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Dual substrate oxidations by *Azotobacter vinelandii* membranes *

T.Y. Wong and Robert J. Maier

The McCollum-Pratt Institute and Department of Biology, The Johns Hopkins University, Baltimore, MD (U.S.A.)

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Azotobacter vinelandii **membranes oxidized H_2 and malate simultaneously. However, the O_2 uptake rate of *A. vinelandii* membranes with both H_2 and malate provided was about the same as with malate alone. In contrast, during dual substrate oxidation of H_2 and succinate, the O_2 uptake activity was approximately the same rate as the summation of the individual H_2-dependent and succinate-dependent O_2 uptake rates. During dual substrate oxidation of H_2 and succinate, each substrate contributed about equal amounts of reductant as electrons. The above results can be explained on the basis of the oxidases utilized for the various substrates. Cyanide inhibition studies demonstrated that during dual substrate oxidation of H_2 and succinate, the terminal oxidases used (cytochrome *o* and *d*) are the combination of the principal oxidases used by each substrate alone. Cyanide inhibition curves of respiration indicated that the reason for the competition between H_2 and malate for oxidase activity is most likely due to the use of cytochrome *d* by both of these substrates. Use of chlorpromazine, which inhibits the cytochromes *c* to cytochrome *o* branch of electron flow, also indicated that the terminal oxidases used for succinate plus H_2 are the combination of the separate principal oxidases (cytochromes *o* and *d*) used by each substrate alone. Succinate-dependent O_2 uptake was highly sensitive to chlorpromazine, and this was attributed to inhibition of the cytochrome *o* branch. Chlorpromazine inhibition phases of malate oxidase and the dual substrate condition malate plus H_2 were similar. The chlorpromazine inhibition experiments indicated that cytochromes *d* and *o* are utilized under both malate alone and malate plus H_2 conditions. In all cases cytochrome *d* appeared to be the oxidase responsible for electron flow from H_2.**

Introduction

As in many other bacteria, the *Azotobacter vinelandii* electron-transport pathway is a complex, branched system. One branch, from cytochromes *b* and *c* to a_1 and *o* is involved in energy conservation, but is considered to be the minor branch for electron flow [1–4]. The other branch, from cytochrome *b* to cytochrome *d*, is thought to be a major pathway of electrons to O_2, but does not appear to produce ATP [1,3–6]. It has been postulated [1,6] that the cytochrome $b \rightarrow d$ branch serves to keep the intracellular O_2 concentration down, thus protecting the O_2-labile nitrogenase from inactivation.

The role of the oxidases in accepting electrons from different organic substrates in *A. vinelandii* is not clear. Many studies indicate that the bulk of the electron flux from physiological substrates terminates with cytochrome *d* rather than with cytochromes *o* and a_1 [1,3,5,7]. However, some studies suggest that the cytochromes $b \rightarrow o$ plus a_1

* This is contribution number 1361 from the McCollum-Pratt Institute and Department of Biology.

Correspondence: R.J. Maier, The McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, MD 21218, U.S.A.

pathway is important for respiration and growth of *A .vinelandii* cultured in low O_2 [8]. Also, from cyanide inhibition studies, Jurtshuk and coworkers [7,9] suggested that the cytochromes $c \rightarrow o$ plus a_1 branch is the major oxidase branch for succinate oxidation. Photodissociation experiments led Yang [10] to conclude that cytochrome *d* is the major oxidase for electrons from NADH. None of the previous studies considered the respiratory competition effects due to the simultaneous oxidation of two substrates. Such a study is possible, due to different sensitivities of oxidase activities to cyanide inhibition.

Another substrate of interest is H_2. The H_2-oxidizing system uses electrons from the H_2 that is evolved by the nitrogenase reaction [6,11,12] and this oxidation system is also cytochrome dependent [13]. Recent spectral studies have indicated that cytochromes *b*, *c* and *d* were involved in H_2 oxidation in *A. vinelandii* membranes [14,15]. However, cytochrome *o* was not reduced by H_2 [14]. Inhibitor studies with cyanide [14] and chlorpromazine [15] also suggested the selective use of cytochrome *d* by the H_2-oxidizing pathway. Cytochrome *d* was selectively utilized for H_2 oxidation even under fully reduced conditions [14]. To analyze further how different oxidases are involved in the oxidation of different substrates, for this study we have measured O_2 uptake rates of membranes with different substrates provided. We have found evidence that electrons generated from H_2 oxidation and malate oxidation compete for O_2; respiratory rates of malate-oxidizing membranes do not increase when H_2 is added and oxidized. However, studies with membranes supplied with both H_2 and succinate showed there was no competition for O_2; rather the O_2-uptake rate was additive. These results can be explained on the basis of the primary oxidases used by each substrate to activate molecular oxygen.

Materials and Methods

Cell growth and preparation of membranes for the N_2-fixing *A. vinelandii* strain CA (obtained from P.E. Bishop, Raleigh, NC) was like that described previously [14]. H_2 and O_2 uptake rates were simultaneously monitored amperometrically as described [14,16]. A potassium phosphate buffer solution (50 mM, pH 7.2) was flushed with N_2 gas and was then used to fill the (5.5 ml volume) electrode chamber. Membrane samples (final 0.1–0.42 mg membrane protein) were injected into the chamber followed by the injection of a total of 0.5 mM of either malate or succinate and the specific activities of O_2 consumption were recorded. Appropriate amount of H_2-saturated buffer and O_2-saturated buffer were then injected into the chamber as indicated in Figs. 1 and 2, and the total O_2 consumption and H_2 consumption were recorded [14]. For Figs. 1 and 2 some oxygen was allowed to remain in the buffer in the chamber at the beginning of the experiment, as indicated at zero time in those figures. All substrates were added in saturating amounts for O_2 uptake activity. There was no O_2 uptake in the absence of substrate. All experiments were repeated at least three times with the results similar to those shown. Portions of H_2- and O_2-saturated buffer were used as standards before and after each experiment (50 μl volume containing 37.7 nmol H_2 or 63.3 nmol O_2). Cyanide inhibition experiments were performed as described previously [14,17]. Membrane samples (20 μl of 20 mg protein per ml suspension) were injected into the amperometric chamber, and O_2 consumption was monitored until the chamber was completely anaerobic. The H_2 concentration was adjusted to 40 μM, by injection of H_2-saturated potassium phosphate buffer solution. Reactions were then started by injecting 50 μl (63.3 nmol) of O_2-saturated buffer into the chamber. Cyanide was then injected into the electrode chamber as an aqueous solution to respiring membranes. There was no H_2 uptake once the O_2 was depleted, and the ratio of H_2 consumed to O_2 was approx. two (data not shown). Injecting more than 50 μl of O_2-saturated buffer did not increase the rate of H_2 uptake. Both the O_2 and H_2 uptake rates were linear during the assay periods. Dixon plots ($1/V$ vs. [cyanide]) were used to calculate the K_i values. The correlation of all lines constructed with linear regression was better than 0.95. All amperometric assays were performed at room temperature.

Chlorpromazine inhibition experiments were performed similarly to those described previously [15], and in the legends to Figs. 5 and 6. One exception to the previous method [15] was the

length of time (10 min) for the drug to equilibrate with the membrane sample in the amperometric chamber. Also, lower chlorpromazine levels (less than 1 μm) were used in the present study. The drug was added to the (0.7 mg protein) membrane sample in the amperometric chamber and allowed to equilibrate for 10 min prior to injection of substrate (malate or succinate). The O_2 uptake rate was then determined 5 min after the injection of substrate (H_2, malate, succinate). The carbon substrates malate or succinate were added to a concentration of 1.0 mM, and H_2 to a concentration of 40 μM. Specific activities (nanomol O_2 consumed per min per mg protein) are given for each experiment in the figure legends.

Results

Fig. 1 shows O_2 uptake activity with H_2 and malate provided as substrates. There was no O_2 uptake upon the injection of the membranes into the chamber (see arrow A); this shows there was no oxidizable endogenous substrates in the membrane preparation. The addition of malate (arrow B) resulted in a rapid consumption of O_2. The specific activity of this malate-dependent oxidation by the membrane fraction was about 104 nmol O_2 per min per mg protein. When H_2 was then injected into the chamber (arrow C), it was consumed at a rate of approx. 52 nmol per min

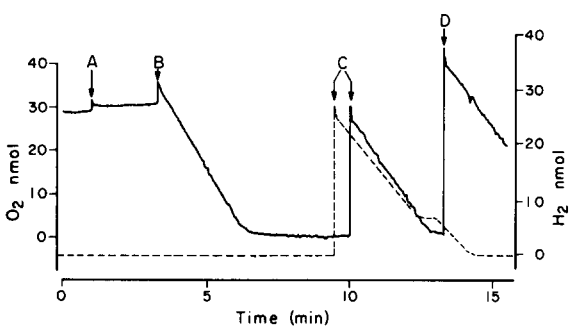

Fig. 1. Direct amperometric recording of H_2 and O_2 uptake in the presence of malate by membranes of *A. vinelandii*. Arrows: A, injection of 0.11 mg membrane protein. B, addition of sodium malate (0.5 mM). C, simultaneous injection of 50 μl H_2 and 20 μl O_2 saturated potassium phosphate buffer solutions. The tracing for H_2 is displaced 0.5 min to the left of the O_2 pen. D, injection of 30 μl O_2 saturated buffer. Solid line, O_2 uptake; dashed line, H_2 uptake.

per mg protein. However, the total O_2 uptake rate with malate plus H_2 (98 nmol per min per mg protein) was only about the same as with malate alone. This indicates competition for electron-transport components. Since the ratio of the H_2 uptake rate to O_2 uptake rate (without other substrates) of these membranes was always two, it was possible to calculate the fraction of O_2 consumed by H_2 oxidation. This was equal to half of the H_2 oxidation rate recorded by the H_2 electrode. The total O_2 uptake, as well as the contribution to O_2 uptake by each of the substrates (H_2, malate, succinate) from experiments like the one shown in Fig. 1 are shown in Table I. The 52 nmol H_2 uptake recorded therefore represent 26 nmol of O_2 consumption via H_2 oxidation (see Table I). Therefore, during H_2 plus malate oxidation, most of the O_2 consumption is due to electrons from malate. The H_2 uptake was totally dependent on the availability of O_2 as the depletion of O_2 (area just prior to D) also resulted in the elimination of H_2 uptake.

Many experiments with our washed membrane preparations demonstrated an H_2/O_2 uptake-rate ratio of 2. Therefore, in the experiment of Fig. 1, we could be certain that when H_2 was added (arrow C) and H_2 and O_2 simultaneously consumed, that H_2 did not abolish malate oxidation.

TABLE I

O_2 UPTAKE WITH EACH SUBSTRATE AND DUAL SUBSTRATE CONDITION

All values are in nmol O_2 uptake per min per mg membrane protein. The numbers are calculated from the experiments shown in Figs. 1 and 2 and as described in the text. The hydrogen-dependent O_2-uptake activity without other substrates of these membranes was approx. 21 nmol O_2 oxidized per min per mg protein.

Substrate	Total O_2 uptake	O_2 consumption due to:		
		H_2	malate	succinate
Malate	104 [a]	–	104 [a]	–
H_2 plus malate	98 [a]	26 [c]	72 [b]	–
Succinate	27 [a]	–	–	27 [a]
H_2 plus succinate	52 [a]	23 [c]	–	29 [b]

[a] Values measured directly from chart recorder tracing.

[b] Total O_2 uptake minus O_2 uptake due to H_2 in the presence of both substrates.

[c] Calculated from H_2 uptake value measured directly in the presence of both substrates.

This was because the H_2/O_2 uptake ratio in the presence of malate was less than 2 (calculated from area of Fig. 1 after arrow C).

Fig. 2 shows the results of another dual-substrate experiment like Fig. 1, except that H_2 and succinate were the reductants. In this experiment, H_2 was the first substrate added in order to demonstrate the O_2-uptake contribution just by H_2. The order of substrate addition (H_2, malate, succiante) for these experiments, however, did not affect the result. The O_2 uptake by H_2 oxidation alone was 21 nmol per min per mg protein. The succinate-dependent O_2 uptake rate of Fig. 2 (arrow C) was 27 nmol O_2 consumed per min per mg protein (see Table I). At arrow D, H_2 and O_2 were added simultaneously to succinate-respiring membranes. In contrast to the Fig. 1 results (H_2 plus malate), during dual-substrate oxidation of H_2 and succinate the total O_2 uptake rate was much higher than with either H_2 or succinate alone (specific activity, 52 nmol O_2 per min per mg protein). This rate was about the same as the summation of the individual H_2-dependent O_2-uptake rate and the succinate-dependent O_2-uptake rates. The H_2-dependent O_2 uptake when both

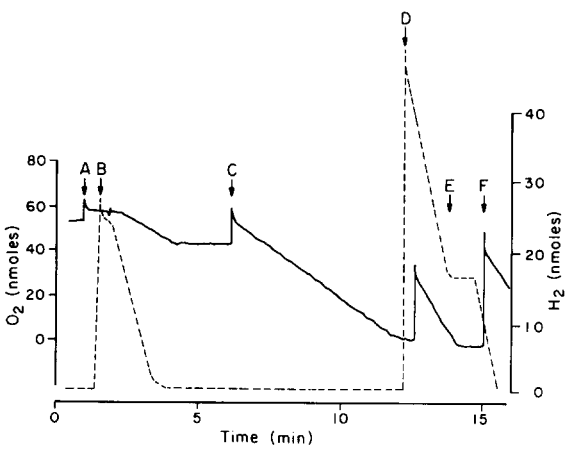

Fig. 2. Direct amperometric recording of H_2 and O_2 uptake in the presence of succinate by membranes of *A. vinelandii*. Arrows: A, injection of 0.37 mg membrane protein. B, injection of 40 μl H_2-saturated potassium phosphate buffer. C, addition of sodium succinate (0.5 mM). D, simultaneous injection of 80 μl H_2 and 25 μl O_2-saturated buffer solutions. E, area where O_2 exhausted. F, injection of 30 μl O_2-saturated buffer. Solid line, O_2 uptake; dashed line, H_2 uptake. The tracing for H_2 is displaced 0.5 min to the left of the O_2 pen.

substrates (H_2 plus succinate) were used was measured as 23 nmol per min per mg protein (see Table I). Therefore, the contribution of each substrate (H_2 and succinate) to the total O_2 uptake was about equal (see Table I), in contrast to the dual substrate H_2 plus malate result.

Jurtshuk et al. [18,19] showed that succinate oxidation in *A. vinelandii* is more sensitive than malate oxidation to inhibition by cyanide. These investigators suggested that the cytochrome $c \rightarrow o$ plus a_1 branch of the electron-transport chain was the major terminal oxidase involved during succinate oxidation. The H_2-oxidizing system of *A. vinelandii* is relatively insensitive to cyanide; this was attributed to the use of cytochrome *d* as the terminal oxidase [14]. It was possible that the cooperative respiratory effect observed with the dual substrates H_2 and succinate was due to the utilization of some separate electron-transport pathways by each substrate, while the competitive effect of malate and H_2 oxidation was due to the utilization of the same electron-transport component(s). We investigated the possibility that the results of Figs. 1 and 2 were due to H_2 and malate competing for the same terminal oxidase, whereas H_2 and succinate use primarily different oxidases. Evidence to support this was determined by performing cyanide and chlorpromazine inhibition experiments of O_2 uptake activity with the multiple substrates.

Jones and Redfearn concluded that cytochrome *d* is relatively insensitive to cyanide in *A. vinelandii* [4], whereas cytochrome *o* has a high binding affinity for cyanide [20]. Fig. 3 shows the effect of cyanide on the total O_2 consumption with H_2 and succinate together as substrates, and with each substrate alone. The cyanide inhibition curve of succinate oxidation was monophasic, having a K_i value of about $1.7 \cdot 10^{-7}$ M. Based on previous cyanide inhibition results [1,3,4,19,7,9,21,14], we attribute this K_i to cytochrome *o*. The distinguishing feature of the H_2 plus succinate curve was that this inhibition curve was clearly biphasic. One of the phases corresponds to the succinate alone curve, with a K_i value also of approx. $2 \cdot 10^{-7}$. The higher K_i value was about $9.0 \cdot 10^{-5}$ M. Based on previous cyanide inhibition results, we attribute the higher K_i to cytochrome *d* [4,7,14,20,22]. The effect of cyanide on the H_2-de-

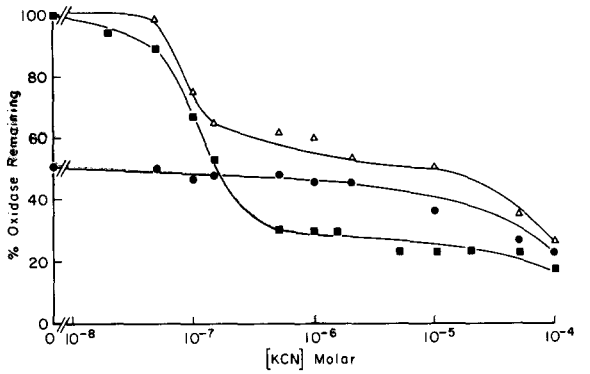

Fig. 3. Effect of CN on the O_2 uptake activity with succinate (■), H_2 (●), and succinate plus H_2 (△) as substrates. Assay conditions were as described previously [14]. Dixon plots ($1/v$ vs. [cyanide]) were used to calculate K_i values reported in the text. H_2 consumption was also monitored in these experiments. The H_2-dependent O_2 uptake rate was calculated as one-half of the H_2 uptake rate recorded by the H_2 electrode. 100% activities were (in nmol O_2 consumed per min per mg protein) succinate oxidation, 22; succinate plus H_2 oxidation, 51.

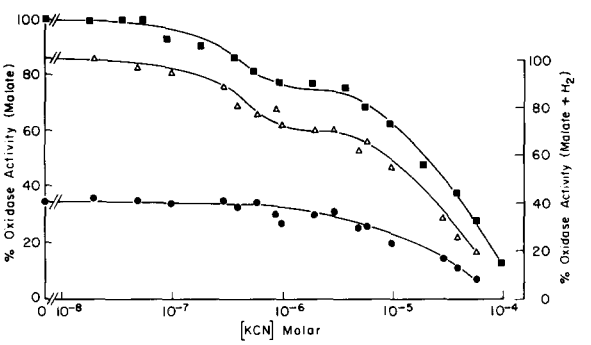

Fig. 4. Effect of CN on the O_2-uptake activity with malate (■), H_2 (●), and malate plus H_2 (△) as substrates. All assay conditions were as described for Fig. 3. 100% activities were (in nmol O_2 consumed per min per mg protein) malate oxidation, 200; malate plus H_2 oxidation, 210.

pendent respiration, as calculated (see Fig. 3 legend) from the dual substrate result is also shown in Fig. 3. This curve was very similar to the cyanide inhibition curve for H_2 oxidation determined directly and reported previously [14] and had a K_i of about $8 \cdot 10^{-5}$ M. This K_i is in good agreement with our previous K_i value of cyanide for H_2 oxidation of 135 μM [14], and probably represents cyanide binding to cytochrome *d*. The results indicate that during mixed-substrate oxidation of H_2 and succinate, the terminal oxidases utilized (cytochromes *o* and *d*) are the combination of the individual oxidase utilized by each substrate alone.

From Table I, we concluded that H_2 and succinate contributed about equally to the respiratory activity when both reductants were provided. From Fig. 3, it is clear that inhibition of the cyanide-sensitive pathway (cytochrome *o*) reduces the O_2 uptake activity with both substrates present about 50%. Since we attribute cytochrome *o* use to the succinate portion of respiration, there is good agreement between the conclusions of Table I and the cyanide sensitivity phases of corresponding oxidase activity of Fig. 3.

Fig. 4 shows results of the effect of cyanide on the O_2 consumption rate with H_2 and malate as substrates. The cyanide inhibition curve of the dual substrate experiment of Fig. 4 was very similar to the cyanide inhibition curve of malate alone. This result indicates that the two substrates utilize similar oxidases. The curve with malate alone as substrate was multiphasic having a lower K_i value at about $4 \cdot 10^{-7}$ M and a higher K_i value of about $5 \cdot 10^{-5}$ M. Cytochromes *d*, a_1 and *o* have been reported as the terminal oxidases for malate oxidation in *A. vinelandii* [1,18,19], and the K_i values we determined are likely due to cytochromes *o* [20] and *d* [22]. The results show that during dual substrate oxidation of H_2 and malate, the higher K_i value oxidase was shared by both substrates. The K_i of the H_2-dependent oxidase activity of Fig. 4 was calculated as approx. $7 \cdot 10^{-5}$ M. The oxidase which is the least sensitive to cyanide [21,22] we attribute to cytochrome *d*. The reason for the competition between H_2 and malate for O_2 then is likely due to the use of cytochrome *d* by both substrates. The effect of cyanide on O_2-dependent H_2 oxidation in the absence of other substrates was also determined on the membrane preparations used for Figs. 3 and 4; as reported previously [14] H_2 oxidation was not very sensitive to cyanide. We observed K_i values for H_2 uptake of 110–135 μM. Therefore the K_i for H_2 determined directly agrees reasonably well with the calculated K_i (70 μM) of Fig. 4.

Previously we demonstrated that chlorpromazine was an inhibitor of O_2-dependent succinate, malate, and NADH oxidation, but not of hydrogen oxidation by *A. vinelandii* membranes [15].

Chlorpromazine did not significantly inhibit the reduction of cytochromes *c* or *d*, nor did it inhibit malate reductase [15]. Carbon monoxide difference spectral experiments indicated that the site of action of chlorpromazine is in the cytochromes *c* to cytochrome *o* branch, as cytochrome *o* failed to form a complex with CO in the presence of the inhibitor [15]. We therefore determined oxidase activities of *A. vinelandii* membranes with malate and with malate plus H_2 to determine the contribution of cytochrome *o* to these pathways. Titration of malate oxidase activity with low concentrations of chlorpromazine (see Fig. 5) revealed some inhibition of malate-dependent respiration at concentrations of inhibitor greater than 0.1 μM. The curve of malate plus H_2 oxidase activity vs. chlorpromazine revealed a curve very similar to the malate alone curve. At 1 μM chlorpromazine, malate-dependent O_2 uptake was inhibited about 30%, whereas malate plus H_2 was inhibited about 35%.

Since CO-difference spectra previously demonstrated that chlorpromazine inhibits the cytochrome *o* pathway [15] the malate oxidase and dual substrate malate plus H_2 oxidase activities most likely do not use cytochrome *o* as the major oxidase. As described previously [14,15] the H_2-dependent respiratory activity showed little inhibition by chlorpromazine (see Fig. 5). It was concluded [15] that this is due to electrons from H_2 utilizing mostly the cytochrome *d* oxidase branch even under reduced conditions. The current results indicate the extent of terminal oxidases (cytochromes *d* and *o*) undergoing electron transport are similar for both the malate and malate plus H_2 substrate conditions. The results provide additional support for the conclusion, obtained from the cyanide results, that the competition between H_2 and malate for O_2 is likely due to the use of cytochrome *d* by both substrates in *A. vinelandii* membranes.

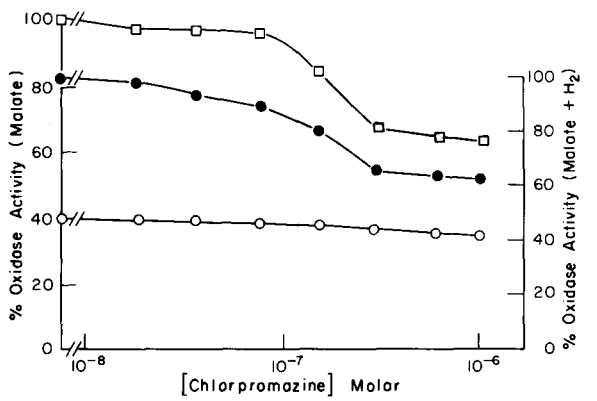

Fig. 5. Effect of chlorpromazine on the O_2-uptake activity with malate (□) H_2 (○) and malate plus H_2 (●) as substrates. Assay conditions were similar to those described previously [14,15] and as described in the text. Membrane samples (20 μl 35 mg protein per ml) was injected into the (5.5 ml volume) amperometric chamber containing potassium phosphate buffer solution (50 mM, pH 7.2). The inhibitor was added at the concentration indicated, and allowed to equilibrate with the membranes in the chamber for 10 min. Then malate (1.0 mM) or malate (1.0 mM) plus H_2 (40 μM) was added, and the O_2 uptake rate determined after 5 min. Approx. 120 nmol O_2 was injected to initiate the assay. Uninhibited specific activities (nmol O_2 uptake per min per mg protein) were: malate oxidation, 145; malate plus H_2 oxidation, 151. The H_2-dependent O_2-uptake rate was calculated as one-half of the H_2-uptake rate recorded by the H_2 electrode.

Similar chlorpromazine inhibition experiments on succinate and succinate plus H_2 oxidation by *A. vinelandii* membranes were performed. Our previous results indicated that succinate oxidation by *A. vinelandii* membranes was slightly more sensitive to 10 μM chlorpromazine than was malate oxidation [15]. Now we have compared succinate and succinate plus H_2 respiratory activities as a function of lower concentrations of chlorpromazine (see Fig. 6), like the malate-dependent O_2 uptake experiments of Fig. 5. Succinate-dependent

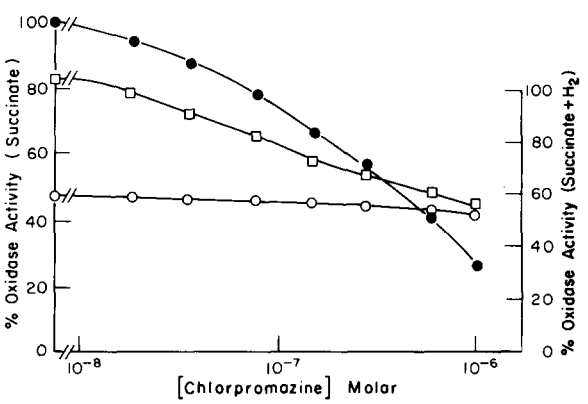

Fig. 6. Effect of chlorpromazine on the O_2-uptake activity with succinate (●), H_2 (○), and succinate plus H_2 (□) as substrates. All conditions were as described in the Fig. 5 legend. Succinate was added to a concentration of 1.0 mM. Uninhibited specific activities (nmol O_2 uptake per min per mg protein) were: succinate oxidation, 44; succinate plus H_2 oxidation, 99.

O_2 uptake was highly sensitive to the inhibitor, reaching approx. 70% inhibition at 1.0 μM chlorpromazine. Therefore under our conditions succinate oxidation must be dependent on cytochrome *o* as a major oxidase. Succinate plus H_2 was also chlorpromazine sensitive; at 1.0 μM chlorpromazine it was 45% inhibited. Therefore both succinate and succinate plus H_2 utilize cytochrome *o*. H_2 oxidation alone does not (see Fig. 6), as this activity was chlorpromazine-insensitive. The results provide more evidence that the additive effect of respiratory activity by succinate plus H_2 is due to the oxidases (cytochrome *o* and *d*) involved.

Discussion

In agreement with results of others [1–4,8,18,19] our results indicate that different terminal oxidases can serve for different physiological substrates. However, none of these studies considered the simultaneous oxidation of two substrates, nor did they address the substrate H_2. First, it cannot be assumed that H_2 oxidation utilizes the same cytochrome pathways as other reductants for H_2 oxidizing bacteria [23]. In some H_2-oxidizing bacteria other than *Azotobacter,* selective use of oxidases by H_2 has been implicated; for example, differences in the involvement of cytochrome *o* compared to cytochrome aa_3 was suggested for *Paracoccus denitrificans* [24]. Also, *Arthrobacter* strain 11/x contains an additional oxidase, cytochrome *d*, when cells are cultured lithotrophically with H_2 [23,25]. The mechanism by which specific oxidases are utilized by certain reductants is not known. *Rhizobium japonicum* utilizes additional oxidases for H_2 oxidation symbiotically that are not present in cultures grown under free-living conditions [17]. In our studies, when *A. vinelandii* membranes oxidized succinate primarily a cyanide-sensitive oxidase was utilized. Our chlorpromazine inhibition experiments [15] indicate this oxidase is cytochrome *o*. Consequently, another substrate (H_2), which utilizes cytochrome *d* as oxidase, can apparently be simultaneously oxidized with an increase in total respiration. In conditions in which a substrate utilizes both cytochromes *o* and *d* (such as with malate), H_2-dependent O_2 uptake still occurs, but the total respiratory activity does not increase. The present study relies only on the use of inhibitors to indicate the terminal oxidases utilized by the substrates. To confirm and clarify the role of these oxidases as terminal oxidases for each substrate, mutants lacking specific oxidases are needed. Additionally, the isolation of specific dehydrogenase complexes with other electron-transport components and their reconstitution into liposomes will likely be another useful approach.

Acknowledgements

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