** : their irreversible inhibition was evidenced by convenient kinetic methods : dependance with time, dilution and protection by substrates. K_i and k_i values correspond to inhibition where an intermediate non covalent complex is involved, k_{2nd} corresponding to a bimolecular process without intermediate.Values of k_{2nd} and k_i/K_i (comparable since of the same unit) show that phosphorylated α-enone correspond to the best inhibitors. The most striking fact is to observe that in both series, neutral esters are more active than the corresponding charged phosphate or phosphonate, more close to the substrate GAP structure : the esters **2**, **3**, **14** and **15** exhibit an higher activity than the charges bearing analogues **1** and **13**. The reverse should be expected considering the interaction of the polar head with the protein.

Covalent binding of these esters with the protein was confirmed by NMR with GAPDH and compound **2** ($\delta^{31}P$ = -1.6 ppm in triethanolamine buffer).The NMR spectrum of the incubation solution for 20 mn of the enzyme and an excess of **2** in the buffer TEA (followed by dialysis on Sephadex G15 gel) shows a peak at + 1.5 ppm ; a peak at the same chemical shift is observed for the adduct of propane thiol on **2** in TEA.Slow hydrolysis of **2** in the reaction gives a peak at 0.5 ppm which cannot be confused whith the peak at 1.5 ppm,clearly corresponding to the inhibitor covalently bound to the enzyme.

Modelling studies with the 3D structure of the GAPDH enzyme, now available (4) allow to account for the higher activities obtained with the selected compounds of table 1, and also for the higher activity of neutral molecules versus polar ones. Indeed the best fit is obtained with the binding of the inhibitors at the inorganic phosphate and not the glyceraldehyde-phosphate binding site ; this process brings in close vicinity of the phosphorus head two hydrophobic residues (Alanine-135 and Threonine-225). Irreversible inactivation occurs through covalent binding of the essential cysteine -165 residue involved in the formation of the intermediate thio-acetal of the enzymatic reaction. Further studies particularly protein sequencing will be performed to confirm these results.

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