Biochimica et Biophysica Acta 807 (1985) 320–323
Elsevier

BBA 40048

BBA Report

Chlorpromazine inhibition of electron transport in *Azobacter vinelandii* membranes *

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(Received November 16th, 1984)

Key words: Chlorpromazine; Electron transport; Malate oxidation; (*A. vinelandii*)

Chlorpromazine was a potent inhibitor of O_2-dependent malate oxidation, but not of H_2 oxidation in *Azotobacter vinelandii* membranes. However, chlorpromazine did not significantly affect the activity of malate reductase or the reduction of cytochromes *c* and *d*. In the presence of chlorpromazine, cytochrome *o* failed to form a complex with CO. The site of action of chlorpromazine seems to be in the cytochromes *c* to cytochrome *o* branch, the pathway utilized by malate, succinate and NADH, but not by H_2.

Membranes of *Azotobacter vinelandii* contain a multitude of electron-transport components. These include several flavin-dependent dehydrogenases, ubiquinone Q_8 and at least six detectable cytochromes, forming a complex, branched electron-transport pathway [1,2]. The cytochrome *c* to cytochrome $a_1 + o$ branch is involved in energy conservation, while the other branch, from cytochrome *b* to cytochrome *d*, is thought to be the major pathway of electrons to O_2, but does not appear to produce ATP. A recent study [3] in our laboratory showed that, while L-malate oxidation utilized both branches, H_2 was able to reduce cytochrome *d*, but not able to reduce cytochrome *o*. Cyanide inhibition studies also supported the conclusion that H_2 oxidation used the cytochrome *b* to cytochrome *d* branch rather than cytochrome *o* as terminal oxidase. We also found [3] that chlorpromazine was a potent inhibitor of L-malate oxidation, but H_2 oxidation was not very sensitive to chlorpromazine inhibition. However, we did not know the site of action of chlorporamizine. Other previous studies showed that chlorpromazine was a potent inhibitor of lactate oxidation in *A. vinelandii* membranes, but it did not inhibit the flavoprotein-containing lactate dehydrogenase enzyme [4]. We now show that the major site of chlorpromazine inhibition is at the cytochrome *c* to cytochrome *o* branch of the electron-transport chain.

Membranes from *A. vinelandii* strain CA (from P.E. Bishop, North Carolina State University, Raleigh, NC) were prepared as described previously [3]. Oxidase activity were measured amperometrically. The assay conditions were similar to those described with minor modifications [5]. To test the effect of chlorpromazine, the drug was first added to the air-oxidized form of the membrane sample in the amperometric chamber. A total of 5 mM of either L-malate, succinate or NADH was then added. The reaction was monitored for 10 min. H_2-dependent oxidation was monitored amperometrically as described previously [3]. Fig. 1 shows the recorder tracing directly demonstrating the inhibition during a 10 min period by chlorpromazine on L-malate oxidation.

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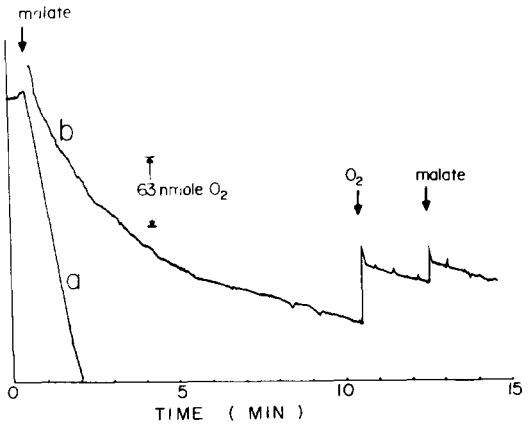

Fig. 1. The inhibition effect of chlorpromazine on L-malate-dependent O_2 uptake by *A. vinelandii* membranes. Trace a shows the oxidation rate of L-malate without chlorpromazine, trace b with chlorpromazine. After 5 min, the specific activity of L-malate oxidation decreased from 184 to 28 nmol O_2 per min per mg protein in the presence of 100 μM chlorpromazine.

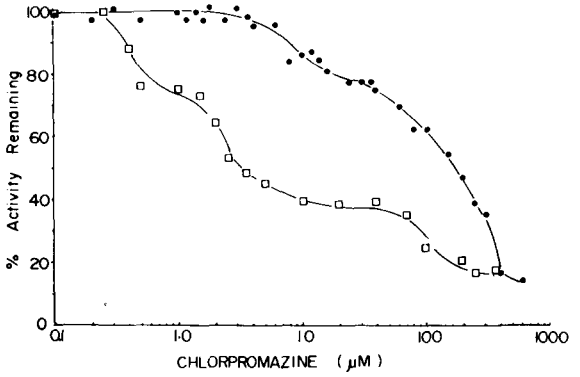

Fig. 2. Effects of chlorpromazine concentrations on O_2-dependent (●———●) and methylene blue-dependent (u) L-malate oxidation. The reaction chamber contained 5 mM L-malate in air-saturated buffer. The reaction mixture for methylene blue-dependent L-malate oxidation was the same as above, except that 2 mM KCN and 1 mM methylene blue was included in the reaction mixture. The inhibition was quantitated 5 min after malate addition.

The initial rate of malate oxidation in the presence of chlorpromazine was essentially the same as the sample without chlorpromazine. However, the rate of L-malate-dependent O_2 uptake decreased with time, and was much slower than the unhibited rate after 5 min. This slower but linear rate was not due to oxygen or L-malate limitation, as adding these substrates to the chamber did not increase the oxidation rate (see Fig. 1). There were no inhibitory effects of chlorpromazine on the air-oxidized membranes. Prolonged incubation with chlorpromazine (up to 60 min) in the absence of substrate did not significantly change the inhibition curve shown in Fig. 1.

We compared the effects of the chlorpromazine concentrations on O_2-dependent L-malate oxidation and methylene blue-dependent L-malate oxidation, the latter being a measure of L-malate reductase. As shown in Fig. 2. O_2-dependent L-malate oxidation was much more sensitive to chlorpromazine than the methylene blue-dependent L-malate oxidase. This result indicated that the major site of chlorpromazine inhibition was not at the L-malate dehydrogenase site.

We also studied the reduction kinetics of individual types of cytochrome by L-malate. The semi-microquartz cuvette (10 mm pathlength) contained 1 ml membrane sample (2 mg protein per ml), 100 μM chlorpromazine and 5 mM L-malate. After incubating at room temperature for approx. 5 min, the sample was mixed for 20 s to reoxidize the cytochromes. The reductions of cytochrome *c* and cytochrome *d* were individually monitored at wavelengths pairs of 551–538 nm and 630–615 nm, respectively [2]. Fig. 3A shows the L-malate-dependent reduction of cytochrome *d*, with and without chlorpromazine. The addition of chlorpromazine (100 μM) did not decrease or significantly delay the reduction of this cytochrome oxidase. Fig. 3B shows a similar experiment in which the reduction of cytochrome *c* was monitored. In the presence of 100 μM chlorpromazine, the rate of cytochrome *c* reduction was unaffected. However, slightly more cytochrome *c* was reduced in the presence of the inhibitor. We can conclude that chlorpromazine does not significantly affect cytochrome *d* or *c* reduction.

Carbon monoxide forms a complex with the reduced form of terminal oxidases. If chlorpromazine inhibits cytochrome *c* reduction, but still allows the reduction of cytochrome *d*, the (malate-reduced plus CO)-minus-(malate-reduced) spectrum in the presence of chlorpromazine should reveal a spectrum characteristic only of cytochrome *d*. Fig. 4 shows such a difference spectrum with (Fig. 4a) or without (Fig. 4b) chlorpromazine.

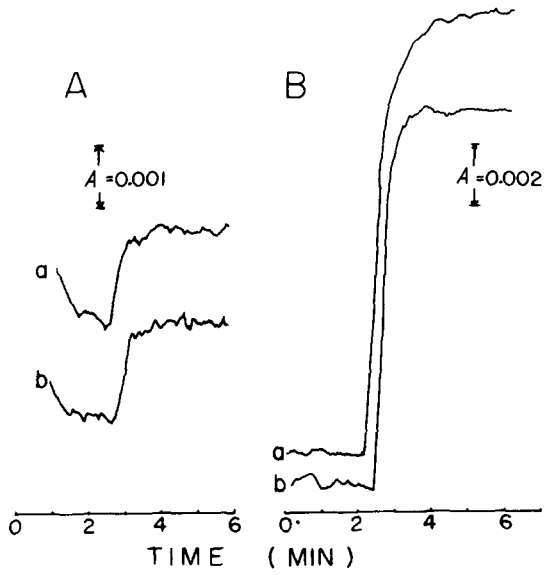

Fig. 3. Kinetic studies of cytochrome reduction by L-malate. (A) The reduction of cytochrome *d* (630–615 nm) and (B) the reduction of cytochrome *c* (551–538 nm). Traces (a) represent the reduction of the cytochrome in the presence of 100 μM chlorpromazine, and traces (b) represent the reduction of the cytochrome without the addition of chlorpromazine.

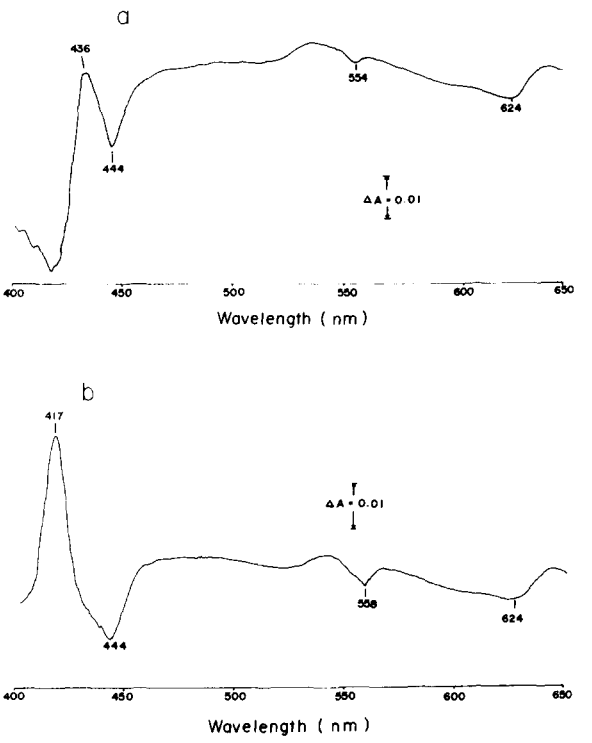

Fig. 4. (Carbon monoxide + malate-reduced)-minus-(malate-reduced) difference spectra of *A. vinelandii* membranes in the presence of 100 μM chlorpromazine (Fig. 4a), and without the addition of chlorpromazine (Fig. 4b).

Cytochrome *d* (trough at 624 nm) was observed in both scans (Fig. 4a and b). However, in the experiment without chlorporomazine (Fig. 4b) cytochrome *o* (trough at 558 nm and a distinctive peak at 417 nm) was also observed. This observation means that cytochrome *o* was not reducible in the presence of chlorpromazine.

We previously showed that H_2 oxidation and L-malate oxidation differed in their sensitivity to chlorpromazine [3]. Now we have compared the sensitivities of H_2, L-malate, succinate and NADH oxidation to chlorpromazine (see Table I). H_2 oxidation was not inhibited at all by 10 μM chlorpromazine and slightly by 100 μM chlorpromazine. In contrast, the oxidation of all the other substrates were markedly inhibited by chlorpromazine. H_2 oxidation in *A. vinelandii* membranes does not utilize the cytochrome *o* pathway [3], and is therefore insenstive to chlorpromazine. Malate, succinate and NADH all utilize both cytochrome *d* and cytochrome *o* as terminal oxidases [1,2,5,6], and we have shown they are much more sensitive to chlorpromazine. This inhibitor should be useful for further studies on the electron transport chain of *A. vinelandii*.

This research was supported by grant 84-CRCR-1-0-435 from the U.S. Department of Agriculture Competitive Grants Program.

TABLE I

EFFECT OF CHLORPROMAZINE ON SUBSTRATE OXIDATION BY *A. VINELANDII* MEMBRANES

The initial specific activities of H_2, L-malate, succinate and NADH oxidation rates were 95, 186, 80 and 227 nmol O_2 per min per mg protein, respectively.

Substrate (5 mM)	Percentage of inhibition	
	by 10 μM chlorpromazine	by 100 μM chlorpromazine
H_2	0	20
malate	40	75
succinate	46	80
NADH	33	85

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