GLADIOLIC ACID AND RELATED AROMATIC ORTHO-DIALDEHYDES, A NOVEL CLASS OF MITOCHONDRIAL INHIBITORS. INACTIVATION OF CYTOCHROME C

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SUMMARY: Gladiolic acid (4-methoxy-5-methyl-o-phthalaldehyde-3-carboxylic acid), an antifungal metabolite produced by Penicillium gladioli (McCull. & Thom), was found to inactivate the catalytic properties of horse heart cytochrome c by reacting with functionally-critical lysine residues in the heme protein. Cyclopaldic acid, quadrilineatin, flavipin and 3-formylopianic acid, aromatic o-dialdehydes structurally related to gladiolic acid (GA), also inactivated cytochrome c. Dihydrogladiolic acid did not complex with cytochrome c. Three moles of GA reacted per mole of cytochrome c reduced the ability of the protein to function with beef heart cytochrome oxidase and pig heart NADH-cytochrome c reductase by 85% and 100%, respectively. The ferricytochrome c-GA complex weakly inhibited the interaction of ferrocytochrome c with the acidic protein of cytochrome oxidase. The reaction between GA and cytochrome c appeared to proceed further than a simple Schiff base formation and the complex was not reversed by dialysis. Gladiolic acid acted as an "apparent" inhibitor of cytochrome oxidase of sweet potato mitochondria by inactivating cytochrome c in situ. Oxidative phosphorylation associated with cytochrome oxidase activity (Site III) was strongly inhibited.

Gladiolic acid (keto form: 4-methoxy-5-methyl-o-phthalaldehyde-3-carboxylic acid; lactol form: 4-formyl-3-hydroxy-7-methoxy-6-methylphthalide) is an antifungal metabolite produced by Penicillium gladioli McCull. & Thom (1,2,3,4). Structurally-related dialdehydes are cyclopaldic acid, quadrilineatin and flavipin, metabolites, respectively, of the fungi Penicillium cyclopium Westl. (5), Aspergillus quadrilineatus Thom & Raper (6) and Aspergillus flavipes (Bainier & Sartory) Thom & Church (7). Flavipin is also produced by Epicoccum nigrum (8). A study of the mode of action of these compounds, primarily on plant mitochondria, has revealed them to be a novel class of inhibitors of electron transport and oxidative phosphorylation. In addition, the dialdehydes were discovered to be highly reactive with lysine residues of cytochrome c. This contribution describes some initial experiments on the inactivation of the catalytic properties of horse heart cytochrome c by gladiolic acid (GA) and related aromatic ortho-dialdehydes. The effects of GA and flavipin on cytochrome oxidase activity and Site III oxidative phosphorylation in sweet potato mitochondria are also described.

Materials and Methods

Fungus cultures were obtained from the Commonwealth Mycological Institute, Kew, Surrey, England. Gladiolic acid and dihydrogladiolic acid, cyclopaldic acid, quadrilineatin and flavipin, respectively, were isolated and crystallized from culture filtrates of P. gladioli IMI 38,567, P. cyclopium IMI 89,312, A. quadrilineatus IMI 89,349 and A. flavipes IMI 89,347 essentially as described by previous workers (2,4,5,6,7). Gladiolic acid was also prepared by periodate oxidation of dihydrogladiolic acid (4). 3-Formylopianic acid was synthesized by the method of Brown and Newbold (9). Ortho-phthalaldehyde was obtained from the Aldrich Chemical Co. Sigma Chemical Co. supplied NADH, NADH-cytochrome c reductase (pig heart, type I) and ferricytochrome c (horse heart, type III). Eastman Organic Chemicals supplied N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD).

Cytochrome oxidase was prepared from beef heart mitochondria as described by Yonetani (10). The A_{445} nm (reduced): A_{422} nm (oxidized) ratio was 1.18. The oxidation or reduction of cytochrome c was assayed at 550 nm with a Beckman DB-G recording spectrophotometer. Cytochrome c was standardized using extinction coefficients of 0.9×10^4 (liter/mole/cm) for ferricytochrome c and 2.81×10^4 (liter/mole/cm) for ferrocytochrome c at 550 nm. Ferricytochrome c was reduced with $Na_2S_2O_4$ and bubbled with air to remove the slight excess of dithionite. Sweet potato (Ipomea batata) mitochondria were prepared by the method of Wiskich and Bonner (11). Cytochrome oxidase activity in sweet potato mitochondria was assayed manometrically using ascorbate-TMPD as the reductant. Orthophosphate was estimated according to Sumner (12) and mitochondrial nitrogen was determined by the method of Lang (13). The dialdehydes were added to the reaction systems in microvolumes of 50% ethanol. Equal volumes of ethanol were included in the controls.

Results

Gladiolic acid, cyclopaldic acid, 3-formylopianic acid, quadrilineatin, flavipin and o-phthalaldehyde all reacted rapidly with oxidized (and reduced) cytochrome c at pH 9.0 and modified its ability to function with beef heart cytochrome oxidase. The ϵ-NH_2 groups of lysine are the only chemical functions in cytochrome c which are available to react with an aldehyde (14). As seen in Fig. 1, cytochrome c was inactivated >95% when reacted with GA in a molar ratio of 4:1 (4 moles of GA per 1 mole of horse heart cytochrome c, each mole containing 19 moles of free ϵ-NH_2 groups of lysine (14)). The reaction between cytochrome c and GA at low molar ratios appeared to go to completion or virtual completion since GA could not be detected as the 2,4-dinitrophenylhydrazone derivative after the cytochrome c-GA complex was removed from the reaction solution by precipitation with trichloroacetic acid (results not shown). Two moles of cyclopaldic acid reacted per mole of cytochrome c (2 moles per 19 moles of free ϵ-NH_2 lysine

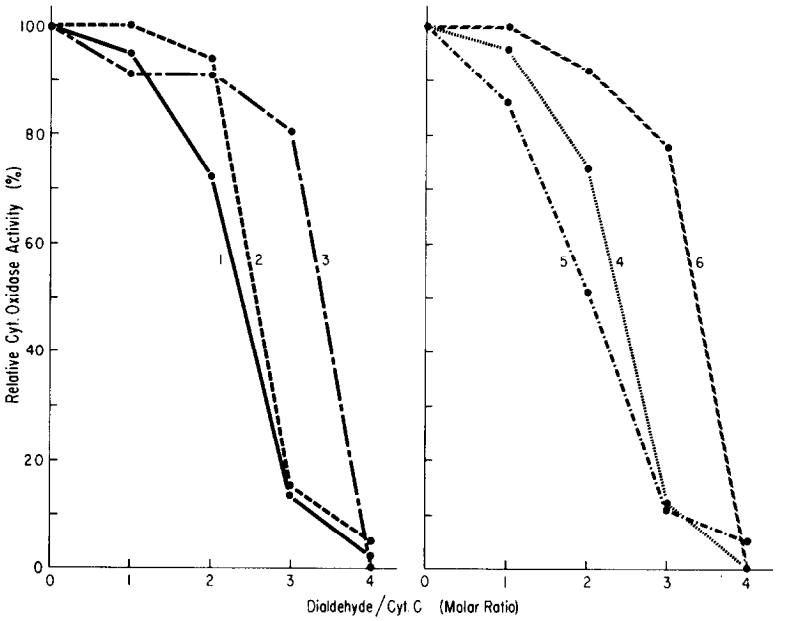

Figure 1.: Inactivation of the catalytic activity of cytochrome c with purified beef heart cytochrome oxidase by GA and related aromatic ortho-dialdehydes. Curves 1 to 6 represent, respectively, GA, flavipin, o-phthalaldehyde, 3-formylopianic acid, cyclopaldic acid and quadrilineatin. The dialdehydes and ferricytochrome c (5×10^{-4} M) were reacted at the different molar ratios in 0.05 M Na borate buffer, pH 9.0 (Clark & Lubs) for 30 min. The enzyme reaction system consisted of K_2HPO_4-KH_2PO_4, 0.05 M, pH 7.2, Na borate, 3.3×10^{-3} M; ferrocytochrome c or dialdehyde-reacted ferrocytochrome c ($Na_2S_2O_4$), 3.3×10^{-5} M (with respect to ferrocytochrome c) and 10 µl of cytochrome oxidase. Volume, 3.0 ml; temp., 25°C. Cytochrome c was omitted from the reference cuvette. The activity is expressed as a percent of the control rate with unmodified cytochrome c. Correction was made for autoxidation of the ferrocytochrome c-dialdehyde complexes.

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groups) reduced the reactivity with cytochrome oxidase by 50%. Flavipin and 3-formylopianic acid were comparable in effect to GA, while quadrilineatin and o-phthalaldehyde were less effective and precipitated ferricytochrome c when reacted with the protein at a molar ratio of 4:1. The ferrocytochrome c-dialdehyde complexes at 4:1 molar ratios were oxidized very slowly by cytochrome oxidase (see footnote, Fig. 2). Beef heart cytochrome oxidase *per se* did not appear to be inhibited by either GA or the ferricytochrome c-GA complex (Table I and Fig. 3). In the experiment of Table I, advantage was taken of the slower rate of reaction between ferrocytochrome c and GA at pH 7.2 than 9.0, so that the cytochrome oxidase rapidly oxidized the ferrocytochrome c before appreciable amounts of the latter could react with GA.

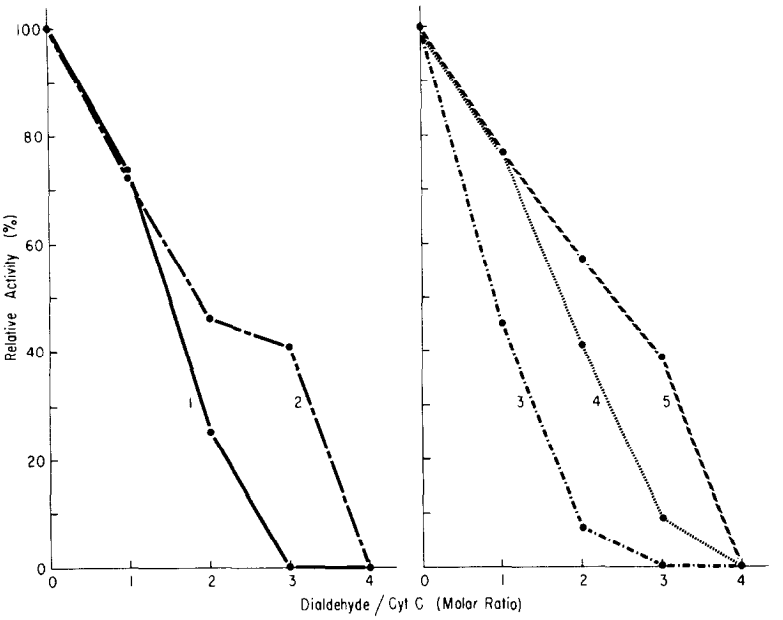

Figure 2.: Reactivity of ferricytochrome c-aromatic ortho-dialdehyde complexes with pig heart NADH-cytochrome c reductase. Curves 1 to 5 represent, respectively, GA, o-phthalaldehyde, cyclopaldic acid, 3-formylopianic acid and quadrilineatin. The dialdehydes were reacted with ferricytochrome c as specified in Fig. 1. The reaction system was set up as follows: tris buffer, 0.012 M, pH 8.5; cytochrome c-dialdehyde complex (in 0.2 ml of 0.05 M Na borate buffer), 3.9 x 10^{-5} M; beef heart cytochrome oxidase, 40 µl. Volume, 2.54 ml; temp., 25°C. The cytochrome oxidase was added to oxidize (slow rate) the fraction of cytochrome c which had been reduced during the reaction with each dialdehyde. When the absorption peak at 550 nm disappeared, 3.3 x 10^{-4} M KCN (final concn.) was added to completely inhibit the cytochrome oxidase, followed by 1.0 x 10^{-4} M (final concn.) NADH and 0.5 mg of NADH-cytochrome c reductase (Sigma activity:1 mg reduces approx. 0.1 µmole of cytochrome c per min. at pH 8.5 at 25°C). Total volume, 3.04 ml. The reference cell contained 3.3 x 10^{-5} M ferricytochrome c and the enzyme was omitted. The activity is expressed as a percent of the control rate with native cytochrome c.

Three moles of GA reacted per mole of ferricytochrome c completely eliminated the activity of the heme protein with NADH-cytochrome c reductase from pig heart (Fig. 2) and 2 moles of cyclopaldic acid reacted per mole of cytochrome c lowered the rate of reduction by 95%. Ortho-phthalaldehyde, 3-formylopianic acid and quadrilineatin were less effective than GA and cyclopaldic acid and showed approximately the same order of cytochrome c inactivation with NADH-cytochrome c reductase as for cytochrome oxidase (Fig. 1). The inactivity of the ferricytochrome c-GA complex with NADH-cytochrome c reductase was not reversed by dialysis (Table II).

The results of Fig. 1 indicated a greater degree of inactivation of cyto-

TABLE I

Effect of GA and cytochrome c-GA complex on the activity of beef heart cytochrome oxidase

addition	μmoles ferrocytochrome c oxidized/min.
Cyt. ox. + ferrocytochrome c	0.08
+ Cyt. ox. + 10^{-3} M GA + ferrocytochrome c	0.07
++ Cyt. ox. + ferricytochrome c-GA (1:5) + ferrocytochrome c	0.078

+ Cytochrome oxidase was incubated with 1.0×10^{-3} M GA for 7 min. in K_2HPO_4-KH_2PO_4 buffer, 0.05 M, pH 7.2, and the reaction was initiated by adding ferrocytochrome c. The reaction system had KH_2PO_4-K_2HPO_4, 0.05 M, pH 7.2; ferrocytochrome c, 3.5×10^{-5} M; cytochrome oxidase, 20 μl and ethanol, 2.66% v/v. Volume, 3.0 ml; Temp., 25°C. The reference cuvette did not contain ferrocytochrome c.

++ Ferricytochrome c (5.0×10^{-4} M), was reacted with 2.5×10^{-3} M GA (1:5 molar ratio) in 1 ml of Na borate buffer, 0.05 M, pH 9.0. The cytochrome oxidase was incubated in phosphate buffer with 0.1 ml of the ferricytochrome c-GA complex (1.67×10^{-5} M with respect to the final concentration of cytochrome c) before the addition of 3.5×10^{-5} M ferrocytochrome c. Reaction conditions as above except for 1.67×10^{-3} M Na borate.

TABLE II

Dialysis of the ferricytochrome c-GA complex and activity in the NADH-cytochrome c reductase system

addition+	μmoles ferricytochrome c reduced/min.	
	undialyzed	dialyzed
Native cytochrome c	0.015	0.015
ferricytochrome c-GA	0.0	0.0

+ Ferricytochrome c (1.43×10^{-4} M) was reacted for 15 min. with 2.5×10^{-3} M GA in 0.05 M Na borate buffer, pH 9.0 and dialyzed overnight against 300 ml (several changes) of 0.05 M Na borate buffer. The native cytochrome c was similarly dialyzed. The reaction system contained tris buffer, 0.01 M, pH 8.5; Na borate, 6.6×10^{-3} M; native ferricytochrome c or ferricytochrome c-GA complex, 1.53×10^{-5} M (with respect to ferricytochrome c); NADH, 1×10^{-4} M and 0.5 mg of pig heart NADH-cytochrome c reductase (Sigma activity: 1 mg reduces approx. 0.16 μmoles of cytochrome c per min at pH 8.5 at 25°C. Volume, 3.0 ml; temp., 25°C. Ferricytochrome c was omitted from the reference cuvette.

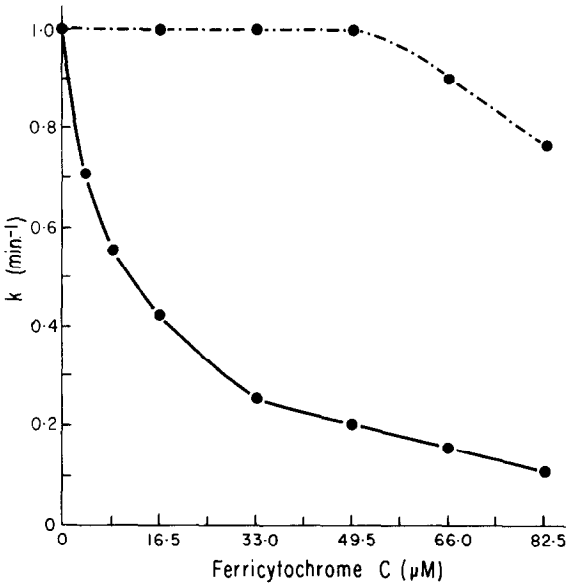

Figure 3.: Effect of ferricytochrome c and ferricytochrome c-GA complex on the first order rate constant (k) for the oxidation of ferrocytochrome c by beef heart cytochrome oxidase. The reaction system contained KH_2PO_4-K_2HPO_4, 0.033 M, pH 7.2; Na borate, 0.01 M; ferricytochrome c (solid line) or ferricytochrome c-GA complex (1:4 molar ratio; dotted line), μM as given; ferrocytochrome c, 1.66 x 10^{-5} M and 10 μl of cytochrome oxidase. Volume, 3.0 ml; temp., 25°C. The cytochrome c-GA complex was first reacted with cytochrome oxidase for varying time periods to oxidize the fraction of cytochrome c that was reduced when complexed with GA (followed at 550 nm). Ferrocytochrome c was then added. Cytochrome oxidase incubated in buffer for equivalent time periods showed no activity loss. The reference cuvette contained appropriate amounts of ferricytochrome c and ferrocytochrome c was omitted.

chrome c by carboxylic, (GA, cyclopaldic acid and 3-formylopianic acid) as opposed to non-carboxylic (quadrilineatin, o-phthalaldehyde) dialdehydes with the exception of the phenolic flavipin. The inactivity of the ferrocytochrome c-GA complex with cytochrome oxidase may be due to a lowering of the basicity of the protein and the removal of a positively charged site required for oxidase-cytochrome c interaction. Succinylation of cytochrome c, for instance, reduces its reactivity with cytochrome oxidase and succinylated cytochrome c does not form an active complex with cytochrome oxidase (15,16). Likewise, the cytochrome c-GA complex (1:4 molar ratio) appears to interact very weakly with cytochrome oxidase as shown in Fig. 3. While the basic ferricytochrome c inhibits the interaction of ferrocytochrome c with the acidic protein of cytochrome oxidase (17,18 and Fig. 3), the ferricytochrome c-GA complex had only a small effect on the rate of oxidation of ferrocytochrome c by cytochrome oxidase.

The ferricytochrome c-GA complex has a strong absorption maximum at 260 nm

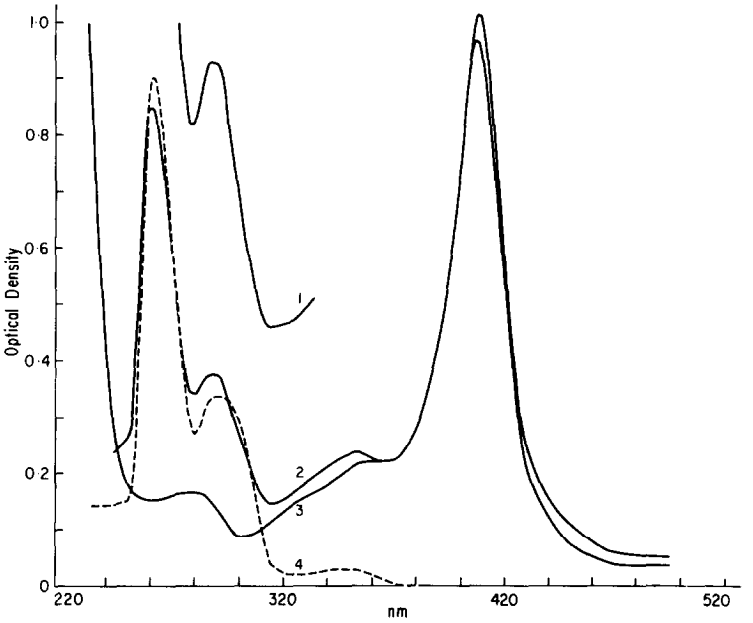

Figure 4.: Absorption spectra of lysine-GA and ferricytochrome c-GA complexes. Curve 1, cytochrome c, 2.1 x 10^{-5} M; GA, 2.0 x 10^{-3} M. Curve 2, cytochrome c, 1.1 x 10^{-5} M; GA, 2.0 x 10^{-3} M. Curve 3, cytochrome c, 1.1 x 10^{-5} M. Curve 4, lysine, 1.0 x 10^{-4} M; GA, 2.0 x 10^{-3} M. Reaction buffer, 0.05 M Na borate, pH 9.0 Volume, 3.0 ml. The GA was added last to the sample cuvette. The reference cuvette contained 0.05 M Na borate buffer, pH 9.0 and 2.0 x 10^{-3} M GA (when GA was in the sample cuvette). The spectra were obtained with a Beckman DB-G spectrophotometer.

and weaker maxima at 290 and 343 nm (Figs. 4 and 5). Similar absorption maxima were exhibited by a complex between lysine and GA. The Soret peak at 410 nm was shifted to a higher wavelength when ferricytochrome c was reacted with GA, indicating a reduction of the cytochrome (Fig. 4). Ferricytochrome c was reduced approximately 50% when reacted with a large molar excess of GA and characteristic absorption peaks appeared at 523 nm and 550 nm. A reduction of ferricytochrome c was noted with each o-dialdehyde, particularly the phenolic flavipin, and the complexes were autoxidizable.

Table III shows the inhibitory effect of GA and flavipin on the oxidation of ascorbate-TMPD (cytochrome oxidase activity) and site III oxidative phosphorylation in sweet potato mitochondria. Oxidative phosphorylation was inhibited more strongly than electron flow by both dialdehydes and flavipin was an especially potent inhibitor of site III phosphorylation. Cyclopaldic acid, quadrilineatin, 3-formylopianic acid and o-phthalaldehyde were also inhibitory but not dihydrogladiolic acid, isophthalaldehyde and terephthalaldehyde. The ortho-dialdehyde grouping is, therefore, required for inhibition.

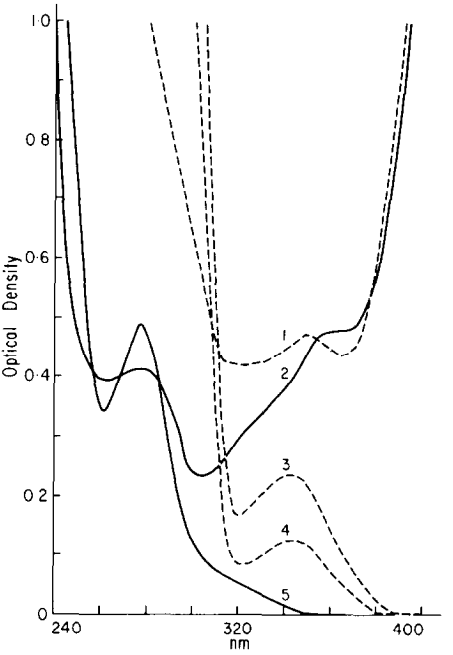

Figure 5.: Absorption spectra of lysine-GA and ferricytochrome c-GA complexes. Curve 1, cytochrome c, 1.87×10^{-5} M; GA, 3.5×10^{-4} M. Curve 2, cytochrome c, 1.87×10^{-5} M. Curve 3, lysine 5.0×10^{-4} M; GA, 5.0×10^{-4} M. Curve 4, lysine, 3.5×10^{-4} M; GA, 3.5×10^{-4} M. Curve 5, GA, 3.5×10^{-4} M. The reference cell contained GA for curves 1, 3 & 4. The reaction conditions were as given in Fig. 4.

Discussion

The present investigation appears to constitute the first report of a chemical modification of cytochrome c by naturally-occurring compounds. Furthermore, GA can seemingly inactivate cytochrome c in the mitochondrion and act as an "apparent" inhibitor of cytochrome oxidase (Table III). Since GA does not significantly impair the reduction of cytochrome c by ascorbate-TMPD and does not inhibit mammalian or plant cytochrome oxidase (Table I and unpublished observations with mung bean mitochondria using the Aminco-Chance dual wavelength spectrophotometer) it must react with cytochrome c *in situ* and block the interaction between the heme protein and cytochrome oxidase, possibly in the same manner as it does between solubilized beef heart cytochrome oxidase and ferrocytochrome c. Gladiolic acid is not a site-specific inhibitor of cytochrome c function but rather, a multi-site mitochondrial inhibitor which affects electron flow at several points in the transfer chain and inhibits oxidative phosphorylation. It acts neither as a typical uncoupler nor as an energy transfer inhibitor (manuscript in preparation).

The inactivation of cytochrome c by GA is probably a reflection of both

TABLE III

Inhibition of ascorbate-TMPD oxidation and Site III oxidative phosphorylation in sweet potato mitochondria by gladiolic acid or flavipin

	Gladiolic acid			Flavipin		
Concn.+ (mM)	µatoms O_2 uptake	µmoles Pi esterified	P/O	µatoms O_2 uptake	µmoles Pi esterified	P/O
0	16.6	12.6	0.76	17.6	12.4	0.71
1.0	1.6 (90)	0.0	0.0 (100)	2.8 (84)	0.0	0.0
0.5	4.6 (72)	0.0	0.0	--	--	--
0.25	6.6 (60)	0.0	0.0	6.7 (62)	0.0	0.0
0.10	13.6 (18)	4.9	0.36 (53)	10.1 (43)	0.0	0.0
0.05	16.5 (0)	12.2	0.74 (0)	15.3 (13)	0.0	0.0
0.025	16.1	12.2	0.76	17.6 (0)	2.5	0.14 (
0.0125	--	--	--	17.1	8.4	0.49 (
0.01	--	--	--	16.7	11.0	0.71 (

+ The reaction system in the Warburg vessels consisted of mannitol, 2.2 x 10^{-1} M; glucose, 2 x 10^{-2} M; KH_2PO_4-K_2HPO_4, 1 x 10^{-2} M, pH 7.2; $MgSO_4$, 6 x 10^{-3} M; NaF, 1 x 10^{-2} M; ascorbate 1.2 x 10^{-2} M; TMPD, 1.2 x 10^{-3} M; ADP, 1 x 10^{-3} M; hexokinase, 1 mg and ethanol, 1.3% v/v. Volume, 3.0 ml, temp., 30°C. Sweet potato mitochondria, 0.25 mg N (GA), and 0.216 mg N (flavipin), were incubated with each dialdehyde for 10 min. before ascorbate-TMPD was tipped in. The reaction was terminated at 15 min. with 0.5 ml of 20% trichloroacetic acid. Oxygen uptakes wer corrected for autoxidation of ascorbate-TMPD. The figures in parentheses denote % inhibition.

a decrease in the basicity of the protein as well as a modification of several lysine residues, including one near the heme group, essential for interaction with cytochrome oxidase (19). Thus, the affinity of cytochrome oxidase for the ferrocytochrome c-GA complex may be greatly decreased over the affinity for native cytochrome c (19). The same lysine residue(s) may also be involved in the interaction of ferricytochrome c with NADH-cytochrome c reductase. Except for trinitrophenylation, any modification such as acetylation, succinylation or guanidylation of a small number (3 or less) of cytochrome c lysine residues has not produced much or any inactivation of protein function (15). Trinitrophenylation of 3 ϵ-NH_2 lysine groups of cytochrome c inactivated the molecule with cytochrome oxidase by 77% (19) while the reaction of GA with not more than 3 mole proportions of lysine residues of cytochrome c reduced the activity by approximately 85% (Fig. 1). The reaction between the ϵ-NH_2 lysine groups of cytochrome c and GA must proceed further than a simple Schiff base formation, since the cytochrome c-GA complex was not reversed by dialysis in contrast to the Schiff base complex formed between salicylaldehyde and cytochrome c (20). The reduction of ferricytochrome c upon reaction with GA indicates an oxidation-reduction process.

The modification of cytochrome c by GA is currently being explored to determine which critical lysine residues react with the dialdehyde to cause a functional alteration of the protein.

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