THE INHIBITION OF *AZOTOBACTER VINELANDII* TERMINAL OXIDASES BY CYANIDE

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1. Introduction

The complex, branched respiratory system of the nitrogen-fixing aerobe *A. vinelandii* contains 3 spectrally-distinct cytochromes which can bind carbon monoxide and may therefore be capable of acting as terminal oxidases, viz cytochromes *o*, a_1, and a_2 [1, 2]. Carbon monoxide action spectra of respiring whole cells or isolated respiratory membranes of *A. vinelandii* have indicated that cytochrome a_2 (*d*) is the major functional oxidase associated with the oxidation of physiological substrates, whereas oxidation of the high potential, artificial substrates ascorbate-TMPD* and ascorbate-DCPIP occur via cytochrome oxidases a_1 and *o* respectively [2, 3]. Very recently, however, the ability of cytochromes a_1 and *o* to act as terminal oxidases in *A. vinelandii* has been questioned, mainly on the grounds of their low redox potentials [4].

This paper described the effect of the classical cytochrome oxidase inhibitor, potassium cyanide, on the respiratory activity of *A. vinelandii* membranes oxidising a variety of substrates. The results support the concept that this organism contains three cytochrome oxidases which can be functionally isolated by the use of different electron donors and which can be distinguished by their quantitatively and/or qualitatively different responses to cyanide.

* *Abbreviations*: TMPD, tetramethylphenylenediamine; DCPIP, dichlorophenolindophenol.

2. Materials and methods

A. vinelandii was batch-cultured at high aeration on nitrogen-free medium and harvested just past the turnover point into oxygen-limited growth [5]; phosphorylating respiratory membranes were prepared and assayed for oxygen uptake and cytochrome content as described previously [5–7]. The concentration of cytochrome a_1 was calculated from the absorption at approximately 595 nm in the reduced *minus* oxidised difference spectrum using the millimolar extinction coefficient for cytochrome a_1 $m\mathrm{M}\epsilon = 4.6$; [8]). Protein was assayed by the modified biuret method [9]. Potassium cyanide was freshly prepared for each set of experiments by neutralisation with 1 N acetic acid.

The terminal oxidases were regarded as catalysing bi-substrate reactions involving reducing equivalents and molecular oxygen [10]. Oxygen uptake rates were measured at high (i.e. essentially saturating) oxygen concentrations and the concentration of the reduced substrate was varied either directly (by varying the concentration of the electron mediators TMPD or DCPIP) or indirectly (through the oxidation of several physiological substrates by respiratory chain dehydrogenases of different activity [7, 11, 12].

3. Results and discussion

The oxidation of physiological substrates by *A. vinelandii* respiratory membranes (predominantly via cytochrome oxidase a_2 [2, 3] was substantially less sensitive to cyanide than was the oxidation of the

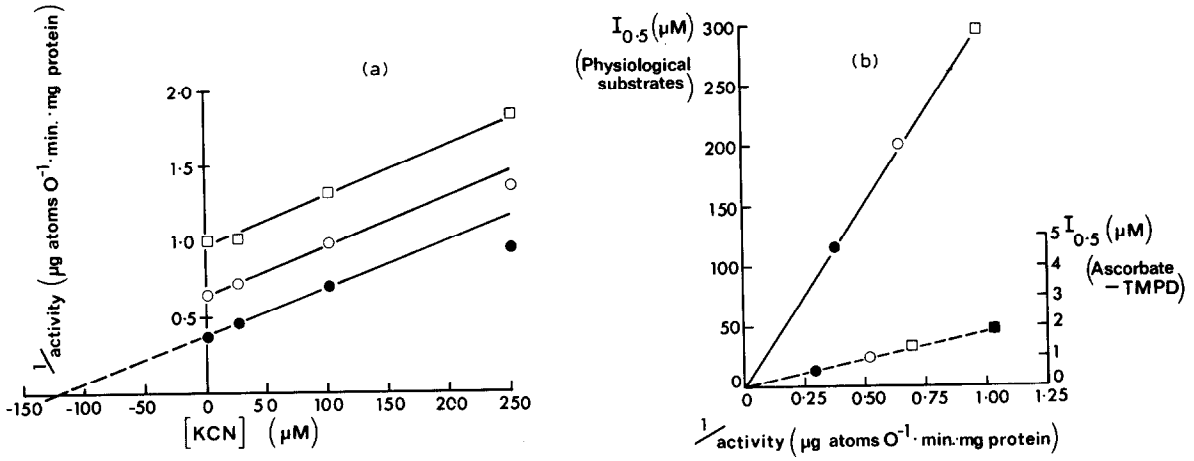

Fig. 1. The effect of cyanide on the oxidation of physiological substrates by *A. vinelandii* respiratory membranes. Oxygen uptake was measured polarographically at 30°C under state 3 conditions as described previously [7]. Respiratory membranes *plus* reaction mix were incubated with cyanide for 1 min prior to the addition of substrate; initial oxygen uptake rates were recorded: (a) Dixon plot ($^1/_v$ *versus* [cyanide]); substrates, NADH (□); malate (○) and NADH *plus* malate (●); 0.322 mg protein/2.4 ml assay volume; (b) $I_{0.5}$ *versus* $^1/_v$ (———) substrates: NADH (□); malate (○) and NADH *plus* malate (●); data from fig. 1(a). (– – – –) substrate: ascorbate *plus* varying concentrations of TMPD, 0.44 mM (■), 0.71 mM (□), 1.77 mM (○), infinite (●); data from fig. 2(b).

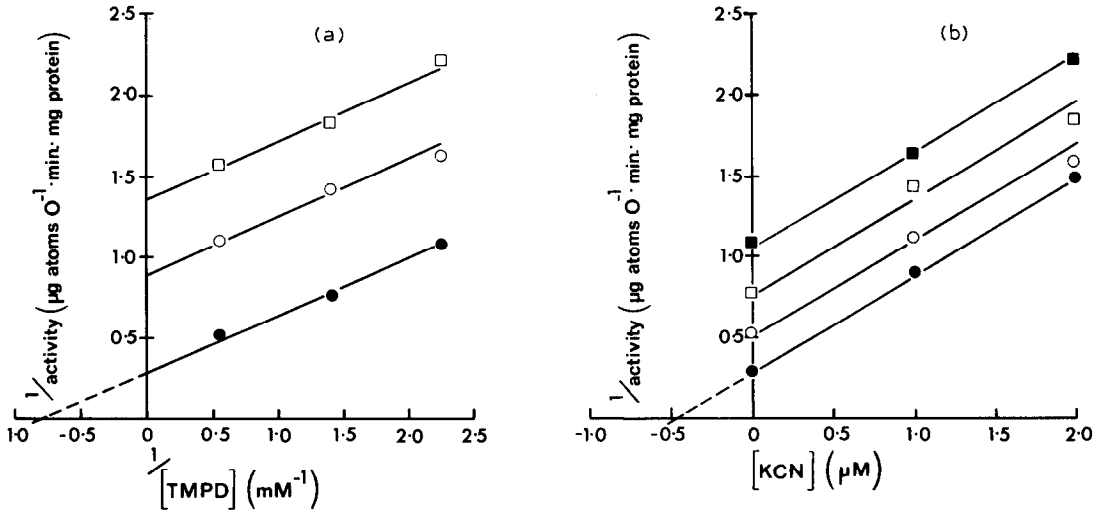

Fig. 2. The effect of cyanide on the oxidation of ascorbate-TMPD by *A. vinelandii* respiratory membranes. Cyanide inhibition of oxygen uptake was measured as for fig 1: (a) Lineweaver–Burk plot ($^1/_v$ *versus* ^{1}/[TMPD]) at varying concentrations of cyanide: nil (●); 1 μM (○), 2 μM (□); 0.290 mg protein/2.4 ml assay volume; (b) Dixon plot ($^1/_v$ *versus* [cyanide]) at varying concentrations of TMPD: 0.44 mM (■); 0.71 mM (□); 1.77 mM (○); infinite (●); 0.290 mg protein/2.4 ml assay volume.

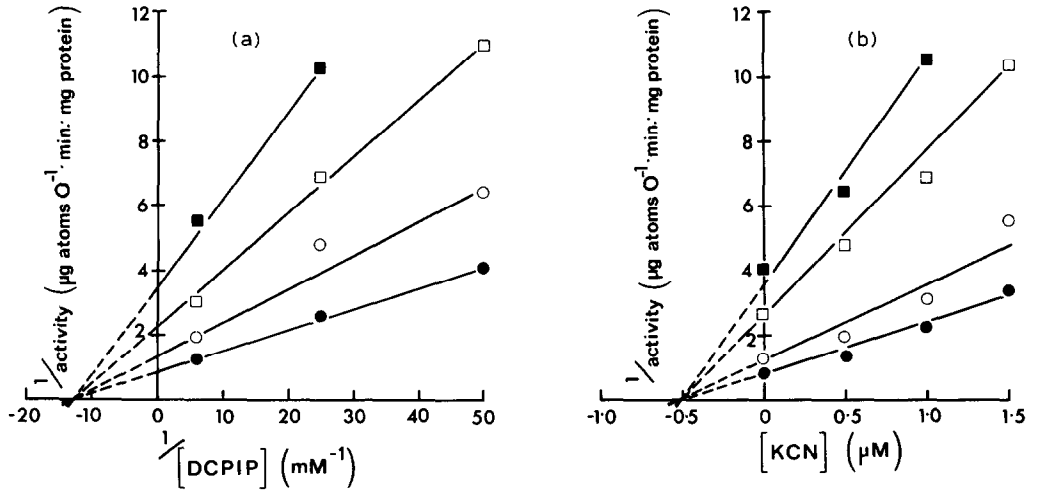

Fig. 3. The effect of cyanide on the oxidation of ascorbate-DCPIP by *A. vinelandii* respiratory membranes. Cyanide inhibition of oxygen uptake was measured as for fig. 1: (a) Lineweaver–Burk plot ($^1/v$ *versus* $^1/$[DCPIP]) at varying concentrations of cyanide: nil (•); 0.5 µM (○); 1.0 µM (□); 1.5 µM (■); 0.639 mg protein/2.4 ml assay volume; (b) Dixon plot ($^1/v$ *versus* [cyanide]) at varying concentrations of DCPIP: 20 µM (■); 40 µM (□); 160 µM (○); infinite (•); 0.639 mg protein/2.4 ml assay volume.

artificial, high potential substrates ascorbate-TMPD and ascorbate-DCPIP (via cytochromes a_1 and o respectively [3]. These result agree with those of earlier investigations [2, 5, 13–15]. Thus, at cyanide concentrations greater than approximately 5 µM, oxygen uptake with NADH, malate or NADH *plus* malate as substrate, was considered to occur solely via cytochrome a_2. Under these conditions, linear parallel lines were observed in plots of $^1/v$ *versus* [cyanide] (fig. 1 (a)) and indicated pure, uncompetitive inhibition of cytochrome a_2. This was confirmed when $I_{0.5}$ was plotted against $^1/v$ for each substrate and a straight line which passed through the origin was obtained (fig. 1 (b), solid line). For uncompetitive inhibition $I_{0.5} = K_i$ only when $v = V_{max}$. Thus, since the observed oxygen uptake rate with NADH + malate as substrate (2.71 µgatom oxygen·min^{-1}·mg protein^{-1}) probably reflected the maximum rate of electron flux through the central Q-b_1 region of the respiratory chain rather than the V_{max} of the oxidase a_2 [7], the K_i of cyanide for cytochrome a_2 was probably considerably less than the observed minimum $I_{0.5}$ value of 115 µM. Although the aerobic steady state reduction level of cytochrome a_2 is considerably lower than that of cytochrome b_1 [2], it is difficult to envisage a V_{max} for cytochrome a_2 greater than 20 µgatom oxygen·min^{-1}·mg protein^{-1} (equivalent to a turnover number of approximately 1900 e·sec^{-1}) and the K_i is therefore unlikely to be less than 15 µM cyanide.

The inhibition by cyanide of ascorbate-TMPD oxidation (cytochrome a_1) was also of the pure, uncompetitive type as indicated by the family of linear, parallel lines which were obtained in plots of $^1/v$ *versus* $^1/$[TMPD] (fig. 2 (a) and of $^1/v$ *versus* [cyanide] (fig. 2 (b)). This was confirmed when $I_{0.5}$ was plotted against $^1/v$ and a straight line which passed through the origin was obtained (fig. 1 (b); ———); the slope of this line was considerably less than that obtained during the oxidation of physiological substrates and reflected that much greater sensitivity to cyanide of cytochrome oxidase a_1 than cytochrome a_2. At an infinite concentration of TMPD the V_{max} was 3.50 µgatom oxygen·min^{-1}·mg protein^{-1} (equivalent to a turnover number of cytochrome a_1 of 865 e·sec^{-1}) and the K_i ($\equiv I_{0.5}$) for cyanide was 0.46 µM, i.e. at least one order of magnitude less than that of cytochrome a_2. In contrast, cyanide inhibited the oxidation of ascorbate-DCPIP (cytochrome o) in a pure, non-competitive manner (fig. 3 (a), (b)). At an infinite concentration of DCPIP the V_{max} was 1.16 µgatom oxygen·min^{-1}·mg protein^{-1} (equivalent to a turnover number for cytochrome o of 357 e·sec^{-1}) and the

K_i for cyanide was 0.51 μM, i.e. almost identical to that of cytochrome a_1.

The effect of cyanide on the oxidation by *A. vinelandii* respiratory membranes of physiological substrates, ascorbate-TMPD or ascorbate-DCPIP is clearly quantitatively and/or qualitatively different and suggests the presence of three functional terminal oxidases (identified by carbon monoxide action-spectra as cytochromes a_2, a_1, and *o* respectively [13]). The pure, uncompetitive inhibition of cytochrome oxidases a_1 and a_2 by cyanide (albeit with widely different K_i values) suggests that this inhibitor combines principally with ES to yield a stable oxidase–inhibitor complex. However, whether ES represents the reduced [11, 16] or oxygenated [17] forms of the oxidases remains in doubt. Pure uncompetitive inhibition of all three oxidases was observed using carbon monoxide (C.W. Jones, unpublished data), an inhibitor which combines only with the reduced forms. The pure, non-competitive inhibition of cytochrome *o* affords little clue to the mode of action of cyanide on this oxidase.

A. vinelandii cytochrome oxidases *o* and a_1 are clearly much more sensitive than cytochrome a_2 to cyanide, a conclusion which has already been drawn for at least two other *o* a_1 a_2 multiple-oxidase systems [18, 19]. Cyanide inhibition kinetics similar to those found with *A. vinelandii* have also been observed for cytochrome oxidases a_1 and *o* from other bacteria (D.J. Meyer and C.W. Jones, unpublished data).

The low redox potentials of *A. vinelandii* cytochromes *o* and a_1 [4] obviously offer no barrier to these cytochromes acting as terminal oxidases, but would imply that they might terminate respiratory chains of low oxidative phosphorylation efficiency. There is good evidence to suggest that this may be true for cytochrome oxidase a_1 [5, 20], but there appears to be conflicting evidence in the case of cytochrome *o* [5, 20–22].

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