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THE RESPIRATORY SYSTEM OF THE MARINE BACTERIUM *BENECKEA NATRIEGENS*

II. TERMINAL BRANCHING OF RESPIRATION TO OXYGEN AND RESISTANCE TO INHIBITION BY CYANIDE

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SUMMARY

1. Cell-free extracts of the marine bacterium *Beneckea natriegens*, derived by sonication, were separated into particulate and supernatant fractions by centrifugation at 150 000 × *g*.

2. NADH, succinate, D(−)- and L(+)-lactate oxidase and dehydrogenase activities were located in the particles, with 2- to 3-fold increases in specific activity over the cell free extract. The D(−)- and L(+)-lactate dehydrogenases were NAD^+ and $NADP^+$ independent. Ascorbate–*N*,*N*,*N*′,*N*′-tetramethylphenylenediamine (TMPD) oxidase was also present in the particulate fraction; it was 7–12 times more active than the physiological substrate oxidases.

3. Ascorbate–TMPD oxidase was completely inhibited by 10 μM cyanide. Succinate, NADH, D(−)-lactate and L(+)-lactate oxidases were inhibited in a biphasic manner, with 10 μM cyanide causing only 10–50 % inhibition; further inhibition required more than 0.5 mM cyanide, and 10 mM cyanide caused over 90 % inhibition. Low sulphide (5 μM) and azide (2 mM) concentrations also totally inhibited ascorbate–TMPD oxidase, but only partially inhibited the other oxidases. High concentrations of sulphide but not azide caused a second phase inhibition of NADH, succinate, D(−)-lactate and L(+)-lactate oxidases.

4. Low oxidase activities of the physiological substrates, obtained by using non-saturating substrate concentrations, were more inhibited by 10 μM cyanide and 2 mM azide than high oxidase rates, yet ascorbate–TMPD oxidase was completely inhibited by 10 μM cyanide over a wide range of rates of oxidation.

5. These results indicate terminal branching of the respiratory system. Ascorbate–TMPD is oxidised by one pathway only, whilst NADH, succinate, D(−)-lactate and L(+)-lactate are oxidised via both pathways. Respiration of the latter substrates occurs preferentially by the pathway associated with ascorbate–TMPD oxidase and which is sensitive to low concentrations of cyanide, azide and sulphide.

Abbreviations: TMPD, *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine; PMS, phenazinemethosulphate; DCIP, 2,6-dichlorophenolindophenol.

6. The apparent K_m for O_2 for each of the two pathways was detected using ascorbate–TMPD and NADH or succinate plus 10 μM cyanide respectively. The former pathway had an apparent K_m of 8–17 (average 10.6) μM and the latter 2.2–4.0 (average 3.0) μM O_2.

INTRODUCTION

Mitochondrial respiratory systems are usually highly sensitive to inhibition by cyanide [1]. Cyanide resistant respiratory systems have, however, been observed in plants [2, 3], yeasts and fungi [4–7], protozoa [8, 9] and algae [10]. Cyanide resistance in these organisms is due to the presence of a cyanide-insensitive "alternate oxidase": in addition to cytochrome oxidase. Branching of the respiratory system occurs at the flavoprotein level, before the site of action of antimycin A. The alternate oxidase can be inhibited by hydroxamic acids [2]. Thus salicylhydroxamic acid, cyanide or antimycin A alone have little effect on the rate of oxygen uptake, but salicylhydroxamic acid plus cyanide (or antimycin A) cause complete inhibition of respiration. The cyanide and antimycin A sensitive pathway contains a fairly normal complement of cytochromes and is coupled to oxidative phosphorylation. The nature of the alternative oxidase is unknown but, since it can also be inhibited by iron-chelating agents (α,α-dipyridyl, thiocyanate and 8-hydroxyquinoline) in addition to hydroxamic acids, it may contain a non-heme iron protein [2, 3]. Induction of the alternate oxidase may be related to copper deficiency [6, 7].

There are a wide range of bacterial cytochromes known to interact with CO and thus have a possible oxidase function; these include cytochromes of the *a*-type (a_1, a_3), *b*-type (*o*), *c*-type and *d*-type (a_2) (c.f. refs. 11–13, 21). Combinations of up to four of these possible oxidases occur in bacteria [11], suggesting terminal branching of the respiratory system. Despite this, there have been few studies of terminal branching to O_2 of the respiratory systems of prokaryotic organisms [14]. Perhaps the best studied system has been that of *Azotobacter vinelandii*, which may be a special case because of the oxygen scavenging requirement for protection of nitrogenase [15–17]. Most bacterial respiratory systems are highly sensitive to cyanide [18], though cyanide insensitivity has been observed in some species [14, 15, 19, 20].

In this paper we demonstrate the presence of a terminally branched respiratory system to O_2 in the marine bacterium *Beneckea natriegens* with branching, unlike those of the eukaryotic organisms mentioned above, occurring after the site of action of antimycin A. Previous studies from this laboratory have shown that this bacterium contains a complex respiratory system with four, or possibly five, CO-binding cytochromes, including an unusual high potential, *c*-type cytochrome, each of which could be acting as a terminal oxidase [21, 22].

MATERIALS AND METHODS

Bacteria

B. natriegens strain 111 [23] was grown at 35 °C with high aeration on the DL-lactate, minimal salts medium containing 0.4 M NaCl as described previously [21], with harvesting in the stationary phase (15 h growth). Cells were disrupted by

sonication and the cell free extract, after low-speed centrifugation to remove the debris, was fractionated into particulate and supernatant fractions by centrifugation at 150 000 $\times g$ for 90 min. The particles were washed once in 10 mM $MgCl_2$–50 mM Tris–HCl (pH 7.5) before resuspension in the same buffer.

Oxidase activities

Respiration was measured polarographically at 35 °C in an O_2 electrode (Rank Bros., Bottisham, Cambridge). The reaction mixture contained 2.9 ml of 50 mM Na_2HPO_4–NaH_2PO_4 (pH 7.8) buffer and a suitable concentration of cell free extract, particulate or supernatant fraction protein. The reaction was initiated by the addition of 0.1 ml substrate to give final concentrations of 2.5 mM NADH, 30 mM succinate, 30 mM D(−)-lactate, 30 mM L(+)-lactate or 1.5 mM ascorbate plus 1.0 mM *N,N,N',N'*-tetramethylphenylenediamine (TMPD). The ascorbate–TMPD oxidase was corrected for the low non-enzymatic oxidation of ascorbate–TMPD. Water-insoluble inhibitors were added in 50 μl methanol; control experiments showed that 50 μl methanol had no effect on respiration. KCN was made up in aqueous solution at pH 7.8. Inhibitors were prepared fresh for each experiment.

The apparent K_m for O_2 was obtained by measuring O_2 uptake at varying initial O_2 concentrations. The electrode chamber, containing 2.9 ml of buffer, was fitted with a serum cap through which were inserted one long and one short syringe needles; the tip of the shorter needle was above the reaction solution. N_2 was bubbled into the reaction chamber, until the desired O_2 concentration was obtained, as indicated on the recorder. By using a recorder with stepped inputs in the range 1–100 mV and changing one of the stepped resistors (5 $\times$ 470 Ω) of the Rank Bros. polarising circuit unit to 1000 Ω, full scale deflection on the recorder could be obtained for initial O_2 contents from air-saturated down to 1.0 μM. Some drift was noticed at low initial O_2 concentrations due to gas leakage into the chamber but this was usually low enough to be neglected, and could be easily corrected for when required.

A peak-to-peak noise level of less than 2 % full scale deflection was obtained at an initial 1.0 μM O_2 concentration. Output from the electrode with respect to O_2 concentration was linear down to at least 1.0 μM O_2. The experiments were performed by reducing the O_2 content of the buffer and, after allowing the output to stabilize, starting the reaction by addition from a microsyringe of 0.1 ml of particles and substrate, which had been preincubated long enough to have gone anaerobic.

Chemicals

CO and high purity nitrogen were obtained from the British Oxygen Company; NADH, phenazine methosulphate (PMS), 2,6-dichlorophenolindophenol (DCIP), succinate, D(−)-lactate, and L(+)-lactate were obtained from Sigma Chemical Company; TMPD was obtained from Eastman Kodak Company. All other reagents were the finest grade available and glass distilled water was used throughout.

RESULTS AND DISCUSSION

Distribution of respiratory activities

Table I shows the distribution of NADH, succinate, D(−)-lactate, L(+)-lactate and ascorbate–TMPD oxidase activities in the cell-free extract, particulate and

TABLE I

DISTRIBUTION OF OXIDASE ACTIVITIES

The assay method is described in the text.

Substrate	Oxygen uptake (μatom/min per mg protein)		
	Cell-free extract	Particles	Supernatant
NADH	0.168	0.308	0.045
Succinate	0.090	0.211	0.003
L(+)-lactate	0.035	0.106	0.003
D(−)-lactate	0.072	0.192	0.005
Ascorbate+TMPD	0.960	2.605	0.902

supernatant fractions. The activities are all 2 or 3 times greater in the particulate fraction than the cell free extract. Apart from some ascorbate–TMPD oxidase there is little oxidase activity in the supernatant fraction. NADH oxidase activity in the particulate fraction was consistently 50–100 % greater than succinate oxidase, whilst the rate of oxidation of ascorbate–TMPD was 7–12 times greater than NADH oxidase.

The assay of NADH, succinate, L(+)-lactate and D(−)-lactate dehydrogenase activities using DCIP or DCIP plus PMS [24] showed that they are also located mainly in the particulate fraction (Weston, J. A., unpublished). No D(−)-lactate or L(+)-lactate dehydrogenase activities could be found using NAD^+ or $NADP^+$ as acceptors, suggesting that these dehydrogenases are NAD^+ and $NADP^+$ independent.

The distribution of the oxidase and dehydrogenase activities mainly in the particulate fraction corresponds to the location of the cytochromes in the particulate fraction [22]. The location of some CO-binding *c*-type cytochrome in the supernatant fraction [21] may explain the significant ascorbate–TMPD oxidase activity remaining in this fraction.

Inhibitor studies

Ascorbate–TMPD oxidase was almost completely inhibited by 10 μM cyanide (Fig. 1). Cyanide inhibition of NADH, succinate, D(−)-lactate and L(+)-lactate oxidases was biphasic (Fig. 2). These oxidases were 10–50 % inhibited by 5 μM cyanide, with no further inhibition until the cyanide concentration was greater than 0.5 mM; 10 mM cyanide caused 90–100 % inhibition (Fig. 2). There was a similar pattern of total inhibition of ascorbate–TMPD oxidase by low concentrations of azide (2 mM) and sulphide (10 μM), but only partial inhibition of succinate and NADH oxidases by equal concentrations of these inhibitors. Higher concentrations of sulphide, like cyanide, caused almost complete inhibition of the succinate and NADH oxidases (> 90 % inhibition by 20 mM). Higher concentrations of azide (up to 100 mM) did not cause further inhibition of NADH oxidase, but further inhibited the oxidation of succinate by interaction with succinate dehydrogenase.

These results show that there are two oxidases operative in the particles, one inhibited by low concentrations of cyanide, azide and sulphide and the other inhibited

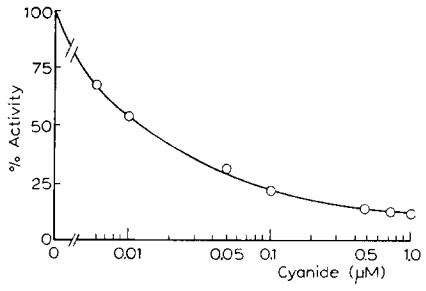

Fig. 1. The inhibition of ascorbate–TMPD oxidase by cyanide. Respiration was measured at 35 °C as described in the text. The reaction mixture was preincubated for 5 min with cyanide before initiating the reaction by addition of 1.5 mM ascorbate plus 1.4 mM TMPD. The uninhibited oxidase activity was 1.9 μatoms oxygen uptake/min per mg protein.

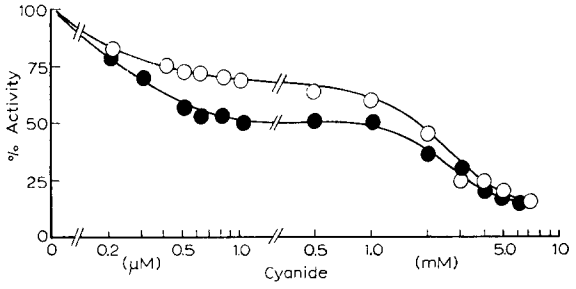

Fig. 2. The inhibition of succinate oxidase by cyanide. The reaction mixture was incubated at 35 °C with cyanide for 5 min before addition of 0.8 mM succinate (● — ●) or 30 mM succinate (○ — ○). The uninhibited oxidase activities were 0.08 and 0.20 μatoms oxygen uptake/min per mg protein, respectively.

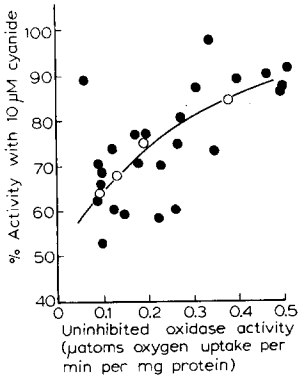

Fig. 3. Effect of oxidase activity on the degree of inhibition by 10 μM cyanide. (●) The degree of inhibition observed for oxidation of NADH, succinate, L(+)-lactate or D(−)-lactate, with each point representing a separate experiment with a different batch of particles. (○) The inhibition observed (from left to right) by L(+)-lactate, D(−)-lactate, succinate and NADH using one batch of particles for all four substrates (average of five experiments).

by higher concentrations of cyanide and sulphide, with ascorbate–TMPD acting only on the cyanide sensitive pathway. Other explanations of results of this type have been thoroughly discussed by Bendall and Bonner [3] with respect to their work on cyanide insensitive respiration in plant mitochondria: their conclusions are equally applicable to the present data. Our results differ from those reported for most eukaryotic systems in that there is no second-phase inhibition by high cyanide concentrations in the latter organisms. Patterson has briefly reported the presence of multiple oxidases of varying cyanide sensitivity in *Pseudomonas aeruginosa* [25].

The degree of inhibition by cyanide in the plateau region of the inhibition curves for the oxidation of NADH, succinate, D(−)-lactate and L(+)-lactate was related to the overall oxidase activity (Fig. 3): the greater the oxidative capacity, the lower the degree of inhibition by cyanide.

Lowering the oxidase rate with any one substrate, by reducing the substrate concentrations below saturating levels, also resulted in a higher degree of inhibition by cyanide in the plateau region (Fig. 2). This suggests that reducing equivalents pass preferentially down the ascorbate–TMPD pathway with the "excess" oxidation occurring via the other pathway. Since ascorbate–TMPD can be oxidized much faster than any of the physiological substrates, the rate-limiting step must be before its site of entry into the system.

Respiration of glucose, D(−)-lactate or L(+)-lactate by intact cells of *B. natriegens* was also resistant to cyanide (70 % activity in the presence of 3 mM cyanide). Growth was not inhibited by inclusion of 1.0 mM cyanide in the medium.

Ascorbate–TMPD oxidase was strongly inhibited by CO (80 % inhibition by 0.5 mM CO). Lowering the rate of ascorbate–TMPD oxidase (by lowering the TMPD concentration) resulted in a decrease in inhibition, and at ascorbate–TMPD oxidase rates similar to the values for oxidation of NADH or succinate there was only 10–15 % inhibition. On the other hand, inhibition by 10 μM cyanide was unaffected by the rate of ascorbate–TMPD oxidation.

NADH and succinate oxidases were found to be inhibited to a variable degree by CO, with 25–55 % inhibition in separate experiments. When 10 μM cyanide or 2 mM azide was also present an increase in inhibition of 5–25 % occurred. It was thus difficult to ascertain the site of action of CO, and its usefulness as an inhibitor was limited (c.f. ref. 15). For NADH and succinate oxidases it probably acts by partially inhibiting both pathways, though mainly inhibiting the cyanide resistant pathway: the extra degree of inhibition caused by cyanide or azide is due to complete blockage of the cyanide sensitive pathway.

The alternative oxidase of eukaryotic mitochondria can be inhibited by hydroxamic acids [2] and by iron chelating agents [3]: inhibition of respiratory activity requires the presence of hydroxamic acid plus cyanide (or antimycin A, as branching occurs before its site of action).

Oxidation of NADH, succinate, D(−)-lactate or L(+)-lactate by the particulate fraction of *B. natriegens* is not inhibited by the hydroxamic acids salicylhydroxamic acid (3 mM) or *m*-chlorobenzhydroxamic acid (3 mM), nor by the iron-chelating agents α,α-dipyridyl, 8-hydroxyquinoline or thiocyanate. There is no increase in the inhibition caused by 10 μM cyanide or 2 mM azide when salicylhydroxamic acid or *m*-chlorobenzhydroxamic acid are added. Ascorbate–TMPD oxidase is also unaffected by the hydroxamic acids and iron-chelating agents.

Oxidation of NADH, succinate, D(−)-lactate or L(+)-lactate but not ascorbate–TMPD is inhibited by antimycin A (50 % by 20 μg/ml and > 90 % by 100 μg/ml).

Antimycin A, therefore, acts before the site of branching in *B. natriegens*, unlike the situation found in eukaryotic microorganisms and plant mitochondria that contain an alternate oxidase [1, 3–10], where branching occurs at the flavoprotein level. Furthermore, the lack of inhibition of the *B. natriegens* cyanide-resistant oxidase by hydroxamic acids [1] suggests that the nature of the alternate oxidase of this organism may be different to the other alternate oxidases.

Catalase activity and H_2O_2 formation

Lenhoff and Kaplan [27] have claimed that terminal branching of the respiratory chain of *Pseudomonas fluorescens* occurs, with one branch forming H_2O_2 which is then oxidised via a peroxidase, utilising reducing equivalents from the other branch of the respiratory system. *Pseudomonas ovalis* Chester may also form peroxide as a major end product of respiration [28], as does *Ascaris lumbricoides* [29]. It is thus possible that one of the respiratory branches of *B. natriegens* may involve peroxide rather than water formation from oxygen and that this is then utilized by the other pathway via a peroxidase.

Measurements of peroxide formation [26] indicate that peroxide is not a product of respiration by the particulate fraction of *B. natriegens*. Catalase activity is found mainly in the supernatant fraction, but there is some in the particulate fraction, thus complicating assays for peroxide formation, and production of traces of peroxide [30] may be obscured; however, peroxide is not a major end product of respiration. Furthermore, the molar stoichiometry of NADH disappearance to O_2 uptake indicates a 2 : 1 relationship during the progress of the NADH oxidase reaction (without, or plus 10 μM, 500 μM or 5 mM cyanide). Catalase activity is totally inhibited by 500 μM cyanide. The particulate fraction derived from cells harvested in the early log phase contains no catalase activity but has similar oxidase activities and inhibitor patterns to stationary-phase particles: again no peroxide formation can be detected.

The affinity of O_2

Meyer and Jones [31, 32] have recently suggested that there are different efficiencies of phosphorylation related to respiration via various types of bacterial cytochrome oxidases at differing O_2 tensions. It is therefore possible that one pathway of respiration of *B. natriegens* is able to act at lower O_2 concentrations than the other.

In order to test whether one pathway had a greater affinity for oxygen than the other, experiments were done to measure the apparent K_m for O_2 of the separate pathways. The affinity of bacterial oxidases for O_2 has previously been measured by determining the "critical O_2" concentration [32–34]. In this technique a single O_2 uptake trace is followed through the point at which the trace deviates from linearity (zero order kinetics) to give a curve (first order kinetics): analysis of the latter enables estimation of the K_m for O_2. Preliminary experiments in our laboratory showed that, unless an electrode plus recorder of exceptionally rapid response time is used, incorrect values are obtained except at extremely low rates of initial O_2 uptake (when gas leakage problems might occur). We have therefore measured the affinities for O_2 using varying starting O_2 concentrations and measuring the initial uptake for each

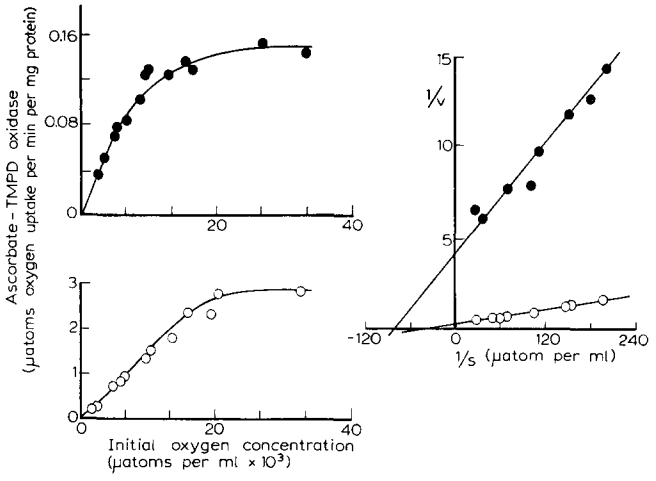

Fig. 4. The apparent K_m for oxygen via the cyanide-sensitive pathway, using 1.5 mM ascorbate plus 1.4 mM TMPD (○) or 0.4 mM TMPD (●) as substrate. The technique used is described in the text.

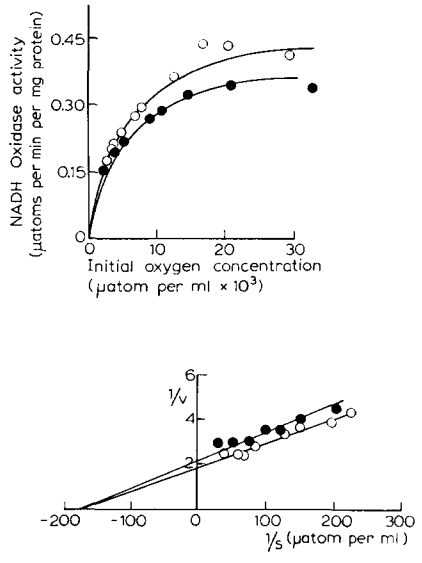

Fig. 5. The apparent K_m for oxygen via the cyanide-resistant pathway or both pathways, using a 5 mM NADH as substrate in the presence of 10 μM cyanide (● — ●) or without cyanide (○ — ○), respectively. The method is described in the text.

condition.

The cyanide-sensitive pathway was assayed using ascorbate–TMPD (Fig. 4), the cyanide-resistant pathway was assayed using NADH plus 10 μM cyanide (Fig. 5) or succinate plus 10 μM cyanide, and both pathways were assayed by NADH (Fig. 5) or succinate in the absence of inhibitor. In each case reciprocal plots show simple Michaelis–Menten kinetics. In a series of experiments, the cyanide-sensitive pathway

TABLE II

THE AFFINITY FOR O_2 OF THE DIFFERENT BRANCHES OF THE RESPIRATORY SYSTEM

The values given are each obtained from separate experiments on different samples of particles.

Pathway	Substrate	Apparent K_m for O_2 (μM)
Cyanide-sensitive pathway	1.5 mM ascorbate–1.4 mM TMPD	10.0, 9.4, 17.0
	1.5 mM ascorbate–0.4 mM TMPD	8.5, 8.0
Cyanide-resistant pathway	2.5 mM NADH (+ 10 μM KCN)	2.2, 2.8, 3.0
	30 mM succinate (+ 10 μM KCN)	4.0, 3.1
Both pathways	2.5 mM NADH	2.2, 2.8, 3.5, 3.7
	30 mM succinate	3.3, 3.3, 4.4, 3.1

had an apparent K_m value in the range 8.0 to 17.0 μM O_2 (average 10.6 μM) (Table II). The apparent K_m value for O_2 of the cyanide resistant pathway was 2.2 to 4.4 μM O_2 (average 3.0 μM). The apparent K_m value for O_2 when both pathways were operative was, as expected, that of the pathway with the greater affinity for O_2 (the cyanide-resistant pathway). The apparent K_m values O_2 were not greatly affected by lowering the oxidase activities of the pathways by using non-saturating substrate concentrations (Fig. 4 and Table II).

Thus, the cyanide-resistant pathway has about a 3.5-fold greater affinity for O_2 than the other pathway. However, these values are probably not sufficiently divergent for one pathway to have a specific role as the sole oxidant at low prevailing oxygen concentrations (c.f. ref. 32). The apparent K_m value for O_2 of the cyanide-resistant pathway is comparable to values previously found for other bacterial systems [32, 34] but the cyanide-sensitive pathway has a lower affinity for O_2.

The presence of a mixture of inverted and right side out vesicles of the particulate fraction of *B. natriegens* could lead to an alternative, though less probable interpretation of the above results. For example, if NADH and succinate are oxidized only by inverted vesicles (since the dehydrogenases are probably located on the inside of the cytoplasmic membrane), ascorbate–TMPD oxidized only by right side out vesicles and cyanide acts only on one side of the membrane with low permeability across the membrane, then differential sensitivity to cyanide inhibition might occur. However, the biphasicity of the cyanide inhibition curves, the rate dependancy of the inhibition by 10 μM cyanide, the simple Michaelis–Menten kinetics for the apparent K_m values for O_2 and the different apparent K_m values for O_2 for ascorbate–TMPD compared to NADH and succinate would argue against such an interpretation. Furthermore, a recent report suggests that sonic vesicles of Gram-negative bacteria are largely inverted [35].

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