New Inhibitors of the Quinol Oxidation Sites of Bacterial Cytochromes *bo* and *bd*†

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ABSTRACT: A screen has been performed of possible inhibitors of the quinol oxidation sites of the two terminal oxidases of *Escherichia coli*, cytochromes *bo* and *bd*. Aurachin C and its analogues were found to be particularly effective inhibitors of both enzymes, whereas aurachin D and its analogues displayed a selectivity for inhibition of cytochrome *bd*. In addition, a tridecyl derivative of stigmatellin was found to inhibit cytochrome *bo* at concentrations which were without significant effect on cytochrome *bd*. Titration of membrane-bound cytochromes *bo* and *bd* with aurachin C gave an observed dissociation constant in the range of 10^{-8} M. A similar observed dissociation constant was determined for aurachin D inhibition of cytochrome *bd*. For both enzymes, their kinetic behavior during a series of substrate pulses indicates that it is reduction of the enzyme by quinol, and not reaction with oxygen, which is inhibited. It is concluded that the aurachins are powerful inhibitors of the quinol oxidation sites of bacterial cytochromes *bo* and *bd*. The effects of aurachin C on cytochrome *bo* were investigated in more detail. The number of inhibitor binding sites on the purified enzyme was determined by titration to be 0.6 per enzyme. At an inhibitor/oxidase ratio of 1.0, electron donation into the enzyme from added quinol is extremely slow, making it very unlikely that there is more than one quinone-reactive site. Aurachin C caused a potent inhibition of electron donation from a pulse of quinol. In contrast, it was without effect on cyanide or carbon monoxide binding to the reduced enzyme, on cyanide binding to the oxidized enzyme, on the optical spectra of the heme groups, or on the kinetics of oxygen reduction after photolysis of carbon monoxide from the reduced enzyme. We conclude that binding of aurachin C specifically inhibits the quinol oxidation site and does not directly affect the properties of the binuclear center.

Specific inhibitors have been useful as probes of structural and mechanistic aspects of a wide range of enzymes, including those of respiration and photosynthesis. Most useful are those which are specific and which bind tightly to their target site so that only stoichiometric amounts are required. In the case of respiratory and photosynthetic electron transfer chains, a wide range of synthetic and natural compounds have been described (von Jagow & Link, 1986; Link et al., 1993; Trebst, 1980). Many of these compounds are likely to interact at or close to the sites on proteins for binding of the natural quinones, ubiquinone and plastoquinone.

Cytochromes *bo* and *bd* are two terminal respiratory oxidases found in *Escherichia coli* and many other bacteria. Both enzymes catalyze the oxidation of ubiquinol by molecular oxygen to produce quinone and water (Minghetti & Gennis, 1988), although their proteins are structurally unrelated. Cytochrome *bd* is predominant when the oxygen concentration in the growth medium is low, whereas cytochrome *bo* predominates when the oxygen concentration is high (Cotter et al., 1990). Cytochrome *bo* contains five subunits encoded by the *cyo* operon (Au & Gennis, 1987) and is a member of a large superfamily of proton-pumping oxidases which includes eukaryotic cytochrome *c* oxidase. Subunit I is 40% identical to the subunit I of bovine cytochrome *c* oxidase and contains a low spin heme and a heme−copper binuclear center. A likely common core structure of these enzymes has been proposed, based on genetic and biophysical studies (Hosler et al., 1993; Calhoun et al., 1993; Brown et al., 1993). A possible model of the core structure of the unrelated cytochrome *bd*, which contains three heme groups but no copper, has also been proposed based upon similar types of studies (Fang et al., 1989; Ingledew et al., 1992).

Both of these bacterial enzymes oxidise ubiquinol, in contrast to the cytochrome *c* oxidases which oxidise cytochrome *c*. In the cytochrome *c* oxidases, subunit II has residues which have been implicated in the binding of cytochrome *c* (Capaldi, 1990) and contains an additional copper center (Stevens et al., 1982) which is likely to be the initial acceptor of electrons from cytochrome *c* (Hill, 1991; Nilsson, 1992). These features are not present in subunit II of cytochrome *bo*, even although there is extensive sequence homology overall with subunit II of cytochrome *c* oxidases. At present, little is known about the quinol oxidation site of either bacterial oxidase. For cytochrome *bo*, a semiquinone signal, attributed to the semiquinone form of the substrate bound in the quinol oxidation site, has recently been described (Salerno and Ingledew, in press). In the case of cytochrome *bd*, limited proteolysis experiments have indicated that the quinone binding site may be formed in part by its subunit I (Dueweke & Gennis, 1991). However, there is no clear global sequence motif for quinone binding sites

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in general, and the location of the quinol binding site(s) in both enzymes remains unclear. In the case of cytochrome *bo*, there has been a recent suggestion that there may be two independent quinone-reactive sites on the enzyme which can form a basis for a proton translocation mechanism similar to that in the *bc* complexes (Musser et al., 1993).

In this work, we have screened a range of possible quinone site antagonists for their inhibitory effect on cytochromes *bo* and *bd*, and a variety of inhibitors have been identified. Aurachin C and its derivatives are particularly effective on both enzymes, whereas aurachin D and derivatives act relatively specifically on cytochrome *bd*. Aurachins are natural products, extracted from the myxobacterium *Stigmatella aurantiaca* (Kunze et al., 1987). They and some synthetic analogues have been found to be potent inhibitors of complex I and complex III of the mitochondrial respiratory chain (Kunze et al., 1987; Oettmeier et al., 1994; Friedrich et al., 1994). They also affect photosystem II and the cytochrome *bf* complex of thylakoids, and the cytochrome bc_1 complex of *Rhodospirillum rubrum* (Oettmeier et al., 1990). We show here that these compounds are extremely potent inhibitors of the quinol oxidation sites in both cytochromes *bo* and *bd* and that binding is far tighter than that of any previously described inhibitors which are likely to affect the quinone binding site, such as HQNO,[1] piericidin A, and UHDBT (Kita et al., 1986; Matsushita et al., 1984).

MATERIALS AND METHODS

Cell Growth and Enzyme Preparation. Cytochrome *bo* was derived from strain RG145 of *Escherichia coli*. This strain overexpresses cytochrome *bo* and lacks cytochrome *bd*. Cytochrome *bd* was studied in membranes derived from strains GL101 or GL105 which lack cytochrome *bo* (Au & Gennis, 1987). Strains were the kind gift of Professor R. B. Gennis. Cells were grown at 30 °C in Luria broth supplemented with 50 mM phosphate buffer (pH 7.2) and 100 μg mL^{-1} ampicillin. Larger cultures (15 L) were vigorously aerated. Cells were harvested in late log phase, washed, and resuspended in 50 mM phosphate buffer (pH 7.2). The cells were disrupted and membranes isolated by centrifugation of the lysate at 15000*g* for 30 min. Membranes were washed with urea and cholate as described by Matsushita et al. (1986). Cytochrome *bo* was solubilized using 1% octyl β-D-thioglucopyranoside and 1% Triton X-100 and purified on DEAE-Sepharose CL-6B essentially as described in Moody et al. (1993a).

Quinol Oxidase and NADH−Ubiquinone Oxidoreductase Activities. Quinol oxidase activities were measured from oxygen consumption rates using a Clark-type oxygen electrode. Membranes were resuspended in 2.5 mL of a medium at 23 °C containing 50 mM potassium phosphate and 0.5 mM EDTA at pH 7.5 and 0.05% (w/v) lauryl maltoside. The reaction was initiated by addition of 1.5 mM duroquinol. Purified cytochrome *bo* was assayed in the same manner, but in a buffer of 50 mM potassium phosphate and 2 mM EDTA at pH 7.0 and 23 °C.

NADH−ubiquinone oxidoreductase activity was measured in RG145 membranes resuspended to a final cytochrome *bo* concentration of 30 nM in a buffer of 50 mM potassium phosphate and 2 mM EDTA at pH 7.0 and 23 °C and with 1 mM neutralized potassium cyanide added to inhibit the oxidase. 100 μM NADH was added, and the reaction was started by addition of 40 μM decylubiquinone. Reduction of the quinone to quinol was monitored with the wavelength pair 280−295 nm.

Spectrophotometric and Kinetic Measurements. Difference spectra and dual wavelength transient kinetics were monitored in the same sample with a single beam instrument built in-house. Cyanide and carbon monoxide were photolyzed from the fully reduced, liganded forms of purified cytochrome *bo* with a frequency-doubled Nd/YAG laser (Spectron Physics) which produced a 10 ns flash at 532 nm with an energy in excess of 100 mJ/pulse. Photolysis and the subsequent kinetics of rebinding were monitored at 416−430 nm for carbon monoxide (Brown et al., 1994) or at 424−434 nm for cyanide (Mitchell & Rich, 1994).

The fast kinetics of reaction of fully reduced cytochrome *bo* with oxygen at 23 °C were monitored essentially by the flow-flash method which has been applied originally to cytochrome oxidase (Gibson & Greenwood, 1963) and, more recently, to cytochrome *bo* (Svensson & Nilsson, 1993). Purified cytochrome *bo* was dissolved to 0.8 μM in 0.6 mL of 50 mM potassium phosphate and 2 mM EDTA at pH 7.0; then 10 mM sodium ascorbate and 1 μM phenazine methosulfate were added. The sample was gassed with carbon monoxide, placed in a sealed cuvette, and left at room temperature for several minutes until the fully reduced, carbon monoxide-ligated form had been generated quantitatively. This was confirmed by monitoring the spectrum of the enzyme. Then, 0.15 mL of air-saturated buffer was injected into the solution, and reaction with oxygen was initiated by laser flash photolysis of the carbon monoxide compound, as above. Kinetics of oxidation were followed at 422 nm since a large change occurs at this wavelength as the enzyme changes from the fully reduced, CO-ligated form to the final product (assumed to be an oxyferryl species (Moody et al., 1993b; Cheesman et al., 1994)).

Steady state turnover behavior of cytochromes on slow time scales (Figure 2) was monitored by a triple-wavelength procedure that minimized sloping base-line changes. In this method, a sample wavelength and two reference wavelengths on either side of it are monitored cyclically. The difference between the sample wavelength and the (weighted) average of the two references provides a monitor of cytochrome changes that is largely unaffected by changes of base-line slope.

Chemicals. A preliminary report of the synthesis of the quinolones and their 1-hydroxy derivatives appeared in Reil et al. (1994). All inhibitors were dissolved in ethanol and quantitated gravimetrically. Duroquinol was prepared from the quinone as previously described (Rich, 1981). Stock solutions of up to 250 mM were made in dimethyl sulfoxide to which 10 mM HCl had been added.

RESULTS

Screening of Possible Inhibitors of Cytochromes bo and bd. In order to identify possible inhibitors, a wide range of compounds which are known to affect quinone sites in other

[1] Abbreviations: K_d, dissociation constant; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; duroquinol, 2,3,5,6-tetramethyl-*p*-benzohydroquinone; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; MOA-stilbene, (*E*)-β-methoxyacrylate−stilbene or (*E*,*E*)-methyl 3-methoxy-2-(styrylphenyl)propenoate.

Table 1: Inhibitory Effects of a Range of Compounds on the Quinol Oxidase Activities of Cytochromes *bo* and *bd*[a]

compound	final concn	cytochrome *bd* (% inhibn)	cytochrome *bo* (% inhibn)
potassium cyanide	10 mM	94	100
sodium azide	4.2 mM	0	40
hydroxylamine	3 mM	0	90
piericidin A	100 μM	70	80
heptylhydroxyquinoline *N*-oxide (HQNO)	50 μM	90	100
nonylhydroxyquinoline N-oxide (NQNO)	1.1 μM	37	82
(1,5-dimethylhexyl)-quinazolinamide[b]	100 μM	88	23
(1-methyldecyl)quinazolinamide[b]	100 μM	85	24
MOA-stilbene[c]	100 μM	13	23
antimycin A	50 μM	80	18
valinomycin	100 μM	0	0
rotenone	5 μM	0	0
tridecylstigmatellin[c]	1 μM	0	73
stigmatellin	200 μM	14	94
nigericin	100 μM	44	35
DNP-INT	10 μM	0	0
benzylhydroxamic acid (BHAM)	100 μM	0	0
salicylhydroxamic acid (SHAM)	200 μM	0	0
dibromothymoquinone (DBMIB)	100 μM	38	82
myxothiazol	125 μM	0	24
mucidin (strobilurin A)	44 μM	0	0
funiculosin	60 μM	10	0
undecylhydroxydioxobenzo-thiazole (UHDBT)[d]	2 μM	0	52
	20 μM	18	97
aurachin A	700 μM	27	56
aurachin C	214 nM	90	90
aurachin D	400 nM	93	5
	5 μM	98	57

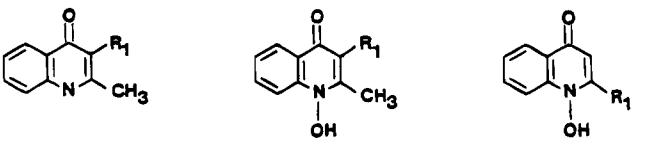
[a] Inhibition of oxygen consumption rates in the presence of 1.5 mM duroquinol was determined as described in Materials and Methods. Membranes prepared from strains GL101 or GL105 were used for cytochrome *bd* experiments; membranes from RG145 were used for cytochrome *bo*. The final enzyme concentration was 100 nM in all cases. Compounds as indicated are the the kind gifts of [b]Prof. R. M. Hollingworth, MI; [c]T. Wiggins, formerly of Zeneca Agrochemicals, U.K.; [d]Prof. B. Trumpower, Hanover, NH.

systems were first screened at high concentrations. Some inhibitors which are likely to act at the binuclear center were included for comparison (Table 1). A number of new inhibitors were identified. Of particular interest was the marked inhibition of both oxidases with aurachin C. In addition, it appeared that aurachin D had a potent inhibitory effect on cytochrome *bd* at a concentration which had little effect on cytochrome *bo*. A related compound, aurachin A, was far less effective on either oxidase. A further compound of interest was tridecylstigmatellin, which at 1 μM concentration had a significant inhibitory effect on cytochrome *bo* without affecting cytochrome *bd*.

Since aurachins C and D showed powerful inhibitory effects, a range of synthetic analogues based upon the aurachin structures were tested both for potency and for specificity (Table 2). Compounds related to the structure of aurachin C (type II and type III derivatives) were found to be potent inhibitors of both oxidases. In the case of derivatives related to the aurachin D structure (type I compounds in Table 2), specificity was again observed for inhibition of cytochrome *bd*. Little systematic variation of inhibitor potency was observed as the chain length of the R group increased from C_8 through to C_{12}.

The potencies of aurachin C, aurachin D, and decylaurachin D were examined in more detail by titration of the quinol oxidase activities in membrane samples (Figure 1A).

Table 2: Inhibitory Effects of a Range of Synthetic Analogues of Aurachin C and Aurachin D on the Quinol Oxidase Activities of Cytochromes *bo* and *bd*[a]



I II III

compd type	R_1 group	cytochrome *bd* ($\log_{10} I_{50}$)	cytochrome *bo* ($\log_{10} I_{50}$)
I	$(CH_2)_7CH_3$	7.7	4.7
I	$(CH_2)_8CH_3$	7.7	5.0
I	$(CH_2)_9CH_3$	7.5	5.0
I	$(CH_2)_{10}CH_3$	7.4	4.9
I	$(CH_2)_{11}CH_3$	7.8	4.6
II	$(CH_2)_7CH_3$	7.3	7.2
II	$(CH_2)_9CH_3$	7.4	7.3
II	$(CH_2)_{11}CH_3$	7.5	7.6
III	$(CH_2)_7CH_3$	6.8	6.8
III	$(CH_2)_9CH_3$	7.0	7.6
III	$(CH_2)_{10}CH_3$	6.8	7.2
III	$(CH_2)_{11}CH_3$	6.8	7.1

[a] Inhibition of oxygen consumption was determined as in Table 1. In this case, final concentrations of enzymes in the resuspended membrane samples were 30 nM. Inhibition is expressed as the negative $\log_{10}$ of the concentration in molarity required for half-maximal inhibition.

Both enzymes were potently inhibited with an inhibition of 50% at a titer of around 0.5 molecule of inhibitor/enzyme. Simulation of these data indicated observed dissociation constants of around 10 nM in both cases. For aurachin D and its decyl derivative, potent inhibition of cytochrome *bd* was observed at concentrations which had little or no effect on cytochrome *bo* (Figure 1A), in agreement with the data in Tables 1 and 2. Observed binding constants for inhibition of cytochrome *bd* by aurachin D and its decyl derivative were also close to 10 nM.

The quinol oxidase activity of purified cytochrome *bo* was also titrated with aurachin C (Figure 1B), and a curve was simulated using an equation for a tightly binding inhibitor (Rich et al., 1991) and fitted to the data by linear regression analysis. A best fit was obtained with a curve based on a stoichiometry of 0.6 molecule of inhibitor/enzyme and an observed dissociation constant, K_d, for aurachin C of 7 nM. This is substantially tighter binding than that reported for other inhibitors such a HQNO (K_d of 2 μM and 7 μM for *bo* and *bd*, respectively (Kita et al., 1986)), piericidin A (K_d of 2 μM and 15 μM for *bo* and *bd*, respectively (Kita et al., 1986)), or UHDBT (K_d of 0.3 μM for *bo* (Matsushita et al., 1984)).

Membrane samples were also titrated with tridecylstigmatellin (Figure 1C). This confirmed that the compound was relatively selective for cytochrome *bo*, since cytochrome *bo* was inhibited 50% by a concentration of 0.5−1 μM whereas cytochrome *bd* was inhibited by less than 5% with 10 μM tridecylstigmatellin.

Effects of inhibitors on the NADH−ubiquinone oxidoreductase activity of membrane samples derived from the RG145 strain of *E. coli* were also examined. Neither aurachin C, decylaurachin D, nor tridecylstigmatellin inhibited this reaction at concentrations which were maximally effective on the oxidase reactions, indicating that their effects may be specific for the oxidases in whole cells.

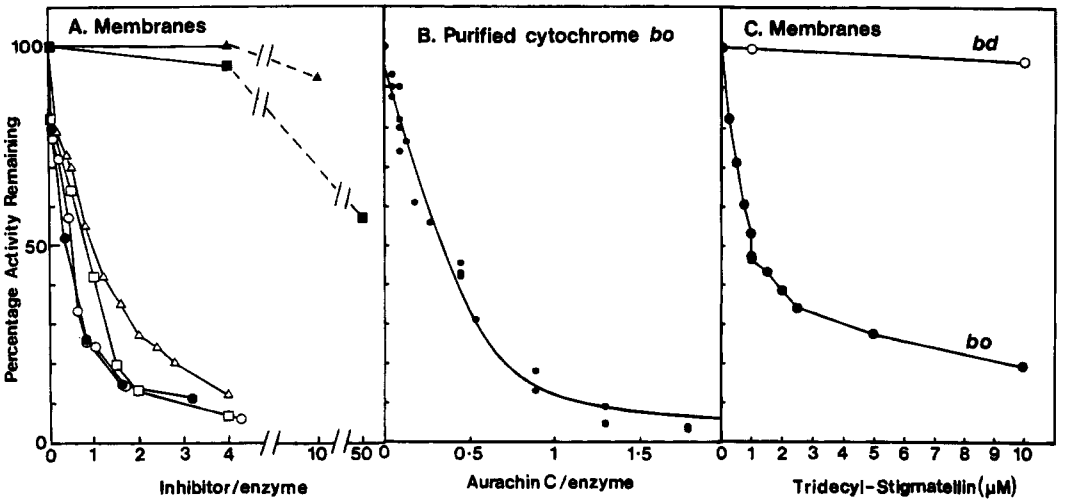


FIGURE 1: Titration of quinol oxidase activities of cytochromes *bo* and *bd* with aurachins. The rates of oxygen consumption were measured in the presence of various concentrations of aurachin C with 1.5 mM duroquinol as substrate. In (A) and (C), membranes were resuspended to a final oxidase concentration of 100 nM. Control turnover numbers were 140 and 225 electrons s^{-1} for cytochromes *bo* and *bd*, respectively. In (A), symbols represent the following: aurachin C titration of *bo* (●) or *bd* (○) membranes; aurachin D titration of *bo* (■) or *bd* (□) membranes; decylaurachin D titration of *bo* (▲) or *bd* (△) membranes. In (C), symbols represent tridecylstigmatellin titration of *bo* (●) or *bd* (○) membranes. In (B), purified cytochrome *bo* was dissolved to 120 nM and control turnover number was 60 electrons·s^{-1}. The solid line is a simulation for a tight binding inhibitor. It was best fitted to the data by least-squares analysis, without constraints on maximum and minimum rates, and gave values of 0.6 inhibitor/enzyme and a dissociation constant of 7 nM (see text).

The Site of Inhibition of Cytochromes bo and bd by Aurachin C. In order to investigate the locus of inhibition of aurachin C on cytochromes *bo* and *bd*, a protocol was established in which its effects on the major partial reactions of the catalytic cycle could be determined (Figure 2). This was achieved by continuous monitoring of optical changes in the Soret region during a series of substrate pulses. In the first instance, a small pulse of quinol was added to the aerobic enzyme (point A in Figure 2). This caused a very fast attainment of a steady state condition, presumably composed in both cases of a mixture of the oxygen intermediates and a small amount of reduced heme. The spectral changes associated with this transition to the aerobic steady state were much larger in cytochrome *bo* than in cytochrome *bd* (compare change induced at point A in Figure 2, panels A and B). At these enzyme concentrations, the quinol was consumed in less than 1 min, after which time the sample relaxed back toward the base-line condition (in the case of cytochrome *bo*, the oxyferryl intermediate is very stable (Moody et al., 1993b; Moody & Rich, 1994) so that the trace does not relax fully back to the base line in this time). A second larger quinol pulse, sufficient to cause reduction of all of the oxygen, was then given at point B. This again rapidly established the same steady state transiently until the oxygen in the medium was consumed, at which time the heme groups became reduced in both enzymes. When the anaerobic reduction level had stabilized, oxygen was rapidly mixed into the buffer (point C). This caused a reoxidation back to the steady state within the 1−2 s mixing time of the experiment, which was rapidly followed by heme re-reduction as the system again became anaerobic. Aurachin C was then added to the same anaerobic samples (point D). The level was kept fairly low (0.7 μM) so that some slow residual turnover could still occur to cause anaerobiosis. Since oxygen was added with the inhibitor, the enzymes became transiently oxidized and again reverted to the fully reduced state as the oxygen was consumed. This oxygen depletion took much longer than in the control because of the inhibition of turnover. A second oxygen pulse (point E) resulted in fast reoxidation to the steady state, followed again by heme re-reduction on anaerobiosis.

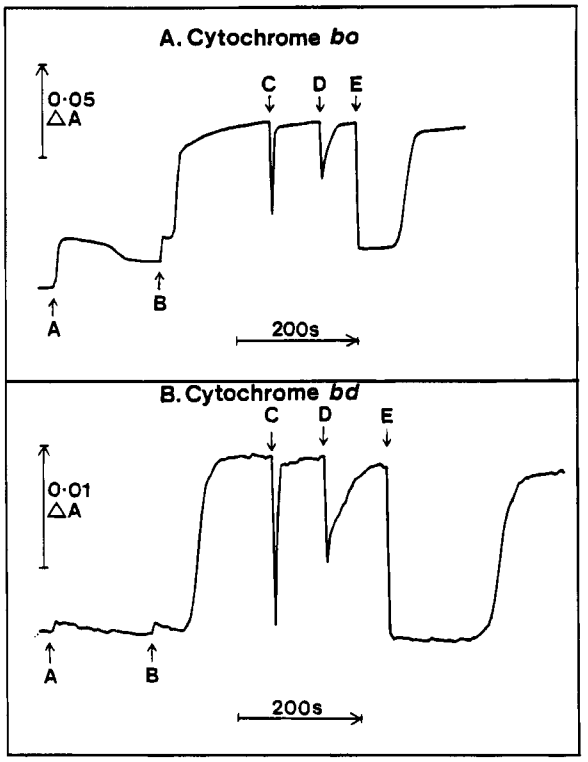


FIGURE 2: Effects of Aurachin C on the steady state turnover behavior of cytochromes *bo* and *bd*. Steady state changes of the oxidases were monitored optically as described in the Materials and Methods. The cuvette contained 0.25 μM purified cytochrome *bo* (A) or 0.12 μM cytochrome *bd* membranes (B) in a medium of 50 mM potassium phosphate and 2 mM EDTA at pH 7.0 and containing 0.05% lauroylsarcosine. Generation of turnover intermediates and heme reduction were monitored by a triple wavelength procedure at 426 *minus* (408 + 444)/2 nm for cytochrome *bo* (top trace) or at 432 *minus* (412 + 452)/2 nm for cytochrome *bd* (bottom trace). The following additions were made at the points indicated: A, 200 μM duroquinol; B, 1 mM duroquinol; C, oxygen; D, 0.7 μM aurachin C (plus a small amount of oxygen); E, oxygen.

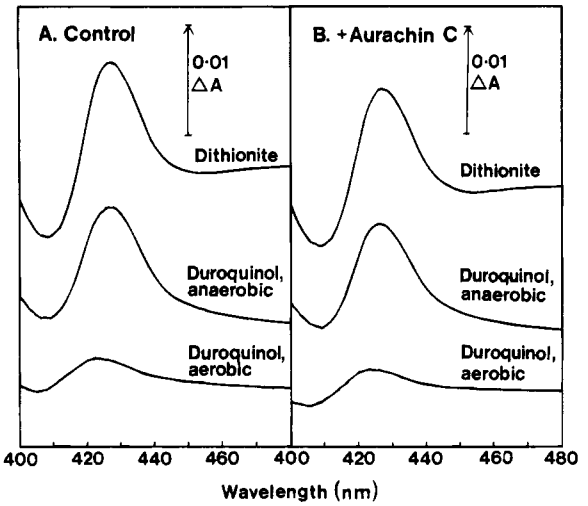


FIGURE 3: Steady state optical difference spectra of cytochrome *bo* during a series of substrate pulses in the absence (A) or in the presence (B) of aurachin C. An experiment similar to that of Figure 2A was performed, but with 0.9 μM cytochrome *bo*, and spectra were recorded as follows: (i) immediately after the addition of 200 μM duroquinol in the presence of oxygen, i.e., in the aerobic steady state; (ii) in the presence of 1 mM duroquinol and after anaerobiosis; (iii) after dithionite addition. In (B), the same spectra were recorded in the presence of 0.9 μM aurachin C, and in this case care was taken that sufficient time was allowed for a stable steady state to develop. All spectra are difference spectra *versus* an aerobic sample in the absence of quinol substrate.

The extra time taken to reach anaerobiosis after the oxygen pulse at point E clearly shows for both enzymes that their quinol oxidase activities are severely inhibited. However, reaction of the binuclear center of either enzyme with oxygen is unaffected by aurachin C, as judged by the lack of inhibition of oxidation kinetics at point E. Hence, the donation of electrons into the enzyme from quinol substrate must instead be inhibited in both cases.

Further Examination of the Steady State Behavior of Cytochrome bo and the Effect of Aurachin C. In order to provide a fuller description of the steady state changes being observed in the data obtained with cytochrome *bo* in Figure 2A, the full spectra of the optical changes which were being monitored are shown in Figure 3. In the aerobic steady state with quinol (immediately following point A in Figure 2A), the spectrum has a peak and trough at around 424 and 406 nm, respectively. This is consistent with the presence of the oxyferryl ($F^{\bullet}$) oxygen intermediate which is formed by the donation of two electrons from quinol substrate followed by reaction with oxygen to produce an $Fe^{4+}-O^{2-}$ species (which has a peak and trough at 420 and 401 nm, respectively) (Moody et al., 1993b; Cheesman et al., 1994; Watmough et al., 1994; Moody & Rich, 1994), together with a very small contribution from reduced heme *b* (with a peak and trough at 427 and 408 nm). On depletion of quinol, the heme rapidly reoxidizes, but the $F^{\bullet}$ intermediate decays only very slowly (Moody et al., 1993b), explaining why full decay back to the base line in Figure 2A is not observed. On anaerobiosis in the presence of excess duroquinol, a spectrum consistent with reduction of more than 95% of both hemes is observed, with no P/F form contribution. Addition of dithionite (top trace in Figure 3A) causes little additional heme reduction but does remove a base-line slope by reducing duroquinone back to duroquinol (compare top and middle traces in Figure 3A).

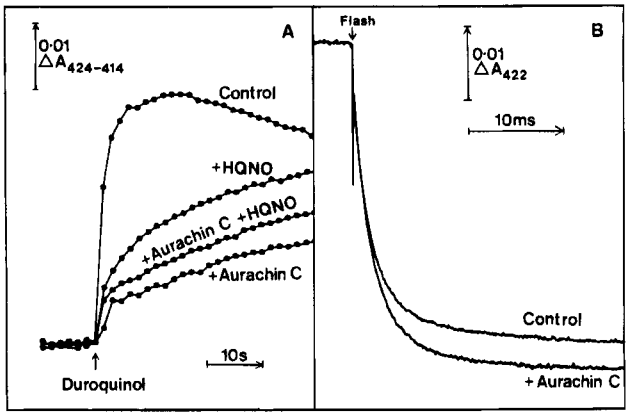


FIGURE 4: Time resolution of the effects of aurachin C on the reduction of cytochrome *bo* by duroquinol and its oxidation by molecular oxygen. In (A), the rate of approach to the steady state after addition of 200 μM duroquinol in the presence of oxygen was monitored at 424−414 nm. The cuvette contained 0.9 μM cytochrome *bo* in 50 mM potassium phosphate and 2 mM EDTA at pH 7.0 and with 200 units/mL catalase and 50 units/mL superoxide dismutase also added to remove any traces of peroxide or superoxide which might be present in the quinol solution. The control was repeated in the presence of 50 μM HQNO, 8.6 μM aurachin C, or both. In (B), the rate of oxidation of reduced cytochrome *bo* was monitored at 422 nm, after photolysis of carbon monoxide from a reduced sample in the presence of oxygen (see Materials and Methods). The cuvette contained 0.8 μM cytochrome *bo* in 50 mM potassium phosphate and 2 mM EDTA at pH 7.0 and with 0.05% (w/v) lauroylsarcosine also present. 10 mM sodium ascorbate and 1 μM phenazine methosulfate were added, and the sample was gassed with carbon monoxide for 3 min and then sealed. After incubation for sufficient time for the reduced-carbon monoxide compound to fully develop, 0.15 mL of air-saturated buffer was rapidly mixed into the sample. Carbon monoxide was laser photolyzed within 1 s of oxygen addition, and kinetics of the oxygen reaction were monitored at 422 nm. The control was repeated in the presence of 1.8 μM aurachin C.

The presence of aurachin C had no effect on the sizes of positions of these spectra (Figure 3B), provided that sufficient time was allowed for the steady state to fully develop (see Figure 4). This shows that, once the electrons have entered the enzyme from quinol, the oxygen intermediates can still form in the normal manner in the binuclear center in the presence of inhibitor.

Essentially the same results were obtained with HQNO, although in this case much higher concentrations were required for the same magnitude of effect (data not shown).

Specificity of the Effects of Aurachin C on the Quinol Oxidation Site in Cytochrome bo. In order to further time resolve the inhibitory effects of aurachin C on the quinol oxidation and the oxygen reduction reactions of cytochrome *bo*, experiments similar to those in Figure 2 were performed, but on faster time scales. The effects of aurachin C and HQNO on the kinetics of the approach to steady state induced by 200 μM duroquinol are shown in Figure 4A. The approach to the steady state is dramatically inhibited by aurachin C and is also inhibited to a lesser extent by higher concentrations of HQNO. The half-time of reduction in the control was less than 1 s. In the presence of the inhibitors, the half-time of reduction was more than 10 s with HQNO and even longer with aurachin C. Similar effects were observed when decylubiquinol was used in place of duroquinol (data not shown). As already described above (Figure 3), the final stable spectrum of the aerobic steady state is the same in the absence and the presence of aurachin C,

indicating that the same mixture of intermediates occurs even though the attainment of this mixture takes much longer when inhibitor is present.

The kinetics of oxidation of reduced cytochrome *bo* were also resolved in the millisecond time range by monitoring optical changes at 422 nm after laser photolysis of carbon monoxide from a reduced carbon monoxide-ligated sample in the presence of oxygen (Figure 4B). In the control sample, heme oxidation was observed with a half-time of 1.4 ms, and this rate was the same in the presence of aurachin C.

A range of other tests were performed with cytochrome *bo* to establish whether regions of the enzyme other than the quinol oxidation site were affected by the inhibitor. In the first instance, spectra of the enzyme were taken in the absence and presence of 8.5 μM aurachin C in order to determine whether any inhibitor-induced spectral shifts occurred, as is the case in some other systems with other quinone-site inhibitors (Slater, 1973; Ohnishi et al., 1988; Kamensky et al., 1985). In the (reduced *plus* inhibitor) *minus* (reduced) difference spectrum, the amplitude of the signal obtained in the Soret region was less than 1% of the amplitude of the reduced *minus* oxidized difference spectrum. Since the magnitude of such changes in other systems can be as high as 7−8%, we take this to indicate that no significant spectral shift is occurring in reduced cytochrome *bo*. No significant inhibitor-induced spectral shifts were observed in the oxidized form of the enzyme either. We also failed to observe any significant spectral shifts when 20 μM tridecylstigmatellin or 20 μM HQNO was used instead of aurachin C.

The effects of aurachin C were determined on the rates of recombination of the reduced binuclear center of cytochrome *bo* with carbon monoxide (Brown et al., 1994) and with cyanide (Mitchell & Rich, 1994) after laser photolysis of the ligand from the reduced enzyme. In no case was there any indication of a significant change of observed rate constant of rebinding, binding constant, spectral change, or photolysis yield compared to the control behavior. Similarly, HQNO was also tested with carbon monoxide recombination and also failed to elicit any change.

Finally, the effect of aurachin C on the binding of cyanide to oxidized cytochrome *bo* was tested by monitoring formation of the cyanide compound at 426−406 nm on addition of 10 mM cyanide. We used a heterogeneous control oxidized enzyme (Moody et al., 1993a) which had biphasic cyanide binding kinetics, with observed rate constants of cyanide binding of 0.09 (60%) and 0.005 (40%) s^{-1} at pH 7 and 10 mM cyanide. Neither the rates, the extents, nor the spectrum of this change was altered when the experiment was repeated in the presence of 2 μM aurachin C.

DISCUSSION

A variety of inhibitors of bacterial cytochromes *bo* and *bd* have been described previously (Kita et al., 1986; Matsushita et al., 1984). Of these, cyanide, hydroxylamine, and azide are likely to act at the sites which carry out the oxygen reduction chemistry, whereas HQNO, piericidin A, and UHDBT are more likely to act on the quinol oxidation sites, since they are known to act as quinone antagonists in other systems (Roberts et al., 1978; von Jagow & Link, 1986). None of these inhibitors have such powerful actions, however, as those observed here with aurachins C and D. Derivatives of these compounds, in which the natural polyisoprenyl side chain is replaced by a simpler saturated aliphatic side chain, are also highly effective (Table 2), showing that the ring structure itself is the critical part of the structure. It would seem most likely that carbonyl and N or NOH moieties are placed at a distance similar to the hydroxyl group separation of the natural quinol substrates. Hence they could utilize the hydrogen bonding network which would normally promote quinol binding, so that they would then act as competitive inhibitors of the substrate quinol. The finding that the apparent binding constant is little changed with increasing chain length (and therefore with increasing lipid/water partition coefficient) is likely to arise because even with the shortest chain length of C_8 the molecules are likely to be almost entirely partitioned into the membrane phase. In any case, the observed I_{50} values in many cases are approaching the value of the enzyme concentration of 30 nM, so that a more careful study (such as in Figure 1A) would have to be performed in order to determine true dissociation constants. Specificity of the aurachin D type of cytochrome *bd* is intriguing. The shorter separation of the hydrogen bonding points must preclude tight binding to cytochrome *bo* but must still be capable of being accommodated in the *bd* quinol oxidation site. In the case of tridecylstigmatellin, the oxidase specificity is less surprising since this molecule is a more complex structure and recognition of surrounding protein structure seems likely, at least in part, to contribute to overall binding propensity.

Examination of the effects of aurachin C on the partial reactions of cytochromes *bo* and *bd* (Figures 2 and 4) shows clearly that this compound is acting by inhibiting their quinol oxidation sites. In the case of cytochrome *bo*, no secondary effects on other parts of the enzyme could be discerned (this is likely to be the case also with cytochrome *bd*, although this was not tested so thoroughly). This includes a lack of perturbation of the optical spectra of either heme, and a lack of effect on the ligand binding properties or oxygen reactivity of the binuclear center. Direct binding to the quinol oxidation site is reasonable in view of the similarity of the aurachin structure to that of the quinol substrates, as noted above, and in view of the fact that it is known to act at quinone sites in other quinone-utilizing enzymes (Oettmeier et al., 1990).

The lack of effect of aurachin C on the mixture of intermediates at steady state in the presence of quinol and oxygen (Figures 2 and 3) further confirms that the binuclear center itself is operating normally once it has received electrons. Since we have shown that formation of the oxyferryl intermediate requires the uptake of two protons from the medium (Moody & Rich, 1994), it can be further concluded that the proton transfer pathway into the binuclear center is not inhibited by this compound. It might also be pointed out that our data show for the first time that the route of entry of the binuclear center ligands carbon monoxide and cyanide cannot be through the quinol binding site since their rebinding kinetics are unaffected by the binding of inhibitor; this point concerning the flight path of ligands through the protein structure is one of increasing relevance to models of the chemistry of coupling of electron and proton transfer in these enzymes (Rich, 1994).

HQNO has already been reported to inhibit cytochrome *bo* at higher concentrations (Kita et al., 1986), and its site of action has been investigated. It was suggested that its

action was primarily on the site of oxidation of the enzyme by molecular oxygen (Withers & Bragg, 1989). However, our own results show that it is acting in a manner similar to aurachin C on the quinol oxidation site and has no effect on the binuclear center. In any case, an inhibitory effect on the quinol oxidation site is expected since HQNO affects the quinone binding sites of many other quinone-utilizing enzymes, including mitochondrial bc_1 (von Jagow & Link, 1986) and chloroplast *bf* complexes (Jones & Whitmarsh, 1988; Rich et al., 1991). The discrepancy in results might arise from the high concentrations of HQNO which were used (Withers & Bragg, 1989), which could induce additional, nonspecific secondary effects in some instances. The claim of a spectral shift of heme groups induced by HQNO (Withers & Bragg, 1989), which we did not observe, is probably attributable to the different rates of attainment of steady state conditions in the control and inhibited states.

Binding of aurachin C is sufficiently tight to allow titration of the number of quinone binding sites. For purified enzyme, the data indicate a titer of 0.6 aurachin C binding site/cytochrome *bo* monomer. This substoichiometric titer probably results at least in part from a small error in the quantitation of enzyme. For this, we used an extinction coefficient of 20.5 mM^{-1} cm^{-1} at 560−580 nm for the reduced *minus* oxidized enzyme (Minghetti et al., 1992). It is likely from our data (unpublished) that this extinction coefficient should be adjusted upward by 25% so that the inhibition titer would be closer to a value of 1 inhibitor site/enzyme. Such a value of extinction coefficient would be in accord with more model protoheme compounds. Another trivial reason for part of the substoichiometric titer might arise from a fraction of the purified enzyme being unable to bind the inhibitor. At a nominal titer of 1 aurachin C/enzyme, we find that electron donation from quinol into the enzyme is extremely slow. These results strongly indicate that there is not more than one quinol-reactive site per enzyme and argue against a recent model in which two independent quinone-reactive sites per enzyme were proposed (Musser et al., 1993).

Since aurachin D and its derivatives are relatively specific for cytochrome *bd* whereas tridecylstigmatellin is relatively specific for cytochrome *bo*, the use of a combination of inhibitors will be particularly useful in determining the flux rate through the two enzymes when both are present in the same membranes. In addition, the identification of such potent inhibitors for the quinol sites opens up the possibility of the generation of random inhibitor-resistant mutants, a technique which has been used particularly effectively in the characterization of the quinone-reactive sites in other systems, especially photosystem II and the *bc* complexes (Trebst, 1991; Gennis et al., 1993; Colson, 1993; Robertson et al., 1990; Howell et al., 1987).

REFERENCES

Au, D. C.-T., & Gennis, R. B. (1987) *J. Bacteriol. 169*, 3237−3242.

Brown, S., Moody, A. J., Mitchell, R., & Rich, P. R. (1993) *FEBS Lett. 316*, 216−223.

Brown, S., Rumbley, J. N., Moody, A. J., Thomas, J. W., Gennis, R. B., & Rich, P. R. (1994) *Biochim. Biophys. Acta 1183*, 521−532.

Calhoun, M. W., Thomas, J. W., Hill, J. J., Hosler, J. P., Shapleigh, J. P., Tecklenburg, M. M. J., Ferguson-Miller, S., Babcock, G. T., Alben, J. O., & Gennis, R. B. (1993) *Biochemistry 32*, 10905−10911.

Capaldi, R. A. (1990) *Annu. Rev. Biochem. 59*, 569−596.

Cheesman, M. R., Watmough, N. J., Gennis, R. B., Greenwood, C., & Thomson, A. J. (1994) *Eur. J. Biochem. 219*, 595−602.

Colson, A.-M. (1993) *J. Bioenerg. Biomembr. 25*, 211−220.

Cotter, P. A., Chepuri, V., Gennis, R. B., & Gunsalus, R. P. (1990) *J. Bacteriol. 172*, 6333−6338.

Dueweke, T. J., & Gennis, R. B. (1991) *Biochemistry 30*, 3401−3406.

Fang, H., Lin, R.-J., & Gennis, R. B. (1989) *J. Biol. Chem. 264*, 8026−8032.

Friedrich, T., Ohnishi, T., Forche, E., Kunze, B., Jansen, R., Trowitzsch, W., Höfle, G., Reichenbach, H., & Weiss, H. (1994) *Biochem. Soc. Trans. 22*, 226−230.

Gennis, R. B., Barquera, B., Hacker, B., Van Doren, S. R., Arnaud, S., Crofts, A. R., Davidson, E., Gray, K. A., & Daldal, F. (1993) *J. Bioenerg. Biomembr. 25*, 195−209.

Gibson, Q. H., & Greenwood, C. (1963) *Biochem. J. 86*, 541−554.

Hill, B. C. (1991) *J. Biol. Chem. 266*, 2219−2226.

Hosler, J. P., Ferguson-Miller, S., Calhoun, M. W., Thomas, J. W., Hill, J., Lemieux, L., Ma, J., Georgiou, C., Fetter, J., Shapleigh, J., Tecklenburg, M. M. J., Babcock, G. T., & Gennis, R. B. (1993) *J. Bioenerg. Biomembr. 25*, 121−136.

Howell, N., Appel, J., Cook, J. P., Howell, B., & Hauswirth, W. W. (1987) *J. Biol. Chem. 262*, 2411−2414.

Ingledew, W. J., Rothery, R. A., Gennis, R. B., & Salerno, J. C. (1992) *Biochem. J. 282*, 255−259.

Jones, R. W., & Whitmarsh, J. (1988) *Biochim. Biophys. Acta 933*, 258−268.

Kamensky, Y., Konstantinov, A. A., Kunz, W. S., & Surkov, S. (1985) *FEBS Lett. 181*, 95−99.

Kita, K., Konishi, K., & Anraku, Y. (1986) *Methods Enzymol. 126*, 94−113.

Kunze, B., Höfle, G., & Reichenbach, H. (1987) *J. Antibiot. 40*, 258−265.

Link, T. A., Haase, U., Brandt, U., & von Jagow, G. (1993) *J. Bioenerg. Biomembr. 25*, 221−232.

Matsushita, K., Patel, L., & Kaback, H. R. (1984) *Biochemistry 23*, 4703−4714.

Matsushita, K., Patel, L., & Kaback, H. R. (1986) *Methods Enzymol. 126*, 113−122.

Minghetti, K. C., & Gennis, R. B. (1988) *Biochem. Biophys. Res. Commun. 155*, 243−248.

Minghetti, K. C., Goswitz, V. C., Gabriel, N. E., Hill, J. J., Barassi, C. A., Georgiou, C. D., Chan, S. I., & Gennis, R. B. (1992) *Biochemistry 31*, 6917−6924.

Mitchell, R., & Rich, P. R. (1994) *Biochem. Soc. Trans. 22*, 703−707.

Moody, A. J., & Rich, P. R. (1994) *Eur. J. Biochem.* (in press).

Moody, A. J., Rumbley, J. N., Gennis, R. B., Ingledew, W. J., & Rich, P. R. (1993a) *Biochim. Biophys. Acta 1141*, 321−330.

Moody, A. J., Rumbley, J. N., Ingledew, W. J., Gennis, R. B., & Rich, P. R. (1993b) *Biochem. Soc. Trans. 21*, 255S.

Musser, S. M., Stowell, M. H. B., & Chan, S. I. (1993) *FEBS Lett. 327*, 131−136.

Nilsson, T. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6497−6501.

Oettmeier, W., Dostatni, R., Majewski, C., Höfle, G., Fecker, T., Kunze, B., & Reichenbach, H. (1990) *Z. Naturforsch. 45*, 322−328.

Oettmeier, W., Masson, K., Soll, M., & Reil, E. (1994) *Biochem. Soc. Trans. 22*, 213−216.

Ohnishi, T., Brandt, U., & von Jagow, G. (1988) *Eur. J. Biochem. 176*, 385−389.

Reil, E., Soll, M., Masson, K., & Oettmeier, W. (1994) *Biochem. Soc. Trans. 22*, 62S.

Rich, P. R. (1981) *Biochim. Biophys. Acta 637*, 28−33.

Rich, P. R. (1995) *Aust. J. Plant Physiol.* in press.

Rich, P. R., Madgwick, S. A., & Moss, D. A. (1991) *Biochim. Biophys. Acta 1058*, 312−328.

Roberts, H., Choo, W. M., Smith, S. C., Marzuki, S., Linnane, A. W., Porter, T. H., & Folkers, K. (1978) *Arch. Biochem. Biophys. 191*, 306−315.

Robertson, D. E., Daldal, F., & Dutton, P. L. (1990) *Biochemistry 29*, 11249−11260.

Slater, E. C. (1973) *Biochim. Biophys. Acta 301*, 129−154.

Stevens, T. H., Martin, C. T., Wang, H., Brudvig, G. W., Scholes, C. P., & Chan, S. I. (1982) *J. Biol. Chem. 257*, 12106−12113.

Svensson, M., & Nilsson, T. (1993) *Biochemistry 32*, 5442−5447.

Trebst, A. (1980) in *Methods in Enzymology* (Colowick, S. P., & Kaplan, N. O., Eds.) Vol. 69c, pp 675−715, Academic Press, New York.

Trebst, A. (1991) in *Herbicide resistance in weeds and crops* (Casely, J. C., Cussans, G. W., & Atkin, R. K., Eds.) pp 145−164, Butterworth-Heinemann Ltd., Oxford.

von Jagow, G., & Link, T. A. (1986) *Methods Enzymol. 126*, 253−271.

Watmough, N. J., Cheesman, M. R., Greenwood, C., & Thomson, A. J. (1994) *Biochem. J. 300*, 469−475.

Withers, H. K., & Bragg, P. D. (1989) *Biochem. Cell Biol. 68*, 83−90.

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