INTERACTIONS OF 2-FURYLETHYLENES WITH THIOL ENZYMES*

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Furylethylenes are inhibitors of glyceraldehyde 3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), glutathione reductase (GR; EC 1.6.4.2), and, in part, also malate dehydrogenase (EC 1.1.1.37). These four enzymes served as model thiolproteins employing the SH groups of the embodied cysteine for catalytic activity. The inhibition degree of the afore-mentioned enzymes is proportional to the reactivity of furylethylenes towards thiols. The inhibition of GAPDH and GR with 1-(5-nitro-2-furyl)-2-nitro--2-methoxycarbonylethylene (*I*) was characterized by inhibition constants K_i and also, its type was estimated. A direct chemical modification of SH groups of cysteine residues was found in GAPDH, a fact that could be closely connected with the inactivation of the enzyme.

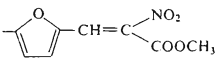
Furylethylenes (vinylfurans), the biologically active compounds[1-6] exert a pronounced inhibitory effect on energy metabolism of both prokaryotic[7] and eukaryotic[8] cell types. As evidenced[8], the exclusion of energy generating processes, leading to inhibition of biosynthetic processes and finally to growth stopping in animal and yeast cells is the consequence of glycolysis blockade. Inhibition of glycolysis is caused by an inactivation of key enzymes in this metabolic pathway, namely glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), hexokinase (EC 2.7.1.1), and 6-phosphofructokinase (EC 2.7.1.11). Furylethylenes were found to react with thiols[9,10] and since all three enzymes employed the SH groups for catalytic activity, it was decided to study interaction of these compounds with thiol enzymes in more detail.

EXPERIMENTAL

Chemicals. Furylethylenes *I*—*III* were synthesized in the Department of Organic Chemistry, Faculty of Chemical Technology, Slovak Institute of Technology, Bratislava. Stock solutions

* Part III in the series Biochemically Important Reactions of 2-Furylethylenes; Part II: This Journal, *47*, 1659 (1982). Abbreviations used throught this paper: ADH alcohol dehydrogenase, GAP glyceraldehyde 3-phosphate, GAPDH, glyceraldehyde 3-phosphate dehydrogenase, GAPDH glucose 6-phosphate dehydrogenase, GR glutathione reductase, GSSG oxidized glutathione, MDH dehydrogenase.

in dimethyl sulfoxide were used for all experiments.



R = NO_2 (*I*); Br (*II*); H (*III*)

The final concentration of the solvent in the reaction mixture never exceeded 1%. Glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle (GAPDH; EC 1.2.1.12, 10 mg prot./ml), glucose 6-phosphate dehydrogenase from yeasts (G6PDH; EC 1.1.1.49, 1 mg/ml), malate dehydrogenase from pig heart (MDH; EC 1.1.1.37, 5 mg/ml), glutathione reductase from yeasts (GR; EC 1.6.4.2; 1·7 mg prot./ml), and also auxiliar and indication enzymes, substrates, coenzymes and buffer constituents for determination of enzyme activities were commercial products of Boehringer, Mannheim, FRG. All experiments were carried out in redistilled water.

The effect on enzymes. To obtain basic data of substances *I*—*III*, the inhibitors were incubated with the respective enzyme (GAPDH, MDH, GR) at 25°C for 2 h after which its activity was determined. The extent of inhibition was estimated on comparison with the control. At the concentration and conditions given, dimethyl sulfoxide did not exert any considerable inhibitory effect. The content of the individual incubation mixtures was as follows: GAPDH (0·1 mol l^{-1} carbonate–hydrocarbonate buffer of pH 8·6, 66·5 μg prot./ml (21·7 nkat/ml)), G6PDH (0·1 mol . l^{-1} triethanolamine buffer of pH 7·6, 4·0 μg prot./ml (37·1 nkat/ml)), MDH (0·1 mol l^{-1} phosphate buffer of pH 7·0, 2·5 μg prot./ml (50·0 nkat/ml)) and GR (0·1 mol l^{-1} tris(hydroxymethyl)aminomethane buffer of pH 8·0, 8·4 μg prot./l (47·4 nkat/ml)). The final concentration of inhibitors was 5 . 10^{-4} mol l^{-1} in all experiments. The effect of GR was investigated with and without $NADPH_2$ (3·8 . 10^{-3} mol l^{-1}).

Estimation of inhibition constants K_i *for* GAPDH *and* GR. A solution of the inhibitor in dimethyl sulfoxide (final concentration max. 1%) was pipetted directly into the spectrophotometric cell (1 cm, 2 ml) containing the complete reaction mixture for determination of the enzyme activity. The reaction was started by addition of the enzyme. The final concentration of GAPDH and GR was 3·3 μg/2 ml and 1·2 μg/ml, respectively. The initial rate of enzyme reactions was examined at various concentrations of the inhibitor and substrates (GAP, NAD^+, P_i, or GSSG) specified directly with the interpretation of results obtained (Fig. 1).

Estimation of enzyme activities. The rate of catalytic reduction of NAD ($NADP^+$), and oxidation of NADH + H^+ ($NADPH + H^+$) was estimated from the changes of absorbance at 340 nm in a 1 cm-quartz cell upon time with a Specord UV VIS (Zeiss, Jena) spectrophotometer at 25°C (ref.[11]). Data read from the linear regions of curves showing the changes in concentration of $NADH_2$ ($NADPH_2$) upon time (maximum up to 5 min after starting the reaction) were taken for determination of K_1. The activity of individual enzymes was calculated providing that $A^{1cm}_{340nm} = 0{\cdot}1$ corresponds to 0·0161 μmol $NADH_2$ ($NADPH_2$) in 1 ml ($\varepsilon = 6{\cdot}22 . 10^{-3}$ l . . mol^{-1} cm^{-1}). Composition of the reaction mixtures for activity determination of the individual enzymes was as follows: for GAPDH carbonate–hydrocarbonate (0·1 mol l^{-1}) buffer of pH 8·6, EDTA (0·2 mmol l^{-1}), Na_2HPO_4 (50 mmol l^{-1}), NAD^+ (1·5 mmol l^{-1}), GAP (1·3 mmol l^{-1}). For GR tris (hydroxymethyl)aminomethane (0·1 mmol l^{-1}) buffer of pH 8·0, EDTA (5 mmol l^{-1}), GSSG (5·4 mmol l^{-1}), and $NADPH_2$ (0·2 mmol l^{-1}). For MDH phosphate (0·1 mmol l^{-1}) buffer of pH 7·4, aspartate (0·4 mmol l^{-1}), α-oxoglutarate (1·0 mmol l^{-1}), $NADH_2$ (0·2 mmol . l^{-1}), and glutamic oxalacetic transaminase (2·5 μg ml^{-1}). For G6PDH triethanolamine

(50 mmol l^{-1}) buffer of pH 7·6, EDTA (5 mmol l^{-1}), $NADP^+$ (0·3 mmol l^{-1}), and glucose 6-phosphate (0·5 mmol l^{-1}).

Determination of the amino acid content. The content of amino acids in GAPDH hydrolysates obtained after incubation the compound *I* being added, or not was determined with an automatic amino acid analyzer (AAA Hd 1200, ZSNP, Žiar nad Hronom, Czechoslovakia). Solution of GAPDH (10 mg prot. in 1 ml of 3·2 mol . l^{-1} $(NH_4)_2SO_4$) was repeatedly (3×) dialyzed in cold water (100 ml) for 6 h and the inhibitor (5 . 10^{-6} mol) was added. The mixture was then incubated at 25°C for 2 h, and oxidized with a 8-fold volume excess of the oxidation mixture (30% H_2O_2, 1 part, 88% HCOOH, 9 parts) at 0°C for 30 min (oxidation of the embodied cysteine to cysteic acid[12]). The residue was hydrolyzed after evaporation under diminished pressure at 100°C with 6M-HCl (10 ml) for 20 h at 110°C. The hydrolysate was evaporated to dryness *in vacuo*. The experiment without the inhibitor proceeded in an analogous manner. Both residues after evaporation were separately dissolved in a citrate buffer (pH 2·2) and analyzed with an automatic amino acid analyzer.

RESULTS AND DISCUSSION

Furylethylenes exert an emphasized inhibitory effect on thiol enzymes. This results from experiments examining the influence of 1-(5-nitro-2-furyl)-2-nitro-2-methoxy-

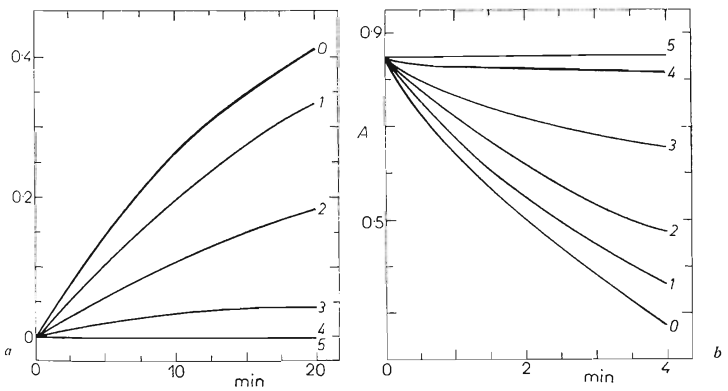


FIG. 1

Influence of the catalytic activity of glyceraldehyde 3-phosphate dehydrogenase (*a*) and glutathione reductase (*b*) with 1-(5-nitro-2-furyl)-2-nitro-2-methoxycarbonylethylene (*I*). The activity is indicated by changes in absorbance at 340 nm due to formation of $NADH_2$, or to consumption of $NADPH_2$ in the process of catalysis of the appropriate enzyme. The concentrations of furylethylene used: 0 *0* 5 . 10^{-6} *1*, 1 . 10^{-5} *2*, 5 . 10^{-5} *3*, 1 . 10^{-4} *4*, 5 . 10^{-4} *5* mol l^{-1}

carbonylethylene (*I*), 1-(5-bromo-2-furyl)-2-nitro-2-methoxycarbonylethylene (*II*), and 1-(2-furyl)-2-nitro-2-methoxycarbonylethylene (*III*) on the activity of four selected model SH enzymes, namely glyceraldehyde 3-phosphate dehydrogenase, glucose 6-phosphate dehydrogenase, glutathione reductase, and malate dehydrogenase. All these enzymes employed the SH groups of the embodied cysteine for its catalytic activity[13,14]. The effect of furyl ethylenes on glutathione reductase was investigated in the absence, or in the presence of $NADPH_2$. As already known[15], the SH group of the catalytic centre of glutathione reductase is sensitive towards SH reagents in the presence of its coenzyme only. N-Ethylmaleimide (*IV*) regarded as a classic SH reagent, and engaged in many studies concerning the characterization of SH groups of proteins with catalytic property[13,14] was also examined in order to compare the inhibitory activity. The enzymes were incubated at 25°C for 2 h with or without inhibitors ($5 . 10^{-4}$ mol l^{-1}), and their activity estimated. The % of inhibition was determined in relation to the control.

The results (Table I) show the high inactivation activity of derivative *I* and of its Br-analogue *II* on GAPDH, G6PDH, and GR. The extent of inhibition in all enzymes, due to the respective derivatives, increases with their reactivity towards thiols. The reactivity of furylethylenes *I*–*III* with thiols is characterized by second order rate constants (Table I) determined for reaction with 2-mercaptoethanol as a model thiol[9]. Another finding following from Table I is that glutathione reductase is sensitive towards furylethylenes *I*–*III* only in the presence of $NADPH_2$.

TABLE I

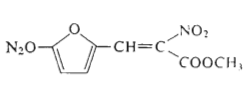
Inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glucose 6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH) and glutathione reductase (GR) after a 2 h-incubation with furylethylenes *I*–*III* and N-ethylmaleimide *IV* ($c = 5 . 10^{-4}$ mol l^{-1}) at 25°C

Inhibitor	Inhibition, %					k^a
	GAPDH	G6PDH	MDH	GR without $NADPH_2$	GR with $NADPH_2$	$l\ mol^{-1}\ s^{-1}$
I	>99·9	90·3	25·5	<0·1	96·0	$4·5 . 10^6$
II	98·2	71·3	22·8	<0·1	84·0	$2·7 . 10^5$
III	85·5	4·6	0·1	<0·1	14·0	$9·2 . 10^4$
IV	>99·9	40·3	2·7	37·5	>99·9	$3·7 . 10^4$

[a] The second order rate constants determined for the reaction of *I*–*III* with 2-mercaptoethanol[9] and of *IV* with glutathione[16].

The background of enzyme inhibition with furylethylenes was studied in more detail with GAPDH and GR; 1-(5-nitro-2-furyl)-2-nitro-2-methoxycarbonylethylene (*I*) was employed for being the most effective inhibitor. This compound was added in various concentrations with the complete reaction mixture for activity determination of the respective enzyme directly into the spectrophotometric cell. The reaction was started by addition of the enzyme and monitored spectrophotometrically. The results obtained with GAPDH and GR are plotted in Fig. 1. As seen, compound *I* slows down, and even fully stops (proportionally to its concentration) reactions catalyzed by the corresponding enzymes. The extent of inhibition is time dependent in both cases.

A more exact characterization of GAPDH inhibition by compound *I* was done by determination of inhibitory constants K_i in respect to all three substrates, *i.e.* glyceraldehyde 3-phosphate (GAP), NAD^+ and inorganic phosphate (P_i). Results are summarized in Fig. 2. Inhibition of GAPDH with compound *I* is therefore, in respect to NAD^+ competitive, and in relation to GAP and P_i noncompetitive. The inhibition of GR was characterized by inhibitory constant K_i in relation to one substrate only, *i.e.* to the oxidized glutathione (GSSG). The characterization for the other substrate ($NADPH_2$) was not done for the restricted possibilities to chose various concentrations of $NADPH_2$ as a varying substrate. K_i was found to be 17·7 μmol l^{-1}, the inhibition is noncompetitive. Inhibition constants for GAPDH and GR determined in the same conditions with N-ethylmaleimide (*IV*) were K_i = = 270·0 μmol l^{-1} (GAP) and K_i = 273·0 μmol l^{-1} (GSSG).



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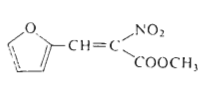

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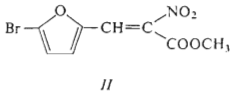

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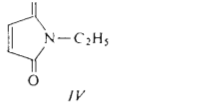

IV

Further experiments were focused to a detailed characterization of binding of compound *I* to GAPDH. This enzyme was chosen for these experiments because the nature of its SH groups and their role in catalysis are being investigated. This enzyme (isolated from a rabbit muscle, M_r 144 000, ref.[17]) is composed of 4 identical subunits. The total number of SH groups per one enzyme subunit is 4. The essential SH group in each subunit belongs to Cys-149 (ref.[18]). These SH groups are acceptors of acyl groups of the substrate (glyceraldehyde 3-phosphate) in the process of enzyme catalysis what is accompanied with the formation of covalent acyl thioester bond.

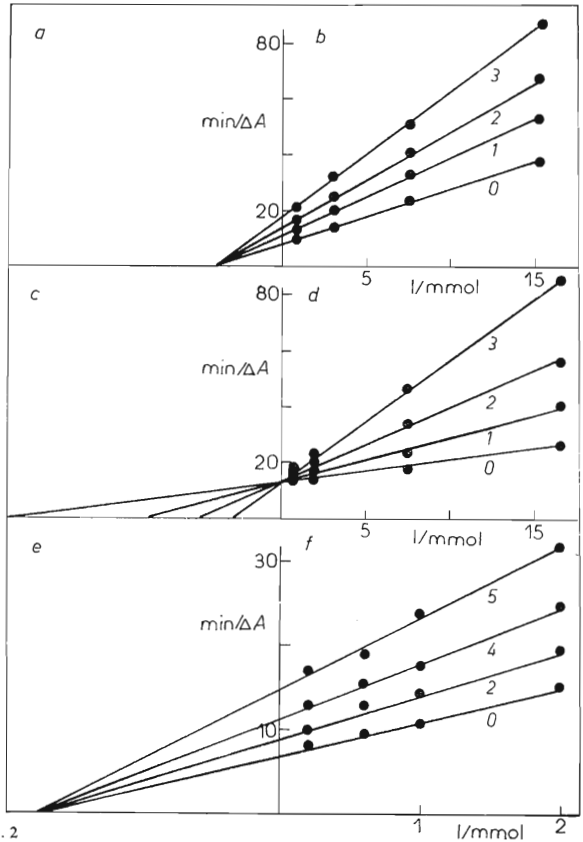

FIG. 2

Characterization of the inhibition nature of glyceraldehyde 3-phosphate dehydrogenase with 1-(5-nitro-2-furyl)-2-nitro-2-methoxylcarbonylethylene (*I*). The initial stedy state velocity reduction of $NADH_2$ in the process of enzyme catalysis measured spectrophotometrically in the absence (*0*) and in the presence of furylethylene in concentration $6 \cdot 10^{-6}$ (*1*), $8 \cdot 10^{-6}$ (*2*), $1 \cdot 10^{-5}$ (*3*), $2 \cdot 10^{-5}$ (*4*), $4 \cdot 10^{-5}$ (*5*) mol l^{-1}. Varying substrates were glyceraldehyde 3-phosphate (GAP), NAD^+, and inorganic phosphate (P_i). *a*: GAP; $K_i = 1{\cdot}3 \cdot 10^{-5}$ mol l^{-1}, *c*: NAD^+; $K_i = 4{\cdot}9 \cdot 10^{-6}$ mol l^{-1}, *e* P_i $K_i = 3 \cdot 10^{-5}$ mol l^{-1}

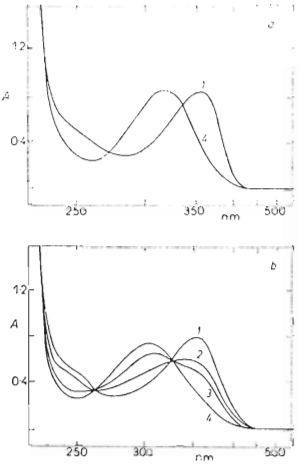

FIG. 3

A spectrophotometric proof of reaction of 1-(5-nitro-2-furyl)-2-nitro-2-methoxycarbonylethylene (*I*) with glutathione (*a*) and glyceraldehyde 3-phosphatehydrogenase (*b*) in carbonate–hydrocarbonate buffer of pH 8·6. Concentration of furylethylene $1{\cdot}2 \cdot 10^{-5}$ mol l^{-1}, of glutathione $8{\cdot}3 \cdot 10^{-4}$ mol l^{-1} and of the enzyme $2{\cdot}9 \cdot 10^{6}$ mol l^{-1} ($4{\cdot}6 \cdot 10^{-5}$ mol of SH groups per liter). *1* the spectrum of furylethylene, *2*, *3* spectra of reaction mixtures with the enzyme after 10 s, and 5 min, *4* spectra of reaction mixtures with glutathione (*a*) and enzyme (*b*) after 30 and 60 min after initiation of the reaction. Temperature 25°C. Spectra were measured in a 3 cm cells

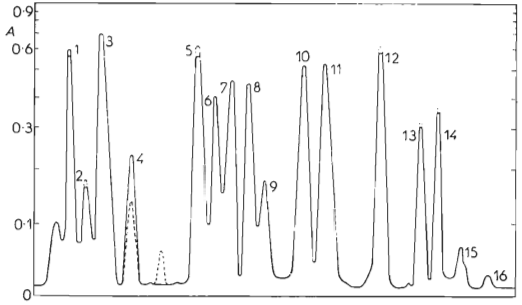

FIG. 4

Content of amino acids of glyceraldehyde 3-phosphate dehydrogenase before (full line) and after (dashed line) the action of 1-(5-nitro-2-furyl)-2-nitro-2-methoxycarbonylethylene (*I*) determined in the enzyme hydrolysate. Incubation of the enzyme ($2{\cdot}5 \cdot 10^{-5}$ mol l^{-1}) with the inhibitor ($2 \cdot 10^{-3}$ mol l^{-1}) 2 h at 25°C. The amino acid composition determined by an automatic analyser **1** Lys, **2** His, **3** NH_4^+, **4** $CYSO_3H$, **5** Asp, **6** Thr, **7** Ser, **8** Glu, **9** Pro, **10** Gly, **11** Ala, **12** Val, **13** Ileu, **14** Leu, **15** Tyr, **16** Phe

Their modification leads to a total loss of the enzyme activity[19]. Therefore, furylethylenes *I*–*III* might have caused inactivation of GAPDH by modification of their catalytically active SH groups. This fact is indicated by the similarity of absorption (UV and VIS) spectra of the reaction products of *I* with glutathione as a model low-molecular thiol and with GAPDH (Fig. 3). The free SH group of the embodied cysteine of this tripeptide (γ-L-glutamyl-L-cysteinylglycine) can probably imitate in very rough approximation the SH groups of the embodied cysteine in GAPDH. The reactions were proceeded in the carbonate buffer pH 8·6 at 25°C. Spectra were measured in a 3 cm-quartz cells. Concentration of *I* was in both experiments $1{\cdot}2 \cdot 10^{-5}$ mol l^{-1}, that of glutathione $8{\cdot}3 \cdot 10^{-4}$ mol l^{-1}, that of GAPDH $2{\cdot}9 \cdot 10^{-6}$ mol l^{-1}, and that of SH groups $4{\cdot}6 \cdot 10^{-5}$ mol l^{-1}. Reaction of furylethylenes *I*–*III* with thiolproteins (Fig. 3*b*) can well be monitored spectrophotometrically, analogously as with low-molecular thiols. This makes it possible to characterize kinetically the modification reactions and to look for the relations between the extent of modification and of inactivation of the enzyme. Such a study can be advantageous for investigating relationships between the structure of the active centre and the catalytic activity of SH enzymes.

A direct proof concerning the modification of SH groups in GAPDH after the action of compound *I* was obtained through analysis and by comparison of the amino acid composition of the modified and unmodified enzymes. Compound *I* caused more than a 30% decrease of the cysteine content. Simultaneously, a new peak was observed on the analyser record (between cysteic and aspartic acids), which can be associated with a ninhydrine-positive reaction product of the furylethylene under study and cysteine. The content of other amino acids was virtually unchanged. Record illustrating these facts is reproduced in Fig. 4. Compound *I* modified therefore only the SH groups of the embodied cysteine. Modification of NH_2 groups of lysine, or N-terminal amino acid did virtually not take place at the given conditions (inhibitor : SH = 5 : 1). This finding is in accordance with the results of kinetic measurement of reactions of furylethylenes with thiols and amines which showed that the reactivity to SH groups is approximatelly by 4 order of magnitude higher than that to NH_2 groups[9].

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