Inhibition of Glyceraldehyde-3-Phosphate Dehydrogenase by Phosphorylated Epoxides and α-Enones†

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ABSTRACT: Pentalenolactone and koningic acid are antibiotics known for their potent inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. The reactive groups present in these antibiotics are, respectively, an epoxide and an α-enone, which form covalent bonds with an active-site cysteine residue of the enzyme. This information was used for the design of two series of glyceraldehyde 3-phosphate analogues with similar reactive groups that could function as potential irreversible inhibitors of glyceraldehyde-3-phosphate dehydrogenase. Inactivation kinetics, NMR analysis, protection experiments, and titration of free cysteine residues together indicate that the inhibitors bind to the active site of the enzyme and form a covalent bond with the active-site cysteine residue of the enzyme. Binding probably takes place at the inorganic phosphate site of the enzyme and may lead to a conformational change. Comparison of the reactivities of the inhibitors for the glycosomal enzyme from the protozoan parasite *Trypanosoma brucei* and the rabbit muscle enzyme revealed that some of them had a preference for the trypanosome enzyme. When their effect was measured on the multiplication of trypanosome *in vitro* cultures, one inhibitor appeared to exhibit an inhibitory effect at a concentration significantly lower than the trypanocidal drugs, pentamidin and (difluoromethyl)ornithine, that are routinely used in the treatment of African sleeping sickness.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12),[1] a key enzyme in glycolysis, catalyzes the oxidative phosphorylation of the triose glyceraldehyde 3-phosphate to form 1,3-diphosphoglycerate in the presence of NAD^+ and inorganic phosphate (Harris & Waters, 1976). In our ongoing search for inhibitors of the glycolytic enzymes (Betbeder et al., 1990; Alric et al., 1991; Opperdoes et al., 1990; Willson et al., 1992, 1993), we describe here the activity of a number of triose phosphate analogues capable of inactivating GAPDH by forming a covalent bond with the enzyme. In the design of these new inhibitors we were led by the structural information available for two naturally occurring inhibitors of GAPDH: pentalenolactone and koningic acid.

Pentalenolactone (Figure 1), an antibiotic sesquiterpene isolated from *Streptomyces areae*, is a potent inhibitor of GAPDH from various sources (Cane & Sohng, 1989; Lambeir et al., 1991). This antibiotic has a highly hydrophobic character and contains two reactive groups: an epoxide and an α-enone. It has been shown to bind irreversibly to the enzyme from rabbit muscle, while the binding to the enzymes from yeast, *Escherichia coli*, *Bacillus stearothermophilus* and *Trypanosoma brucei* is reversible. In the case of the rabbit muscle enzyme, cysteine 149 is supposed to be the target for this compound to which it binds in a covalent manner (Cane & Sohng, 1989). In the case of the *T. brucei* enzyme, the antibiotic would bind near the substrate-binding site but the structure of the enzyme–inhibitor complex has not been established.

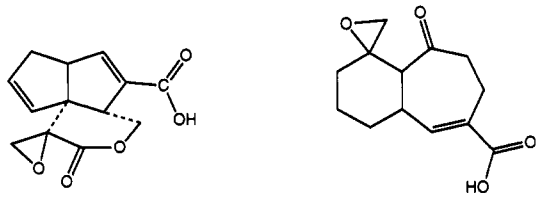

FIGURE 1: Chemical structures of two naturally occurring antibiotic inhibitors of the enzyme GAPDH: pentalenolactone (left) and koningic acid (right).

The other antibiotic, koningic acid (Figure 1), is also a sesquiterpene produced by the fungus *Trichoderma koningii* and is an equally potent inhibitor of GAPDH. It bears as reactive group an epoxide (Sakai et al., 1991). Koningic acid is a better inhibitor of eukaryotic than of bacterial-type GAPDHs, but the reason for this difference has not yet been established (Kato et al., 1992). Epoxides such as glycidol and glycidol phosphate have been shown to irreversibly inhibit the glycolytic enzymes triosephosphate isomerase (Rose & O'Connell, 1969) and yeast GAPDH (McCaul & Byers, 1976) that both act on triose phosphates.

We have investigated the possibility that analogues of glyceraldehyde 3-phosphate, containing either an epoxide or an α-enone group, would be able to form a covalent bond with GAPDH. For this purpose, large sets of compounds were

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[1] Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase, EC 1.2.1.12; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate; P_i, inorganic phosphate; P_s, phosphate group of the substrate glyceraldehyde 3-phosphate; 1,3-diPG, 1,3-diphosphoglycerate.

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synthesized (Lauth et al., 1993) and their inhibitory effects on GAPDH from rabbit muscle were studied by classical enzyme kinetic methods. Those compounds that gave an irreversible inactivation of the enzyme are studied in more detail in this paper, particularly with respect to their kinetic behavior and their differential effects on two GAPDHs, those of rabbit muscle and of *Trypanosoma brucei*, a protozoan hemoflagellate that is the causative agent of African sleeping sickness in man.

The latter organism is characterized by an unusual compartmentation of its glycolytic pathway which is confined within a microbody called the glycosome (Opperdoes, 1987). It has a glycolytic flux which is among the highest ever reported for an organism and the glycosomal GAPDH is characterized by a number of unique structural and kinetic features (Lambeir et al., 1991; Michels et al., 1991). Moreover, inhibition of glycolysis of *T. brucei* has been shown to lead to an immediate depletion of the cell of ATP, followed by a rapid disappearance of the parasites from the host's bloodstream (Clarkson & Brohn, 1976; Fairlamb et al., 1977). Together these properties render the glycosomal GAPDH of the trypanosome a promising target for drug intervention.

MATERIALS AND METHODS

Enzymes, Substrates, and Inhibitors. Glycosomal GAPDH was isolated from the bloodstream form of *T. brucei* as described (Misset et al., 1987). Rabbit muscle GAPDH and glyceraldehyde 3-phosphate were purchased from Boehringer GmbH, Germany, or from Sigma Chemical Co. Fresh glyceraldehyde 3-phosphate was prepared by hydrolysis of the dimethyl acetal according to the instructions by the manufacturer. The concentration of D-glyceraldehyde 3-phosphate was determined enzymatically with triosephosphate isomerase and glycerol-3-phosphate dehydrogenase (enzymes purchased from Boehringer GmbH). The NADH concentration was calculated from its absorbance using an ϵ_{340} = 6.22 mM^{-1} cm^{-1}. The synthesis of the inhibitors used in this study has been described elsewhere (Lauth et al., 1993).

Inactivation Studies. The inactivation of GAPDH by the compounds was measured in the direction of NADH formation. The reaction mixture (1 mL) contained 0.1 M triethanolamine hydrochloride buffer, pH 7.6, 1 mM EDTA, 0.1 M KCl, 10 mM potassium phosphate, 2 mM NAD, and 0.8 mM D-glyceraldehyde 3-phosphate. All reactions were carried out at 25 °C and the formation of NADH at 340 nm was measured with a Perkin-Elmer λ 9 spectrophotometer equipped with a kinetic accessory unit. The activities of the compounds on GAPDH were measured after various times of preincubation of the enzyme with the compound in the assay buffer, followed by the addition of NAD^+, glyceraldehyde 3-phosphate, and inorganic phosphate to start the reaction. Possible effects of inhibitors on the absorbance of NADH were verified by running reactions without enzyme. The percentage of remaining activity was calculated using as reference a control incubation without inhibitor but containing the same volume of solvent used for the inhibitor; at concentrations of the solvent below 10%, no significant effect on the enzyme activity was observed.

For determination of product concentration at infinity $[P]_\infty$ according to the method of Liu and Tsou (1992), which requires large quantities of substrates and enzymes, the study was performed in the reverse direction, from 1,3-diphosphoglycerate to GAP. Measurements were made in the presence of all substrates: 0.1 M TEA/HCl buffer, pH 7.6, 1 mM EDTA, 0.1 M KCl, 10 mM potassium phosphate, 2 mM 1,3-diPG, and 0.42 mM NADH; concentrations of inhibitor 4 were 0 (control), 2.4, 3.6, 4.8, 7.2, 9.6, and 12 mM. The enzyme (0.1 mg/mL at final concentration) was added to the reaction mixture to start the reaction.

Data Analysis. The time-dependent inactivation of GAPDH by the selected compounds followed pseudo-first-order kinetics. Plots such as those presented in Figures 3 and 4 resulted from experiments run in triplicate; individual initial rates were kept only when they corresponded to values obtained with correlation coefficient equal to or better than 0.998. For such inactivation, three pathways can be envisaged: (1) formation of a reversible enzyme–inhibitor complex, followed by inactivation of the enzyme through formation of a covalent bond; (2) simultaneous complex formation and inactivation, and (3) binding of the inhibitor leading to a conformational change which is then followed by an inactivation step. The three pathways of inactivation can be expressed as follows:

pathway 1 $$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E\text{–}I \xrightarrow{k_i} E\text{–}I^*$$

where E represents the free enzyme, I the inhibitor, E–I a dissociable complex, and E–I* the inactivated irreversible inhibitor–enzyme complex. The kinetic equation corresponding to pathway 1 in steady-state conditions has been derived by Meloche (1967):

$$t_{1/2} = \frac{\ln 2}{k_i}\left(1 + \frac{K_i}{[I]}\right)$$

where k_i is the inactivation rate constant and K_i is the dissociation constant of E–I, defined as $(k_{-1} + k_i)/k_{+1}$. The half-life of inactivation ($t_{1/2}$) plotted as a function of the reciprocal of inhibitor concentration represents a straight line that intercepts the ordinate at the positive value of $\ln 2/k_i$ and the abscissa at the negative value $-1/K_i$.

pathway 2 $$E + I \xrightarrow[k_{2nd}]{} E\text{–}I^*$$

For pathway 2 the kinetic equation can be simplified to

$$1/t_{1/2} = \frac{k_{2nd}}{\ln 2}[I]$$

The plot of the reciprocal of the half-life of inactivation versus inhibitor concentration results in a straight line passing through the origin, the slope of which corresponds to the value k_{2nd}. The involvement of a conformational change in the inactivation process can be determined by the method of Liu and Tsou (1992). Binding to the enzyme and the resulting conformational change are described as follows:

pathway 3 $$E \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E_x + I \xrightarrow{k_{+4}} EI^*$$

where E_x represents a form of the enzyme that differs in conformation from E and that binds the inhibitor to form the inactivated inhibitor–enzyme complex EI*. For this type of inactivation Liu and Tsou (1992) have derived the following equation:

$$[P]_\infty = k_2K_1K_2[A][B][E_0]\left\{\frac{k_{-3}}{k_{+3}k_{+4}[I]} + \frac{k_{-3}}{(k_{+3} + k_{-3})k_{+3}}\right\}$$

where A and B are the substrates and K_1 and K_2 are their respective affinity constants. Kinetic differentiation between enzyme inactivation through complex formation involving a

conformational change or not is obtained by plotting the final concentration of the product formed against the reciprocal of the inhibitor concentration. The plot of $[P]_\infty$ against $1/[I]$ gives a straight line with a positive slope that intercepts the ordinate at a positive value in the case where a conformational change is involved.

Nuclear Magnetic Resonance. Nuclear magnetic resonance (NMR) experiments were carried out using a Bruker Fourier transform ^{31}P NMR spectrometer operating at 81.015 MHz. Sample volumes of 2 mL in 10-mm diameter tubes were used, with 85% phosphoric acid as reference. After the incubation period (10 min), the excess inhibitor was removed by filtration on a Sephadex G15 gel. The test tube contained initially 24 mM of inhibitor in 0.2 mL of dimethyl sulfoxide (DMSO), 1.8 mL of 0.1 M triethanolamine buffer, pH 7.6, and 0.2 mg of GAPDH.

Titration of Sulfhydryl Groups. For the titration of the number of reactive thiol groups present in GAPDH, the enzyme was incubated with inhibitor until 90% inactivation had been reached. Excess of inhibitor was subsequently removed by dialysis against 1 L of buffer for 17 h at 4 °C. Thiol content of the enzyme was determined in 0.1 M triethanolamine buffer (pH 7.6), 1 mM EDTA, 0.2% SDS (necessary to clear up the solution) with 0.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by titrating the release of the 5-thio-2-nitrobenzoate anion at 412 nm (ϵ_M = 13.6 mM^{-1} cm^{-1}) at 25 °C.

Computer modeling was carried out by using the MM3-BIOSYM program.

Studies on Live Trypanosomes. The bloodstream form of *T. brucei* stock 427 was isolated from male Wistar rats by cardiac puncture and cells were separated from blood elements as described previously (Misset et al., 1987). The effect of the compounds on the multiplication of bloodstream-form trypanosomes was assayed using an *in vitro* culture system of *Trypanosoma equiperdum*, an organism closely related to *T. brucei*, as previously described (Baltz et al., 1985). The rate of O_2 consumption at 25 °C was measured polarographically using a Clark-type oxygen electrode. In a typical experiment, *T. brucei* bloodstream trypanosomes (0.6 mg protein) and various concentrations of compounds were added to 1 mL of phosphate-buffered saline (pH 8.0) containing 5 mM glucose. The rate of O_2 consumption was recorded as a function of time, relative to a control without inhibitor but supplemented with an equal amount of solvent.

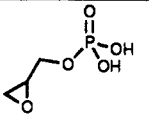
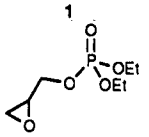
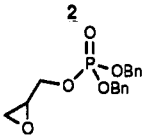
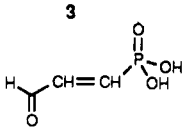
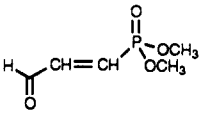
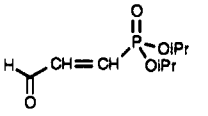
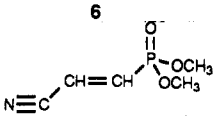
RESULTS

The compounds selected for this study and their effects on the GAPDHs of *T. brucei* and rabbit muscle are summarized in Table 1. Compounds **1–3**, which are glycidol phosphate and its esters, have as reactive group an epoxide. Compounds **4–7** are all phosphonates with a conjugated double bond (α-enones). Within each group the surroundings of the phosphorus atom were varied. Compounds **1** and **4** carry a negative charge, whereas the others are esters with varying hydrophobic character.

Inactivation Kinetics with Compounds 4 and 5. Figures 3 and 4, given as an example, show that the kinetics of inactivation of both the *T. brucei* and rabbit muscle GAPDHs by compound **5** followed a pseudo-first-order mechanism. Although both enzymes were irreversibly inhibited, the kinetics of this process apparently followed different pathways. A replot of the inactivation curve for the *T. brucei* GAPDH indicated that this enzyme was inactivated upon binding of **5** (according to pathway 2) and thus only k_{2nd} could be determined (cf. Table 1). A similar analysis carried out for the rabbit muscle enzyme revealed that **5** first formed a reversible complex with the enzyme, followed by the formation of a covalent bond. In this case both k_i and K_i could be determined (cf. Table 1). As they were determined, the K_i values appeared to be consistent with the K_m values of the substrates: 0.08 and 0.15 mM for GAP on the rabbit muscle and the *T. brucei* GAPDHs, respectively, and 0.06 and 0.45 mM for NAD^+ (Lambeir et al., 1991).

According to Table 1, inactivation of both rabbit muscle and *T. brucei* GAPDH by compound **4** was also preceded by the formation of a reversible complex between the enzyme and the inhibitor. However, analysis of the kinetics of product formation during enzyme inactivation indicated that GAPDH had undergone a conformational change before it reacted with **4**. The plot of $[P]_\infty$ in millimolar against $1/[I]$ (millimolar^{-1}) gave a straight line [$y = (0.44 \pm 0.04)x + (0.013 \pm 0.003)$] that intercepted with the ordinate at a positive value. This intercept has a dimension of a concentration (in the range of 10^{-5} M). Similar results were obtained with GAPDH from *T. brucei*. The same analysis for compound **5** was not possible. In the presence of saturating concentrations of substrate, GAPDH was completely protected from inactivation by **5**. Even after 1 h of incubation of the enzyme with 1.5 mM of **5** the enzyme remained 100% active.

Table 1: Effect of Compounds on Rabbit Muscle and *T. brucei* GAPDH: Structure and Inactivation Constants[a]

		K_i (mM)	k_i (min^{-1})	k_i/K_i (M^{-1} min^{-1} × 10^{-2})
	Rm	22.22	0.29	0.13 ± 0.004
	Tb	125	1.73	0.13 ± 0.005
1				
	Rm	1.36	0.69	5.07 ± 0.18
	Tb	0.9	0.41	4.55 ± 0.18
2				
	Rm	7.82	1.86	2.37 ± 0.07
	Tb	5	1.15	2.30 ± 0.09
3				
	Rm	3.33	0.33	1.05 ± 0.03
	Tb	4.54	4.5	10 ± 0.4
4				
	Rm	0.06	0.73	121 ± 3.6
	Tb			792 ± 30[b]
5				
	Rm			116 ± 3.5[b]
	Tb			30 ± 1.2[b]
6				
	Rm	4.44	1.06	2.4 ± 0.07
	Tb	1.85	0.66	3.56 ± 0.14
7				

[a] See also Figures 3 and 4. Rm = rabbit muscle GAPDH; Tb = *T. brucei* GAPDH. [b] k_{2nd}.

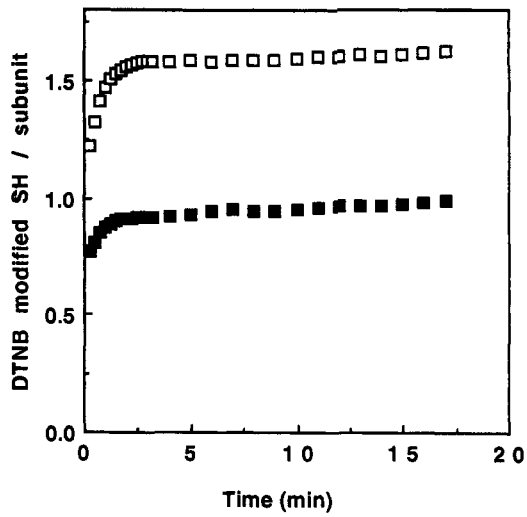


FIGURE 2: Titration of the number of reactive cysteines in rabbit muscle GAPDH with DTNB. The upper trace shows the titration of the enzyme (10 μM) without inhibitor. The lower trace shows the titration of the enzyme after reaction with 0.6 mM compound **5** until 90% inactivation was obtained, after which the enzyme was freed from excess inhibitor (experiments performed in the presence of 0.02% SDS).

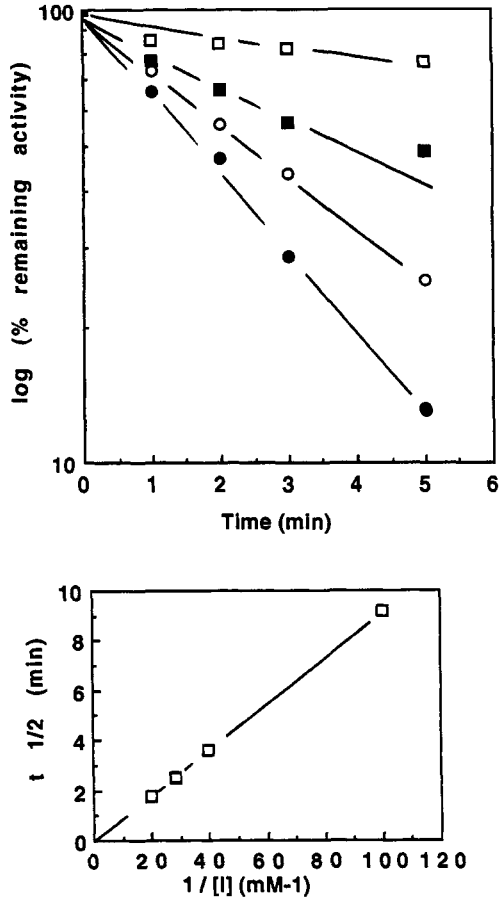


FIGURE 3: Inactivation of *T. brucei* GAPDH by compound **5**. Inactivation with time at different inhibitor concentrations: 0.01, 0.025, 0.035, and 0.050 mM (lower panel: secondary plot and determination of the inactivation constant k_{2nd}).

Properties of the Epoxide Inhibitors 1–3. The three epoxides were tested as racemic mixtures. Comparison of the inactivation rate of rabbit muscle GAPDH by the racemic and the *R* and *S* enantiomers of compound **1** revealed no significant differences; at 9 mM inhibitor concentration, values

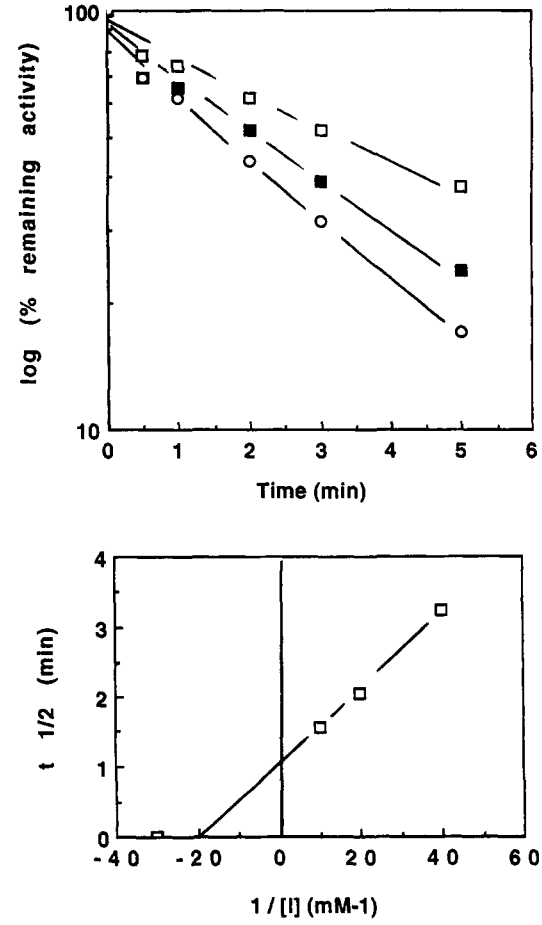


FIGURE 4: Inactivation of rabbit muscle GAPDH by compound **5**. Inactivation with time at different inhibitor concentrations: 0.025, 0.050, and 0.100 mM (lower panel: secondary plot and determination of the inactivation constants K_i and k_i).

are respectively 0.126 ($\pm$ 0.01) min^{-1}, 0.115, and 0.106. Similar results were obtained with two *R* and *S* enantiomers of an ester analogous to **2** and **3**, namely, the *R* and *S* diphenylglycidol phosphates (at 2 mM, respectively, 0.419 and 0.338 min^{-1}).

The epoxides first formed a reversible complex with both the *T. brucei* and the rabbit muscle enzyme, prior to their inactivation. This contrasts with previous data on the reaction of yeast GAPDH with glycidol phosphate, which would not involve a reversible complex (McCaul & Byers, 1976). The two enzymes displayed important differences toward the negatively charged organic phosphate **1**. The rabbit muscle enzyme had a K_i value 5 times higher for **1** than the *T. brucei* enzyme, but the former was inactivated at a 5 times slower rate. Thus the resulting reactivity (k_i/K_i) was the same for both enzymes (Table 1). Surprisingly, the uncharged and thus more hydrophobic phosphate esters **2** and **3** were not able to discriminate between the two homologous enzymes, which suggests that a difference in charge in the neighborhood of the binding site could have been responsible for the observed difference with **1**. Moreover, when the k_i/K_i values for compounds **1–3** were compared, it turned out that the charged compound **1**, which much more resembled the substrate glyceraldehyde 3-phosphate, was a much less efficient inhibitor than the bulky phosphate esters **2** and **3**. This higher activity of the esters was the result of their smaller K_i values for both the enzymes, rather than from a faster inactivation rate.

Properties of the α-Enone Inhibitors 4–7. In the α-enone series, as with the epoxide series, the charged phosphonate **4**

Table 2: Protection by Substrates of *T. brucei* and Rabbit Muscle GAPDH from Inactivation by Compound **5**[a]

substrate varied	concn required (mM)		% protection
	rabbit muscle GAPDH	*T. brucei* GAPDH	
GAP	0.12	0.5	100
NAD^+	1	2	90
P_i	5	10	100

[a] The respective enzymes were incubated in the presence of 5 mM inhibitor with incubation times from 1 to 5 min. Concentrations used were for GAP, 0.05–2.5 mM; for NAD^+, 0.2–4 mM; for P_i, 2–20 mM. (K_m values for GAP are 0.08 and 0.15 mM, for P_i 5 and 6 mM, and for NAD^+ 0.06 and 0.45 mM for the rabbit muscle and *T. brucei* enzymes, respectively.)

Table 3: Effect of Inhibitors on the Multiplication of Bloodstream Trypanosomes in in Vitro Culture and on Respiration[a]

compound	LD_{100} (μM)	I_{50} (mM)	K_{2nd} (M^{-1} min^{-1})
2	150	5	37
3	300	1	92
5	10	0.6	180
6	0.3	0.6	200

[a] Cultures were started with 2×10^4 cells of *T. equiperdum* and cell multiplication monitored by counting the number of trypanosomes after 4, 22, and 48 h. Oxygen consumption was monitored on isolated *T. brucei* cells in an oxygen electrode. LD_{100} is the concentration of inhibitor that led to a complete inhibition of cell growth. I_{50} is the concentration at which respiration was inhibited by 50% after 5 min of preincubation of cells with the inhibitor. K_{2nd} represents the rate of inactivation of oxygen uptake.

was much less active on the two enzymes than the bulky hydrophobic phosphonate esters **5**–**7**. As described above for compound **5**, also for the other compounds, the pathway of enzyme inactivation was dependent on the source of the enzyme (Table 1). Despite these differences in pathway, the reactivities of the compounds can easily be compared by evaluating the k_i/K_i and the k_{2nd} values, which are expressed in the same units. The reactivity of **4** and **5** toward the two enzymes was significantly different. Both compounds had a higher activity with the *T. brucei* enzyme than with that of rabbit muscle enzyme. For compound **4**, this was due to a higher inactivation rate (k_i). The chain length between the phosphorus atom and the third carbon appeared to be critical for activity of these phosphonates. By adding one additional oxygen atom (Lauth et al., 1993), the analogous compounds completely lost their activity (not shown).

Protection by Substrates. As mentioned above, compound **5** was unable to inactivate the two enzymes in the presence of saturating concentrations of substrates. Table 2 shows that all three of the substrates, D-glyceraldehyde 3-phosphate, NAD^+, and P_i, at sufficiently high concentrations to saturate the enzyme, were able to protect the enzymes from inhibition by 2 mM **5**. A similar protection for glycidol phosphate by glyceraldehyde 3-phosphate and NAD^+ has previously been described for yeast GAPDH (McCaul & Byers, 1976). Identical results were obtained with **4** (at 6 mM) and with **6** and **7** (at 2 mM).

Nature of the Covalent Link between Inhibitor and Enzyme. To confirm the covalent nature of the binding of the inhibitors to the proteins and to identify the nature of the covalent link, the interaction of representative compounds from both series with the rabbit muscle enzyme was studied using phosphorus NMR. When an excess of **2** was incubated for 15 min with the enzyme, after which the unreacted ligand was removed, the chemical shift of the phosphorus atom of the phosphate (δ = –0.16 ppm in triethanolamine) had changed to a value of +1.5 ppm. An identical chemical shift of +1.5 ppm was observed for the formation of the adduct of propanethiol with compound **2** under identical conditions. Similar results were obtained with the α-enone **5**: the chemical shift of the phosphorus atom changed from +16 to +21 ppm when it became covalently bound to the protein and an identical shift was observed in the case of the formation of the adduct of **5** with propanethiol.

Titration (in the conditions indicated in the experimental part) of the number of reactive cysteines in rabbit muscle GAPDH with DTNB prior to and after preincubation of the enzyme with compound **5** revealed that one cysteine per catalytic site of the enzyme had reacted with the inhibitor (Figure 2). Together these experiments indicate that the inhibitors formed a covalent bond with a thiol group of a cysteine in the enzyme.

Effect of the Inhibitors on Glycolysis and the Multiplication of Trypanosomes. All compounds were tested for their effect on the multiplication of *T. equiperdum* in an *in vitro* culture system (Baltz et al., 1985). Inhibitors **1**–**3** of the epoxide series exhibited no significant activity below concentrations of 150–300 μM. However, the α-enone derivatives proved to be much more active. The best result was obtained with compound **6**. At a concentration of 0.3 μM, multiplication of the trypanosomes was completely inhibited. The activity of **6** compared favorably with that obtained for two well-known trypanocidal reference drugs: pentamidin (LD_{100} = 2 mM) and (difluoromethyl)ornithine (LD_{100} = 100 μM) under the same conditions (Table 3).

There exists a linear correlation between the rate of glycolysis and the rate of oxygen consumption in bloodstream form trypanosomes (Fairlamb, 1989). To verify whether the above-described inhibitory effect on the multiplication of trypanosomes was the result of a direct inhibition of the enzyme GAPDH and thus on glycolysis, we measured the effect of the inhibitors on the respiration of intact cells using an oxygen electrode. Indeed, all compounds tested inhibited respiration in a time-dependent manner (Table 3). Again the compounds **5** and **6** from the α-enone series were the most effective ones.

DISCUSSION

A first selection of potentially irreversible inhibitors of GAPDH described in this study was based on the premise that the effect of such compounds could not be reversed upon dilution of the inhibitor–enzyme complex (Lauth, 1992). The irreversible nature of the inhibition by these compounds was confirmed in this paper by the fact that (i) all compounds exhibited pseudo-first-order inhibition kinetics and (ii) the chemical shift in ^{31}P NMR of the phosphorus atom in some inhibitors observed upon their binding to the protein was identical to the shift resulting from the formation of a covalent adduct between the inhibitors and propanethiol. The involvement of a cysteine residue as the thiol donor for the formation of the covalent bond was further confirmed by titration of the number of free cysteines present per catalytic subunit. The reactive cysteine is most likely the active-site Cys 149 and the covalent bond formed must be that between the cysteine S and the C_1 atom of the epoxides **1**–**3**, as is to be expected for an attack of nucleophiles on an epoxide (Parker & Isaacs, 1959). The same formation of covalent bond was previously suggested by McCaul and Byers (1976) for the interaction of glycidol phosphate with yeast GAPDH and a similar reaction was proposed by Sakai et al. (1991) in the case of koningic acid. Therefore, we expect the Cys 149 to react also with the C_1 atom of the α-enones **4**–**7**.

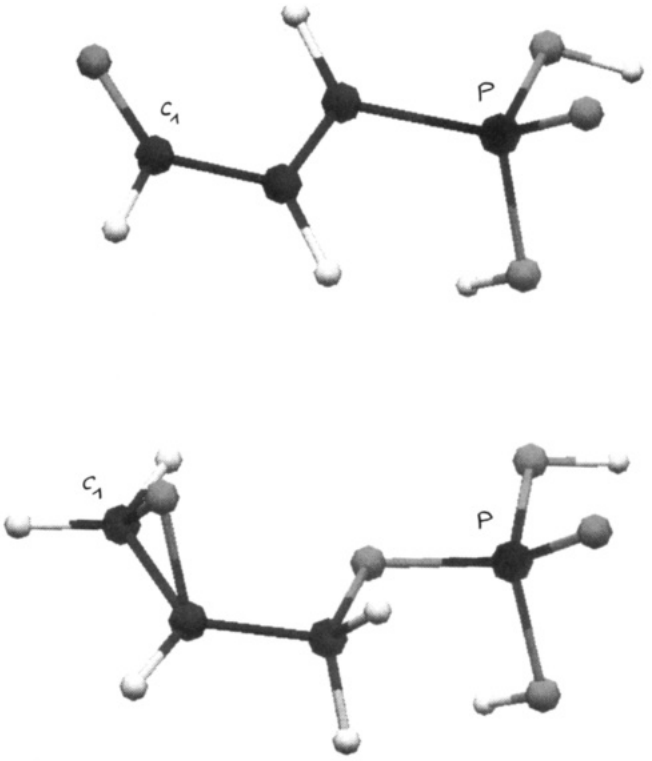


FIGURE 5: Modeling of the energetically most favorable conformations of compounds **1** and **4**: distances between the C_1 and P atoms are 4.15 and 4.25 Å, respectively.

The entire structure of the epoxide compounds **1–3** is required for the inhibition of GAPDH. Their fragments, such as the glycidol bearing a hydrophobic tosylate group, or the phosphoric ester dibenzyl phosphate, had no inhibitory activity with GAPDH (not shown). Also in the case of the α-enone derivatives the entire structure is required. This is inferred from the fact that the half-life of inactivation of rabbit muscle GAPDH by the nonphosphorylated α-enone compound acroleine was 16 min at a concentration of 0.1 mM (not shown), while this is a few seconds in the case of compound **5** at a concentration of only 0.05 mM.

The chain length of the compounds is of critical importance for inhibition as can be concluded from a comparison of the two series of compounds. In the epoxide series, the phosphates **1**, **2**, and **3** were all active, while the corresponding phosphonates (where the oxygen atom between phosphorus and the carbon chain has been suppressed; Lauth, 1992) had no activity. The contrary was true in the second series of α-enone compounds (**4–7**). Here all the phosphonates were active, while the corresponding phosphates, which have an additional oxygen atom between the carbon chain and the phosphorus atom, were inactive or poor reversible inhibitors (not shown). It thus seems that a precise distance between the phosphorus atom and the carbon 1 is essential for inhibition. Molecular modeling of the structures of the two charged compounds **1** and **4** gave as minimized geometries the structures shown in Figure 5. The P–C_1 distances in the compounds **1** and **4** were 4.85 and 4.15 Å, respectively. A distance of about 4.2 Å must be the critical distance. This can be inferred from the following arguments: (i) addition of an oxygen atom in **4** to make the corresponding phosphate completely abolished activity; (ii) for the epoxide, reducing the 4.85-Å P–C_1 distance of the extended conformation to the critical value of 4.25 Å in a non-ground-state conformation of the inhibitor would increase the energy by only 1 kcal/mol. Other factors might be of some importance: indeed, from a phosphate to a phosphonate, the oxygen atom replaced by a CH_2 group may intervene in binding or repulsive interactions and the pK of the OH groups are increased by 1 unit (Freedman et al. 1957).

No significant differences in reactivity were found between the *R* and *S* enantiomers of the epoxides **1** and **3**. This indicates that the active-site cavity accommodating the reactive epoxide group allows sufficient flexibility for reaction with the active-site Cys 149.

Substitutions at the phosphorus atom within the two series of compounds revealed that the hydrophobic esters have a higher affinity for GAPDH than have the negatively charged unesterified compounds **1** and **4**. The difference in K_i value for compounds **1** and **2**, as measured with the rabbit muscle enzyme, indicates an increase in binding energy of about 1.5 kcal/mol. This result was unexpected for compounds that were designed to function as substrate analogues and that were supposed to bind to the glyceraldehyde 3-phosphate site. Our observations, therefore, may indicate that these inhibitors bind at a location different from this site, where a hydrophobic contribution would be more favorable. On the other hand, the fact that the enzyme is protected from inhibition by both the substrate glyceraldehyde 3-phosphate and the cofactor NAD^+ would suggests that binding yet occurs at or near the active site. McCaul and Byers (1976) have reported that the inactivation of the yeast enzyme by glycidol phosphate is not only prevented by glyceraldehyde 3-phosphate and by NAD^+ but also enhanced by the presence of 3.2 mM NADH. This suggests that the reduced form of the cofactor induces a conformational change in the catalytic domain allowing the accommodation of the inhibitor in the active site. Corbier et al. (1989) have suggested that initially the C(3) phosphate group of the substrate, as well as of glycidol phosphate, do not bind at the substrate P_s site but at the nearby P_i site. At some stage in the catalytic sequence the C(3) phosphate of the acyl group would be transferred from one anion-binding site to the other, most likely during the coenzyme exchange step. The subsequent reaction of P_i with the intermediate thioester could only occur when P_i would be in a hydrophobic environment; otherwise its nucleophilicity would be to low [pK_a = 7.2 and 12.3 in water, while 14 and 23 in acetonitrile (Ramirez et al., 1980)]. Binding of our neutral esters in this hydrophobic environment of the P_i binding site may be the explanation for their efficient inhibition.

T. brucei GAPDH shows strong similarities with other GAPDHs with respect to its P_s and P_i sites (Read et al., 1987; Biesecker et al., 1977; Michels et al., 1991). Model building suggested that compound **5** could have a preference for binding to the P_i site rather than to the P_s site (Ch. Verlinde, unpublished observations). By positioning the phosphorus atom of **5** in the same position as the phosphorus atom of glyceraldehyde 3-phosphate, the energy constraint was too high to form a thiohemiacetal. By contrast, positioning of the phosphate in the P_i site rendered this reaction energetically possible. Moreover, the conformational change imposed by the binding of Cys 165 to the carbonyl group of the inhibitor, brought the uncharged amino acid Pro 135 and the carbon chain of Thr 225 in the vicinity of the phosphate head. This also may explain why the neutral esters **2**, **3**, and **5–7** performed better than the charged analogues **1** and **4**.

Our kinetic analyses have revealed that, prior to, or simultaneously with, the formation of the covalent bond between inhibitor **4** and enzyme, a conformational change occurs in the enzyme. A conformational change has also been suggested for glycidol phosphate and yeast GAPDH (McCaul & Byers, 1976). It is most likely that this change in

conformation represents the movement of the C(3) phosphate from the P_i site to the P_s site, although it cannot be excluded that other conformational changes play a role. Comparison of the crystal structure of the apo-GAPDH from *Bacillus stearothermophilus* with that of the holoenzyme revealed that coenzyme binding is accompanied by an important conformational change, which modulates in part the formation of the anion binding sites. This structural transition also involves residues 207–209, which together constitute the P_i binding fold (Skarzynsky & Wonacott, 1988). However, a precise localization of the inhibitor in the active site of the enzyme would require X-ray analysis of the crystallized enzyme–inhibitor complex. So far, attempts to crystallize trypanosome GAPDH complexed with compound **2** or **5** have failed to give diffracting crystals.

We have not studied in detail the involvement of a conformational change for the other inhibitors. However, the structural resemblances of the inhibitors let us predict that conformational changes, prior to or simultaneously with the formation of the covalent bond, will occur in most of the other inhibitor–enzyme combinations.

Despite the high degree of similarity in structure of the glyceraldehyde 3-phosphate binding site and the P_i site that exists between the *T. brucei* and the other GAPDHs (Michels et al., 1991), it is encouraging that several of our compounds displayed some selectivity with respect to the trypanosome enzyme. The charged compounds **1** and **4** had no significant activity on intact cells, and this is not surprising since charged compounds in general do not easily permeate through membranes and thus are not able to reach the target enzyme. However, the more hydrophobic inhibitors all exerted an almost instantaneous inhibition of glycolysis. This inhibition, as evidenced by the cessation of oxygen uptake by the parasite, is probably the consequence of a direct inhibition of GAPDH. Inhibition of GAPDH directly affects the trypanosome's only pathway to generate metabolic ATP. Thus a factor of 10 in selectivity for some of the phosphorylated α-enones renders these compounds promising inhibitors. They are simple structures as compared to pentalenolactone and koningic acid, which are highly toxic (Duszenko & Mecke, 1986; Kato et al., 1992). Another encouraging observation is that our most active compound **6** had a better effect on *T. equiperdum* in *in vitro* experiments than the recommended anti-trypanosome drugs pentamidin and (difluoromethyl)ornithine.

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