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THE RESPIRATORY CHAIN OF *AZOTOBACTER VINELANDII*

III. THE EFFECT OF CYANIDE IN THE PRESENCE OF SUBSTRATES

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SUMMARY

1. Cyanide (100 μM) causes a rapid disappearance of the band (648 nm) of oxidized cytochrome *d* in particles of *Azotobacter vinelandii* oxidizing NADH. The rate of disappearance of the band can be related to the rate of inhibition of the oxygen consumption.

2. The kinetics of the disappearance of the 648-nm band of cytochrome *d* with excess cyanide in the presence of substrates deviate from first-order, indicating that at least two conformations of the enzyme are involved.

3. The rate of binding of cyanide to cytohrrome *d* increases the larger the rate of turnover of the oxidase. From this it is concluded that cyanide binds preferentially to the enzymically active oxidized conformation of cytochrome *d*.

4. The instantaneous inhibition of the oxidation of ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) by cyanide is related neither to the binding of cyanide to cytochrome *d* as determined spectrophotometrically, nor to the rate of inhibition of the oxidation of NADH. This indicates that different oxidases are involved in the oxidation of NADH and of ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), in line with the conclusion of Jones and Redfearn (1967) Biochim. Biophys. Acta 143, 340–353.

5. From experiments in which the change in redox states of cytochrome b_1 and *c*-551 were related to the rate of binding of cyanide to cytochrome *d*, it is concluded that the cytochromes b_1 and *d* are located in the main pathway to oxygen, whereas cytochrome *c*-551 functions in the branch via cytochrome *o*.

6. After prolonged incubation of particles with cyanide in the presence of NADH a residual activity (5%) was found in which a *b*-type cytochrome is involved. This shows the existence of a third but minor pathway to oxygen.

Abbreviations: HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DCIP, 2,6-dichlorophenol indophenol.

INTRODUCTION

Microspectroscopic studies of aerobically-grown micro-organisms have revealed the disappearance of the band of oxidized cytochrome *d* upon addition of cyanide without the appearance of the band of the reduced enzyme [1–6]. In a previous paper [6], the kinetics of the binding of cyanide to oxidized cytochrome *d* in particles of *Azotobacter vinelandii* were studied. It was proposed that cyanide binds preferentially to the enzymically active conformation of oxidized cytochrome *d*, denoted cytochrome d_x, which is in equilibrium with the non-active conformation absorbing at 648 nm [6, 7].

In our laboratory Van Buuren et al. [8–11] showed that the rate of cyanide binding to cardiac cytochrome *c* oxidase is affected by the redox state of the enzyme. Some indications in the literature suggested that the rate of binding of cyanide to cytochrome *d* also depends on the presence of substrates. In a previous paper [6] we showed that the reaction of cyanide with cytochrome *d* in oxidized particles is very slow. Furthermore, it could be demonstrated, in accordance with the results of Negelein and Gerischer [1], that cyanide does not affect the spectrum of reduced cytochrome *d*, but when oxygen was introduced to reduced particles a fast binding of cyanide to cytochrome *d* was observed [6]. The same observation was made by Repaske and Josten [12] who found a trough at 648 nm in the difference spectrum of particles of *A. vinelandii* incubated with NADH, oxygen and cyanide minus oxidized particles incubated with cyanide alone. This and also similar experiments by Eilermann [13] suggested a faster binding of cyanide to cytochrome *d* in the presence of substrates.

Jones and Redfearn [14] proposed that cyanide is an effective inhibitor of cytochrome *o* but that it is less sensitive towards cytochrome *d*, thus explaining the differences in the inhibition of the oxidation of ascorbate plus 2,6-dichlorophenol indophenol (DCIP) and NADH by cyanide. However, no spectroscopic studies were carried out by them to support these suggestions. We have, therefore, investigated the effect of cyanide on the rate of the spectral changes of cytochrome *d* in particles of *A. vinelandii* during turnover conditions with various substrates. Furthermore, the relationship of the amount of enzymically active oxidized conformation of cytochrome *d* to the rate of binding of cyanide was studied by varying the respiratory activity either by activation or inhibition.

METHODS

Phosphorylating particles were prepared as described by Pandit-Hovenkamp [15] and stored in 40 mM phosphate buffer (pH 7.2), 0.25 M sucrose, 40 mM KCl at 77 °K. When measuring spectra, the oxygen consumption or redox states of the cytochromes, particles were diluted with 30 mM phosphate buffer (pH 7.6), 5 mM $MgCl_2$, 1 mM EDTA.

Spectra were recorded on a Perkin–Elmer spectrophotometer Model 356. In order to study some spectroscopic properties of cytochrome *d*, difference spectra were taken with in the reference cell cyanide-incubated particles, in which cytochrome *d* was present as cyano-cytochrome *d*. Cyano-cytochrome *d* was prepared by the action of 2 mM cyanide in the presence of ascorbate (4 mM), *N*,*N*,*N*′,*N*′-tetramethyl-*p*-

phenylenediamine (TMPD) (0.25 mM) and oxygen. As shown earlier [6] the spectrum of cyanide-treated particles is almost flat between 600 and 700 nm. The spectra of cytochrome *d* were normalized as described previously [6, 7], taking 600 and 700 nm as reference points. The intensity of the oxidized absorption band (648 nm) was calculated from these spectra using the difference between $A_{648\,nm}$ and $A_{625\,nm}$. In order to follow simultaneously the oxygen concentration the sample cuvette was equipped with a Clark-type electrode. Longer periods of aerobic incubation with cyanide in the presence of reducing substrate were achieved using a medium saturated with oxygen and maintaining the temperature at 16 °C. When the medium became anaerobic, 10 μl of a 1 % solution of H_2O_2 was added to the reaction mixture in which 20 μg catalase/ml was present. Due to leakage of oxygen via the top-stirrer of the specially constructed apparatus, the recording of the oxygen consumption is less reliable for very accurate activity measurements. Therefore, the rate of inhibition of the oxygen consumption by cyanide was checked in a separate experiment on a Gilford oxygraph, equipped with a first derivative device.

The changes in the redox state of the cytochromes *c*-551 and b_1 were followed using an Aminco–Chance dual-wavelength spectrophotometer, at the wavelength pairs 551–536 nm and 559–573 nm, respectively. In these experiments the oxygen concentration in the cell was also followed by means of a Clark-type electrode and longer periods of aerobic incubation were obtained as described above.

In order to study the rate of binding of cyanide to cytochrome *d* under various turnover conditions, the rate of oxidation of NADH or malate were regulated either by inhibition with 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), which brings about an increased intensity of the absorbance at 648 nm of cytochrome *d* (unpublished), or by activation of the particles. Activation of the oxidation of NADH was achieved by addition of 1 mM NADH to a concentrated suspension of particles (about 25 mg protein/ml), followed by bubbling air for 30 s. Such preincubated particles have double the NADH oxidation activity of non-treated particles.

Chemicals were Analar grade, mainly obtained from British Drug Houses, except those used for the culture medium which were less highly purified.

RESULTS

Binding of cyanide in the presence of NADH

The difference spectrum of oxidized particles of *A. vinelandii*, measured against particles preincubated with cyanide (cf. Methods), is characterized by an absorption band at 648 nm. Addition of cyanide to particles oxidizing NADH results in a decrease of the intensity of this band [6]. The kinetics of this process, determined by monitoring spectra with short intervals, are illustrated in Fig. 1, where the logarithm of the absorbance at 648 nm is plotted as a function of time. It can be seen that the initial rate of the decrease in absorbance at 648 nm depends on the cyanide concentration, and that the kinetics deviate from first-order. The latter observation suggests that an intermediate [8–11] is involved in the reaction of cyanide with cytochrome *d* [6]. The apparent first-order rate constant of the binding of cyanide (100 μM), as calculated from the tangent of the initial part of the curve (Fig. 1), has a higher value in the presence of substrates ($3 \cdot 10^{-2}\ s^{-1}$ at 16 °C) than with the same concentration of cyanide alone ($1.5 \cdot 10^{-4}\ s^{-1}$ at 32 °C) [6]. This indicates that the reaction of

cyanide with cytochrome *d* is faster during turnover conditions, i.e. under conditions where the enzymically active oxidized conformation of cytochrome *d* (cytochrome d_x) is present at a higher concentration [7].

It was reported [14] that a high concentration of cyanide (2 mM) causes an instantaneous inhibition of the oxidation of NADH. However, at lower cyanide concentration (0.1 mM) an increase in the rate of inhibition of the oxygen consumption was found in the experiment of Fig. 1 in which the oxygen concentration was measured simultaneously (not shown). Furthermore, it was found that the minimal activity (5%) was reached when the 648-nm band became undetectable. This indicates that the decrease of the absorption band of oxidized cytochrome *d* at 648 nm and the inhibition of the oxygen consumption are in some way related. The residual activity (5%) was resistant to prolonged incubation with cyanide even at higher concentrations of cyanide, pointing to the presence of a cyanide-insensitive side-path to oxygen.

If cytochrome d_x is the species which reacts fast with cyanide [6], it may be expected that particles inhibited by HQNO, and thus giving rise to a decrease of the d_x conformation (unpublished), will show a slower binding of cyanide to cytochrome *d*. This is confirmed by the experiments of Fig. 2, where the decrease of the absorption at 648 nm is monitored as a function of time after the addition of cyanide to HQNO-inhibited particles oxidizing NADH. As can be seen from the first-order plot (Fig. 2),

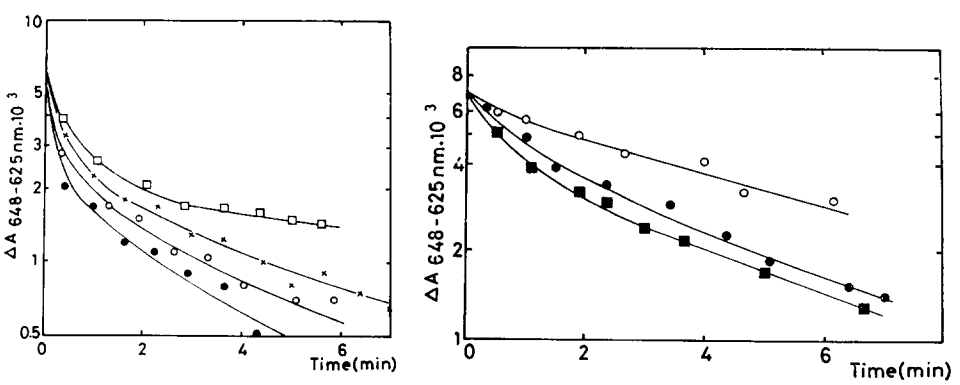

Fig. 1. Time course of the reaction of cyanide with cytochrome *d* in particles oxidizing NADH, plotted semilogarithmically. Particles (0.62 mg protein/ml, Prepn 1*) were suspended in an oxygen-enriched phosphate–$MgCl_2$–EDTA solution (pH 7.6) at 16 °C, containing 20 μg catalase/ml. After preincubation with NADH (2.5 mM) for 20 s, the reaction was started by the addition of the appropriate amount of cyanide to the sample cuvette. The reference cell contained a particle suspension in which cytochrome *d* was cyanide-liganded (see Methods). Spectra were scanned between 600 and 700 nm with a sweep of 25 s. Intensities of the absorbance of cytochrome *d* at 648 nm were calculated from normalized spectra, as described in Methods and refs 6, 7. Oxygen concentration was measured simultaneously, but not shown. In order to maintain the medium aerobic 10 μl H_2O_2 (1%) was added, when necessary. Cyanide concentration: □–□, 100 μM; ×–×, 200 μM; ○–○, 300 μM; ●–●, 500 μM.

Fig. 2. Time course of the reaction of cyanide with cytochrome *d* in particles oxidizing NADH in the presence of HQNO, plotted semilogarithmically. Conditions and procedure as described in Fig. 1. The addition of 10 μM HQNO causes an inhibition of the oxygen consumption of 85%. Cyanide concentration: ○–○, 50 μM; ●–●, 100 μM; ■–■, 200 μM.

* Particles obtained from different batches of bacteria have been numbered accordingly.

the apparent rate constant for binding of cyanide (100 μM) calculated from the tangent at the time of addition, decreases about 4 times ($k = 7 \cdot 10^{-3}\ s^{-1}$ at 16 °C) when compared with the value found in the absence of HQNO (Fig. 1). This suggests that the formation of a cytochrome *d*-cyanide complex is dependent on the rate of turnover of cytochrome *d*, thereby affecting the amount of cytochrome d_x.

In accordance with this, the most rapid rate of binding of cyanide to cytochrome *d* was observed with activated particles oxidizing NADH, which showed an intensity of the 648 nm band of 55% that of oxidized particles (not shown). Unfortunately with these particles anaerobiosis was already achieved in the presence of 100 μM cyanide in about 1 min, so that no apparent rate constant could be calculated from successively recorded spectra. Therefore, we studied the binding of cyanide by following the inhibition of the oxygen consumption.

Since the rate of oxidation of cytochrome *d* is extremely rapid when compared to the rate of binding of cyanide, the value of the first derivative of the oxygen consumption is proportional to the concentration of free enzyme. Fig. 3 shows the logarithm of the first derivatives of the oxygen consumption as a function of time after subsequent additions of NADH and cyanide to activated and non-treated particles (activities, 3.4 and 2.2 μatoms $O \cdot min^{-1} \cdot mg^{-1}$ protein, respectively). As can be seen, addition of cyanide (100 μM) causes a decrease of the rate of oxidation which deviates

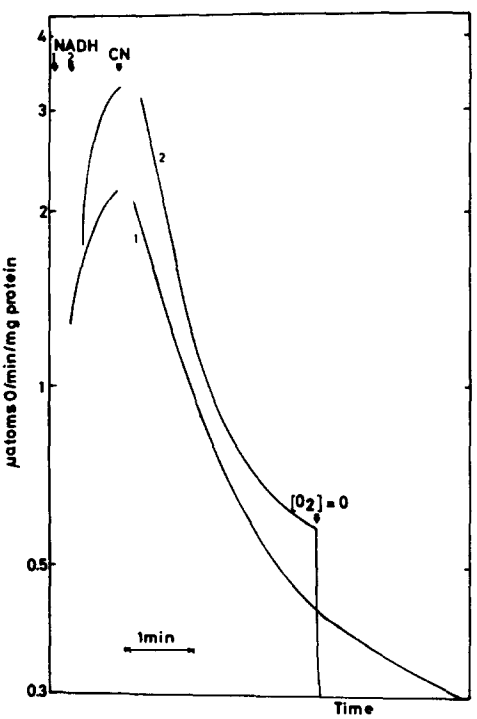

Fig. 3. The effect of cyanide on the first derivative of the oxygen consumption as a function of time in activated and non-treated particles. Particles, 0.9 mg protein/ml, Prepn 2. Reaction temp. 22 °C. For further details see Methods. Arrows indicate addition of NADH (1 mM) or cyanide (100 μM). 1 and 2, non-treated and activated particles, respectively.

from first-order kinetics. It is evident that the value of the apparent rate constant for binding of cyanide, as calculated from the first part of the curve, is slightly greater for activated ($k = 2.1 \cdot 10^{-2}\ s^{-1}$ at 22 °C) than for non-treated particles ($k = 1.5 \cdot 10^{-2}\ s^{-1}$ at 22 °C). This indicates again that the formation of the cytochrome *d*-cyanide complex is more rapid under conditions where more of the enzymically active conformation of cytochrome *d* is present.

Jones and Redfearn [14] studied the effect of cyanide on the redox state of the cytochromes b_1 and *c*-551, in particles oxidizing NADH or succinate. However, they did not report on the changes in the redox state of these cytochromes during longer aerobic incubation, which is accompanied by an increasing inhibition of the respiratory activity (see Fig. 3). Fig. 4 illustrates the change of the redox state of the cytochromes b_1 and *c*-551 as a function of time upon addition of 100 μM cyanide to particles, that are in an activated, non-treated and HQNO-inhibited state and showing various respiratory activities with NADH (2.3, 1.3 and 0.6 μatoms O/min per mg protein, respectively). Under all these conditions the absorbance of cytochrome *c*-551 ($\Delta A_{551-536\,nm}$) increased instantaneously by about 15% upon addition of cyanide. This rapid reduction does not correspond to the inhibition of the oxygen consumption, when measured simultaneously (not shown). The rapid reduction (Fig. 4) is followed by a second phase during which the redox state further increases until nearly 90% of cytochrome *c*-551 is reduced, while the oxygen consumption was inhibited by 95%. In contrast to this the absorbance of cytochrome b_1 ($\Delta A_{559-573\,nm}$) shows only a gradual increase of the redox state until a value of 78% reduction of the dithionite-reduced particle is reached, and the oxygen consumption was maximally inhibited. From these results it is concluded that the binding of cyanide to cytochrome *d*, as shown by the decrease of the absorbance at 648 nm (Figs 1 and 2) and the inhibition of the oxygen consumption (Fig. 3), is reflected by an increase of the redox states of the cytochromes b_1 and *c*-551. Furthermore, as can be expected from previous

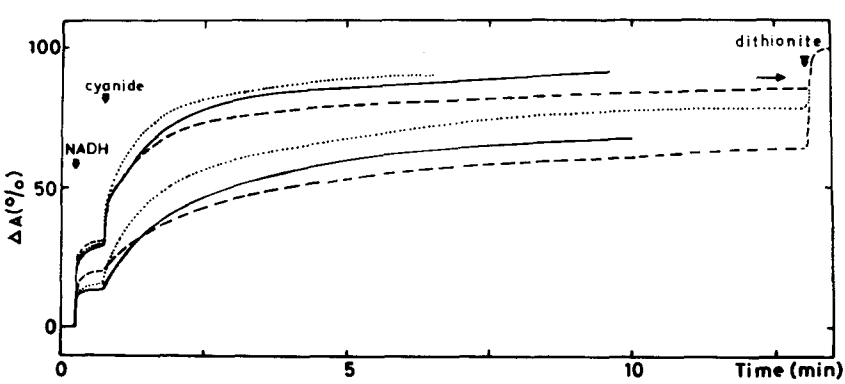

Fig. 4. The effect of cyanide on the redox state of cytochromes b_1 and *c*-551. Particles, 0.44 mg protein/ml, Prep. 3. Conditions and procedure as described in Methods and Fig. 1, temp. 25 °C. Vertical arrows indicate successive additions of 6 mM NADH and 100 μM cyanide. Horizontal arrow indicates redox state of cytochrome b_1 at anaerobiosis. 100% reduction was achieved by the addition of a few grains of $Na_2S_2O_4$. Three upper tracings represent cytochrome *c*-551; three bottom tracings represent cytochrome b_1. ——, non-treated particles, 1.3 μatoms O · min^{-1} · mg^{-1} protein; – – –, in the presence of 5 μM HQNO, 0.6 μatoms O · min^{-1} · mg^{-1} protein; ·····, activated particles, 2.3 μatoms O · min^{-1} · mg^{-1} protein.

results, Fig. 4 shows that the rate of the changes in the redox state of the cytochromes *c*-551 and b_1 upon addition of cyanide increases at higher rates of turnover.

It is interesting to note that the redox state of cytochrome b_1 (78%), obtained when maximal inhibition of the oxygen consumption (95%) was reached, remains 10% below that found at anaerobiosis (88%). Such an effect is not observed with cytochrome *c*-551. Therefore, it is proposed that a *b*-type cytochrome is involved in the cyanide-insensitive oxidation of NADH.

Binding of cyanide in the presence of ascorbate plus TMPD

When a low concentration of cyanide (100 μM) is added to particles of *A. vinelandii* oxidizing ascorbate in the presence of TMPD or DCIP, an instantaneous inhibition of the oxygen consumption takes place [14, 16], whereas with NADH higher concentrations of cyanide are required [14]. It has been suggested by Jones and Redfearn [14] that cytochrome *d* is involved in the oxidation of NADH, but not in that of ascorbate and DCIP. Therefore, they proposed a second cyanide-sensitive oxidase located in a branch of the respiratory chain. Fig. 5A shows that the absorbance at 648 nm decreases slowly when cyanide is added to particles in the presence of ascorbate and TMPD, although an instantaneous inhibition of the oxygen consumption was measured (not shown). This indicates that the formation of a cytochrome *d*–cyanide complex is not related to the rate of inhibition of the respiratory activity, in accordance with the proposal of Jones and Redfearn [14]. Fig. 5A also shows the deviation from first-order kinetics of the binding of cyanide as was found when NADH was used as substrate (see Fig. 1). The value for the apparent first-order rate constant at 100 μM cyanide, as calculated from the initial part of the curve ($k = 3 \cdot 10^{-3}\ s^{-1}$ at 16 °C) is smaller than with NADH in the presence of HQNO

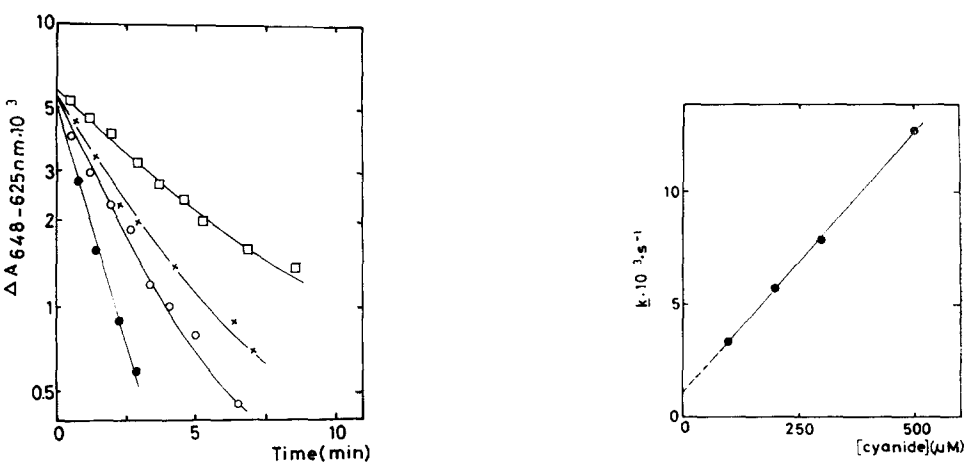

Fig. 5A. Time course of the reaction of cyanide with cytochrome *d* in particles in the presence of ascorbate plus TMPD, plotted semilogarithmically. Conditions and procedure as described in Methods and Fig. 1. After preincubation with ascorbate (7 mM) plus TMPD (0.25 mM) the reaction was started by the addition of the appropriate amount of cyanide. Cyanide concentration: □–□, 100 μM; ×–×, 200 μM; ○–○, 300 μM; ●–●, 500 μM.

Fig. 5B. Apparent first-order rate constants as a function of cyanide concentration. The first-order rate constants were calculated from the initial part of the curves in Fig. 5A.

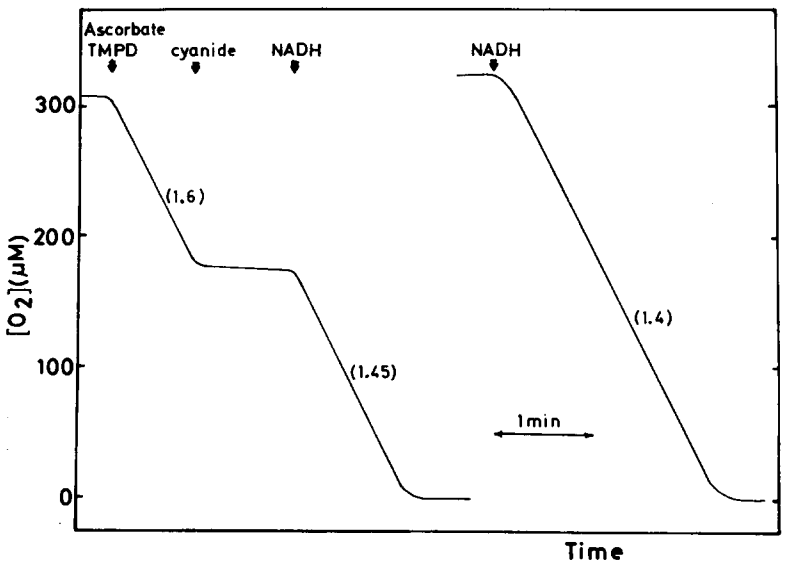

Fig. 6. The effect of NADH on the oxygen consumption of particles incubated with cyanide in the presence of ascorbate plus TMPD. Particles, 0.23 mg protein/ml, Prepn 4; temp. 16 °C. Arrows at left tracing indicate successive additions of ascorbate (7 mM) plus TMPD (0.25 mM), cyanide (100 μM) and NADH (1 mM). Arrow at right tracing indicates addition of 1 mM NADH only. Numbers in brackets indicate rate of oxidation (μatoms O · min^{-1} · mg^{-1} protein).

(cf. Fig. 2), although it is still considerably larger than the rate constant found in the absence of substrate [6]. From this we conclude that in the cyanide-inhibited ascorbate-TMPD system, the rate of turnover of cytochrome *d* is very low.

Fig. 5B shows that the values of the apparent first-order rate constants, obtained from Fig. 5A, are linearly dependent on the cyanide concentration. From the slope of the straight line a second-order rate constant can be calculated with a value of 23 $M^{-1} \cdot s^{-1}$ at 16 °C. The k_{off}, estimated from the intersection point with the ordinate, has a value of approximately $1 \cdot 10^{-3}$ s^{-1}. Both values are substantially larger than those reported [6] for the binding of cyanide to cytochrome *d* in the absence of substrate ($k_{on} = 0.7$ $M^{-1} \cdot s^{-1}$ and $k_{off} = 5 \cdot 10^{-5}$ s^{-1} at 32 °C).

The observation that the addition of cyanide to particles oxidizing ascorbate and TMPD does not cause an instantaneous formation of cyano–cytochrome *d* suggests that the electron pathway used for NADH oxidation must still be functional. This is confirmed by the experiment illustrated in Fig. 6 where the oxygen consumption was measured as a function of time. It is clear that the rate of oxidation of NADH by particles that are preincubated (1 min) with ascorbate, TMPD and cyanide (100 μM) is the same or even higher than the oxidation of NADH by untreated particles. After prolonged incubation with ascorbate, TMPD and cyanide, however, it was found (not shown) that the rate of oxidation of NADH decreases as a function of incubation time and reaches a minimal activity when the band at 648 nm of cytochrome *d* has disappeared. These experiments demonstrate that different oxidases are involved in the oxidation of ascorbate plus TMPD and of NADH, in line with the suggestion of Jones and Redfearn [14].

DISCUSSION

The low rate of binding of cyanide to oxidized cytochrome *d* in the absence of reducing substrates has been explained by us [6] as due to a preferential binding of cyanide to the enzymically active oxidized conformation of the enzyme [7], denoted cytochrome d_x [6]. This explanation has been confirmed by the observations in this paper that the rate of binding of cyanide is more rapid at higher rates of turnover of cytochrome *d*, i.e. with increasing amounts of the enzymically active conformation of cytochrome *d*.

It is interesting to note that Van Buuren et al. [8–11] demonstrated that the rate of the binding of cyanide to mammalian cytochrome *c* oxidase also increases at higher turnover rates. The faster binding was explained by them as being due to a conformational change of the oxidase into a more open conformation, thereby exposing the cyanide-sensitive site. In analogy to this model, we propose that the enzymically active conformation of cytochrome *d* (d_x) has a more open conformation, facilitating the binding of cyanide, whereas the constituent absorbing at 648 nm has a more closed conformation.

The decrease of the intensity of the absorption band at 648 nm after the addition of cyanide (Fig. 1) corresponds with a gradual increase in inhibition of the oxidation of NADH (Fig. 3) and a gradual increase in the redox state of cytochrome b_1 (Fig. 4). These results show that cytochrome *d* is involved in the oxidation of NADH, a conclusion in line with that reported earlier [14]. On the other hand, cytochrome *c*-551 shows a biphasic change in its redox state upon addition of cyanide. The rapid increase in its redox state can be explained by a nearly complete inhibition of the oxidase in the branch of the respiratory chain involving the cytochromes *c*-551 and *o* [14]. Since the slow phase of the change of the redox state of cytochrome *c*-551 corresponds to that of cytochrome b_1 it may be concluded that when the respiration via the branch is inhibited, the cytochromes *c*-551 and b_1 are close to equilibrium.

Our observations at high oxygen concentration on the binding of cyanide to cytochrome *d* are not in line with the conclusion of Arima and Oka [17] that the effect of cyanide can be reversed by oxygen. However, the difference spectra shown by these authors illustrate a disappearance of the absorbance at 648 nm (trough) after prolonged incubation with cyanide and oxygen, which can be interpreted as a binding of cyanide to cytochrome *d*.

It was shown that no correlation exists between the inhibition by cyanide of the oxidation of ascorbate plus TMPD and the binding of cyanide to cytochrome *d*. This indicates that cytochrome *d* is not involved directly in the oxidation of the dye, a conclusion in agreement with a suggestion of Jones and Redfearn [14]. It was shown earlier [6] that cyanide does not shift the absorption band of cytochrome a_1 (598 nm) and that particles devoid of cytochrome a_1 are still capable of oxidizing ascorbate plus TMPD or DCIP [6, 18]. Therefore, it is likely that cytochrome *o*, which can only be detected by its CO difference spectrum, is the cyanide-sensitive oxidase of the side path of the respiratory chain [14]. It is noteworthy that this cytochrome reacts more readily with cyanide than cytochrome *d*, since an immediate inhibition of the oxidation of ascorbate plus TMPD is observed at 100 μM cyanide (Fig. 6, cf. also ref. 14).

Still a third but minor pathway from NADH to oxygen in *A. vinelandii* must be proposed in order to explain the oxygen consumption insensitive to prolonged

incubation with cyanide. In this pathway an auto-oxidizable *b*-type cytochrome may be involved, since the redox state of cytochrome b_1 increases at anaerobiosis although the system is maximally inhibited by cyanide.

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