Biochimica et Biophysica Acta, 634 (1981) 279—288

BBA 47994

MIDPOINT POTENTIALS OF CYTOCHROMES IN VESICLES OF ANAEROBICALLY-GROWN *PARACOCCUS DENITRIFICANS* DETERMINED BY THE INDIRECT COULOMETRIC TITRATION METHOD

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(Received September 30th, 1980)

Key words: Midpoint potential; Cytochrome; Indirect coulometric titration; Potentiometric titration; (Paracoccus denitrificans)

Summary

1. Multiplicity of redox components with spectral properties similar to *b*-type cytochromes was established in vesicles derived from anaerobically-grown *Paracoccus denitrificans*.

2. Multiplicity of *c*-type cytochromes was not apparent either from low temperature spectroscopy or potentiometric titrations.

3. Cytochromes $a + a_3$ and a component, only observable at liquid nitrogen temperature, with a spectral maximum at 582.5 nm were detected.

4. Redox cycling of electron transport components using the indirect coulometric titration method was a convenient means of pairing redox potentials and was reproducible in total absorbance changes, midpoint potentials and spectral maxima.

Introduction

Studies on the free energy relationship between the redox reaction of the electron transport chain from NAD to cytochrome *c* couples and [ATP]/[ADP][P_i] ratio in intact cells of *Paracoccus denitrificans* [1], isolated mitochondria [2] and perfused organs [3] indicate that the first two sites of oxidative phosphorylation are near equilibrium. An accurate determination of the midpoint potentials of the redox couples is required for these studies.

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Simultaneous absorbance and potentiometric measurements, reviewed by Dutton and Wilson [4], have provided information on the number of electron transport components [5], their midpoint potentials [6] and localization of ligand interactions [7].

Kuwana and his associates have developed an indirect coulometric titration method [8,9] and have studied the stoichiometry, n values, and midpoint potentials of purified electron transport components such as cytochrome c oxidase [10]. The indirect coulometric titration (ICT) technique has several advantages over the chemical titration method such as:

1. Solution conditions remain essentially invariant during a titration.
2. Small volume cells can be constructed.
3. Oxygen can be effectively removed before and excluded during a titration.
4. Quantitative increments of charge, on the nanoequivalent level, can be added.
5. Automation is possible.

This paper describes the spectral and potentiometric properties of cytochromes in membrane vesicles derived from anaerobically-grown *Paracoccus denitrificans* using the indirect coulometric titration method.

Materials and Methods

Paracoccus denitrificans was grown in 12 liter volumes of medium [11] containing potassium nitrate (10 g/l). Prior to inoculation with an anaerobically-grown culture, the medium was purged with nitrogen (99.99% purity) until the oxygen concentration, measured with a Yellow Springs Model 53 Oxygen Monitor, was one-fifth atmospheric concentration. After inoculation, the oxygen concentration decreased to the sensitivity limit of the oxygen electrode, estimated to be less than 2 μM oxygen. Nitrogen was passed over the surface and the medium was gently stirred during growth. Cells were harvested during logarithmic growth phase and membrane vesicles prepared by the lysozyme method as described by Tucker and Waddell [12]. Vesicles were suspended in potassium phosphate buffer (0.1 M, pH 7.0) and stored at $-20°C$.

Spectrophotometric (spectral) measurements. A rapid scanning spectrophotometer capable of operating in a double wavelength or derivative mode was used to record spectra [13]. All spectral information was computer stored. The computer was programmed so that spectra could be replotted in various formats. Particularly, difference spectra were plotted using any other spectrum as the baseline. For example, most commonly the baseline would be the components when they were either fully oxidized or reduced [14]. Low temperature spectra were recorded with an American Instrument Co. DW-2 spectrophotometer.

Indirect coulometric titration method. Cell design and degassing procedure for indirect coulometric titrations were essentially the same as that described by Hawkridge and Kuwana [8]. Two platinum wires were epoxied in the cell body. One platinum wire served as the indicator electrode for potentiometric measurements. The other was used to electrochemically generate oxygen at an applied potential of +1.4 volt versus Normal Hydrogen Electrode (NHE). All potentials reported in the text will be corrected to this reference electrode. Hydrogen peroxide formation during incremental oxygen generation was not

apparent using a purified preparation of cytochrome *c* peroxidase as an indicator. The potentiostat was of conventional three electrode design.

Fig. 1 summarizes the experimental approach of an indirect coulometric titration experiment. At a potential of —449 mV, the methyl viologen radical cation was electrochemically generated at the optically transparent tin oxide electrode (OTE) [15]. This cation can, in the case of *Paracoccus denitrificans*, reduce the electron transport chain directly. After charge addition and equilibration, a spectrum was recorded. The procedure was repeated, charge addition, equilibration and spectrum recorded until the cytochromes were completely reduced. The reduced cytochromes were then oxidized, presumably by the terminal oxidase in the vesicles, via the incremental addition of oxygen which was electrogenerated at a platinum electrode operating at +1.4 V.

For potentiometric titrations, additional mediators which are listed in the figure legends, were also used. In reductive titrations, the potential of the solution was poised by incremental charge additions at the tin oxide electrode operated at —449 mV. Spectra were recorded and redox potentials were measured using a platinum foil versus a silver/silver chloride reference electrode. In potentiometric titrations, the other platinum electrode used for oxygen generation was disconnected. In oxidative potentiometric titrations, potentials were poised by incremental, electrochemical oxidation of ferrocenyl methyl trimethylammonium (FMTA)-perchlorate at the tin oxide electrode operated at +627 mV (Fig. 1).

Protein. Protein was estimated using the method of Lowry et al. [16].

Reagents. Analytical grade reagents were obtained commercially. DNAase, lysozyme and RNAase were the products of Sigma (St. Louis, MO).

Results and Discussion

In a reductive/indirect coulometric titration, a spectral maximum appearing at 607 nm indicated the presence of cytochrome $a + a_3$ (data not shown) while the maximum at 559 nm suggested the presence of a high potential chromo-

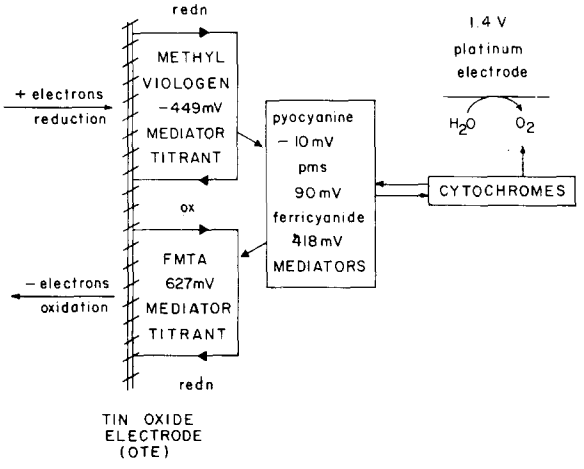
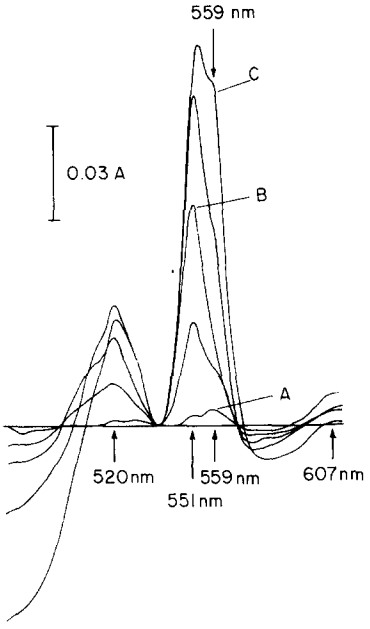


Fig. 1. Diagram depicting the indirect coulometric titration method (see text for explanation).



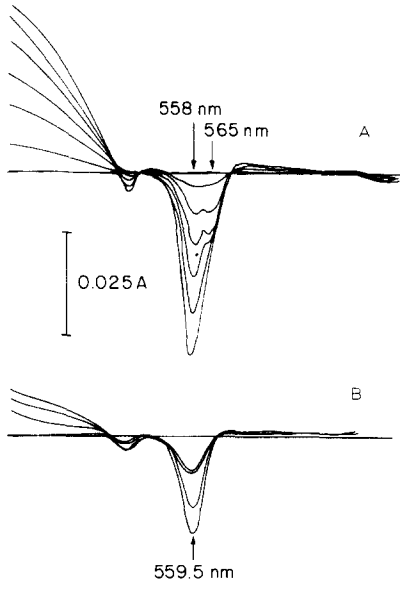


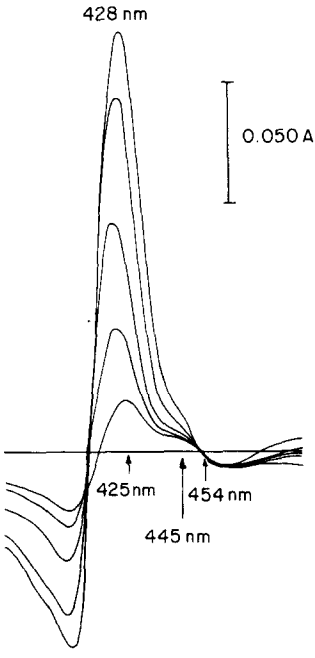
Fig. 2. (Left) Difference spectra obtained after incremental additions of reducing charge. Reference wavelength was 540 nm and a baseline with the cytochromes oxidized was stored in computer memory. Methyl viologen and protein concentration were 0.5 mM and 2 mg protein per ml, respectively. Buffer was potassium phosphate (0.1 M, pH 7.0).

Fig. 3. (Right) A. Difference spectra of the high potential cytochrome (559.5) and the low potential cytochrome (558, 565) obtained using the completely reduced cytochrome spectrum as the baseline. Other conditions were as in Fig. 2. B. Spectral characterization of the cytochrome (559.5). Baseline spectrum in computer memory was the oxidized, low potential, E_{m_7} −85 mV, cytochrome (558, 565).

phore (spectrum A, Fig. 2). The midpoint potentials of cytochrome $a + a_3$ were not determined. A *c*-type cytochrome with a maximum at 551 nm (spectrum B, Fig. 2) appeared as incremental reductive charge was added followed by an absorbance change at 559 nm (spectrum C, Fig. 2). The reduced cytochromes were oxidized subsequently in a reverse sequence, presumably via the oxidase present in the vesicles, by the electrochemically generated oxygen. No differences in the spectral maxima or in the total absorbance were noted beween two successive oxidative and reductive cycles.

When the spectrum of the completely reduced cytochromes was used as the baseline and an oxidative titration performed, a low potential component with maxima at 558 and 565 nm became apparent (Fig. 3A). However, a definite shift in the 558 nm maximum occurred even though the low potential component was not being oxidized. When the spectrum containing the completely oxidized low potential component (maxima at 558 and 565 nm) was used as the baseline, a cytochrome with a maximum at 559.5 nm (Fig. 3B) appeared. This cytochrome (maxima 559.5 nm) contributed 65 to 75% of the total absorbance change at 559 nm.

In the Soret region, maxima at 445 and 425 nm were indicative of cytochromes $a + a_3$ and a *c*-type cytochrome, respectively. The maximum at 425 nm



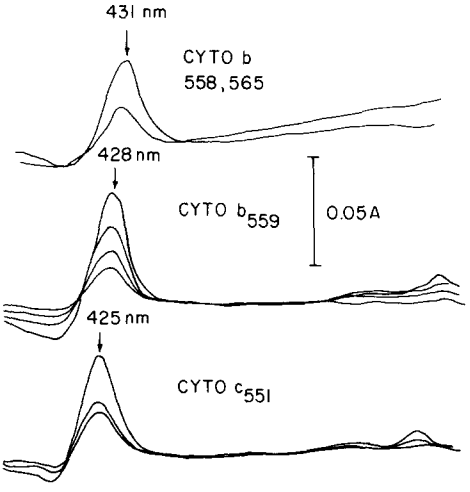


Fig. 4. (Left) Difference spectra of the Soret region obtained after incremental addition of reducing charge. Reference wavelength was 454 nm and a baseline with the cytochromes oxidized was stored in computer memory. Methyl viologen and protein concentration were 0.5 mM and 0.8 mg protein per ml, respectively.

Fig. 5. (Right) Spectral maxima in the Soret region of cytochrome (558, 565) (top), cytochrome (559.5) (middle) and cytochrome (551) (bottom).

shifted to higher wavelengths at the high potential (maximum 559.5 nm), and the low potential (maxima 558, 565 nm) cytochromes underwent reduction (Fig. 4).

When various baselines were chosen and were computer subtracted, the separation of the spectral maxima was possible for the *c*-type cytochrome at 425 nm, for a high potential cytochrome (559.5) with a spectral maximum at 428 nm in the Soret region, and for the dual maxima cytochrome (558, 565) with a Soret maximum at 431 nm (Fig. 5).

In the potentiometric titrations, the *c*-type cytochrome titrated with a midpoint potential of 240 ± 15 mV and had an n value of 0.95 ± 0.03 at pH 7. The data are shown in Fig. 6. The chromophore (559) titrated as an one electron acceptor with a midpoint potential of +355 mV at pH 7 (data not shown).

A sigmoidal curve was obtained in the potentiometric titrations using the wavelength pair 560 minus 540 nm in the E_h range of +100 to −125 mV (Fig. 7). The inflection portion of the curve represents the relative contribution of each component to the total absorbance change [5]. The component with the highest potential in this range, the cytochrome (559.5), contributed approx. 63% of the total absorbance change in agreement with our previous estimate. The sigmoidal curve was resolved into two one-electron acceptors as shown in

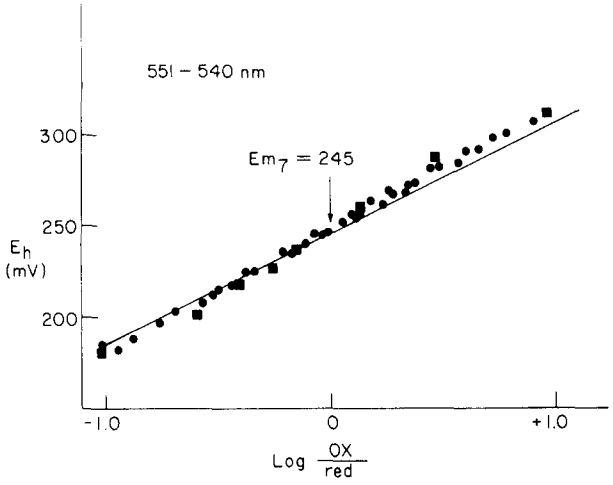


Fig. 6. Absorbance change at 551 minus 540 nm as a function of the oxidation-reduction potentials (E_h). Mediators and concentrations were methyl viologen (0.5 mM), ferrocenyl methyl trimethylammonium perchlorate (0.3 mM), ferricyanide (100 μM), hydroquinone (60 μM), diaminodurene (30 μM), 1,2-naphthoquinone (30 μM), phenazine methosulfate (60 μM) and phenazine ethosulfate (30 μM). Vesicles (2.1 mg protein/ml) were suspended in phosphate buffer (0.1 M, pH 7.0). The circles refer to oxidative and squares refer to reductive titrations. The solid line represents the theoretically calculated line which was drawn with an n = 1 in the Peters-Nernst equation.

Fig. 8. The midpoint potential of the +40 mV component corresponded to cytochrome (559.5). The —85 mV component corresponded to the cytochrome with maxima at 558 and 565 nm. These midpoint potentials were reproducible to within ±15 mV.

Difference spectra, dithionite reduced minus oxidized, which were obtained at near liquid nitrogen temperatures are shown in Fig. 9. The shoulder at 564 nm was assigned to the low potential, E_{m_7} = —85 mV, cytochrome (558, 565). A maxima at 556 and 537, and at 527 to 530 nm were composites of more than one cytochrome. A 582.5 nm maximum, not observed at room temperature,

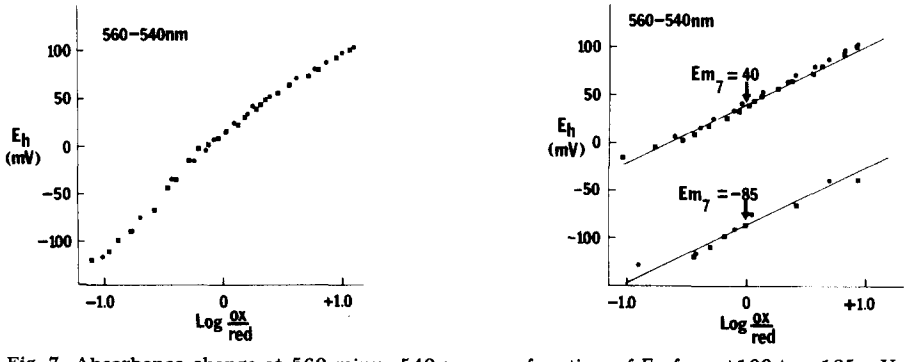


Fig. 7. Absorbance change at 560 minus 540 nm as a function of E_h from +100 to —125 mV. Mediators and concentrations as in Fig. 6 except diaminodurene was omitted and 5-hydroxy-1,4-naphthoquinone (30 μM), pyocyanine perchlorate (6 μM), 2-hydroxy-1,4-naphthoquinone (30 μM) and anthroquinone-2-sulfonate (40 μM) were also used.

Fig. 8. Resolution of the sigmoid curve of Fig. 7 into two, one-electron acceptors.

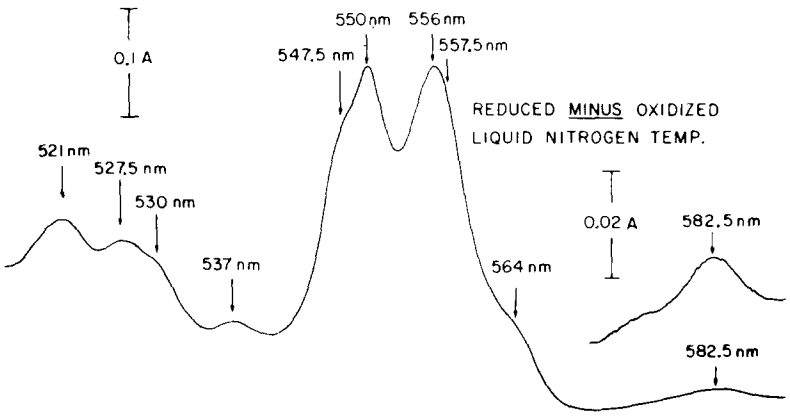


Fig. 9. Low temperature difference spectra of vesicles from *Pc. denitrificans*. Vesicles (2.6 mg/ml) were suspended in 0.1 M, pH 7.0 phosphate buffer. Dithionite was added to the treatment cuvette.

was also present. The spectral band of the *c*-type cytochrome at low temperatures was apparently 'split' with a maximum at 550 nm and a shoulder at 547.5 nm. Such a splitting was reported and used as supportive evidence for the multiplicity of the *c*-type cytochromes by Cox et al. [23].

One can define at least three different cases with *c*-type cytochrome(s) in terms of their number and absorbance properties to rationalize the splitting of the 551 nm band at liquid nitrogen temperatures. In case 1, a single *c*-type cytochrome which exhibits two maxima would be assumed. The absorbance ratio at 550 and 547.5 nm would remain constant during a redox titration if spectra were obtained at low temperatures. In case 2, two cytochromes differing in midpoint potential by 120 mV, each with two maxima which contributed equally to the total absorbance change, was assumed. The ratio of the absorbance at these two maxima in low temperature spectra would remain constant throughout a redox titration. Thus, no distinction between case 1 or case 2 would be possible from spectra obtained at low temperature. The purification of the cytochromes and/or the analysis of the shape of the redox titration curves would be necessary to delineate between cases 1 and 2.

In case 3, two cytochromes, 3A and 3B, differing in midpoint potential by 120 mV, with each cytochrome having one but different wavelength maximum, was assumed. If the higher potential component, 3A, was reduced first, an absorbance ratio greater than 26 would result (see Table I). If 100% of the high potential cytochrome *c* and 50% of the low potential *c*, 3B, were reduced, absorbance ratio would be approx. 2.6. When both cytochromes are completely reduced, the ratio would be 1.3.

Results of low temperature spectroscopy of vesicles paired at various redox potentials are shown in Fig. 10 (top). The ratio of the absorbances at 550 and 547.5 nm were constant over the E_h range 320 to 170 mV. Thus, either case 1 or 2 applies to the data. Since an *n* value close to one was obtained for room temperature redox titrations, either there was: (a) one *c*-type cytochrome or (b) two *c*-type cytochromes with slightly different midpoint potential and/or one of the *c*-type cytochromes was in very low concentration.

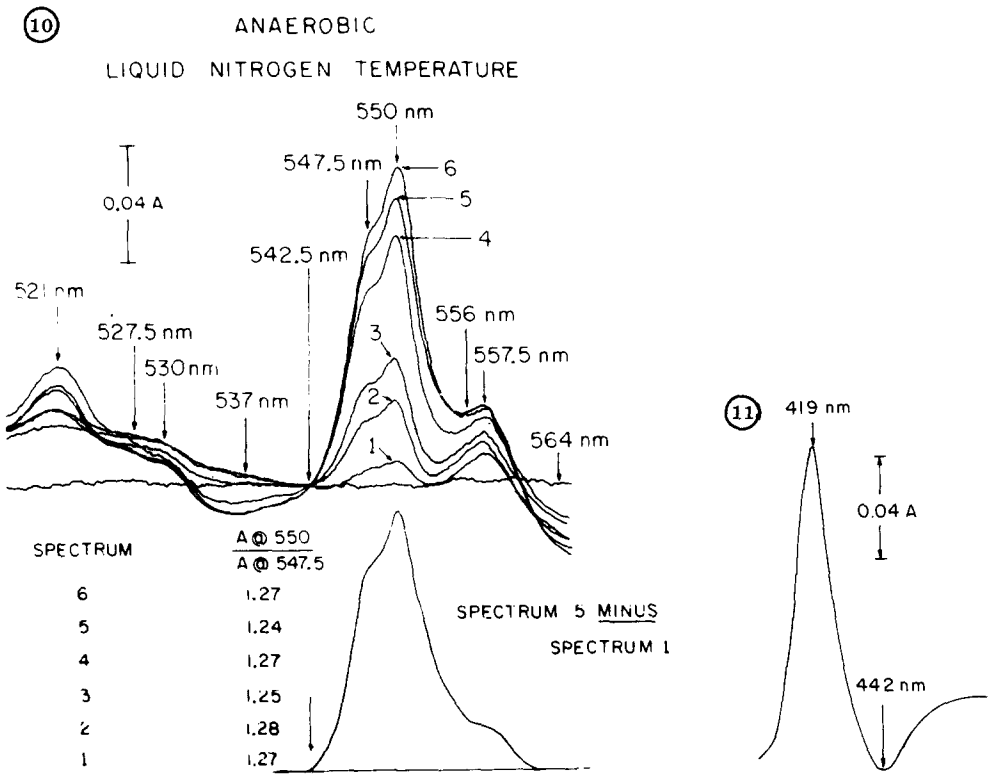


Fig. 10. Low temperature difference spectra of vesicles poised at various redox potentials (top). Ratio of absorbance at 550 to 547.5 nm at various redox potentials (bottom, left). Manual substraction of spectrum 5 minus spectrum 1 (bottom, right). Vesicles (2.6 mg protein/ml) were suspended in 0.1 M phosphate buffer (pH 7.0) containing 20 μM phenazine methosulfate, 60 μM hydroquinone, 50 μM diaminodurene and 50 μM ferricyanide. Redox potentials were adjusted using trace amounts of ferricyanide and dithionite.

Fig. 11. Dithionite reduced + carbon monoxide minus dithionite spectrum. Vesicles (1.2 mg protein/ml) were reduced with dithionite. Treatment cuvette was sparged with carbon monoxide.

Although considerable absorbance change occurred at 557.5 nm, the maximum for chromophore-559, (E_{m_7} = +355 mV), point-by-point subtraction of spectrum 5 minus spectrum 1, indicated most of the absorbance change at 557.5 nm was due to the spectral overlap with the *c*-type cytochrome (Fig. 10, bottom right). The small shoulder was attributed to the incomplete reduction of chromophore-559 when spectrum '1' on Fig. 10 was acquired at 320 mV. Thus, evidence for a *b*-type cytochrome, with an absorption maximum at approximately 560 nm and E_{m_7} = 250 mV as described by Cox et al. was not apparent.

The midpoint potentials and spectral maxima of the components described in our work are summarized in Table II. Evidence for the multiplicity of the *c*-type cytochromes [17,23] or the presence of *b*-type cytochromes with midpoint potentials +250 and 120 [23] are not apparent. The midpoint potential of the *c*-type cytochrome was similar to that reported in a purified preparation [18] suggesting no preferential binding of the oxidized or reduced *c*-type cytochrome as found in mitochondria [19]. The presence of *c*-type cytochromes in

TABLE I

ABSORBANCE RATIOS AT 550 AND 547.5 nm AT LOW TEMPERATURE IN REDOX TITRATION ASSUMING VARIOUS NUMBERS AND SPECTRAL MAXIMUM (*A*) FOR *c*-TYPE CYTOCHROMES

Case	Cytochromes	Maximum (*A*) (nm)		ΔA	$\frac{A\ (550\ nm)}{A\ (547.5\ nm)}$	
1	1, two maxima		550	0.26	Constant in redox titration	1.3
			547.5	0.21		
2	2, each with two maxima	2A	550	0.13	Constant in redox titration	1.3
			547.5	0.10		
	$\Delta E_m \geqslant 120$ mV	2B	550	0.13		
			547.5	0.10		
3	2, each with one maximum	3A	550	0.26	Variable in redox titration	
			High potential		1. Complete reduction of high potential cytochrome *c*	>26
	$\Delta E_m \geqslant 120$ mV	3B	547.5	0.20	2. High potential cytochrome *c* reduced; low potential cytochrome *c* 50% reduced	2.6
			Low potential		3. High and low potential cytochromes *c* reduced	1.3

TABLE II

MIDPOINT POTENTIALS AND SPECTRAL MAXIMA OF REDOX COMPONENTS IN MEMBRANE VESICLES DERIVED FROM ANAEROBICALLY-GROWN *PC. DENITRIFICANS*

Component	Spectral maxima (nm)		E_{m_7} (mV)
	alpha band	Soret band	
Low potential cytochrome	558, 565	431	−85
High potential cytochrome	559.5	428	+40
c-type cytochrome	551	425	+240
Chromophore	559	—	+355

the periplasmic space of *Pc. denitrificans* was not investigated.

The spectral maximum (or maxima in some cases) and midpoint potentials of cytochromes in vesicles derived from anaerobically-grown *Pc. denitrificans* (Table II) were indistinguishable from cytochromes in vesicles derived from cells grown aerobically in the same medium in the absence of nitrate (Kula, T. and Lillich, T., unpublished observations). Thus, we have no evidence from potentiometric and spectral data of additional *c*-type cytochromes with maximum at approx. 560 nm in vesicles of anaerobically-grown cells as suggested by John and Papa [20]. However, a membrane-bound carbon monoxide binding pigment, other than cytochromes $a + a_3$, was present in vesicles of anaerobically-grown cells but not readily demonstrable in vesicles of aerobically-grown cells [21].

The carbon monoxide complex had a maximum at 419 and a trough at 442 nm (Fig. 11). Multiplicity of components contributing to the 419 nm maximum cannot be excluded.

Since we cannot detect the carbon monoxide binding cytochrome, presum-

ably the *b*-type, with a midpoint potential of 120 mV as described by Cox et al. [23], the 419 nm maximum has been tentatively assigned to the interaction of carbon monoxide with a component, maximum 582.5 nm, detected at liquid nitrogen temperature. Detailed potentiometric and spectral data in the presence of carbon monoxide are required to delineate the amount and role of this component (maximum, 582.5 nm) in *Pc. denitrificans* during growth on various substrates and at different stages of growth.

Although differences between the data of Cox et al. [23] and our data were noted, the comparison was incomplete. Growth conditions, method of cell disruption, strain differences, mediators and mediator concentrations were some of the variable parameters.

Acknowledgements

The authors wish to thank Dr. Harvey Wilgus for providing the purified cytochrome *c* peroxidase from baker's yeast. This research was supported by a grant from NIH (GM 19181).

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