THE EFFECT OF CARBOXINS ON HIGHER PLANT MITOCHONDRIA

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1. Introduction

Carboxin (5,6-dihydro-2-methyl-1,4-oxathin-3-carboxanilide), a systemic fungicide, has been shown to be a potent inhibitor of succinate metabolism in fungi (e.g., *Ustilago maydis* and *Rhizoctonia solani* [1,2]). It is also a powerful inhibitor of beef heart succinate dehydrogenase (SDH), apparently interfering with the function of an iron–sulphur group in the enzyme [3].

Studies with whole yeast cells suggest [4] that carboxin is an inhibitor of the alternate (cyanide-insensitive) oxidase of yeast and, by implication, of higher plant mitochondria. These authors claimed that an iron–sulphur component of the SDH complex is the alternate oxidase. More recent studies with plant mitochondria have suggested that SDH is not per se the alternate oxidase, and have implicated a role for ubiquinone, either as the branch point of the alternate path [5] or as the oxidase [6].

This communication reports an investigation of the effects of carboxins on higher plant mitochondria (both CN-resistant and CN-sensitive) and compares these effects with those on rat liver mitochondria. The results indicate that carboxins are inhibitors of both the alternate path and SDH. The plant enzyme has a much lower affinity for the inhibitors than does its mammalian counterpart.

Abbreviations: Carboxin, 5,6-dihydro-2-methyl-1,4-oxathin-3-carboxanilide; PMS, phenazine methosulphate; SHAM, salicylhydroxamic acid; SDA, succinate dehydrogenase; TPP, thiamine pyrophosphate

2. Materials and methods

Untreated potato tubers (*Solanum tuberosum* var. Russet Burbank) were generously provided by Professor H. Timm, University of California, Davis. Sweet potatoes (*Ipomoea batatas*) were purchased from local markets.

Mitochondria were isolated from while potato tubers and sweet potato roots as described [7]. Rat liver mitochondria were prepared as in [8] and sub-mitochondrial particles as in [6].

Oxygen consumption was measured on the oxygen electrode as described [7] with 3.3 ml reaction medium and 0.2 ml mitochondria (approx. 2 mg protein). For plant mitochondria a reaction medium of 0.4 M mannitol, 10 mM TES buffer (pH 7.2), 5 mM KH_2PO_4 and 1 mM $MgCl_2$ was used. The reaction medium for rat liver mitochondria consisted of 0.1 M KCl, 0.25 M sucrose, 15 mM KH_2PO_4, 1 mM EDTA and 10 mM $MgCl_2$. When present, the concentrations of ADP and ATP were 1 mM and 0.2 mM, respectively. Other experimental conditions are described in the figure and table legends.

Carboxin was a gift from Uniroyal Research Laboratory (Guelph, Ontario). 3′-Phenoxycarboxin was a gift from Dr G. A. White, Agriculture Canada, (London, Ontario). Other chemicals were from Sigma Chemical Co. (St Louis, MO).

3. Results

3.1. *Inhibition of succinate oxidation*

Figure 1 shows that carboxins are potent and selective inhibitors of succinate oxidation by rat liver

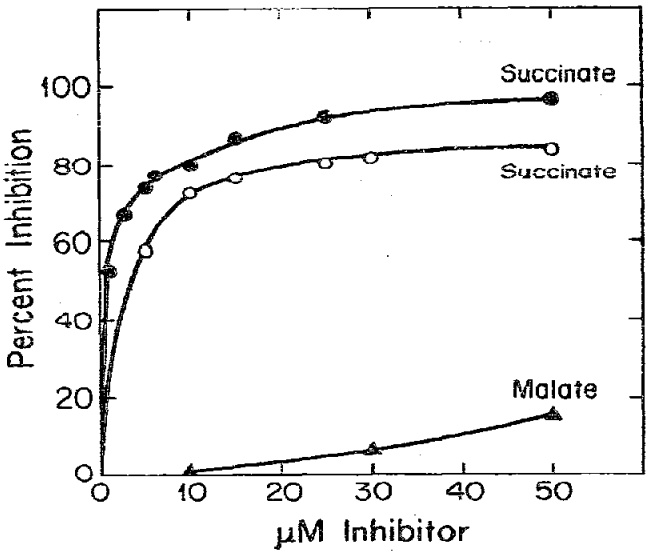

Fig.1. Effect of carboxins on rat liver mitochondria. Oxygen uptake was measured as described in section 2. The reaction mixture contained 10 mM succinate or 10 mM malate + 10 mM glutamate, 1 mM ADP and 0.2 mM ATP in 3.3 ml standard reaction medium. (○—○) Carboxin; (●—●) 3'Phenoxy-carboxin.

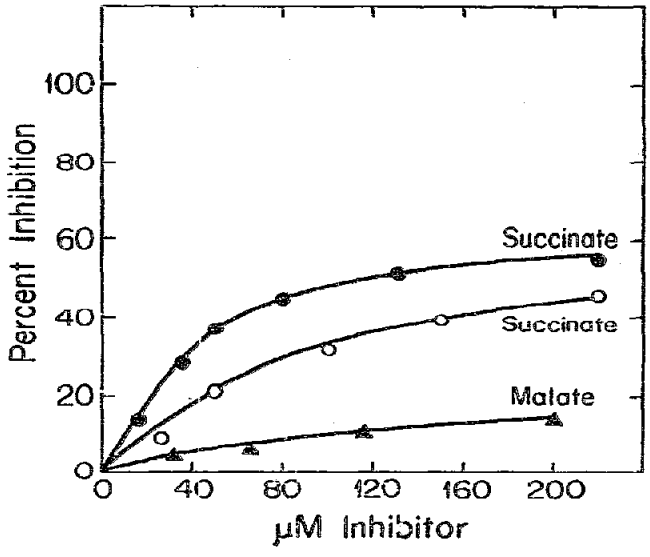

Fig.2. Effect of carboxins on white potato mitochondria. Experimental conditions are described in fig.1. (○—○) Carboxin; (●—●) Phenoxy-carboxin.

mitochondria. 3'-Phenoxy-carboxin is more effective than carboxin with succinate as substrate (K_i values 0.7 µM and 6 µM, respectively). Neither inhibitor has much effect on malate oxidation in the presence of glutamate. These effects are similar to those observed with beef heart submitochondrial particles [3] and with *Micrococcus denitrificans* [9].

Similar trends were observed with cyanide-sensitive white potato mitochondria (fig.2), except that neither compound was as effective against succinate oxidation as in rat liver. The K_i apparent for phenoxy-carboxin is 80 µM while K_i apparent for carboxin is 200 µM. Other plant mitochondria oxidizing succinate show roughly the same sensitivity to carboxin [10,11]. Disruption and fractionation of potato mitochondria does little to increase their sensitivity to carboxin (table 1: succinate oxidase activity of submitochondrial particles is inhibited 68% by 500 µM carboxin), showing that inaccessibility to SDH is not responsible for the low inhibitions. Succinate oxidation may be linked to oxygen consumption through the dye PMS, thus by-passing the electron transport chain. PMS–mediated succinate oxidation by beef heart mitochondria is less sensitive to carboxin than is succinate oxidase, and it has been suggested that this is due to PMS accepting electrons from two separate sites on the SDH complex, one prior to the carboxin-sensitive site [12]. The same seems to be true of the plant system, since PMS-mediated succinate oxidation is inhibited only 20% (table 1). It thus seems that carboxin binds to potato SDH at the same site as on the mammalian enzyme. However, the affin-

Table 1
Effect of carboxin on succinate oxidation by potato submitochondrial particles

Carboxin (µM)	Succinate oxidase (nmol O_2/min)	Succinate–PMS oxidase
0	104	158
250	41	135
500	34	122

Oxygen consumption was measured as described in section 2 with 0.2 mM ATP in the reaction medium. Succinate, 10 mM, PMS, 0.1 mM and 0.5 mg protein were used/assay. When succinate–PMS activity was measured, 2 µM antimycin A was also present to inhibit the cytochrome path

ity of the plant system for the inhibitor is an order of magnitude lower.

3.2. *Inhibition of alternate oxidase*

The respiration of isolated sweet potato mitochondria, with succinate and with NAD-linked substrates, is partially resistant to cyanide [13]. Although the degree of resistance varies with the season and the length of root storage, typically 40–70% of the state 3 rate of O_2 consumption is insensitive to KCN. For example, in the experiments shown in fig.3, the state 3 rate of succinate oxidation was 155 nmol O_2 min^{-1} mg protein^{-1} while the rate in the presence of 0.5 mM KCN was 97 nmol O_2 min^{-1}. When malate (plus pyruvate) was the substrate, the rates in the absence and presence of KCN were 97 and 67 nmol O_2 min^{-1} mg protein^{-1}, respectively.

Substituted hydroxamic acids are selective inhibitors of cyanide-resistant respiration [15]. In the present study, oxygen uptake in the presence of salicyl-hydroxamic acid (SHAM) was considered to be mediated by the cytochrome path, whereas that in the presence of KCN was presumed to be due to alternate path activity (cyanide and SHAM together inhibit oxygen consumption completely). Figure 3 shows the effect of 3′-phenoxy-carboxin on both pathways. Carboxin has similar effects, although considerably higher concentrations were required to elicit the inhibitions shown (data not presented). Phenoxy-carboxin inhibits succinate oxidation in the presence of KCN and SHAM, respectively, although its effect is somewhat greater in the presence of KCN. Presumably inhibition in the presence of SHAM represents the effect on SDH alone. Malate oxidation (with pyruvate present) is only slightly inhibited by phenoxy carboxin in the presence of SHAM. That is, phenoxy-carboxin does not inhibit malate oxidation via the cytochrome path, as shown also with rat liver and white potato mitochondria (fig.1,2). On the other hand, malate (+ pyruvate) oxidation via the alternate oxidase is inhibited approx. 50% by carboxins (fig.3).

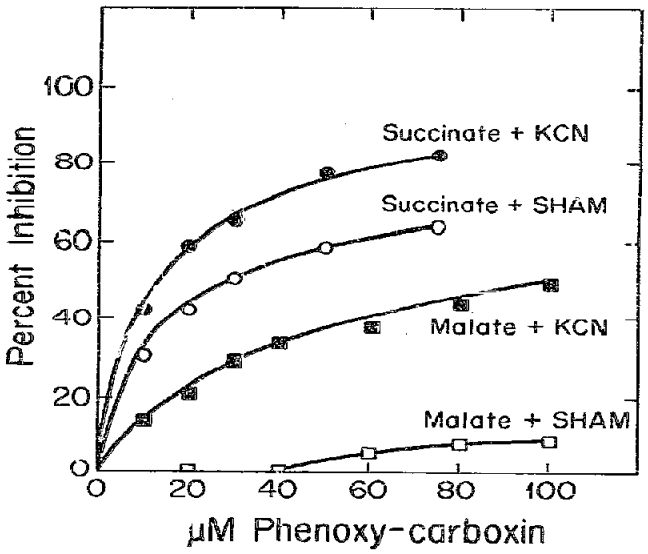

Fig.3. Effect of phenoxy-carboxin on sweet-potato mitochondria. Experimental conditions are as described in fig.1 except that 50 μM TPP and 10 mM pyruvate were included in the reaction medium when malate (1 mM) was substrate.

4. Discussion

Carboxin is proposed as a selective inhibitor of the cyanide resistant path [16]. We favor the suggestion [4] (on evidence which is difficult to evaluate from their experiments on intact yeast cells) that carboxin inhibits both SDH and the alternate path. Part of the SDH complex is suggested as the alternate oxidase [4], thereby accounting for carboxin's effect on both activities. This idea is most unlikely in view of the results presented here, which show that cyanide-resistant malate oxidation is sensitive to phenoxy-carboxin (fig.3). Furthermore, mungbean mitochondrial particles which have been stripped of SDH retain their resistance to cyanide with other substrates [6], showing that SDH is not the alternate oxidase.

Since carboxins inhibit succinate oxidation by cyanide-sensitive white potato mitochondria (fig.2), while SHAM does not (data not shown) and since carboxins inhibit NAD-linked substrate oxidation in the presence of KCN in sweet potato mitochondria (fig.3), it is probable that there are two distinct sites of action for carboxins in cyanide-resistant plant mitochondria.

The greater inhibition of succinate oxidation by carboxin in the presence of KCN – compared with its effect in the presence of SHAM – could mean that the alternate path is more sensitive to carboxin than is SDH. However, the effect of carboxin on malate

(+KCN) suggests otherwise. Hence the greater affect on succinate +KCN points to two inhibitory sites seriatim. This also accounts for the lower K_i apparent for phenoxy-carboxin with succinate +KCN (23 μM) as compared both with succinate + SHAM, and malate + KCN (85 μM).

The lower affinity of plant SDH for carboxin, compared with that from animals and fungi, implies either that the two enzymes are structurally different or that their environment in the inner membrane is different. EPR studies [6,17] also suggest differences between animals and plants in the SDH region of their respiratory chains.

Acknowledgements

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References

[1] Matre, D. E. (1970) Phytopathology 60, 671–676.
[2] Ulrich, J. T. and Mathre, D. E. (1972) J. Bacteriol. 110, 628–632.
[3] Mowery, P. C., Steenkamp, D. G., Ackrell, B. A. C., Singer, T. P. and White, G. A. (1977) Arch. Biochem. Biophys. 178, 495–506.
[4] Lyr, H. and Schewe, T. (1975) Acta Biol. Med. Germ. 34, 1631–1641.
[5] Storey, B. T. (1976) Plant Physiol. 58, 521–525.
[6] Rich, P. R. and Bonner, W. D., jr (1977) Plant Physiol. 59, (6) 59.
[7] Laties, G. G. (1973) Biochemistry 12, 3350–3354.
[8] Weinbach, E. C. (1961) Analyt. Biochem. 2, 335–339.
[9] Tucker, A. N. and Lillich, T. T. (1974) Antimicrob. Agents Chemother. 6, 572–578.
[10] Mathre, D. E. (1971) Pest. Biochem. Physiol. 1, 216–224.
[11] Ulrich, J. T. and Mathre, D. E. (1976) J. Bacteriol. 110, 628–632.
[12] Ackrell, B. A. C., Kearney, E. B., Mowery, P. C., Singer, T. P., Beinert, H., Vinogradov, A. D. and White, G. A. (1976) in: Iron and Copper Proteins (Yasunobu, K., Mower, H. F. and Hayaishi, O. eds) pp. 161–181, Plenum Press, New York.
[13] Tomlinson, P. F. and Moreland, D. E. (1975) Plant Physiol. 55, 365–369.
[14] Solomos, T. (1977) Ann. Rev. Plant Physiol. 28, 279–297.
[15] Schonbaum, G. R., Bonner, W. D. jr, Storey, B. T. and Bahr, J. T. (1971) Plant Physiol. 44, 115–128.
[16] Sherald, J. L. and Sisler, H. D. (1972) Plant Cell Physiol. 13, 1039–1052.
[17] Moore, A. L., Rich, P. R., Bonner, W. D., jr and Ingledew, W. J. (1976) Biochem. Biophys. Res. Commun. 72, 1099–1107.