

Spectroscopic characterization of a high-potential monohaem cytochrome from *Wolinella succinogenes*, a nitrate-respiring organism

Redox and spin equilibria studies

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When purified, a high-potential *c*-type monohaem cytochrome from the nitrate-respiring organism, *Wolinella succinogenes* (VPI 10659), displayed a minimum molecular mass of 8.2 kDa and 0.9 mol iron and 0.95 mol haem groups/mol protein. Visible light spectroscopy suggested the presence of an equilibrium between two ligand arrangements around the haem, i.e. an absorption band at 695 nm characteristic of haem-methionine coordination (low-spin form) coexisting with a high-spin form revealed by a band at 619 nm and a shoulder at 498 nm. The mid-point redox potential measured by visible redox titration of the low-spin form was approximately +100 mV. Binding cyanide ($K_a = 5 \times 10^5 \text{ M}^{-1}$) resulted in the displacement of the methionyl axial residue, and full conversion to a low-spin, cyanide-bound form.

Structural features were studied by 300-MHz ¹H-NMR spectroscopy. In the oxidized state, the pH dependence of the haem methyl resonances (pH range 5–10) and the magnetic susceptibility measurements (using an NMR method) were consistent with the visible light spectroscopic data for the presence of a high-spin/low-spin equilibrium with a transition pK_a of 7.3. The spin equilibrium was fast on the NMR time scale. The haem methyl resonances presented large downfield chemical shifts. An unusually broad methyl resonance at around 35 ppm (pH = 7.5, 25°C) was extremely temperature-dependent [$\delta(323 \text{ K}) - \delta(273 \text{ K}) = 7.2 \text{ ppm}$] and was assigned to the S-CH₃ group of the axial methionine. In the ferrous state only a low-spin form is present. The haem *meso* protons, the methyl group and the methylene protons from the axial methionine were identified in the reduced form. The resonances from the aromatic residues (three tyrosines and one phenylalanine) were also assigned.

Detailed monitoring of the NMR-redox pattern of the monohaem cytochrome from the fully reduced up to the fully oxidized state revealed that the rate of the intermolecular electronic exchange process was approximately $6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ at 303 K and pH = 6.31.

A dihaem cytochrome also present in the crude cell extract and purified to a homogeneous state, exhibited a molecular mass of 11 kDa and contained 2.43 mol iron and 1.89 mol haem *c* moieties/mol cytochrome. The absorption spectrum in the visible region exhibited no band at 695 nm, suggesting that methione is not a ligand for either of the two haems. Recovery of only small amounts of this protein prevented more detailed structural analyzes.

In denitrifying bacteria, nitrate and its reduction products, nitrite, nitric oxide and nitrous oxide, serve as terminal electron acceptors in anaerobic respiration [1]. The electron transport system responsible for the denitrifying process is complex. The different electron acceptors involved are not yet precisely placed in the electron transfer scheme. Independent or branched electron transfer chains lead the electrons towards the different nitrogen oxides during denitrification [1] and a number of *b* and *c*-type cytochromes have been detected [2]. Furthermore, certain bacteria carry out less extensive denitrification than others [3]. Some reduce nitrate incompletely to nitrous oxide. Others reduce nitrite, nitric oxide and nitrous oxide, but not nitrate to dinitrogen [3].

Different still is *Wolinella succinogenes* (VPI 10659), which exhibits capability for dissimilatory reduction of nitrate

through nitrite to ammonia only. It produces no gaseous products but reduces both nitric and nitrous oxides [3]. The electron transport components of this organism thus appear uniquely interesting.

In addition to the previously purified hexahaem *c*-type cytochrome (an ammonia-yielding nitrite reductase) [4] we isolated and purified two other *c*-type cytochromes from nitrate-respiring *W. succinogenes* cells. One is a high-potential monohaem cytochrome present exclusively in the soluble fraction and the other a dihaem cytochrome located in both the soluble and the membrane fractions. The present article describes the purification of these cytochromes and a detailed spectroscopic study of the monohaem cytochrome. Our interest in this cytochrome arises from having noticed its unique physicochemical properties revealed by visible-light absorption and NMR analyzes. The spectroscopic features are compared with those of cytochromes from other dissimilatory nitrate reducers.

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MATERIALS AND METHODS

W. succinogenes (VPI 10659) was grown anaerobically under oxygen-free argon at 37°C in VSY-4 medium (pH 7.6) [5] supplemented with 100 mM nitrate. Cells were harvested by centrifugation after 24 h, ruptured and fractionated as indicated in the text. Products employed in the purification procedures included Ultrigel AcA-44 (obtained from LKB Instruments) and DEAE-Bio-Gel A and Bio-Gel HTP purchased from Bio-Rad Laboratories.

Ultraviolet/visible-light absorption spectra of the cytochromes were obtained with a Varian DMS 90 recording spectrophotometer. The number of *c*-type haem groups per molecule of protein was estimated by the pyridine haemochromogen techniques based on a red $\epsilon_{550\text{ nm}} = 29.1\text{ mM}^{-1}\text{ cm}^{-1}$ of haem *c* [6]. Analytical polyacrylamide disc gel electrophoresis was performed according to the method of Brewer and Ashworth [7]. Minimal molecular masses were determined by SDS/polyacrylamide gel electrophoresis according to the method of Weber and Osborn [8]. Iron content was determined by plasma emission spectroscopy using the Jarrel-Ash model 750 Atomcomp. The modified Biuret method developed by Zamenhof and Chargoff [9] was used for the protein determination using horse cytochrome *c* as the standard. Amino acid analyzes were performed with a Beckman model 120 amino acid analyzer. Protein samples were hydrolyzed in 6 M HCl at 110°C for 24 h, according to the method of Moore and Stein [10]. The values for threonine, serine and tyrosine were corrected for decomposition during hydrolysis.

Electron paramagnetic resonance (EPR) was carried out on a Bruker 200-tt spectrometer, equipped with an ESR-9 flow cryostat (Oxford Instruments Co., Oxford) and a Nicolet 1180 computer where mathematical manipulations were performed.

For the nuclear magnetic resonance (NMR) studies, the protein was dialyzed against deionized water at pH 7.0 at 4°C and lyophilized twice from 99.8% $^2\text{H}_2\text{O}$. The sample was dissolved in 99.8% $^2\text{H}_2\text{O}$ and the pH was adjusted with either NaOH or ^2HCl . Quoted pH values are meter readings uncorrected for the isotope effect. High-resolution NMR spectra were recorded in the Fourier-transform mode on a Bruker CXP 300 spectrometer (300 MHz) equipped with an Aspect 2000 computer. The temperature was controlled to $\pm 0.5^\circ\text{C}$ with a standard Bruker B-VT-1000 variable temperature control unit. All chemical shift values are quoted in parts per million (ppm from internal sodium 3-trimethylsilyl-(2,2,3,3- $^2\text{H}_4$)propionate, positive values referring to low-field shifts. Reduction of the protein was achieved by addition of small amounts of sodium dithionite under an argon atmosphere, to delay auto-oxidation. The samples were reoxidized very slowly by introducing small amounts of air into the NMR tube with a Hamilton syringe through serum bottle caps.

Magnetic susceptibility measurements were performed by an NMR method [11] using concentric cylindrical cells (Wilmad Glass Company Inc., WGS-5BL), the internal capillary containing only the solvent and the marker. The methyl proton resonance of sodium trimethylsilyl (2,2,3,3- $^2\text{H}_4$)-propionate was used as susceptibility marker.

The paramagnetic contribution to the molar magnetic susceptibility (χ_M^p) of the oxidized form was determined as a function of pH. The differences Δf between the resonance positions of the marker in the protein and in the capillary

solution were measured and converted into magnetic susceptibilities using the relation:

$$\chi_M = \frac{3\pi}{4} \frac{\Delta f}{fc} + \chi_0$$

where χ_M is the molar magnetic susceptibility, f is 3×10^8 Hz and c is the concentration of the protein. The solvent and solution densities were assumed to be similar. χ_0 is the magnetic susceptibility of the solvent and was considered to be negligible. A correction value of $\chi_D = 1.7 \times 10^{-3}\text{ cm}^3/\text{mol}$ was used for the diamagnetic contribution of the protein [12].

Purification of the cytochromes

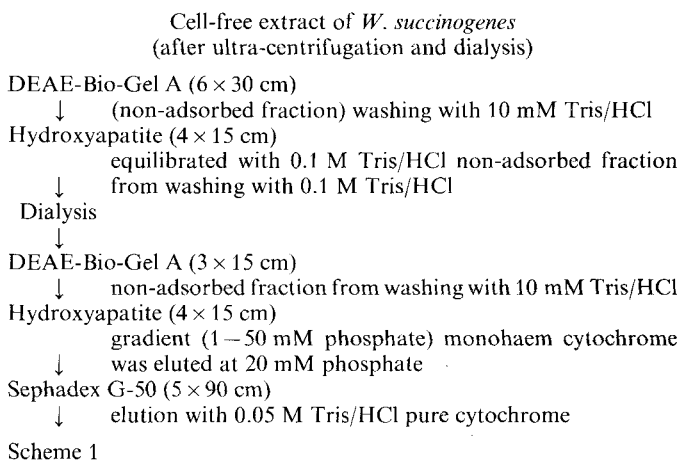
Frozen cells (400 g) were mixed with 10 mM Tris/HCl buffer (pH 7.6 at 4°C) to give 1:1.5 (mass/vol.) suspension and broken by passing three times through a Manton-Gaulin homogenizer at 62 MPa. A few crystals of DNase were added to lessen the viscosity of the extract. After 10 min, the preparation was centrifuged at $13200 \times g$ in a Sorvall RC-5B refrigerated centrifuge for 30 min. The pellet was discarded and the supernatant fluid treated with neutralized streptomycin sulfate (0.5 mg/mg protein). After stirring for 15 min, the preparation was centrifuged at $144000 \times g$ for 2 h. The supernatant containing only soluble proteins served as the source of the high-potential monohaem *c*-type cytochrome, whereas the low-potential *c*-type cytochrome was extracted and purified from the pellet (membrane particles).

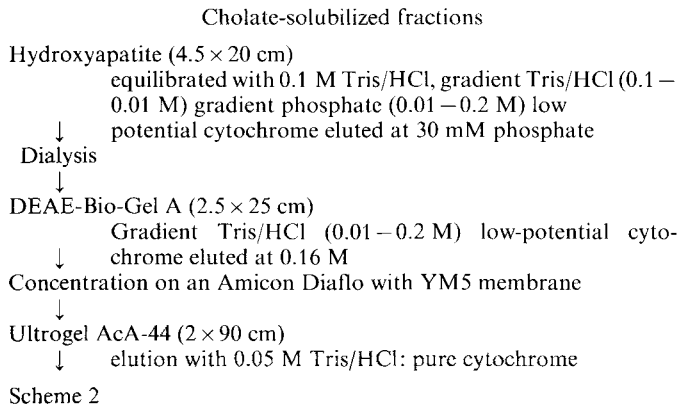
High-potential monohaem cytochrome

The soluble fraction was dialyzed against 10 mM Tris/HCl buffer (pH = 7.6) for 18 h with three changes of dialysis buffer. The monohaem cytochrome was next subjected to the chromatographic purification steps as indicated in Scheme 1, in which all the elution buffer concentrations and columns sizes are indicated.

Low-potential dihaem cytochrome

The membrane fraction was washed three times with 200 ml 0.1 M Tris/HCl buffer and recovered each time by centrifuging at $144000 \times g$ for 1 h. The supernatant fluids containing contaminating soluble proteins were discarded. The pellet from the last wash was suspended in 0.1 M Tris/HCl buffer with a final volume of 200 ml. Sodium cholate was





added (3 mg/ml) and, after the cholate had dissolved, the mixture was incubated at 4°C for 24 h. The preparation was centrifuged and the pellet again suspended and similarly extracted with a higher concentration of sodium cholate (4 mg/ml). The cholate extracts containing the low-potential *c*-type cytochrome and the nitrite reductase were combined and subjected to the chromatographic purification steps indicated in Scheme 2.

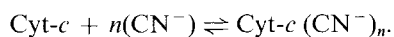
Redox titration of the high-potential cytochrome

The redox titration of the protein was performed under anaerobic conditions in an optical redox cell, modified slightly from the design of Dutton et al. [13]. The potentials were measured with a Fisher Acumet pH meter, model 620. The redox cell containing a volume of 5 ml was kept well stirred and under a continuous stream of argon gas. The final protein concentration was 20 μM. The redox mediator used was 2,6-dichloroindophenol in a final concentration of 2 μM. The potentials were varied by injecting appropriate volumes of deaerated 1 mM sodium dithionite and potassium ferricyanide. The protein was dissolved in 100 mM potassium phosphate buffer, pH 7.6, and titrated in both the oxidative and reductive directions to ensure reversibility. Optical spectra were recorded throughout the titration.

Determination of the binding constant of cyanide to ferric high-potential cytochrome

A 430 μM solution *W. succinogenes* high-potential cytochrome in 0.01 M Tris/HCl, pH = 7, was titrated with potassium cyanide (7 mM). The titration was carried out by injecting different amounts of potassium cyanide solution. The absorbance of the solution at 619 nm was measured after a 15-min incubation. Longer periods of incubation yielded no further changes. The pH value during the titration was maintained at 7.0.

Absorption changes measured at 619 nm were used to determine the ligand stoichiometry and equilibrium constant. The ligand stoichiometry (*n*) and the association constant (*K_a*) were determined considering that for the equilibrium reaction:



The binding parameters can be calculated as follows:

$$\log [(A - A_0)/(A_{100} - A)] = \log K_a + n \log [\text{CN}^-]$$

where *A*₀ is the absorbance of the solution in the absence of cyanide, *A*₁₀₀ is the value of the absorbance of the solution when the cytochrome is completely ligated to CN⁻ and *A* is

the absorbance of the solution for a given concentration of CN⁻.

The concentration of cyanide ion was calculated from the total concentration of cyanide, taking into account the pH and the acid dissociation constant of hydrogen cyanide:

$$[\text{CN}^-] = [\text{CN}^-]_t / (1 + [\text{H}^+]/K_{\text{HCN}})$$

RESULTS

Low-potential dihaem cytochromes

Inspection of the absorption spectra of the purified low-potential dihaem cytochrome indicated that, as the designation implies (Fig. 1 A), sodium dithionite with a low redox potential (broken line) fully reduced this cytochrome, but the higher-potential sodium ascorbate (dotted line) did not. Partial, just perceptible, reduction by sodium ascorbate shown by the small rise in the 550-nm region) indicated that one of the haem groups has a redox potential only slightly higher than of sodium ascorbate. A purity index [*A*_{550–570 nm} (reduced)/*A*_{280 nm} (oxidized)] of 1.69 was calculated for this cytochrome. Assay of a concentrated solution of the low-potential cytochrome by visible-light spectroscopy revealed no band at 695 nm, suggesting that no methionine ligand arrangement occurs for this cytochrome (Fig. 2). For comparison the visible spectra of *Desulfovibria vulgaris* tetrahaem

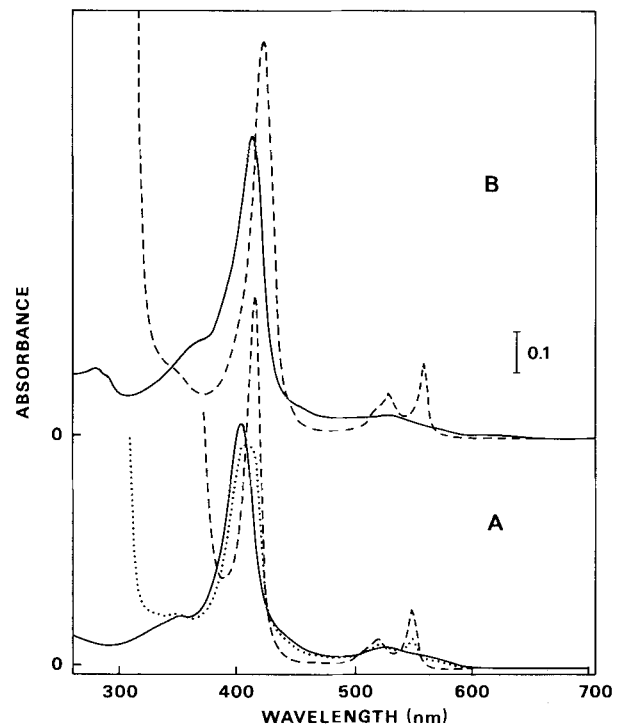


Fig. 1. Absorption spectra of cytochromes from *W. succinogenes*. (A) The low-potential *c*-type: (—) oxidized; (·····) ascorbate-reduced; (---) dithionite-reduced. The protein concentration was 2.5 μM in 0.1 M potassium phosphate buffer, pH 7.6. Solid sodium ascorbate or sodium dithionite was added for reduction of the cytochrome. (B) The high-potential *c*-type: (—) oxidized; (---) ascorbate-reduced. The protein concentration was 8.5 μM in 0.1 M potassium phosphate buffer, pH 7.6. Solid sodium ascorbate was added for reduction of cytochrome

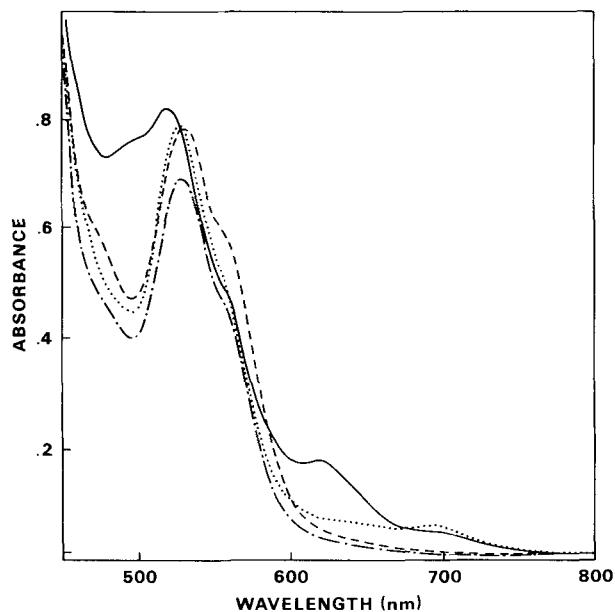


Fig. 2. Absorption spectra at the higher wavelength for cytochromes from *W. succinogenes* and other sources. (.....) Horse heart cytochrome *c*; (---) *Desulfovibrio vulgaris* cytochrome *c*₃; (—) *W. succinogenes* high-potential *c*-type cytochrome; (- · - · -) *W. succinogenes* low-potential *c*-type cytochrome. Horse heart cytochrome *c* has an axial ligand arrangement of the histidine-methionine type and shows a peak in the 695-nm region. *D. vulgaris* cytochrome *c*₃ has a histidine-histidine axial ligand arrangement and does not show the 695-nm peak. The spectrum of the *W. succinogenes* high-potential *c*-type cytochrome resembles that of horse heart cytochrome *c*, whereas that of *W. succinogenes* low-potential *c*-type cytochrome has features similar to those of the *D. vulgaris* cytochrome *c*₃.

cytochrome *c*₃ (*c*-type haem with bis-histidinyl axial coordination) and horse-heart cytochrome *c* (*c*-type haem with methionyl and histidinyl axial ligands) are super-imposed in the figure.

The molecular mass determined by SDS/polyacrylamide gel electrophoresis was 11 kDa. Based on this value, we found 2.43 mol Fe and 1.89 mol haem *c*/mol cytochrome and concluded it to be a dihaem protein.

High-potential monohaem cytochromes

SDS/polyacrylamide gel electrophoresis revealed that the monohaem cytochrome has a minimal molecular mass of 8.2 kDa. Based on this value we found 0.87 mol Fe and 0.95 mol haem *c*/mol cytochrome.

Spectra in Fig. 1B reflect the typical *c*-type cytochrome nature of this protein. A purity index [$A_{552} - A_{570 \text{ nm}}$ (reduced)/ $A_{280 \text{ nm}}$ (oxidized)] of 1.06 was determined. Table 1 compares the absorption maxima and molar absorption coefficients of both cytochromes. In an attempt to elucidate the ligand arrangement around the haem iron atom of the purified cytochrome, spectral details of a concentrated preparation of the oxidized cytochrome were examined (Fig. 2). An absorption maximum at 695 nm indicated the presence of a methionyl residue as an axial haem ligand. In addition to the typical absorption bands of a ferric *c*-type cytochrome, another band was found at 619 nm and a shoulder at 498 nm revealing the presence of a high-spin-state ferric haem iron. After addition of potassium cyanide to the ferricytochrome, these bands no longer appeared. Cyanide induced shifts of

Table 1. Absorption maxima and molar absorption coefficients of *c*-type cytochromes from *W. succinogenes*

The values were determined for purified cytochromes in 0.1 M sodium phosphate buffer, pH = 7.6. The high-potential *c*-type cytochrome was reduced by addition of a few crystals of sodium ascorbate; the low-potential *c*-type cytochrome by addition of a few crystals of sodium dithionite

<i>c</i> -Type cytochrome species	Oxidized		Reduced	
	λ_{max}	ϵ	λ_{max}	ϵ
	nm	$\text{mM}^{-1} \text{cm}^{-1}$	nm	$\text{mM}^{-1} \text{cm}^{-1}$
High-potential monohaem	522.0	7.5	552.0	20.3
	407.2	80.1	523.2	12.6
	280.0	19.2	416.5	104.0
Low-potential dihaem	530.0	20.7	550.6	57.0
	407.5	228.1	552.0	30.5
	280.0	22.1	418.0	349.2

Table 2. Amino acid composition of *c*-type cytochromes from *W. succinogenes*

Amino acid	Residues/molecule of	
	high-potential monohaem cytochrome	low-potential dihaem cytochrome
Lys	11	3
His	1	5
Arg	1	4
Asp	7	13
Thr	3	6
Ser	5	5
Glu	7	12
Pro	1	10
Gly	9	5
Ala	13	12
Cys	2	4
Val	4	5
Met	2	7
Ile	2	4
Leu	6	5
Tyr	3	1
Phe	1	4
Trp	0	0
Total	78	105

other bands as well 407 nm to 412 nm and 522 nm to 532 nm. These effects are comparable to those observed for mammalian cytochrome *c* [14] and for cytochrome *c*₅₅₄ from *Alcaligenes faecalis* [15] and indicate the displacement of the sixth ligand methionine by cyanide.

The amino acid composition of the high-potential monohaem cytochrome differed from that of the low-potential dihaem cytochrome (Table 2). The number of cysteine residues found for each cytochrome appeared sufficient, however, for the attachment of one and two haems in the mono and dihaem proteins, respectively.

Mid-point redox potential

The mid-point oxidation/reduction potential was estimated from measurements of the absorbance changes at 416.5 nm and 552 nm that were followed during the redox titration with dithionite and potassium ferricyanide reoxidation in the presence of dichlorophenolindophenol.

The absorbance changes were plotted as a function of the poised redox potential of the solution as shown in Fig. 3 (only shown for $\lambda = 552$ nm). The experimental data were fitted for one electron in the Nernst equation. The mid-point redox potential determined was $+105 \pm 15$ mV.

Cyanide binding

A 430- μ M solution of *W. succinogenes* high-potential cytochrome was titrated with potassium cyanide at pH 7.0. The changes in the intensity of the band at 619 nm were followed (Fig. 4). The calculated binding constant was 5.2×10^5 M⁻¹ (the fitted slope is 1.21).

The 695-nm band also changed in intensity during the titration indicating that cyanide bound both the high-spin and low-spin forms.

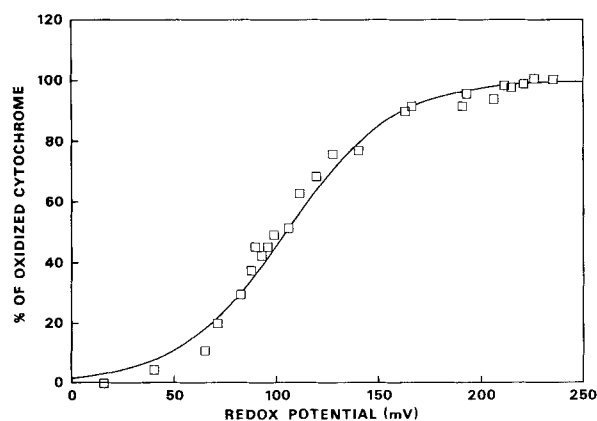


Fig. 3. Redox titration of *W. succinogenes* high-potential cytochrome. Absorbance changes, A_{552} (α -band reduced) minus A_{560} (isobestic point, oxidized/reduced) as a function of the poised redox potential. The theoretical curve shown is calculated from a Nernst equation with $n = 1$

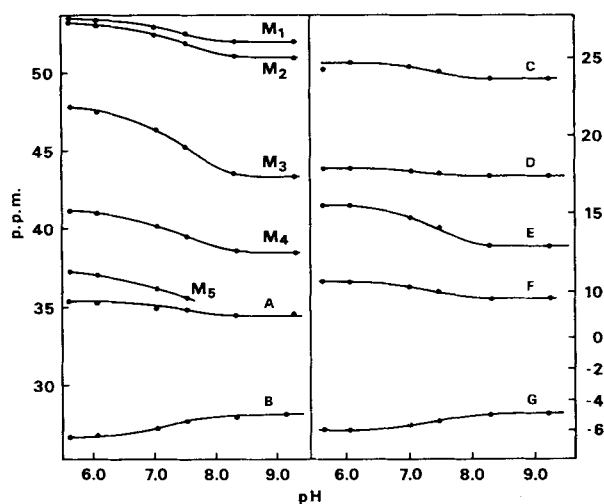
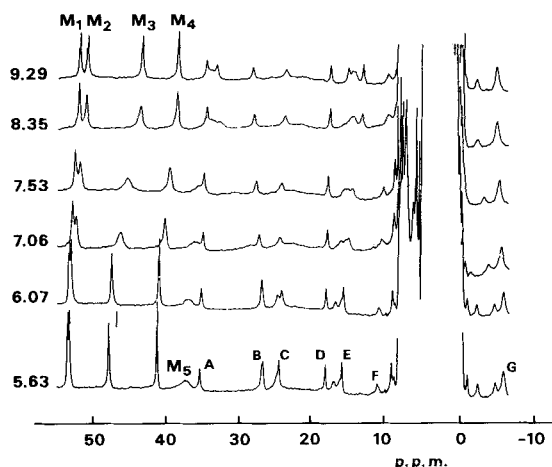


Fig. 5. 300-MHz ¹H-NMR spectra of *c*-type cytochromes of *W. succinogenes*. Left: the high-potential ferricytochrome assayed at 303 K and at the indicated pH values. Right: pH dependence of selected resonances, as seen on left

Temperature and pH influences on ¹H-NMR studies of the high-potential cytochrome

Ferricytochrome

The ¹H-NMR spectra of the ferric form at different temperatures are shown in Fig. 5. As expected for a paramagnetic protein, a large number of resonances were seen downfield from 10 ppm. In analogy with other cytochromes *c*, the four haem methyl groups were expected to present large downfield hyperfine shifts due mainly to contact mechanism. The three-proton intensity resonances M_i ($i = 1, 2, 3, 4$) were assigned to haem methyl groups. The hyperfine shifts of the haem methyl groups were quite large when compared to those of low-spin cytochrome [16] (and references therein).

Varying temperature markedly effected the ferricytochrome spectrum for some selected resonances (Fig. 5). Resonance M_5 was highly temperature-dependent [$\delta(323$ K – $\delta(273$ K) = 7.2 ppm at pH = 7.5] and showed an anti-Curie behaviour. Haem methyl 3 displayed an anti-Curie dependence, haem methyls 2 and 4 were almost temperature-independent, and haem methyl 1 was slightly Curie temperature-dependent.

Examination of the low-field region of the ¹H-NMR spectra of the ferricytochrome at different pH values revealed that all four haem methyl resonances moved downfield with

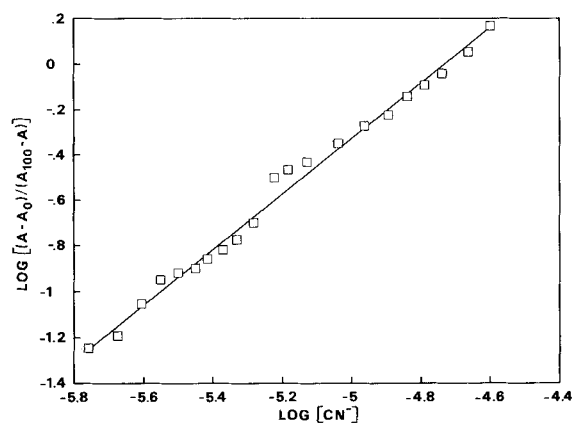


Fig. 4. Log/log plot of the response to binding of cyanide to *W. succinogenes* high-potential *c*-type cytochrome

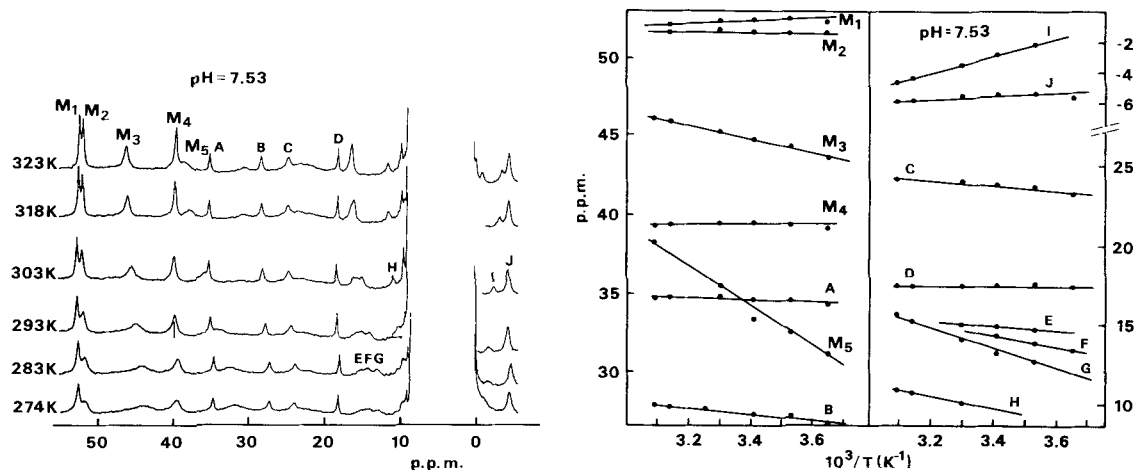


Fig. 6. Influence of assay temperature on the 300-MHz ^1H -NMR spectra. Left: on the high-potential ferricytochrome from *W. succinogenes* (pH = 7.53). Right: chemical shifts of selected resonances, as seen on left

Table 3. Magnetic susceptibility of the high-potential ferricytochrome from *W. succinogenes*

Cytochrome solution was assayed at 308 K by the NMR method described in Materials and Methods

pH	$10^{-3} \times \chi_M^p$	μ
4.90	7.59	4.31
7.50	4.64	3.38
9.98	2.52	2.49

decreasing pH (Fig. 6). A pK value of 7.3 was associated with these variations. Interestingly a line-broadening of the haem methyl peaks occurred in the intermediate range of pH, but only one methyl resonance position was observed throughout the pH range. This indicates fast-to-intermediate chemical exchange between the two spin states on the NMR time scale.

Magnetic susceptibility measurements at several pH values were compatible with an increase of the amount of a high-spin form of the cytochrome at lower pH (Table 3). True $S = 5/2$ and $S = 3/2$ spin systems have well defined magnetic values, i.e. 5.92 and 1.73, respectively. The experimental values obtained for the monohaem cytochrome clearly indicate that the contribution of the high-spin species depended largely on the pH value.

The changes in the visible absorption bands at 619 nm (high-spin) and 695 nm (methionine-low-spin) forms were also monitored at different pH values (Fig. 7). Two inflection points appeared, one at pH 7.3 (decrease of $A_{619 \text{ nm}}$ at higher pH values) and another at pH 10.9. Measurement of the intensity of the 695-nm band is not easily interpretable due to the contribution of the tail of the 620-nm band, but intensity clearly increased at pH values >7 and disappeared at pH values >10 (data not shown). These results are consistent with the pH dependence of the establishment of the high-spin/low-spin equilibrium observed by NMR.

The methyl resonance from the bound methionine axial ligand was not observed in the upfield spectral region. Thus, the high-spin state could be produced by loss of the sixth axial ligand (methionyl sulfur) to the ferric ion. The chemical exchange between the bound and unbound axial ligand could induce a large chemical shift in the methionine methyl resonance [15]. Resonance M_5 is a plausible candidate for this

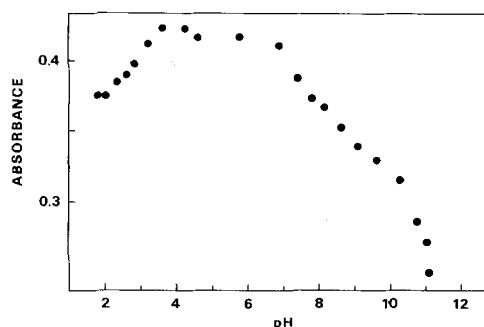


Fig. 7. pH dependence of the intensity of the 619-nm absorbance band of the high-potential ferricytochrome from *W. succinogenes*

S- CH_3 methionine, due to its chemical shift and strong temperature dependence.

Moore [16] reported a linear correlation between the chemical shift of methionine ligand methyl resonance and the sum of the chemical shifts of the accompanying four haem methyl resonances. Using this correlation, the value found for the chemical shift of methionine ligand methyl resonance was close to the one found for resonance M_5 . This supports the previous assignment.

Ferrocyanide

The haem *meso*-proton resonances (9.88, 9.59, 9.30 and 9.25 ppm) and the resonances originated from the bound axial methionine (S- CH_3 at -3.72 ppm and methylene protons at -3.85 , -1.66 and -0.70 ppm) were readily discernible (Fig. 8). The resonances at -1.66 and -0.70 ppm belong to the same methylene protons. The other methylene proton was not identified.

Cyanide complex of ferricytochrome

In the presence of cyanide at 303 K, the ^1H -NMR spectrum of ferricytochrome exhibited hyperfine-shifted methyl resonances at 26.4, 24.6 and 18.9 ppm (data not shown). These observations were consistent with the visible spectroscopic data that also revealed binding of CN^- in the oxidized form converting the haem iron to a low-spin form.

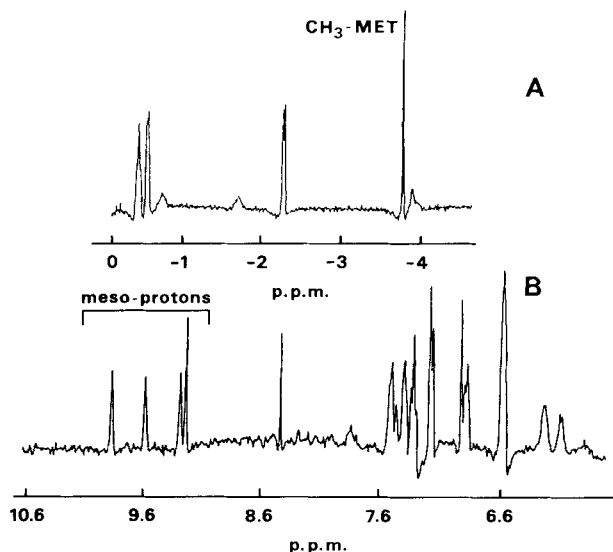


Fig. 8. 300-MHz $^1\text{H-NMR}$ spectrum of the high-potential *W. succinogenes* ferrocytochrome. (A) High-field region with evidence for the appearance of the methyl group of the axial methionine. (B) Low-field region, showing the responses of *meso* and aromatic protons

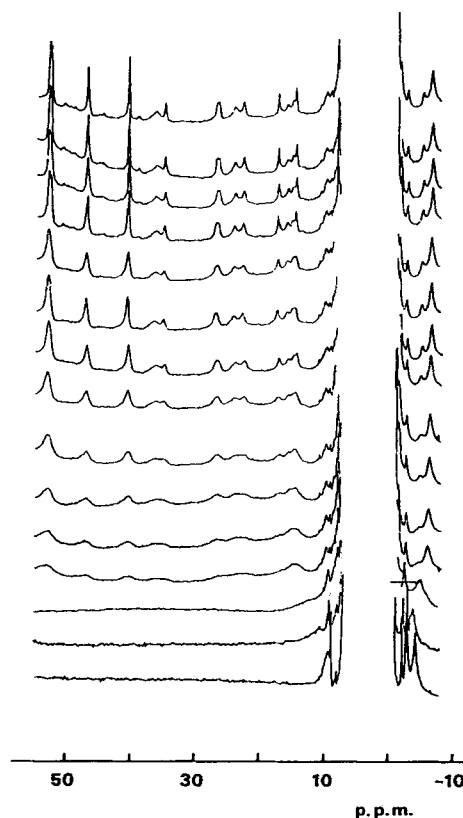


Fig. 9. 300-MHz $^1\text{H-NMR}$ spectra (low- and high-field regions) of high-potential *c*-type cytochrome from *W. succinogenes* at different oxidation-reduction stages proceeding from the fully reduced (lower spectrum) to the fully oxidized state (upper spectrum). The titration was carried out at 303 K, pH = 6.31

Oxidation-reduction equilibria

Fig. 9 shows in detail the 300-MHz NMR reoxidation pattern of *W. succinogenes* monohaem cytochrome followed in the low-field and high-field regions of the spectra.

Drastic modification in the NMR spectrum of the monohaem cytochrome occurred upon proceeding from the fully reduced diamagnetic to the fully oxidized paramagnetic state. This observation provides powerful means of determining the mechanism involved in the electron transfer process.

It is possible to follow the resonance of a methylene proton from the fully reduced state (-1.66 ppm) to the fully oxidized state (-2.24 ppm) since fast chemical exchange was observed. A lower limit for the electron exchange of $3.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (pH = 6.31) can be estimated using the difference in chemical shifts between the oxidized and reduced state. This resonance can be used to estimate the percentage of oxidized and reduced state along the time course of titration. Measuring the line width of the haem methyl resonances in the last steps of reoxidation provides an even better estimation of exchange rates. The chemical shift in these last steps did not change. It is evident that the rate of electron exchange is intermediate-to-slow on the NMR time scale. Using the breadth of these resonances together with the percentage of oxidized state (estimated from the chemical shift of the above-mentioned methylene proton), an exchange rate of $6.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (at 303 K and pH = 6.31) can be calculated.

Identification of the aromatic residues

The aromatic region in the NMR spectra of the *W. succinogenes* high-potential cytochrome during reoxidation (bottom to top) is shown in Fig. 10. Determination of the amino acid composition of the protein revealed the presence of three tyrosines, one phenylalanine and one histidine (Table 2). The sole histidine is the fifth ligand of the haem. Decoupling experiments clearly establish the assignment of one phenylalanine and one tyrosine (see Table 4). Of the other two tyrosines, one seems to give a singlet that shifts to low field when the cytochrome goes from the reduced to the oxidized form.

The other is probably responsible for two of the resonances at 7.54, 7.43 or 6.61 ppm (in the reduced form) which shift to low field when the protein undergoes oxidation. At the present moment it is not possible to identify clearly which of the pairs (7.54, 7.43), (7.54, 6.61) or (7.43, 6.61) correspond to the resonances of this tyrosine (reduced form). In the oxidized form of the cytochrome, these resonances have a large pH dependence, shifting to lower field with increasing pH. The chemical shift of the resonances of this tyrosine between the two oxidation states and lack of resolution as two doublets indicated a close proximity to the haem group. Similarly, the phenylalanine registers the change in the oxidation state, indicating its nearness to the haem.

In agreement with the conclusion that these groups are close to the haem and register the change between high-spin and low-spin (see Fig. 10), the resonances of these two aromatic residues are those most affected by the pH (in the oxidized form).

EPR spectrum of the ferricytochrome

The EPR spectrum of the oxidized form (native) of the high-potential cytochrome (Fig. 11; pH 6.8 and $T = 4.2$ K) mainly featured a $g_{\text{max}} = 3.33$ (half-width 10 mT) and a very broad component around $g = 1.84$ (g_{med}). The g_{min} feature was probably too broad to be detected at high field. These signals are typical of a low-spin haem form. No EPR signals were observed around $g = 6$, indicating the absence of a high-spin haem form.

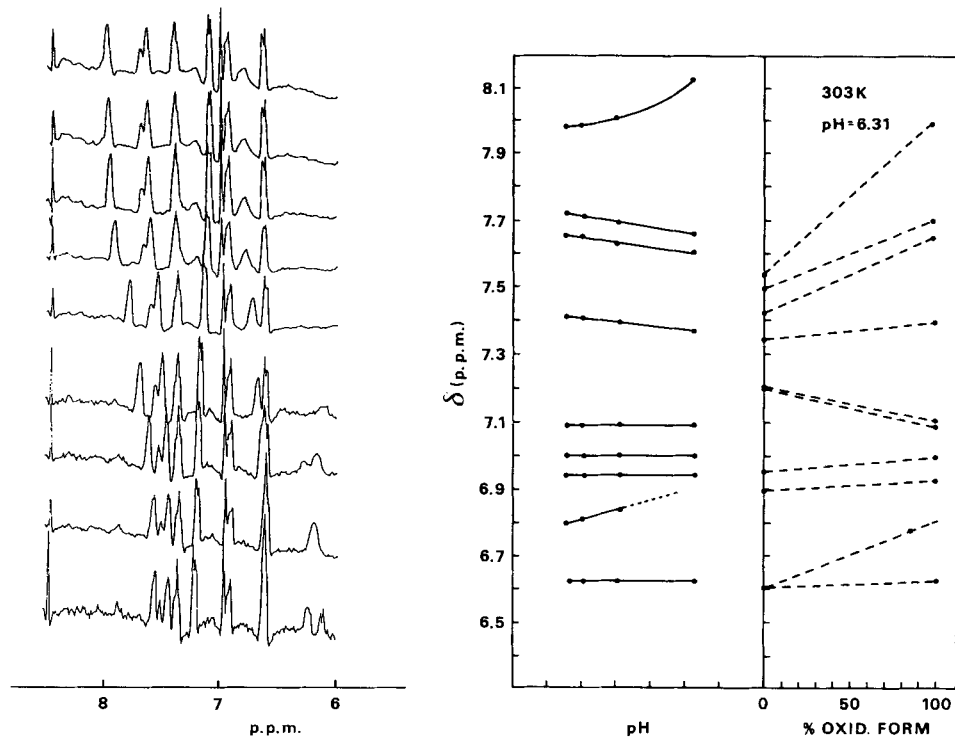


Fig. 10. 300-MHz $^1\text{H-NMR}$ spectra. Left: aromatic region of high-potential *c*-type cytochrome from *W. succinogenes* in several oxidation-reduction stages (fully reduced, lower spectrum; fully oxidized, upper spectrum). The titration was carried at 303 K, pH = 6.31. Right: chemical shift changes of the aromatic proton residues of the oxidized form of the high-potential cytochrome at 303 K as a function of pH and of the redox state at pH 6.31

Table 4. NMR assignments of the aromatic residues of the high-potential cytochrome of *W. succinogenes*

Assignment	δ in	
	oxidized form	reduced form
	ppm	
Tyr	6.61 (doublet)	6.62 (doublet)
	6.90 (doublet)	6.93 (doublet)
Tyr (singlet)	6.96	7.00
Tyr ^a	7.99	7.54
	7.65	7.43
	6.80	6.61
Phe (doublet)	7.21	7.11 (<i>ortho</i>)
(triplet)	7.35	7.40
(triplet) ^a	(7.50)	(7.70) (<i>para</i>)

^a The hyperfine structure is not resolved in these resonances.

DISCUSSION

Because of its unique capacity for performing an interrupted type of denitrification, the electron transport system of *W. succinogenes* (VPI 10659) is interesting in many respects. One relates to the bacterium itself, as the organism obviously possesses a system for utilizing nitrate and nitrite or nitric and nitrous oxides as terminal electron acceptors. The other relates to the physical properties of the enzymes involved in these functions. The difference between the electron transport systems of this organisms and others (e.g. *Escherichia coli* K12 [19], *Achromobacter fischeri* [20] and *Desulfovibrio desulfuricans* ATCC 27774 [21]), which also exclusively yield am-

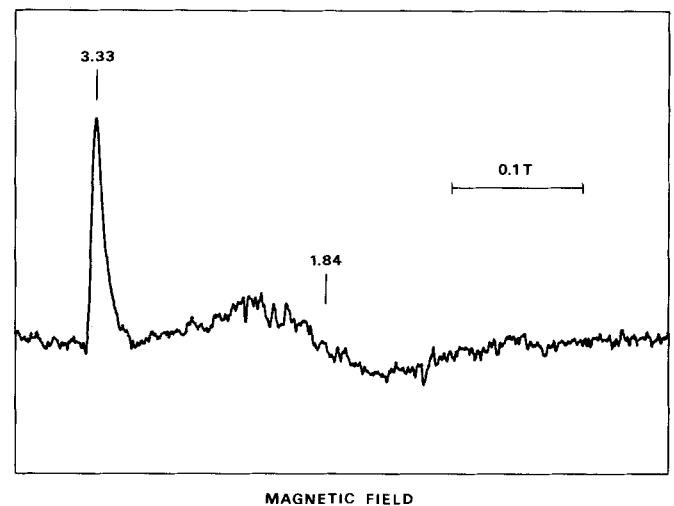


Fig. 11. X-band EPR spectrum of native *W. succinogenes* high-potential cytochrome at 4.4 K. Gain 1.6×10^5 ; microwave power 2 mW, modulation amplitude 1 mT, microwave frequency 9.474 GHz

monia as a product of nitrate respiration and do not reduce nitric oxide or nitrous oxide, may reflect, at least in part, the required adaptation for the utilization of nitric and nitrous oxides as terminal electron acceptors.

Moreover, by studying the electron transport system of an organism which has all but one of the parts of the overall denitrification process, some insight can be obtained into the necessarily more complicated electron transport system operating in bacteria that are complete denitrifiers. Based on the assumption that similar electron transfer proteins are used

in different denitrifying organisms for the same functional reactions, those homologous electron transfer proteins found both in *W. succinogenes* and complete denitrifiers should be those involved in reduction of nitric and nitrous oxides to dinitrogen.

The presence of *b*- and *c*-type cytochromes in cells of *W. succinogenes* grown in a formate/fumarate medium was first reported by Jacobs and Wolin [22]. By redox titration studies these workers established the existence of two *b*-type cytochromes (with redox potentials of -200 and -20 mV) and two *c*-type (with redox potentials of -160 and $+70$ mV).

We have isolated and purified three different *c*-type cytochromes from formate/nitrate-grown *W. succinogenes* cells. A hexahaem *c*-type cytochrome previously reported [4] is equally distributed between the soluble and membrane fractions and reduces nitrite to ammonia. As to the other two, the high-potential one was recovered exclusively in the soluble fraction, whereas the low-potential one was detected in both soluble and membrane fractions.

As *W. succinogenes* cells grown on formate/nitrate medium still have nitric and nitrous oxide reductase activities similar to cells grown on a formate/nitrous oxide medium [3], the organism apparently has an electron transport system capable of performing both NH_3 -forming nitrate respiration and nitric and nitrous oxide respiration. In several studies of nitrite respiration to ammonia [3, 21, 23] the multihaem *c*-type cytochrome nitrite reductase was the only cytochrome observed in the electron transport chain. Experiments that may exclude participation of the high- and low-potential *c*-type cytochromes from participation in nitrite respiration are now needed. Their possible participation in nitric and nitrous oxide respiration should also be investigated.

The roles of the cytochromes isolated from various denitrifying bacteria have been discussed elsewhere [24].

The monohaem nature and small molecular mass of the high-potential cytochrome justifies comparison with cytochrome *c*-551 [25]. However, its lower redox potential makes it unique among high-potential *c*-type cytochromes. Also, the peculiarities of its optical spectrum and its NMR properties indicate the presence of both high- and low-spin states in which methionine occupies the sixth coordination position of the haem iron. The shoulder at 498 nm and the band at 619 nm are reminiscent of the absorption bands of cytochrome *c'* [26]. Since the three-dimensional structure of low-spin *c*-type cytochromes and of cytochrome *c'* are strikingly different [27], the structure of the high-potential cytochrome *c* from *W. succinogenes* should present interesting and exceptional features. The NMR features of this cytochrome are exceptional. There is a spin equilibrium in the ferric form. The high-spin/low-spin equilibrium is pH-dependent with a $\text{p}K_a \approx 7.3$. The transition between the two forms is relatively fast on the NMR time scale, as only four haem methyl groups were observed in the low-field region of the spectrum.

The assignment of resonance M_5 to the methionine methyl resonance fits well with the data published by Moore [16] for other cytochromes. Nevertheless, the position of this resonance in the low-field region close to the haem methyl resonances is unusual and unexpected. In cytochrome *c*₅₅₄ from *Alcaligenes faecalis*, a methyl resonance at 26.7 ppm (pH = 7.1, $T = 298$ K) was also suggested to be the methionine methyl resonance [15]. This last-named cytochrome displayed some NMR properties that resemble those of the *W. succinogenes* high-potential cytochrome. Both cytochromes have unusually far down-field shifts (>40 ppm) with drastic pH dependence. They both bind cyanide in the ferric form

and the chemical shift of the methionine methyl resonance is in the low-field region. If the unusual properties of *W. succinogenes* monohaem cytochrome derive from the high-spin/low-spin equilibrium, such activities would confirm Timkovich's suggestion [15] that a spin equilibrium also occurs in cytochrome *c*₅₅₄ from *A. faecalis*.

Some properties of the high-potential cytochrome resemble those of the *Agrobacterium tumefaciens* cytochrome *c*₅₅₆ and *R. palustris* cytochrome *c*₅₅₆ [28]. These cytochromes belong to the class II cytochrome *c*. The amino acid sequence of the monohaem cytochrome from *W. succinogenes* is not known and its classification cannot be established yet.

For the *Rhodopseudomonas palustris* ferricytochrome *c*₅₅₆ a small percentage of high-spin character was also observed as evidenced by the presence of a weak band at 630 nm [29].

NMR studies on the *R. palustris* and *A. tumefaciens* ferricytochromes *c*₅₅₆ show that both cytochromes exhibit a certain amount of high-spin character that increased with increasing temperature [28]. No methionine methyl assignment was made for these cytochromes (ferric form). By extending the data published by Moore [16] to the class II cytochromes, we could predict that the methionine methyl resonance of *A. tumefaciens* cytochrome *c*₅₅₆ should be at about 16 ppm, and such a resonance is visible in the low-field region of cytochrome *c*₅₅₆ [28].

The data presented indicated that in the reduced form of the high-potential *W. succinogenes* cytochrome only a low-spin form is present. The EPR spectrum of the ferric form also showed that at low temperature only the low-spin form is present. Moreover, the low-spin/high-spin equilibrium is temperature-dependent. It would now be interesting to know the temperature of the transition.

Briefly considering the low-potential *c*-type cytochrome, we find that its properties do not fall into any category so far identified. Despite possession of two haems, its molecular mass (11 kDa) is much smaller than those of the dihaem cytochromes *c*₅₅₂ of *Pseudomonas perfectomarina* (26 kDa) [30] and *Pseudomonas stutzeri* [31] or the *c*₄-like cytochrome (24 kDa) found in several organisms [32]. Even so, the two different redox potentials suggested for the two haem groups of this protein raise the possibility of its functional similarity with the dihaem cytochrome *c*₅₅₂ [31]. Based on previous rationalization, cytochrome *c*₅₅₄, cytochrome *cd*₁ (denitrifying nitrite reductase) and the split- α *c*-type cytochrome from other denitrifiers (but not detected in extracts of *W. succinogenes* cells) may be involved in denitrification reactions not performed by *W. succinogenes*.

A further aspect of interest arises from the recent finding by Macy et al. [33] that *W. succinogenes* can utilize sulfide as an energy source and sulfur as an electron acceptor instead of nitrate. It will be interesting to examine the cytochromes present in the bacteria grown with this newly recognized terminal oxidant in order to determine their eventual specificity in either the nitrate or the sulfur respiratory pathway.

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