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Reaction site of carboxanilides and of thenoyltrifluoroacetone in complex II*

(carboxin/photoaffinity labeling/succinate dehydrogenase)

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ABSTRACT **Oxathiin carboxanilides are systemic fungicides that inhibit the oxidation of succinate by interrupting electron transport between succinate dehydrogenase [succinate:(acceptor) oxidoreductase, EC 1.3.99.1] and coenzyme Q. Kinetic and electron paramagnetic resonance studies have established that the specific binding site of carboxanilides and of thenoyltrifluoroacetone responsible for the inhibition is the same. Although the binding of carboxanilides to membrane preparations of the dehydrogenase is very tight (K_i = 0.01–0.1 μM), it is noncovalent. Identification of the membrane component(s) to which specific binding occurs has therefore required the introduction of a photoaffinity label onto the carboxanilide molecule. By using [*G*-^{3}H]3′-azido-5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide, it was found, in accord with earlier data with other carboxanilides, that unresolved complex II specifically binds about 0.6 mol of the inhibitor per mol of succinate dehydrogenase in equilibrium dialysis experiments. The resolved components of the complex, succinate dehydrogenase and the two binding peptides C_{II-3} and C_{II-4}, failed to bind the inhibitor; however, when these were recombined with reconstitution of coenzyme Q reductase activity, the initial binding titer was restored. Azidocarboxanilide-inhibited complex II was irradiated to generate covalent linkages with the binding site, and the components of the complex were separated on polyacrylamide gel. Most of the specifically bound inhibitor was found in the low molecular weight binding peptides and phospholipids.**

Carboxanilides are potent inhibitors of succinate oxidation in membrane preparations from fungi, bacteria, and animal tissues, blocking electron transfer between succinate dehydrogenase [succinate:(acceptor) oxidoreductase, EC 1.3.99.1] and coenzyme Q (1–4). The kinetics of the inhibition are complex, varying according to the type of membrane preparation and electron acceptor used, and the effects of carboxanilides and of the classical inhibitor thenoyltrifluoroacetone (TTF) are indistinguishable (4–6). The two types of inhibitor displace each other from the specific binding site responsible for the inhibition (6, 7). Carboxanilides are also bound nonspecifically at sites unrelated to the inhibition of succinate oxidation, and this type of binding is so tight that neither TTF nor extensive washing displaces the inhibitor from these loci. In contrast, specifically bound carboxanilide, despite very low K_d values, may be dislodged by washing the particles, with full recovery of catalytic activity (7). This latter property has prevented precise localization of the specific inhibition site, although the observation that specific binding does not occur in complex II preparations from which the dehydrogenase has been extracted suggests that the dehydrogenase is an obligatory component of the binding site (7).

Upon separation of the two low molecular weight binding peptides (C_{II-3} and C_{II-4}) present in complex II from the dehydrogenase, TTF-sensitive coenzyme Q reductase activity disappears; however, when these are recombined with the pure enzyme, the activity is restored (8). Therefore, one or both of these peptides may also play a role in the binding site of the inhibitor. In order to explore this question and to characterize more precisely the membrane components to which carboxanilides become bound during inhibition of succinate-coenzyme Q reductase activity, we have synthesized [*G*-^{3}H]3′-azido-5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide (azidocarboxin), which bears both photoaffinity and isotopic labels. Irradiation of a mixture of complex II and azidocarboxin, with and without TTF present during labeling so as to distinguish specific binding, followed by analysis of the components on polyacrylamide gels, has permitted identification of the components to which the inhibitor is bound.

MATERIALS AND METHODS

Materials. Complex II (9) and reconstitutively active succinate dehydrogenase (10) were isolated by published procedures. Peptides C_{II-3} and C_{II-4} were isolated together from complex II in 50 mM Tris phosphate, pH 7.0/0.5% deoxycholate and reconstituted with succinate dehydrogenase as described (8).

Succinate-coenzyme Q reductase activity (8) and the histidylflavin (11) and protein contents (12) of preparations were determined by published methods. The dehydrogenase of complex II was activated by incubation with 20 mM succinate at 38°C for 7 min.

Synthesis of Azidocarboxin. 3′-Nitrocarboxin, melting point 120–121°C (13), was reduced with activated aluminum in moist ether (14) to the 3-amino compound, melting point 143–145°C. Diazotization and reaction with sodium nitrite according to Galardy *et al.* (15) in subdued light gave the 3′-azidocarboxin, melting point 75–77°C (ethanol). The molar extinction coefficient in ethanol at 246 nm is 2.3×10^4. The azidocarboxin was tritiated in acetic acid with Ni/graphite catalyst and tritiated water overnight at 80°C by New England Nuclear. Extensive purification of their product was required, first by column chromatography on silica gel with 1% (vol/vol) ethanol in benzene as eluant to remove most of the undesired material and then by preparative thin-layer chromatography with silica gel and 1%

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Abbreviation: TTF, thenoyltrifluoroacetone.

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(vol/vol) acetic acid in benzene as developing solvent. All operations involving azidocarboxin were performed in dim light to minimize photodecomposition. The yield of tritiated azidocarboxin was 12% of the amount put through the tritiation procedure; its specific activity was 35.9 Ci/mol (1 Ci = 3.7×10^{10} becquerels).

Methods. Equilibrium dialysis experiments with the reconstituted succinate-coenzyme Q reductase complex or its component parts were conducted anaerobically in 50 mM Tris·HCl, pH 7.6/20 mM succinate/0.1 mM EDTA/0.5% deoxycholate at 4°C for 16 hr in the dark, with a microdialysis unit (Hoeffer Scientific Instruments, Somerville, NJ; 0.25 ml per compartment). The protein concentrations used were: peptide preparation, 0.5 mg/ml; succinate dehydrogenase, 0.8 mg/ml; reconstituted succinate-coenzyme Q reductase, 0.5 mg of peptide plus 0.8 mg of succinate dehydrogenase, per ml, yielding reconstituted complex containing 5 μM flavin. Equilibrium dialysis experiments with complex II were conducted in the absence of deoxycholate and on a larger scale (1 mg of protein in 5 ml of the buffer mixture against 10 ml of buffer mixture). The inhibitor concentration in all cases was 0–50 μM azidocarboxin, with or without 0.2 mM TTF present.

For photoaffinity labeling, preactivated complex II (0.2 mg of protein per ml in 50 mM Tris·HCl, pH 7.6/20 mM succinate) was stirred at 4°C in an open vessel with a 6-mm liquid layer in the presence of 0.2–5 μM azidocarboxin with or without 2 mM TTF. The solution was irradiated from above (3 cm distance) for 12 10-sec intervals with a 9-W ultraviolet lamp (Ultraviolet Products, San Gabriel, CA; model SL2537) with the short-wave filter attached. After irradiation, the preparation was sedimented, washed five times, and resuspended to give 2 mg/ml, and protein and catalytic activities were determined as required. Two aliquots of each sample (50 and 200 μg of protein) were boiled for 2 min with 1.5% (wt/vol) NaDod SO_4/1.5% (vol/vol) 2-mercaptoethanol and subjected to electrophoresis on 9% (wt/vol) polyacrylamide gels (16). One gel (50 μg of protein) was stained with Coomassie blue G-250 to establish the location of the peptides. The other (200 μg of protein) was cut into 2-mm slices, which were dissolved in 10 ml of Soluscint scintillation fluid containing 500 μl of Solusol (National Diagnostics, San Francisco, CA), and radioactivity was determined.

RESULTS AND DISCUSSION

Binding Studies. To provide kinetic evidence that azidocarboxin, like other carboxanilides previously studied (7), competes with TTF for the binding site involved in the inhibition of succinate oxidation, we measured the succinate-coenzyme Q reductase activity of complex II at fixed concentrations of the quinone (2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone) and of azidocarboxin and varying concentrations of TTF. The parallel Dixon plots of the data were consistent with a mutual exclusion effect.

The results of equilibrium dialysis experiments on the binding of azidocarboxin to unresolved complex II and to the complex reconstituted from soluble succinate dehydrogenase and its binding peptides are shown in Fig. 1 *A* and *B*, respectively. Specific binding may be calculated from the difference between the binding curves in the presence and absence of TTF on the assumption that TTF does not interfere to a major extent with the binding of azidocarboxin to unspecific sites. The validity of this assumption is shown by the fact that the K_i value for azidocarboxin in the succinate-coenzyme Q assay (1.5 μM at 38°C) agreed satisfactorily, considering the difference in temperature, with the K_d determined by equilibrium dialysis (1.2 μM at 4°C; see below). It has been shown (7) that the K_i and

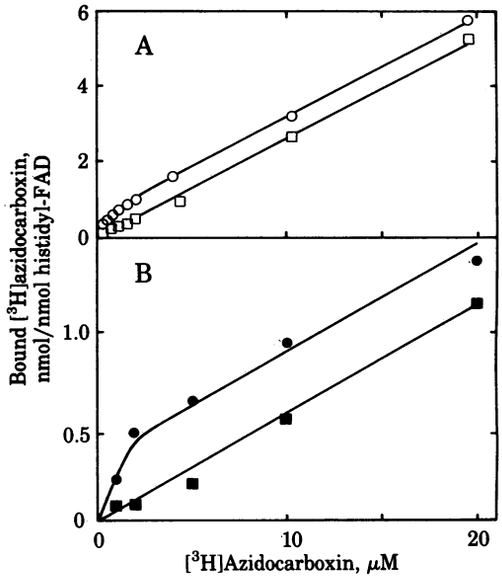

FIG. 1. Effect of TTF on equilibrium binding of [^{3}H]azidocarboxin to complex II and to the reconstituted complex. Equilibrium dialysis was performed. (*A*) Binding of [^{3}H]azidocarboxin to complex II; (*B*) binding to the complex reconstituted from soluble succinate dehydrogenase and the peptides (C_{II-3} and C_{II-4}). ○ and ●, No TTF; □ and ■, with 0.2 mM TTF.

K_d values for another carboxanilide, 2,4,5-trimethyl-3-carboxanilinofuran, determined in the same manner with complex II, are also in good agreement.

A Scatchard plot (not shown) of the data in Fig. 1*A* gave a dissociation constant of 1.2 μM, as already noted, and a binding titer of 0.6 mol of azidocarboxin specifically bound per mol of succinate dehydrogenase (determined as histidylflavin). This is about the same stoichiometry as we have found for the binding of 2,4,5-trimethyl-3-carboxanilinofuran to complex II earlier (0.5 mol/mol) and has been repeatedly confirmed in the present work with different preparations of complex II. Possible reasons for this unusual finding are discussed later.

Resolution of complex II into its components [i.e., succinate dehydrogenase and peptides C_{II-3} and C_{II-4} (8)] destroyed the specific binding site for azidocarboxin (Table 1). When the pure enzyme was recombined with the peptides with full reconstitution of coenzyme Q-reductase activity, specific binding was restored (Fig. 1*B*) with about the same stoichiometry as in untreated complex II (Table 1) and a K_d of 1.2 μM at 4°C. These

Table 1. Specific and nonspecific binding of azidocarboxin to complex II and its components

Sample	Specific binding,* mol/mol flavin	Nonspecific binding,† mol/mol flavin
Complex II	0.6 + 0.10	12.8
Reconstituted system	0.5 + 0.12	2.7
Binding peptides‡	0	1.6
Succinate dehydrogenase	0	0.06

The data come from the equilibrium dialysis experiments described in Fig. 1.

* Specific binding is calculated from the intercept of the Scatchard plot ± the standard error of the estimate.

† In the presence of 50 μM [^{3}H]azidocarboxin.

‡ The amount of binding peptides used was the same as in the reconstituted system. The molar concentration is assumed to be the same.

observations suggest that the reconstituted system possesses the same physical arrangement as complex II, at least as regards the binding site of TTF and carboxanilides.

Table 1 also documents the fact that complex II and the isolated binding peptides, but not the purified dehydrogenase, bind azidocarboxin nonspecifically. This type of binding to unresolved complex II is substantial (12–13 mol per mol of endogenous succinate dehydrogenase at 50 μM of the inhibitor), but is only about one-fifth as much in the reconstituted system despite the similarities in the peptide composition and phospholipid content of the two preparations (8). The lower value for nonspecific binding, also evident in the isolated peptide preparation, may be due to association of the hydrophobic azidocarboxin with deoxycholate micelles. Detergent was included with these samples to impart stability (8), but it was not present in the complex II sample.

Photoaffinity Labeling Studies. Photoactivation of azidocarboxin in pure solution by ultraviolet light under the conditions given in *Materials and Methods* occurred with $t_{1/2}$ = 1.1 min at 4°C, as monitored by the loss of the 245-nm absorbance of the inhibitor (Fig. 2). In the presence of complex II the photolysis is expected to be somewhat slower. Irradiation of complex II in the absence of inhibitor also caused loss of succinate-coenzyme Q activity ($t_{1/2}$ = 13 min under the same conditions; see Fig. 2). Hence, to keep photoinactivation of complex II at or below 10% and yet permit significant conversion of azidocarboxin to the nitrene, we limited the irradiation time to about 5 min.

Table 2 shows the results of a typical experiment in which complex II was irradiated in the presence of 50 μM azidocarboxin with and without TTF and then subjected to electrophoresis. In addition to radioactivity coinciding with protein bands (see ref. 8 for the peptide band pattern), there was a substantial band of radioactivity close to the dye front, where phospholipids

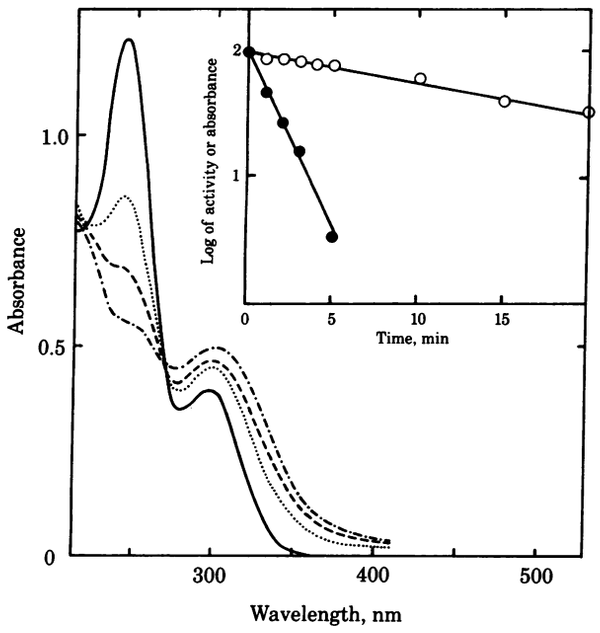

FIG. 2. Effect of ultraviolet light on azidocarboxin and complex II. An aqueous solution of azidocarboxin (54 μM) was irradiated as described for photoaffinity labeling studies, but in the absence of protein. Spectra were recorded in a Cary 19 spectrophotometer at 0 (——), 1 (· · · ·), 2 (– – –), and 3 (·····) min. (*Inset*) Semilogarithmic plot for decay of the percent remaining absorbance at 246 nm (●) or the percent remaining coenzyme Q-reductase activity of complex II irradiated under the same conditions in the absence of azidocarboxin (○).

Table 2. Covalent binding of azidocarboxin to the components of complex II separated by gel electrophoresis*

Component	Azidocarboxin (50 μM)	Azidocarboxin (50 μM) + TTF (2 mM)	Difference
70S subunit	0.26	0.16	0.10
30S subunit	0.13	0.03	0.10
C_{II-3} + C_{II-4}	0.91	0.51	0.40
phospholipids	2.97 (0.06)†	1.21 (0.02)†	1.76 (0.04)†

* Values are given as nmol of azidocarboxin bound per 250 μg of protein (≈1 nmol of histidylflavin).

† Values in parentheses are given per nmol of phospholipid, assuming 52 mol per mol of flavin (8).

migrate. The incorporation is expressed per nmol of complex II, based on the measured histidylflavin content. Peptides C_{II-3} and C_{II-4} are taken together because their separation was incomplete. The differences between the amounts of azidocarboxin bound in the absence and presence of TTF are brought about by two effects: TTF displaces carboxanilides selectively from the specific binding site (7) and, further, because TTF absorbs ultraviolet light, the total amount of azidocarboxin bound in samples containing TTF will be lower because of the lowered yield of nitrene.

When azidocarboxin alone was present, the binding to the large and small subunits of the enzyme and to phospholipid on a molar basis appeared insufficient to account for the irreversible loss of 93% of the catalytic activity in this experiment although, in view of the large molar excess of phospholipids in the preparation, the possibility that specific phospholipid molecules are involved in binding of the inhibitor at the inhibition site cannot be ruled out. After irradiation in the presence of azidocarboxin plus TTF, washing the treated complex II resulted in complete recovery of enzyme activity, indicating that the TTF had fully protected the inhibition site from being covalently bound by azidocarboxin. The decrease in radioactivity at the level of peptides C_{II-3} and C_{II-4} was equivalent to 0.4 mol of azidocarboxin, an amount that cannot be related to the observed 93% inhibition obtained after irradiation in the absence of TTF unless complete inhibition is achieved by only 0.5 mol of specifically bound azidocarboxin per mol of enzyme. Significantly, another carboxanilide, 2,4,5-trimethyl-3-carboxanilinofuran, is similarly bound at the specific site to the extent of 0.5 mol/mol of histidylflavin (7). Possibly, the fact that succinate dehydrogenase is dimeric, at least in solution, is relevant (17).

It is possible to distinguish specific from nonspecific binding without the use of TTF by measuring the dependence of azidocarboxin incorporation into each component on the azidocarboxin concentration. If only nonspecific binding occurred, the incorporation would be linearly dependent on azidocarboxin concentration, whereas for specific plus nonspecific binding a hyperbolic curve would be superimposed on the line. Fig. 3 shows that binding to the 70S and 30S subunits is linearly dependent on the azidocarboxin concentration, but that binding to the peptides and phospholipids is not. Thus, the components of complex II principally involved in binding azidocarboxin are the two low molecular weight peptides. Phospholipids also seem to form part of or to be adjacent to the binding site(s). The 70S and 30S subunits are not close enough to the site to be specifically labeled, despite the fact that they are necessary for the existence of that site.

We conclude from the covalent labeling pattern that the two small peptides of complex II, C_{II-3} and C_{II-4}, are closely involved in the binding of carboxanilides and of TTF at the inhibition

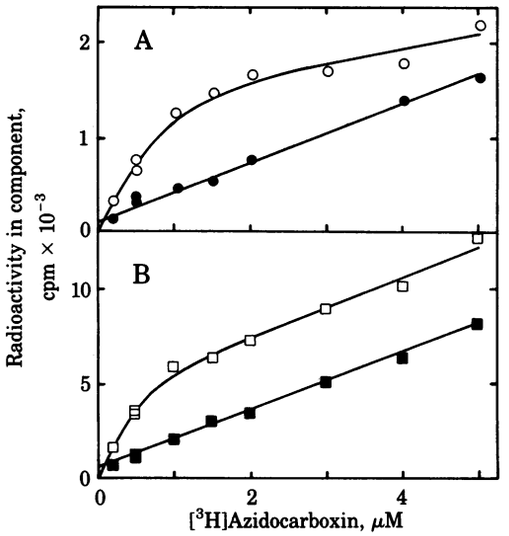

FIG. 3. Covalent binding of [^{3}H]azidocarboxin to components of complex II. Complex II was irradiated in the presence of 0.2–5 μM [^{3}H]azidocarboxin and washed, and its components were separated on 9% polyacrylamide gels. The gels were sliced, and the radioactivity in each protein band or near the dye front (phospholipids) was calculated. ■, 70S subunit of succinate dehydrogenase; ●, 30S subunit of succinate dehydrogenase; ○, peptides C_{II-3} + C_{II-4}; □, phospholipids.

site. Because the peptides do not bind azidocarboxin unless succinate dehydrogenase is also present (i.e., a catalytically competent succinate-coenzyme Q reductase unit is reconstituted), it appears that the combination of the enzyme with the two binding peptides alters the conformation of the latter in such a way that the binding site of carboxanilides and TTF, along with the appearance of coenzyme Q reductase activity, increased the turnover number and enhanced stability (8).

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1. White, G. A. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1212–1219.
2. Ulrich, J. T. & Mathre, D. E. (1972) *J. Bacteriol.* **110**, 628–632.
3. Tucker, A. N. & Lillich, T. T. (1974) *Antimicrob. Agents Chemother.* **6**, 572–578.
4. Mowery, P. C., Ackrell, B. A. C., Singer, T. P., White, G. A. & Thorn, G. D. (1976) *Biochem. Biophys. Res. Commun.* **71**, 354–361.
5. Ackrell, B. A. C., Kearney, E. B., Coles, C. J., Singer, T. P., Beinert, H., Wan, Y. P. & Folkers, K. (1977) *Arch. Biochem. Biophys.* **182**, 107–117.
6. Mowery, P. C., Steenkamp, D. J., Ackrell, B. A. C., Singer, T. P. & White, G. A. (1977) *Arch. Biochem. Biophys.* **178**, 495–506.
7. Coles, C. J., Singer, T. P., White, G. A. & Thorn, G. D. (1978) *J. Biol. Chem.* **253**, 5573–5578.
8. Ackrell, B. A. C., Ball, M. B. & Kearney, E. B. (1980) *J. Biol. Chem.* **255**, 2761–2769.
9. Baginsky, M. L. & Hatefi, Y. (1969) *J. Biol. Chem.* **244**, 5313–5319.
10. Ackrell, B. A. C., Kearney, E. B. & Coles, C. J. (1977) *J. Biol. Chem.* **252**, 6963–6965.
11. Singer, T. P., Salach, J., Hemmerich, P. & Ehrenberg, A. (1971) *Methods Enzymol.* **18B**, 417–427.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
13. White, G. A., Thorn, G. D. & Georgopoulos, S. G. (1978) *Pestic. Biochem. Physiol.* **9**, 165–182.
14. Dann, O. (1943) *Chem. Ber.* **76**, 419–430.
15. Galardy, R. E., Craig, L. C., Jamieson, J. D. & Printz, M. P. (1974) *J. Biol. Chem.* **249**, 3510–3518.
16. Swank, R. T. & Munkres, K. D. (1971) *Anal. Biochem.* **39**, 462–477.
17. Coles, C. J., Tisdale, H. D., Kenney, W. C. & Singer, T. P. (1972) *Physiol. Chem. Phys.* **4**, 301–316.