CARBOXINS: POWERFUL SELECTIVE INHIBITORS OF SUCCINATE OXIDATION IN ANIMAL TISSUES

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Summary: Carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) is a systemic fungicide, reported to inhibit succinate oxidation in certain fungi, particularly Ustilago maydis (corn smut). In the present study the action of carboxin and of other oxathiin derivatives on beef heart succinate dehydrogenase has been investigated. Carboxins inhibited the same activities to the same extent as thenoyltrifluoroacetone (TTF) but at much lower concentrations. For 14 carboxin derivatives the inhibition constants (concentration required to inhibit 50% of the carboxin-sensitive activity) ranged from 2×10^{-8} to 2×10^{-6}M. Like TTF, carboxin derivatives did not inhibit soluble succinate dehydrogenase but inhibited the reduction of coenzyme Q analogs, of 2,6-dichlorophenolindophenol, and of phenazine methosulfate (PMS) in Complex II preparations. The same reactions and succinoxidase activity were also inhibited in inner membranes (ETP). In ETP only $\sim$ 50% of the succinate-PMS activity was carboxin sensitive, the same fraction as is inhibited by TTF or is lost on extraction of coenzyme Q and on incubation with cyanide. While the inhibition of PMS reduction by carboxin was largely or entirely competitive in Complex II, it was predominantly non-competitive in ETP at low concentrations. Some other carboxin derivatives gave mixed inhibition patterns for PMS reduction in ETP even at low inhibitor concentrations. The complex inhibition pattern in the PMS assay seems more compatible with conformation changes affecting activity than with loss of a reaction site for PMS.

Carboxin is a systemic fungicide known to inhibit succinate metabolism in Ustilago nuda and Rhizoctonia solani (1). Investigations with carboxin-sensitive fungi, particularly corn smut (U. maydis)(2,3), with yeast (3), and with membranes from Micrococcus denitrificans (4) have shown that the inhibition site of carboxin responsible for blocking succinate oxidation is in the Complex II region of the respiratory chain. Further evidence that the primary reaction site of carboxin is in the Complex II region has come from genetic studies with carboxin-resistant mutants of U. maydis (5). The characteristics of the inhibition of succinate oxidation by carboxin in fungal preparations (2,3) suggested a striking similarity to the action of TTF. The present paper describes the inhibition of

beef heart succinate dehydrogenase by a series of carboxin derivatives in preparations ranging from inner membranes (ETP) to the purified, soluble enzyme.

MATERIALS AND METHODS

ETP and Complex II and reconstitutively active, butanol extracted succinate dehydrogenase were isolated as previously described (6-8). Activities were measured in fully activated preparations: succinate-PMS reaction with DCIP as terminal acceptor (9), DCIP reduction under the same conditions but without PMS present, succinoxidase polarographically. The reduction of the Q analog, DPB (2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone) was measured with DCIP as terminal acceptor under the conditions of the PMS-DCIP assay (9) but with 39 μM DPB in lieu of variable concentration of PMS and "low K_m" ferricyanide activity (10) spectrophotometrically. DPB was obtained from Dr. Karl Folkers amd carboxin derivatives as in previous work (11). Carboxin and 3'-methylcarboxin were gifts from Dr. J. Wilson, UniRoyal Research Laboratory, Ltd.

RESULTS

Inhibition of the succinate-DPB and succinate-DCIP reductase activities in Complex II. It has been known for many years that TTF completely inhibits the reduction of Q derivatives by succinate in Complex II (12). In the present study Q reductase activity was assayed with DPB, which quantitatively replaces Q_1 in this assay (13). The reduction of DPB by succinate in NO_3^- - activated Complex II was ∿95% inhibited at 60 μM TTF or carboxin, while 3'-methylcarboxin produced comparable inhibition at 7 to 10 μM concentration. Linear Dixon plots, indicating saturation kinetics, were obtained, giving K_i values at 15° as follows: carboxin and TTF, 1.5 to 1.9 μM; 3'-methylcarboxin, 0.3 μM. The succinate-DCIP reductase activity of Complex II was similarly highly sensitive to inhibition. All three inhibitors produced complete inhibition with non-competitive kinetics and linear

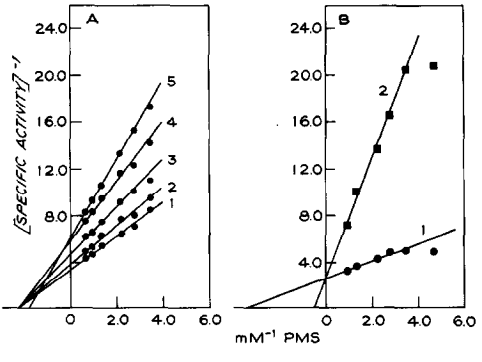

Fig. 1. Effect of 3'-methylcarboxin on membrane-bound succinate dehydrogenase. Ordinate, μmoles of succinate oxidized/min/mg of protein at 38°. A. ETP (turnover number = 19,400 at 38°); inhibitor concentrations: 1, none; 2, 0.05 μM; 3, 0.15 μM; 4, 1.5 μM; 5, 5.0 μM. B. Complex II (turnover number = 10,800); 1, no inhibitor; 2, 0.84 μM 3'-methylcarboxin.

Dixon plots. The K_i values at 15° were: carboxin, 0.7 to 0.9 μM; TTF, 1.1 μM; 3'-methylcarboxin, 0.3 to 0.4 μM.

Inhibition of the succinate-PMS reductase activity in Complex II. Carboxin, 3'-methylcarboxin, and TTF also inhibited the reduction of PMS by succinate in Complex II, but the characteristics of the inhibition were different from the pattern observed in the succinate-DPB and succinate-DCIP reactions. As shown in Fig. 1A, the inhibition is competitive with respect to PMS. The K_i values at 15° were nevertheless very similar to those calculated for DPB and for DCIP reduction: carboxin, 1.4 to 1.5 μM; TTF, 1.1 μM; 3'-methylcarboxin, 0.3 μM. The competitive character of the inhibition of PMS reduction was regularly observed in typical Complex II preparations (preparations having a turnover number of ∿10,000). As shown below, in Complex II samples with higher turnover numbers mixed inhibition was observed.

Inhibition of succinate oxidation in ETP. In ETP preparations low concentrations of carboxin and 3'-methylcarboxin completely inhibited the reduction of DCIP, DPB, and succinoxidase activity. The effect of these compounds on the succinate-PMS reaction in ETP was markedly different, however, from that observed in typical samples of Complex II (Fig. 1B). In ETP the inhibition was non-competitive up to a relatively high concentration of inhibitor, at which point a competitive feature appeared (Fig. 1A). Maximal inhibition was ∿50% (Fig. 2), the same as with TTF. Extraction of Q_{10} (14) or incubation with cyanide (15) also lead to loss of about half of the PMS-DCIP activity in inner membranes. All these treatments reduce the turnover number of ETP (∿ 20,000 ± 1,000) to the value (∿ 10,000) seen in typical Complex II preparations.

A number of carboxin derivatives have been compared for effectiveness in inhibiting

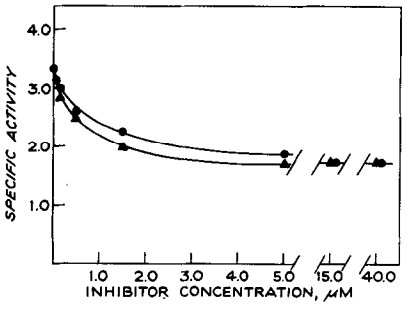

Fig. 2. Titration of succinate-PMS activity of ETP with carboxin (●) and 3'-methylcarboxin (▲) at 38°. The ordinate represents V_{max} (PMS), based on the data of Fig. 1A.

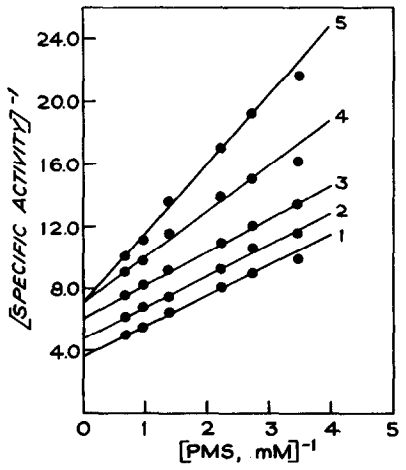

Fig. 3. Effect of 4'-phenoxycarboxin on succinate-PMS activity of ETP at 38°. The turnover number of the ETP used was 18,000; inhibitor concentrations were as in Fig. 1A.

the succinate-PMS reaction in ETP. At low concentrations most of them showed non-competitive kinetics (Fig. 1A) and linear Dixon plots. Some of them produced uncompetitive inhibition and thus curved Dixon plots, however (Fig. 3). The maximal inhibition attained was in the range of 50% for all inhibitors (Table I).

In comparing the inhibitory potency of various carboxins, K_i values were calculated from Dixon plots when these were linear. In addition, the concentrations giving 25% inhibition (I_{25}) and the concentration giving 50% loss of the activity inhibitable by the particular compound (I_{50}) were calculated (Table I). Another basis for establishing a relative scale of potencies is possible by modifying the Hill equation to fit cases where only a part of the measured activity is susceptible to inhibition:

$$\log K - n \log i = \log \frac{zE - T}{z(T-E)} ,$$

where T is the activity of the uninhibited enzyme, E the activity in the presence of a particular inhibitor concentration, i the concentration of inhibitor, and z the reciprocal of the fraction of inhibitor-resistant activity. Values for n (a measure of cooperativity) and of K (next to last column) are included in Table I.

As seen in Table I, with three exceptions where the K values from Hill plots were significantly different from the inhibition constants determined by other means, the four methods gave gratifyingly similar values. All carboxin derivatives were more potent inhibitors than TTF and the apparent K_i values ranged from 2×10^{-8} to 2×10^{-6} M.

TABLE I

COMPARISON OF THE INHIBITORY POTENCY OF CARBOXIN DERIVATIVES AND OF TTF TOWARD SUCCINATE DEHYDROGENASE ACTIVITY OF ETP[a]

Inhibitor	I_{25}[b] (μM)	I_{50}[c] (μM)	Max. Inhibition (%)	n[d]	K[d] (μM)	K[e] (μM)
TTF	7.5	9.6	57	1.00	9.7	9.0
4'-Dimethylaminocarboxin	1.9	2.6	57	0.89	2.4	3.0
4'-Carboethoxycarboxin	1.3	1.5	55	0.53	1.4	
5,6-Dihydro-2-methyl-1,4-oxathiin-3-carboxanilide (Carboxin)	0.90	0.80	47	0.92	0.75	0.78
4'-Benzoylcarboxin	0.85	0.70	47	0.88	0.72	0.80
4'-n-Butoxycarboxin	0.66	0.70	52	1.30	0.60	
3'-Methylcarboxin	0.55	0.60	52	0.64	0.61	0.50
4'-Phenylcarboxin	0.36	0.33	47	1.40	0.22	
4'-n-Butylcarboxin	0.14	0.15	51	0.88	0.18	0.14
4'-Phenoxycarboxin	0.08	0.06	44	1.50	0.02	
5,6-Dihydro-2-methyl-1,4-oxathiin-3-n-decylcarboxamide	0.06	0.09	58	0.76	0.16	0.11
3'-Phenoxycarboxin	0.04	0.05	52	1.20	0.03	0.05
4'-n-Pentylcarboxin	0.03	0.04	54	1.10	0.03	
3'-Octoxycarboxin	0.03	0.02	39	1.50	0.002	
4'-Hexoxycarboxin	0.02	0.02	51	1.01	0.02	

[a]PMS-DCIP assay at 38° (V_{max}). [b]Concentration for 25% inhibition of control activity. [c]Concentration for 50% inhibition of carboxin sensitive activity. [d]K and n values from Hill plot. [e]K_i from Dixon plot.

Effect of carboxins on purified succinate dehydrogenase. Schewe et al. (16) reported that TTF but not carboxin blocks the succinate-PMS reaction in soluble preparations from heart. In our hands neither TTF nor carboxin (6 μM) blocked either the reduction of PMS-DCIP or of ferricyanide ("low K_m" site (10)) in a reconstitutively active, soluble preparation.

Effect of carboxin on succinate dehydrogenase from yeast. PMS reduction by succinate dehydrogenase from aerobic yeast is 50% inhibited by TTF in submitochondrial particles (17). 3'-Oxtoxycarboxin and 4'-hexoxycarboxin inhibited the yeast enzyme non-competitively in submitochondrial particles, giving linear Dixon plots, from which K_i values of 0.54 μM and 3.9 μM, respectively, were calculated. Thus carboxins appear to be less inhibitory to the yeast than to the heart enzyme, although their action on succinate dehydrogenase is qualitatively similar in the two sources.

DISCUSSION

The results presented show that carboxin derivatives inhibit the normal operation of succinate dehydrogenase in membrane preparations from animal tissues and yeast in a manner similar to their action in other fungi (2,3,18). The reactions affected and the maximal inhibitions reached were the same as with TTF.

It has been suggested (18) that carboxins, like the iron chelator TTF, interfere with the functioning of a specific Fe-S component of the enzyme. Support for this idea has come from the observations (13) that carboxins not only inhibit the reoxidation of both the g=1.94 and the Hipip Fe-S centers of the enzyme (particularly the latter) but cause extensive destruction of the Hipip center in Complex II preparations.

The kinetics of the inhibition of PMS reduction are complex. In intact membranes (e.g., ETP) with a turnover number of 20,000 ± 1,000 at 38° in the PMS-DCIP assay, low concentrations of most carboxins give purely or predominantly non-competitive inhibition, as reported by Rossi et al. (14) for TTF. There are exceptions, however: a few carboxins give uncompetitive inhibition at low concentrations (Fig. 3). At relatively high carboxin concentrations a competitive component is always evident. In particles in which the turnover number is decreased to ∿10,000, such as typical Complex II samples (8) or Q-depleted inner membranes (14), the inhibition by carboxins is purely competitive with respect to PMS, as has also been observed with TTF (14). It is suggestive that in Complex II preparations isolated by a modified procedure, yielding a turnover number of ∿13,000, inhibition by 3'-methylcarboxin shows a non-competitive component, so that at V_{max} (PMS) the turnover number declines to 10,000, the value reached on cyanide treatment (Fig. 4).

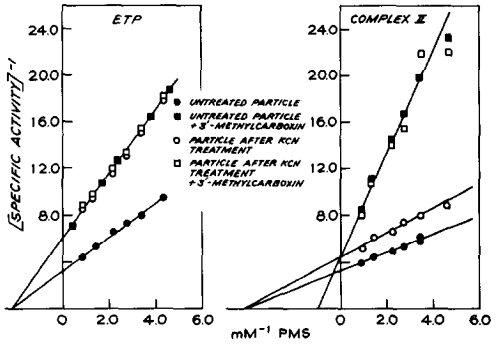

Fig. 4. Comparison of cyanide inactivation and inhibition by 3'-methylcarboxin of succinate-PMS activity in ETP and Complex II. Incubation with cyanide (0.02 M) was for 1 hr at 30°, pH 8.0; 3'-methylcarboxin, where present, was 0.84 μM. A. ETP, turnover number = 19,000 at 38°. B. Complex II, turnover number = 13,200.

The fact that inhibition by carboxins or by TTF, depletion of Q, and inactivation by cyanide all lower the turnover number of the enzyme in ETP to ~ 10,000 and that Q-depleted or cyanide inactivated preparations are not inhibited further by TTF or carboxin (at V_{max} (PMS)) suggests several explanations for the mode of action of TTF and carboxins. (a) These observations are compatible with the idea that one of the two reaction sites of PMS, responsible for ~ 50% of the measured activity (15), is endogenous Q, since all these treatments either deplete the Q pool or block access to it. The two-site hypothesis is not readily reconciled with recent EPR data (13), however, showing that even in Complex II (TN = 10,000) the reoxidation of the Fe-S centers by PMS is too fast to be rate limiting. (b) It has been shown (19) that on incorporation of the soluble enzyme into the membrane its turnover number increases by as much as 50%. This may suggest a potentiation of the succinate-PMS activity by a membrane component (possibly Q (14)), access to which would be blocked by carboxin, as well as by TTF and cyanide. (c) Nelson et al. (20) have proposed that TTF, on interacting with an Fe-S component of succinate dehydrogenase, causes a conformational modification in the enzyme which profoundly alters its interaction with cytochrome b. Since the action of carboxins seems to mimic that of TTF in every respect known, these inhibitors might likewise bring about a conformational change which leads to complete interruption of electron flux via Q and lowered reactivity towards PMS.

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