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THE RESPIRATORY CHAIN OF *AZOTOBACTER VINELANDII*

I. SPECTRAL PROPERTIES OF CYTOCHROME *d*

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SUMMARY

1. Absolute spectra of oxidized particles of *Azotobacter vinelandii* show an asymmetrical band at 648 nm that can be resolved into two broad bands at 635 and 670 nm and a sharp band of cytochrome *d* at 648 nm. After reduction a symmetrical band of reduced cytochrome *d* is observed at 631 nm.

2. The pyridine hemochrome spectrum shows mixed bands of protoheme and mesoheme at 553 and 521 nm and a band of heme *d* at 613 nm.

3. During turnover conditions, cytochrome *d* is partially present in its 648-nm form, yet no reduced component can be detected.

4. Reduction under near-equilibrium conditions and under slow transition from the anaerobic to the aerobic state shows that the 648-nm band is not a direct oxidation product of reduced cytochrome *d* (631 nm).

5. Two explanations for the 648-nm band are given: (i) it is assumed that the band belongs to a copper protein; (ii) the band is ascribed to a charge transfer from a ligand to the ferric heme *d*, thus explaining its sharpness and height.

INTRODUCTION

Cytochrome *d*, also termed cytochrome a_2, is widely distributed in bacteria[1–9]. Its properties were studied by Negelein and Gerischer[2,3] and Keilin[4,5], who showed that the oxidized form has a band at 645–647 nm and the reduced band 628–632 nm. In the early work, it was generally assumed that the function of cytochrome *d* is that of an oxidase[10], and Castor and Chance[11] proved that this is the case in 1959 by showing that CO-inhibited oxidation is restored with light of 637 nm.

From the anaerobically nitrate-grown *Pseudomonas aeruginosa* a soluble cytochrome *cd* has been isolated[12,13], whose function appears to be mainly that of a nitrite reductase[14], although it also reacts with oxygen. It was proposed[15] that cytochrome *cd* contains the same prosthetic group as was found for the aerobically grown *Aerobacter aerogenes*[16]. Newton[17], however, found spectral differences between the heme *d* isolated from the anaerobically nitrate-grown *Micrococcus denitrificans* and the aerobically grown *A. aerogenes*[16].

The soluble cytochrome *cd* has been studied extensively[18,19] but little is known

about cytochrome *d* in an aerobically grown organism. Therefore, as part of our program of research on the obligately aerobic bacterium *Azotobacter vinelandii*[20–23] we investigated the properties of cytochrome *d* in this organism. Its properties are similar to those of cytochrome *d* of *A. aerogenes* with respect to its reaction with cyanide and CO[11,24–26]. Furthermore, it has been concluded by Jones and Redfearn[26] from its CO action spectrum that cytochrome *d* functions as an oxidase in *A. vinelandii*.

In this paper the spectrum of oxidized and reduced cytochrome *d* is studied both in the presence of substrate *plus* oxygen and during anaerobiosis. It will be shown that the 648-nm band represents an indirect oxidation product of reduced cytochrome *d*. The reaction of ligands with cytochrome *d* will be presented in a separate paper.

METHODS

Phosphorylating particles from *Azotobacter vinelandii* were prepared as described by Pandit-Hovenkamp[27]. The particles were washed once with 50 mM Tris sulphate buffer (pH 8.0) and then stored in 40 mM phosphate buffer (pH 7.1)–0.25 mM sucrose–40 mM KCl at 77 °K.

Spectra were recorded on a Perkin–Elmer spectrophotometer Model 356. In order to study absolute spectra of cytochrome *d*, the particles were diluted in 30 mM phosphate buffer (pH 7.6)–5 mM $MgCl_2$–1 mM EDTA. A clay suspension of a proper density, prepared by homogenization and centrifugation, was used as a reference to correct for light scattering. When spectra of cytochrome *d* were studied during turnover conditions, the oxygen concentration in the sample cuvette was followed simultaneously with a Clark electrode.

During reduction the turbidity of the sample decreased steadily. To be able to compare the different spectra after increasing reduction, two wavelengths at which no absorption bands are present (600 and 725 nm, respectively) were chosen as reference points. From these normalized spectra, the percentages of oxidized and reduced absorption bands were calculated by means of a Dupont 310 Curve Resolver.

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

RESULTS

Spectrum of cytochrome d

Fig. 1 shows the absolute spectra of oxidized and reduced particles between 570 and 750 nm. Because reduction of the particles causes a decrease in light scattering, spectra in this paper were normalized taking 600 and 725 nm as isosbestic points. The oxidized particles show a broad asymmetrical band with a peak at 648 nm and a shoulder at 670 nm. Using a curve analyser the band can be resolved into 3 gaussian curves (dotted lines) with peak positions at 635, 648 and 670 nm, bandwidths of 34, 21 and 38 nm, respectively. The 648- and 670-nm absorption bands belong to different components since the ratio of their intensities varies from preparation to preparation. When cytochrome *d* is reduced, a symmetrical band is observed with a maximum at 631 nm and a bandwidth of 23 nm. The ratio of the intensities at 631 and 648 nm is not dependent on the preparation, indicating that they belong to the same or intimately related components. In these particles the band of cytochrome a_1 (peak at 595 nm) is hardly detectable.

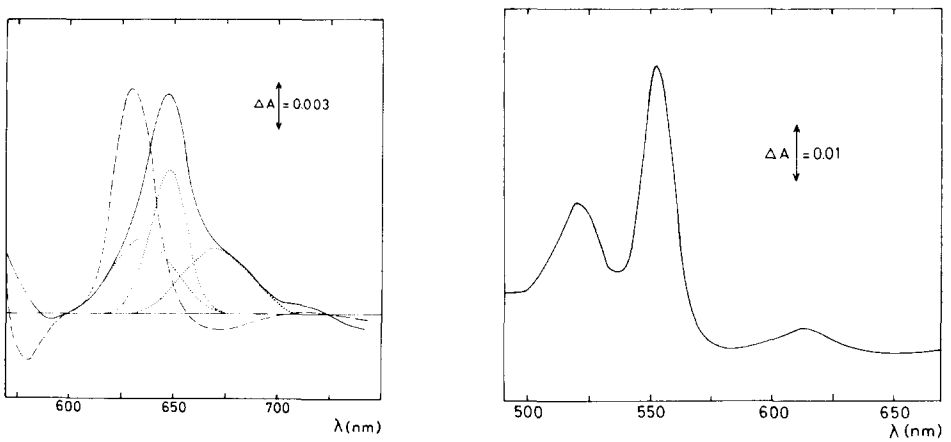

Fig. 1. Spectra of oxidized and reduced particles of *A. vinelandii*. Particles at 0.7 mg protein/ml were suspended in a phosphate–$MgCl_2$–EDTA solution (see Methods). The reference cell contained a sonified clay-powder suspension to diminish the effect of scattering. ———, oxidized; – – – –, reduced with $Na_2S_2O_4$; ·····, computed Gaussian functions of the band given by oxidized particles.

Fig. 2. Pyridine hemochrome spectrum. Particles (2.5 mg protein), suspended in 2 ml phosphate–$MgCl_2$–EDTA solution were reduced with dithionite, after which 0.4 ml 1 M KOH and 0.6 ml pyridine were added. The reference cell contained the phosphate–$MgCl_2$–EDTA solution.

The pyridine hemochrome spectrum of $Na_2S_2O_4$-reduced particles (Fig. 2) shows mixed α and β bands at 553 and 521 nm, respectively, of mesoheme (cytochrome c_4+c_5) and protoheme (cytochrome *b*), and a band of heme *d* at 613 nm. The peak position of the latter is identical with that of heme *d* isolated from *A. aerogenes*[16]. The heme *a* band (587 nm) of cytochrome a_1 is not visible.

Since the intensity of the band at 648 nm in oxidized particles is relatively high compared to that at 631 nm of reduced *d*, the question arises whether the two bands belong to the same or different components. Therefore, we investigated the change in time of the spectrum of cytochrome *d* under anaerobic conditions (Fig. 3). After a lag period, the length of which is dependent on traces of O_2 remaining after evacuation, the particles become slowly reduced by internal substrates. There is first a decrease of the 648-nm band followed by a decrease at 670 nm and then an increase at 631 nm with the simultaneous appearance of an isosbestic point at 640 nm.

The changes in time of absorbances characteristic for the different components of the chain are illustrated in Fig. 4, which clearly shows that the decrease of the 648-nm band precedes the reduction of cytochrome *d* (increase at 631 nm) and of cytochrome c_4+c_5 (increase at 551 nm). The reduction of cytochrome b_1 (increase at 559 nm) is much slower. Because the reduction by internal substrates is slow, the system is probably in near equilibrium. Thus, from left to right the intersection points with the 50% reduction line reflect decreasing redox potentials of the components. From Fig. 4 it can also be concluded that the 648-nm band is not a direct oxidation product of reduced cytochrome *d* (631 nm). Additional evidence for this conclusion comes from a back titration in a Thunberg cuvette (not shown) of dithionite-reduced particles with oxygen pulses produced by electrolysis. This experiment shows a

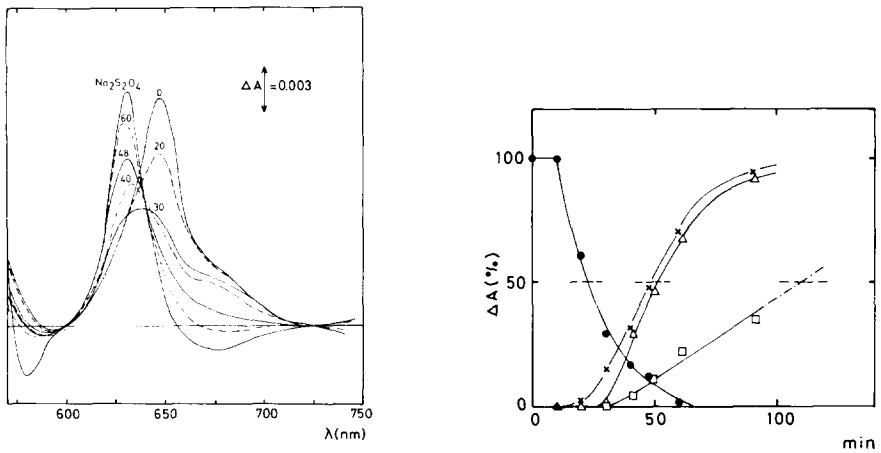

Fig. 3. Spectral changes upon reduction by internal substrates. Particles (0.7 mg protein/ml) suspended in phosphate–$MgCl_2$–EDTA solution were evacuated in a Thunberg cuvette for 8 min. Spectra were scanned after different time intervals (numbers indicate time in min). Scan speed 240 nm/min, temp. 18 °C. The reference cell contained a sonified clay-powder suspension. The spectra are normalized (see Methods).

Fig. 4. The rate of reduction by internal substrates. The % absorbance of bands between 600 and 700 nm were calculated by means of a Curve Resolver (see Methods). Cytochrome c_4+c_5 and b_1 were calculated from the absolute spectra using the wavelength pairs 550–540 nm and 560–570 nm, respectively. ●—●, $A_{648\,nm}$; ×—×, $A_{631\,nm}$; △—△, $A_{550\,nm}$; □—□, $A_{560\,nm}$; – – – –, 50% reduction line. Conditions as in Fig. 3.

disappearance of the 631-nm band without the simultaneous appearance of a band at 648 nm.

Cytochrome d under turnover conditions

The spectral properties discussed thus far were measured under (near) equilibrium conditions, showing a non-relationship between the absorption bands of oxidized (648 nm) and reduced (631 nm) cytochrome *d*. Thus, we turned our attention to the behaviour of these absorption bands under turnover conditions.

The absolute spectrum of cytochrome *d* in the presence of substrate and oxygen is shown in Fig. 5. In order to obtain a longer period of turnover conditions, a medium saturated with pure oxygen was used, while the temperature was decreased to 16 °C. It is shown (Fig. 5) that with NADH (4 mM) as substrate, the intensity of the 648-nm and 670-nm bands are approximately 25% less than in the oxidized particles. The same result was obtained with 3.5 mM malate as substrate. The reduced band at 631 nm is not detectable. It should be mentioned that in this experiment the spectra and oxygen concentration were measured simultaneously. As long as oxygen is present the 648-nm band persists. The appearance of the 631-nm band is only observed after anaerobiosis.

In this experiment the transition from aerobic to anaerobic state was achieved within seconds, making it difficult to follow the absorption bands as a function of the oxygen concentration. We therefore studied the change in the intensities of the oxidized and reduced absorption bands of cytochrome *d* during a slow transition from anaerobic to aerobic conditions.

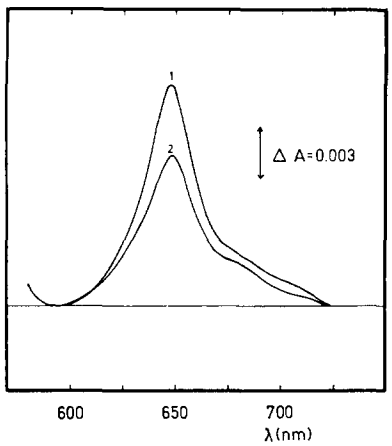

Fig. 5. Spectrum of cytochrome *d* during turnover conditions. Particles (0.7 mg protein/ml) were suspended in a phosphate–$MgCl_2$–EDTA solution saturated with pure oxygen. 1, particles in the absence of substrate; 2, with 4 mM NADH. Spectra were normalized (see Methods). The reference cell contained an appropriate clay-powder suspension.

The oxidized and reduced absorption bands of cytochrome *d* were followed by means of the wavelength pairs 648–605 and 631–605 nm, respectively, on the respirograph of the University of Odense, Denmark, built by H. Degn[28]. As illustrated in Fig. 6, while the oxygen concentration in the gas phase (T_G) is increasing at a steady rate the $A_{631\,nm}$ decreases until, when oxygen becomes detectable in the liquid phase

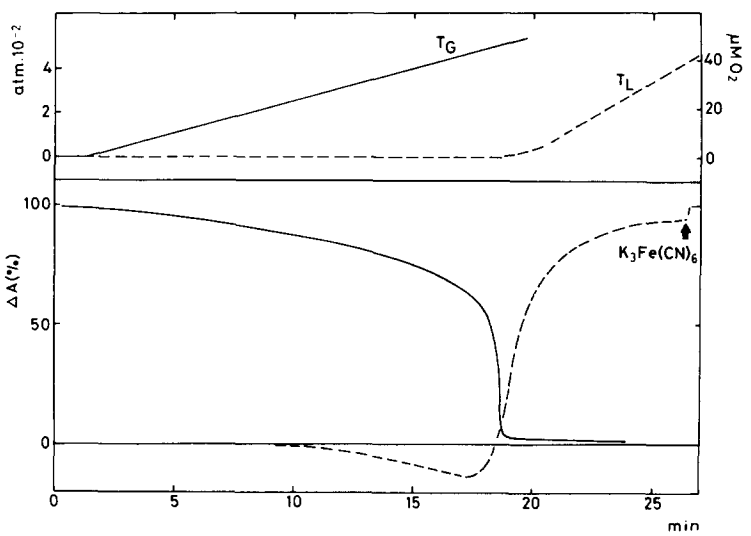

Fig. 6. Effect of transition from anaerobic to aerobic state on the absorption bands of oxidized and reduced cytochrome *d*. Particles (0.5 mg protein/ml) were suspended in phosphate–$MgCl_2$–EDTA solution. Reduction was brought about by 8 mM succinate. Top part: ———, T_G, oxygen concentration in the gas phase; – – – –, T_L, oxygen concentration in the liquid phase. Bottom part: ———, $A_{631\,nm}$ *minus* $A_{605\,nm}$ (% of initial value); – – – –, $A_{648\,nm}$ *minus* $A_{605\,nm}$ (% of value found with 1 mM $K_3Fe(CN)_6$).

(T_L), the 631 nm absorption band has disappeared completely. The 648-nm band appears at this point and increases in intensity with increasing T_L until a steady-state level of 94% of the value obtained with ferricyanide is reached. The decrease of the 648-nm absorption before the increase of T_L is probably due to a contribution of reduced cytochrome *d* at this wavelength. It is, therefore, concluded that in experiments with either limited or excessive amounts of oxygen no direct relationship between $A_{648\,nm}$ (ox) and $A_{631\,nm}$ (red) is found. The observation that the $A_{648\,nm}$ band appears mainly when oxygen becomes detectable in the liquid phase suggests that the $A_{648\,nm}$ component is promoted by higher oxygen concentrations.

DISCUSSION

The spectra of oxidized and reduced cytochrome *d* in particles of *A. vinelandii* show that the position of the absorption bands are identical with those of other aerobically grown bacteria[2–7]. The peak positions of the oxidized and reduced absorption bands of cytochrome *d* differ from those of cytochrome *cd*, isolated from *Ps. aeruginosa* and *M. denitrificans* which shows bands at 625 and 630–635 nm for the reduced and oxidized cytochrome[12–14,17], respectively. The peak at 613 nm of the pyridine hemochrome of cytochrome *d* is at the same position as that of *A. aerogenes*[16], but deviates 5–7 nm from that of the isolated cytochrome *cd*[15,17] (618–620 nm). This clearly shows that cytochrome *d* from an aerobically grown organism differs from an anaerobically nitrate-grown organism, as was earlier proposed by Newton[17].

It is shown in this paper that the disappearance during reduction of the band at 648 nm characteristic in oxidized particles and the appearance of the band at 631 nm of reduced cytochrome *d* are not related to one another. This can be explained in two ways: first, the 648-nm band and the 631-nm band belong to two different components; secondly, oxidation and reduction of cytochrome *d* goes *via* an intermediate form that scarcely absorbs in the red region of the spectrum.

According to the first explanation, the different kinetics are explained by a difference in redox potential between two components. The 631-nm band must belong to heme *d* because it appears upon reduction, it is shifted to 635 nm by CO treatment, and its formation is inhibited by cyanide[2–6]. The 648-nm band can be ascribed to a high-potential component since under near equilibrium conditions, it is reduced before the other cytochromes (Fig. 4). Since the 648-nm band is intense in the oxidized form and disappears on reduction[2–6], it resembles a copper protein, as was suggested by Beinert *et al.*[29]. However, the small width of the band (21 nm) and the low ratio $A_{631\,nm}/A_{648\,nm}$ (1.5), speak against this possibility.

According to the second explanation the difference in kinetics is explained as a difference between two conformations of oxidized cytochrome *d*. The high absorbance of the 648-nm band in the presence of oxygen suggests a high electron density on the heme iron[30]. Therefore, we like to propose that a charge transfer from the ligand to the iron occurs in the 648-nm conformation of cytochrome *d*, thus giving rise to a reduced type spectrum. Such a reduced type spectrum has been shown by Yonetani *et al.*[31] for nitroxide-liganded Type-1 ferrihemoproteins. In this case it was clearly shown by EPR that charge-transfer complexes are formed. In order to explain the kinetics, the 648-nm conformation, possibly promoted by higher oxygen concentrations, must be on a side path of electron transfer. A second oxidized conformation,

scarcely absorbing in the red region is proposed to be directly functional in the chain.

It was shown (Fig. 4) that the redox potential of cytochrome *d* (631 nm) approaches that of cytochrome c_4+c_5. If the redox potentials of isolated and particulate cytochrome c_4 are identical, the value of the redox potential of cytochrome *d*-631 will be about 320 mV.

It is interesting to note that during the slow anaerobic reduction (near equilibrium conditions) cytochrome *O* (560 nm) and cytochrome a_1 (595 nm) were reduced, after cytochromes c_4+c_5 and cytochrome *d* had reached their anaerobic levels. This shows that the redox potentials of cytochromes a_1 and *O* are lower than those of the other cytochromes. Since no low-potential oxidases are known it is questionable whether the cytochromes a_1 and *O* function as oxidases in the respiratory chain (see ref. 26).

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