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Effect of Ligands on Cytochrome *d* from *Azotobacter vinelandii*

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Abstract

Spectra of oxidized and reduced cytochrome *d* in particles of *A. vinelandii* were studied in the presence of the ligands CO, azide, and NH_2OH under oxidizing, reducing, and turnover conditions. Under oxidizing conditions, spectral changes were observed on oxidized cytochrome *d* (absorption maximum at 648 nm) in the presence of CO and NH_2OH showing a shift of the maximum to shorter wavelengths (639 and 645 nm, respectively) and a broadening of the half-band width. Under reducing conditions, spectral changes were observed on reduced cytochrome *d* (absorption maximum at 631 nm) in the presence of CO (absorption maximum at 636 nm), NO, NO^-_2, and NH_2OH (absorption maximum at 642 nm in the presence of dithionite). The spectral changes of cytochrome *d* in the presence of NH_2OH or with dithionite and NO^-_2 were ascribed to the formation of the NO-cytochrome *d* compound. Under turnover conditions CO, NH_2OH, and azide cause a spectral shift of the absorption maximum of cytochrome *d* from 648 nm to 636, 645, and 655 nm, respectively. With NH_2OH and azide a broadening of the half-band width of 7 and 6 nm, respectively, was also observed. The spectral changes caused by CO and NH_2OH were interpreted as a binding of the ligands to cytochrome *d* changing its conformation from the oxidized state absorbing at 648 nm into a more

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stable liganded form. Since azide does not affect the spectral bands of oxidized and reduced cytochrome *d*, the spectral change during turnover in the presence of azide were ascribed to a preferential binding of azide to enzymically active conformation of cytochrome *d* (cytochrome d_x).

Introduction

The respiratory chain of *Azotobacter vinelandii*, which is quite rich in redox components, exhibits very high respiration rates with various substrates and has a very active terminal oxidase (besides cytochromes *o* and a_1) cytochrome *d*. This latter oxidase has never been purified despite numerous efforts by many investigators; redox studies on this enzyme must by necessity be conducted with its membrane-bound form.

In previous papers [1–4] the spectral properties of oxidized and reduced particulate cytochrome *d* were studied, using a clay suspension in order to correct for light scattering. In this way we were able to show the effect of cyanide on the spectral properties of cytochrome *d* and its kinetics of binding to cytochrome *d* under oxidizing and reducing conditions. These experiments have led to the proposal of an enzymically active conformation of cytochrome *d*, denoted by us as cytochrome d_x, which is in equilibrium with the nonactive conformation [1]. It could be shown that only the latter species has an absorption band at 648 nm in its oxidized form. Using electron paramagnetic resonance (EPR) spectroscopy it has also been demonstrated by Kauffman et al. [5] that cyanide reacts with the rhombic, highspin heme species of cytochrome *d*.

Studies of the effect of ligands other than cyanide on the absorption bands of cytochrome *d* are limited. The effect of CO on the spectrum of cytochrome *d*-containing microorganisms was studied mainly by visual microspectroscopy which showed a shift of the absorption band of the reduced enzyme (629–630 nm) to longer wavelength (635–637 nm) upon addition of the ligand [6–9]. A similar effect of CO was found by Tissieres [10] on particles of *A. vinelandii*. Further information on the effects of ligands on cytochrome *d* stems from difference absorption spectroscopy. In this way, effects of CO and NO on the spectrum of reduced cytochrome *d* of *A. vinelandii* [11, 12] were shown.

We present in this paper the effects of some ligands on the absolute absorption properties of cytochrome *d* of *A. vinelandii* under oxidizing and reducing conditions by means of a method described previously [1]. In order to study the effect of ligands on the enzymically active conformation of cytochrome *d*, the experiments were also carried out in the presence of reducing substrates and oxygen.

Methods

Phosphorylating small particles from *A. vinelandii* (grown according to ref. 13) were prepared as described by Pandit-Hovenkamp [13] and stored in 40 mM potassium phosphate buffer (pH 7.2)–0.25 M sucrose–40 mM KCl at −196°C. When measuring spectra, the oxygen consumption, or redox states of cytochromes, particles were diluted with 30 mM postassium phosphate buffer (pH 7.6), 5 mM $MgCl_2$, and 1 mM EDTA.

Microorganisms with an altered cytochrome composition were obtained by growing *A. vinelandii* on a urea-containing medium in the absence of Mo and N_2 according to Knowles and Redfearn [14], thus depressing the formation of *c*- and *d*-type cytochromes. The culture was harvested in the early logarithmic phase when the organism was deficient in cytochrome *d* (cf. Castor and Chance [15]). A similar dependence of the appearance of cytochrome *d* in relation to the growth phase was found for other microorganisms [16]. The reduced *minus* oxidized difference spectrum of phosphorylating small particles showed cytochrome b_1, decreased concentrations of *c*-type cytochromes, and a deficiency of cytochromes a_1 and *d* (not shown) when compared to large-particle preparations.[13].

Spectra were recorded on a Perkin-Elmer spectrophotometer Model 356. When studying the effect of CO on cytochrome *d*, difference absorption spectra were taken with particles with cyanide-liganded cytochrome *d* in the reference cell [3]. The effect of hydroxylamine and azide were studied using a clay suspension in order to correct for light scattering [1, 2]. The spectra were normalized as described previously [2, 3], taking 605 and 700 nm as reference points. In order to follow simultaneously the oxygen concentration and spectra, the sample cuvette was equipped with a Clark-type O_2 electrode. Longer aerobic incubation periods were achieved in the presence of reducing substrate and inhibitors using a medium equilibrated with 1 atm of oxygen and maintaining the temperature at 16°C [3].

The rate of oxygen consumption in the presence of inhibitors was checked in separate experiments on a Gilford oxygraph (cf. the Hill plot).

The changes in the redox state of the cytochromes *c*-551, b_1, and *d* in the presence of CO as a function of the O_2 concentration were measured using an Aminco-Chance dual-wavelength spectrophotometer equipped with a Clark-type O_2 electrode [3].

The concentration of CO in solution was varied by mixing media equilibrated with 1 atm of CO and 1 atm of oxygen in an appropriate ratio.

Protein concentrations were determined as described previously [1] using the biuret method.

Chemicals were of the highest purity available and were obtained from

British Drug Houses except those used for the culture which were less highly purified.

Results

Effect of CO

The effect of CO on the absorption spectrum of particles of *A. vinelandii* in the presence and absence of substrates is shown in Fig. 1. Upon adding CO, the absorption band at 648 nm of oxidized cytochrome *d* decreases in intensity and its maximum shifts to lower wavelength. The magnitude of the decrease in intensity and the shift of the maximum of cytochrome *d* depend on the CO/O_2 ration (not shown), reaching a maximal effect at CO/O_2 ratios higher than 0.5. At these high concentrations of CO, the maximum of the absorption band of cytochrome *d* shifts to 639 nm and the intensity decreases to two-thirds of its original value whereas the component absorbing at 670 nm is unaffected [1]. This effect of CO is probably not due to reduction of cytochrome *d* by internal substrates, since the effect was also observed in the presence of 2 mM ferricyanide (not shown).

After addition of 4 mM NADH to particles preincubated with CO in the presence of oxygen, a symmetrical absorption band is observed at 636 nm (Fig. 1) whose intensity is inversely related to the oxygen concentration. The absorption band at 636 nm is shown in the spectrum of the CO-liganded reduced cytochrome *d* at anaerobiosis, where it was found that the intensity at 636 nm increases 25% without affecting the peak position. This result is in accordance with the observations of Tissieres [10]. It is interesting to note

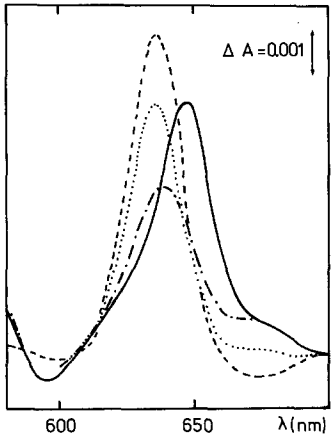

Fig. 1. The effect of CO on the spectrum of cytochrome *d* under oxidizing and reducing conditions. The spectrum was measured in the presence of CO, by mixing 2 ml of buffer equilibrated with 1 atm of oxygen and 1 ml of buffer equilibrated with CO. Particle concentration, 0.61 mg protein · ml^{-1}. NADH at a final concentration 4 mM was added when the effect of CO was measured under turnover conditions. The reference cell contained a particle suspension in which cytochrome *d* was cyanide liganded (cf. ref. 3). Spectra were scanned between 580 and 700 nm with a sweep of 25 sec. For checking whether anaerobiosis was achieved, the oxygen concentration and spectra were measured simultaneously. Spectra: ———, oxidized; - - - -, oxidized in the presence of CO; . . . . , in the presence of CO, oxygen, and NADH, measured directly after the addition of NADH; ----, same conditions as under (. . . .) but after anaerobiosis. The length of the arrow is equal to $\Delta A = 0.001$ *absorbance.*

that under turnover conditions and in the presence of CO, the 648 nm band has disappeared, while the band of the CO-liganded reduced cytochrome *d* has not yet fully appeared. This indicates that part of the cytochrome *d* is in its enzymically active conformation and the residual cytochrome *d* is reduced and CO-liganded.

Since the Soret region is dominated by absorption of the *b*- and *c*-type cytochromes, the effect of CO on this part of the spectrum could be studied best by means of a CO-difference absorption spectrum (Fig. 2). In order to explain in Fig. 2A the characteristic W-shaped Soret band of particles grown under N_2-binding conditions, a CO-difference spectrum was also recorded of particles obtained from bacteria at early log phase grown on an urea-containing medium in the absence of N_2. These particles contain only *b*- and *c*-type cytochromes and do not show the characteristic α-absorption band of cytochromes *d* and a_1. As can be seen from the CO-difference spectrum (Fig.

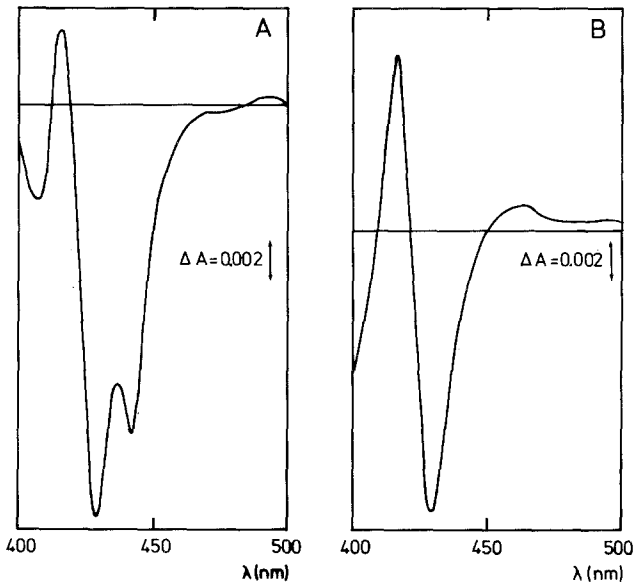

Fig. 2. The effect of CO on the spectrum of reduced particles of *A. vinelandii* deficient in and containing normal amounts of cytochrome *d*. Particles (0.85 mg protein · ml^{-1}) of microorganisms grown under N_2-binding conditions, and particles (1.9 mg protein · ml^{-1}) derived from microorganisms grown on a urea-containing medium, were suspended in potassium phosphate–$MgCl_2$–EDTA solution. After addition of dithionite, CO was bubbled through the sample cuvette for 2 min. The CO-difference spectrum was scanned between 400 and 500 nm with the particles reduced with dithionite in the reference cell. Spectra were corrected using the baseline of oxidized–oxidized. (A), particles of N_2-binding microorganisms containing cytochrome *d*; (B), particles of urea-grown microorganisms deficient in cytochrome *d*.

2B), particles from urea-grown bacteria contain only cytochrome *o* as the CO-binding group, characterized by a peak at 416 nm and a trough at 429 nm. The additional trough at 442 nm found in bacteria grown under N_2-binding conditions can thus be due to either cytochrome a_1 or cytochrome *d*. In the 600–700 nm region hardly any effect of CO was detected at 597 nm (cytochrome a_1), whereas a great effect of this ligand was found on the absorption band of cytochrome *d* (Fig. 1). This suggests that the trough observed at 442 nm in the CO-difference spectrum is caused by a decrease in intensity of the γ band of reduced cytochrome *d* upon binding of CO.

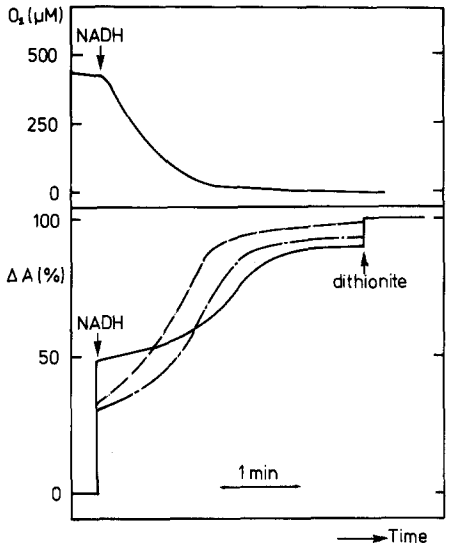

Fig. 3. The redox state of the cytochromes as a function of time in the presence of NADH and CO. Particle concentration 0.038 mg protein · ml^{-1} at a temperature of 25°C. Particles were added to a mixture of 1.2 ml buffer solution, equilibrated with O_2 gas of 1 atm and 1.2 ml buffer solution equilibrated with CO gas of 1 atm. Absorbance changes were measured on an Amin-co-Chance dual-wavelength spectrometer, the cuvette of which was equipped with a Clark-type O_2 electrode (cf. ref. 3). Vertical arrows indicate either addition of NADH (final, 4 mM) or a slight excess of solid dithionite. The upper trace represents the change in time of the oxygen concentration, and the three lower traces represent the redox state of the cytochromes. ———, cytochrome *c*-551, $\Delta(A_{551} - A_{536})$; - - - -, cytochrome b_1, $\Delta(A_{559} - A_{573\ nm})$; ——— ——— ———, cytochrome *d*, $\Delta(A_{636} - A_{605\ nm})$. The length of the arrow is equal to 1 min.

The effect of CO on the redox state of the cytochrome *c*-551, b_1, and *d* in the presence of substrates is shown in Fig. 3. In the presence of CO and the substrates NADH and oxygen, the redox state of the cytochromes does not reach a steady state, that is, the cytochromes become more reduced with decreasing oxygen concentrations. This observation corresponds with that of an increasing inhibition of the rate of oxygen consumption which was measured simultaneously. Furthermore, the experiment shows that in the final phase of the reaction the rate of reduction of cytochrome *d* decreases before that of the cytochromes b_1 and *c*. This suggests that the main site of inhibition by CO is probably situated at or near cytochrome *d* although an effect also on cytochrome *o* cannot be excluded. This result is in accordance with the observations of Jones and Redfearn [11].

Under anaerobic conditions addition of H_2O_2 in the presence of catalase (to release O_2) to CO-incubated particles shows a fast reoxidation of cytochrome *d* and the *b*- and *c*-type cytochromes and a simultaneously restored oxygen consumption, indicating that the binding of CO to cytochrome *d* and its inhibitory effect is easily reversed by oxygen.

Effect of NO, NO_2^-, and NH_2OH

When NH_2OH (300 mM) was added to particles, an instant shift of the band of oxidized cytochrome *d* (648 nm) was observed to 644 nm (Fig. 4). Furthermore, it can be seen that the half-band width has increased 7 nm without changing the height of the absorption maximum. When in the presence of oxygen a reducing substrate was added, the intensity of the band at 644 nm decreased slightly (< 5%) whereas cytochrome a_1 (597 nm) became partially reduced (not shown). After anaerobiosis a spectrum was

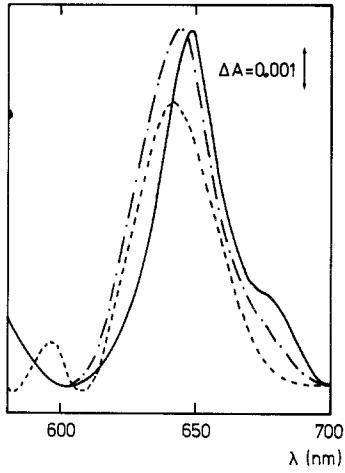

Fig. 4. The effect of hydroxylamine on the spectrum of cytochrome *d* under oxidizing and reducing conditions. Particle concentration 1.37 mg · ml^{-1}. Hydroxylamine (300 mM) was added as a 5 M solution that had been brought to pH 7.6 with HCl. Spectra were recorded under the conditions as described in Methods. Spectra: ———, oxidized; ----, oxidized in the presence of 0.3 M NH_2OH; - - - -, in the presence of 0.3 M NH_2OH and a slight excess of dithionite.

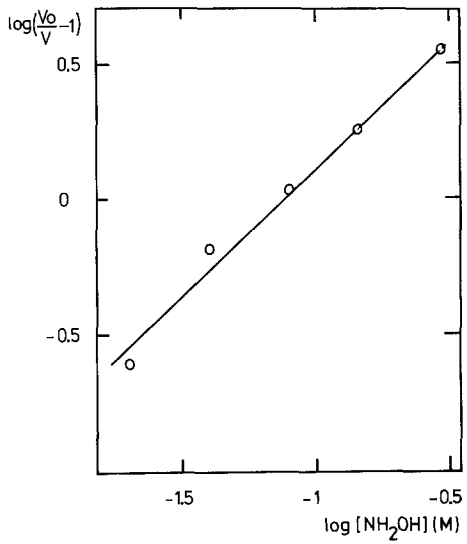

Fig. 5. Hill plot of the inhibition of NADH oxidation by NH_2OH. The rate of oxygen consumption by particles of *A. vinelandii* (1.37 mg protein · ml^{-1}) in the presence and absence of hydroxlamine and the substrates NADH and oxygen were measured on a Gilford oxygraph. V_o, activity in the absence of inhibitor; V, activity in the presence of various amounts of NH_2OH. Temp. 25°C.; NADH, 2.5 mM.

observed lacking the peak of reduced cytochrome *d* at 631 nm. When dithionite was added the maximum shifts to 642 nm and the intensity decreases to 80% of that of oxidized cytochrome *d* (Fig. 4).

In accordance with the spectral effects it was found that the addition of NH_2OH to NADH-respiring particles causes a fast inhibition of the oxygen consumption (not shown). The Hill plot of the inhibition of the oxygen consumption at various hydroxylamine concentrations (Fig. 5) shows a straight line with a slope slightly less that one ($h = 0.93$). The half-maximal inhibition was found to be at 75 mM NH_2OH.

Under anaerobic conditions in the presence of dithionite a spectrum of cytochrome *d* with a peak at 642 nm was also obtained with nitrite or NO (not shown), indicating that the spectral changes observed in the presence of NH_2OH and nitrite are due to the formation of a NO-cytochrome *d* complex. Under these conditions addition of ferricyanide causes a reoxidation of the cytochromes b_1 and *c*-551, but no effect was observed on the absorbance at 642 nm, showing that the ferrocytochrome *d*–NO complex is stable under oxidizing conditions.

Effect of Azide

Incubation of oxidized particles with 300 mM azide in the presence of oxygen did not show an effect on the 648 nm band of oxidized cytochrome *d* (not shown), in accordance with similar observations of Tissieres on *Aerobacter aerogenes* [9]. However, after the addition of NADH (2.5 mM), an instant shift of the 648 nm band is observed to 655 nm. The height of the latter band is slightly lower but its half-band width has increased from 21 to 27 nm (Fig. 6). The oxygen consumption, recorded simultaneously with the spectra, had a respiratory activity of 25% of that found in the absence of azide (activities, 1.98 and 0.94 μatoms O $min^{-1} \cdot mg^{-1}$ protein, respectively). During these turnover conditions the absorption band at 655 nm persists. On anaerobiosis, however, it disappers, and the absorption band of reduced cytochrome *d* appears at 631 nm, which was identical in half-band width and height to that in the absence of azide. On the spectral band of dithionite-reduced cytochrome *d* (631 nm) no effect of azide could be observed even after prolonged incubation (30 min, not shown), suggesting that azide does not bind to the heme of reduced cytochrome *d*. These experiments support our previous proposal (3) that under turnover conditions cytochrome *d* has a more open conformation, facilitating the binding of ligands to its heme.

The magnitude of the shift of the maximum of cytochrome *d* (648 nm) to longer wavelengths (655 mM) depends on the concentration of azide. At a lower azide concentration (20 mM), in the presence of NADH and oxygen, an absorption peak was observed at an intermediate (652 nm) position (Fig. 6). The intensity of the peak is 10% less when compared with that of the

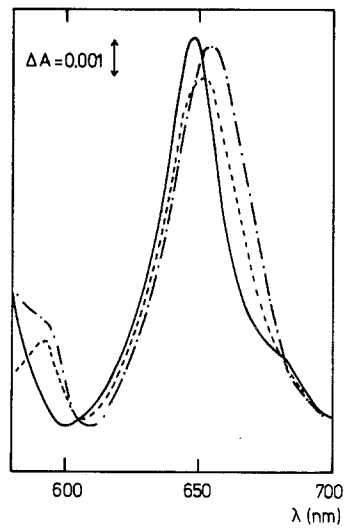

Fig. 6. The effect of azide on the spectrum of cytochrome *d* under oxidizing and reducing conditions. Particle concentration 1.37 mg protein · ml^{-1}. Conditions as described in Fig. 4. Spectra: ———, oxidized; — · — · — ·, in the presence of 300 mM azide, oxygen, and 2.5 mM NADH; ----, in the presence of 20 mM azide, oxygen, and 2.5 mM NADH.

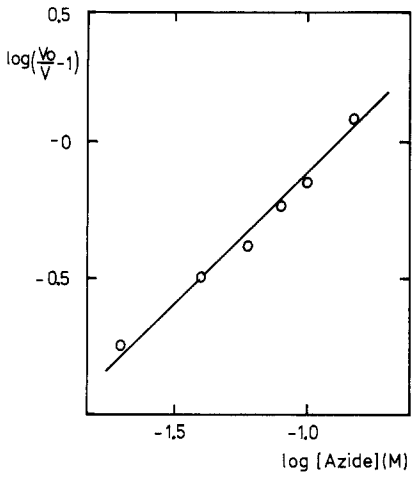

Fig. 7. Hill plot of the inhibition of NADH oxidation by azide. Particle concentration 1.37 mg protein · ml; NADH, 2.5 mM, pH = 7.6. Temp. 16°C. V_o, activity in the absence of inhibition; V, activity in the presence of various concentrations of azide.

bands of 648 or 655 nm. This suggests that at a lower azide concentration cytochrome *d* remains partially in its activated nonliganded conformation (d_x) absorbing little between 600 and 700 nm [2]. At concentrations of azide higher than 300 mM, no further shift of the band at 655 nm could be observed.

As was also observed for the inhibition by hydroxylamine, the inhibition by azide reaches its steady-state level in a few seconds, Also with azide the Hill plot of the inhibition of the oxygen consumption versus the inhibitor concentration (Fig. 7) shows a straight line with a Hill coefficient of nearly 1 ($h = 0.94$). The half-maximal inhibition is found to be at 120 mM azide at pH 7.6.

Discussion

As was also observed by Tissieres [10], reduced cytochrome *d* of *A. vinelandii* reacts with CO showing a shift from 631 to 636 nm. Furthermore, it has been shown that the 648 nm band of oxidized cytochrome *d* shifts to 639 nm in the presence of CO. This is in line with the observation that CO affects the $g = 6$ high-spin ferric EPR signal of cytochrome *d* [5].

Our comparative study of the Soret region of the absorption spectrum of *A. vinelandii* grown under various conditions shows that the maximum at 416 nm and trough at 428 nm observed in the CO-difference spectrum are both due to a *b*-type cytochrome (cytochrome *o*). As was found for cyanide, no significant effect of the ligands CO, NO, or azide could be observed on the band of cytochrome a_1 at 596 nm. The trough at 442 nm can be interpreted as being due to a diminishing in intensity and/or shift of the γ-band of reduced

cytochrome *d* in CO-treated particles, since it was shown that the isolated *d*-type cytochrome of *Pseudomonas aeruginosa* has an absorption maximum in the Soret region with a small intensity when compared with that of the absorption of *c*-cytochromes and which decreased when treated with CO [17, 18].

The effect of NO on the cytochromes of *E. coli* was described by Meyer [12] showing spectral effects by means of a NO-difference spectrum. The characteristic W-shape Soret band was ascribed to cytochromes *d* and *o*. Similar observations were obtained with cytochrome *d* of *A. vinelandii*. The latter observations were confirmed by us in the NO-difference spectra of *A. vinelandii* with minima found at 431 and 439 nm (not shown), indicating that the shift and diminishing of the reduced γ-bands of cytochromes *o* and *d* differ slightly from that in the presence of CO. This is also found in the 650 nm region, where NO shifts the band of reduced cytochrome *d* further to the red than CO. A similar difference between the effect of CO and NO on cytochrome *d* was found in particles of *Streptomyces griseus* [19]. The observation that incubation with NO_2^- and dithionite, or with NH_2OH and subsequent addition of dithionite, leads to the formation of the same NO-ferrous complex of cytochrome *d* can be explained by the formation of NO from a reduction of nitrate or an oxidation of NH_2OH [12].

In the presence of oxygen, both CO and azide cause marked changes in the near-infrared region of cytochrome *d*. CO-induced changes of the coordination sphere of the heme of oxidized cytochrome *d* have also been observed by EPR, where it has been shown [5] that CO increases the rhombicity of the high-spin heme signal at $g = 6$ of cytochrome *d*. These results are in accord with the assignment of the 648 nm absorbance to a charge-transfer band [2] of high-spin ferric heme. It has been suggested by Smith and Williams [20] that a band at this position arises from configuration interaction between a charge-transfer state and a π^* state. The relative amounts of charge transfer and $\pi \rightarrow \pi^*$ character in the band will depend strongly on the axial ligand. A shift of the band to the blue and a decrease in intensity, as found for the CO-induced spectral effects on the 648 nm band of cytochrome *d*, will indicate a larger contribution of the $\pi \rightarrow \pi^*$ character to the band, whereas a shift to longer wavelength, as found for azide, points to more charge-transfer character.

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