The Membrane-bound Cytochromes of an Aerobically Grown, Extremely Thermophilic Bacterium, PS3: Characterization by Spectral Deconvolution Coupled with Potentiometric Analysis

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Cytochromes of the *a*, *b* and *c* types in membranes from the thermophilic bacterium PS3 have been characterized with respect to their spectral, potentiometric and ligand-binding properties. The integrated approach used (a) conventional potentiometric analysis with non-linear least-square analysis, (b) incorporation of the potentiometric procedures into a spectral decomposition protocol at 298 and 77 K, (c) fourth-order finite difference and fourth derivative analysis, and (d) photodissociation studies of the CO-liganded components. Either one *c*-type cytochrome with a split α -band (547, 552 nm at 77 K) or two cytochromes *c* with similar redox potentials (approx. +230 mV) were found, together with at least three *b*-type cytochromes (554, 557 and 561 nm at 77 K). On the basis of its high redox potential (+104 mV), cytochrome *b*₅₅₇ was tentatively equated with the *o*-type oxidase of this organism. A *c*-type cytochrome that formed a photodissociable complex with CO was also detected. The α -band of the previously purified cytochrome *c* oxidase was resolved into two components, having spectral and potentiometric properties remarkably similar to those of the analogous mitochondrial oxidase.

INTRODUCTION

Interest in bacterial respiratory systems has been heightened in recent years by the finding that certain cytochrome oxidases of the *aa*₃-type bear a remarkable resemblance to the cytochrome *c* oxidase (EC 1.9.3.1) of eukaryotic mitochondria, but appear to have much simpler subunit compositions (for a review, see Ludwig, 1980). The best-characterized example is perhaps *Paracoccus denitrificans*, long regarded as a 'mitochondrion-like' bacterium although, under appropriate growth conditions, its respiratory chain also contains the alternative cytochrome oxidases *o* (Sapshead & Wimpenny, 1972) and *d* (Henry & Vignais, 1979) with no obvious mitochondrial counterparts.

From the thermophilic bacterium, PS3 (not firmly identified but believed to be *Bacillus stearothermophilus*; T. Oshima, personal communication), Sone *et al.* (1979) purified a cytochrome oxidase that was claimed to have only one type of polypeptide subunit. The enzyme contained haems *a* and *a*₃, cytochrome *c* and intrinsic copper. Later experiments, however (E. Yang & N. Sone, unpublished; cited by Ludwig, 1980), suggested that an *a*₃ component was missing and that the oxidase comprised cytochromes *a*, *o* and *c* in molar ratios 5:1:5, a novel configuration. This failure to find cytochrome *a*₃ may have resulted from poorly defined growth

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conditions, since in membranes from adequately aerated cultures, Poole (1981) clearly demonstrated the presence of a CO-binding *a*-type cytochrome with absorption maxima at 602 to 605 nm (thus, by definition, *a*₃), cytochrome *o* and a cytochrome *c* that also bound CO and NO. The inability of Fee *et al.* (1978) to observe any perturbation by CO of the optical spectra of PS3 membranes is unexplained. Most recently, Sone & Yanagita (1982) have described a revised purification of the oxidase from vigorously aerated cells and confirmed that it is an *aa*₃ type, but with three subunit types.

There is particular interest in the thermophile PS3, since (1) the purified oxidase reconstituted into liposomes gave the first indication that a terminal bacterial oxidase could act as a proton translocator (Sone *et al.*, 1979), and (2) this oxidase and other energy-transducing proteins so far isolated exhibit remarkable thermostability (e.g. Sone *et al.*, 1979; Goto *et al.*, 1980).

Apart from the existence of *c*, *o* (i.e. *b*) and *aa*₃ types of cytochromes (cytochromes *o* and *aa*₃ being oxidases; Poole *et al.*, 1982) and a preliminary characterization of the purified *aa*₃ oxidase complex, nothing is known about the composition of the respiratory chain of this thermophile.

The aim of the present work was to characterize as fully as possible the membrane-bound cytochromes of PS3 grown under one set of carefully controlled conditions and to assess how similar this respiratory chain is to that of mitochondria. This paper illustrates the use of an integrated approach to the spectral, potentiometric and ligand-binding properties of these cytochromes.

METHODS

Organism, growth conditions and preparation of membranes. The organism was generously donated by Dr T. Oshima (Mitsubishi-Kasei Institute of Life Sciences, Machida-Shi, Tokyo, Japan). The procedures described previously (Poole, 1981) for maintenance and growth were followed, except that in some cases cultures (10 litres) were grown in a 12 litre Biostat-V fermenter (FT Scientific Instruments, Bredon, Glos. GL20 7HH, U.K.). The temperature was 65 °C, and the stirring speed 500 r.p.m.; sterile air was sparged into the medium at 0.5 l min⁻¹ (1 medium)⁻¹. The final cell yield (approx. 1.8 g wet wt l⁻¹) and mean generation time (approx. 20 min) were similar to those obtained in a smaller vessel (Poole, 1981).

Cultures were harvested by continuous-flow centrifugation with either an MSE continuous action rotor in an MSE 18 centrifuge or with an Alfa-Laval separator. Cells pelleted from the resulting slurry by centrifugation at 12 000 *g* (*r*_{av}) for 5 min were washed once under the same conditions with 50 mM-Tris/H₂SO₄ (pH 8.0). Washed membranes were prepared from lysozyme-digested cells as described by Yoshida *et al.* (1975) except that the enzyme concentration was increased from 1 to 2 mg (g wet wt cells)⁻¹. Pelleted membranes were stored below -20 °C until used. Protein content of membrane suspensions was assayed by the biuret method (Herbert *et al.*, 1971) or (for the experiments shown in Figs 4 and 6) using Lowry's method. In each case, the standard was dry bovine serum albumin.

Potentiometric titrations. The procedure described by van Wielink *et al.* (1982) was used with minor modifications. Cytoplasmic membranes were titrated in a medium containing 100 mM-HEPES, 15 mM-MgCl₂, 4 mM-EDTA, 0.4 M-ethanol and the mediators described by van Wielink *et al.* (1982) with the addition of 12.5 µM-*N,N,N',N'*-tetramethyl-*p*-phenylene diamine (TMPD). The pH was 7.0. Titrations were performed at 298 K in either a 3 ml glass cuvette or a glass vessel (nominal capacity 35 ml) depending on whether spectra were to be recorded at 298 K or 77 K, respectively. In either case, the vessel was equipped with a Pt electrode (1 × 5 × 20 mm), an Ag/AgCl reference electrode, a sampling tube, and a line via which the magnetically stirred sample was continuously flushed with argon (O₂ ≤ 0.1 p.p.m.). Reductive titrations were made with additions of a solution of Na₂S₂O₄; in oxidative titrations (always started with a fresh sample), cytochromes were first reduced with NADH and Na₂S₂O₄ before the stepwise addition of an anoxic solution of K₃Fe(CN)₆. Potentials were measured with a Philips digital pH/millivolt meter, PW 9408. For spectral analysis at 77 K of membranes poised at selected potentials, such samples were rapidly and anoxically siphoned into special cuvettes (1.0 ml capacity, path length 2 mm), which were immediately frozen in liquid N₂ and used in the low-temperature accessory (J4-9603, American Instrument Company, 8030 Georgia Ave., Silver Spring, Md. 20910, U.S.A.) of the DW-2a spectrophotometer. Nine scans of each sample were recorded and averaged (see below). Further details of these procedures are given by van Wielink *et al.* (1982).

Analysis of the titration data. For baseline corrections, calculation of peak areas, and scan averaging, the spectrophotometer was real-time and on-line connected to a PDP 11/03 microcomputer (Digital Equipment Co., Maynard, Mass. 01754, U.S.A.). Spectra were stored in a disc-based minicomputer (Hewlett-Packard 21 M-E with RTE IV-B). Spectra recorded at 77 K were normalized with respect to spectra at 298 K, recorded at the same redox potential, to overcome the variable optical pathlength of frozen samples. The normalized 77 K spectra were

analysed by fitting the following equation, which is based on the Nernst and Gauss equations and describes the absorbance (A) in composite α -bands, as a function of wavelength (λ) and oxidation-reduction potential (E):

$$A(\lambda, E) = \sum_{i=1}^n A_{m,i}^* [1 + \exp \{ (E - E_{0,i}') / 25.68 \}]^{-1} \exp \{ -4 \ln 2 \cdot \lambda_{m,i}^2 (\lambda_{m,i} - \lambda)^2 / \lambda^2 \cdot w_{\lambda,i}^2 \}$$

For each of a given number of components (n), four parameters were optimized:

$A_{m,i}^*$, the absorbance maximum of component i at full reduction,

$E_{0,i}'$, the midpoint potential of component i at pH 7.0,

$\lambda_{m,i}$, the wavelength at the absorption maximum of component i ,

$w_{\lambda,i}$, the band width of component i at $A = \frac{1}{2} A_{m,i}$.

The number of components was chosen on the basis of the sum of squared differences between experimental and predicted points.

Fourth-order finite difference and derivative analyses. Two methods were used. In the first, the stored data from the DW-2a spectrophotometer or simulated composite spectra (van Wielink *et al.*, 1982) were analysed according to Savitsky & Golay (1964), with the correction tables of Steiner *et al.* (1972). Alternatively, reduced *minus* oxidized difference spectra recorded at 77 K in a Unicam SP1700 spectrophotometer with cryogenic accessories were transferred to paper tape at 0.1 nm intervals and analysed using the algorithm of Butler & Hopkins (1970) as described in detail by Salmon & Poole (1980) and Poole *et al.* (1980).

Derivative curves were obtained by taking differences between values a specified number of nm apart (the differencing or differentiating interval referred to in Results and Discussion). Consider the absorbance values at 0.1 nm intervals to be numbered sequentially (n). The first derivative values (A') were obtained as absorbance differences between points ω nm apart, with each computed value assigned to the mid-point of the differencing interval. Thus,

$$A' \left(n + \frac{\omega}{2} \right) = A(\omega + n) - A(n)$$

where the numbers in parentheses indicate the positions of the absorbance or derivative values. Higher derivatives (A'' , A''' , A^{IV} , etc.) were obtained by a similar differentiation of the preceding derivative curve (Butler & Hopkins, 1970). For A^{IV} , a set of four differencing intervals (ω , x , y , z) that are similar, but not equal, gives less noise. This and other aspects of the choice of intervals are discussed by Butler & Hopkins (1970) and Butler (1979).

Photodissociation spectra. Membranes were suspended in a buffer that contained 30% (v/v) ethylene glycol, and 50 mM-Tris/H₂SO₄ (pH 8.0 at room temperature) and reduced with 1 mM-NADH or 2 mM-ascorbate + 0.1 mM-TMPD. The low temperature protocol was essentially that described previously (Poole *et al.*, 1979; Poole & Chance, 1981). Flash photolysis was achieved in the sample compartment of a microprocessor-controlled Johnson Foundation dual-wavelength spectrophotometer, by means of a xenon lamp.

Chemicals. The sources of redox mediators are given by van Wielink *et al.* (1982). Yeast extract was from Difco and proteose peptone from Oxoid. All other chemicals were of the highest purity available.

RESULTS AND DISCUSSION

Combined potentiometric and spectral analysis at 298 K

A redox titration of the α -bands of b , c and a -type cytochromes is shown in Fig. 1. At this temperature, the area-potential relationship reveals that at least three b - and/or c -type cytochromes contribute to the 540 to 580 nm region and that the a -type cytochromes absorbing at about 600 nm may be resolved into two components. The parameters from this type of analysis are shown in Table 1. Three major components contributing to the cytochrome b and c region have midpoint potentials of +209, +20 and -166 mV, respectively, whilst the cytochrome oxidase region is resolved into components of +363 and +202 mV. Clearly, in a group of overlapping absorption bands, the goodness of fit between the experimental and predicted points will appear to improve as the number of presumptive components fitted to the data is increased. The analysis was terminated, therefore, when the sum of squared differences between the experimental and predicted points did not improve significantly when the number of components was increased further. In the case of analysis of the area-potential relationship (Fig. 1), the sums of squared differences (in arbitrary units) for fits of one, two, three, four and five components to the data were 205.8, 22.1, 6.3, 4.7 and 6.0, respectively. The three-component fit was therefore adopted, although the narrower band widths of components at 77 K allow greater resolution at this temperature (see below).

Fig. 1. Potentiometric titration of the *b*-type, *c*-type and *a*-type cytochromes in membranes at 298 K. Peak areas of the α -bands of the *b*-type plus *c*-type cytochromes (●) and of the *a*-type cytochromes (○) are plotted versus the redox potential. The redox potential was varied by the addition of NADH, dithionite or ferricyanide in the presence of mediators (see Methods). The protein concentration was 4.8 mg ml⁻¹. The solid line represents the best fit analyses for the titration of cytochromes *b* and *c* (three components) whilst the dotted line is the best fit for the *a*-type cytochromes (two components). Further details of the resolved components are given in Table 1.

Table 1. *Best-fitting parameters for the potentiometric titrations at 298 K of the α -bands of cytochromes in PS3 cytoplasmic membranes*

Percentage area expresses the proportional contribution of a component to the area occupied by the optical absorbance band, $E_{m,7}$ is the midpoint potential of the component at pH 7.0, λ_m is the position of the absorbance maximum and w_λ is the band width at half-height. All values are means of two titrations with standard errors in parentheses.

Cytochromes	From area-potential relationship		From deconvolution of spectra recorded at poised redox potentials			
	Percentage area	$E_{m,7}$ (mV)	Percentage area	λ_m (nm)	w_λ (nm)	$E_{m,7}$ (mV)
<i>b</i> + <i>c</i>	47 (4)	+209 (3)	45 (3)	550.9 (0.4)	9.9 (0.2)	+209 (0)
	22 (2)	+20 (29)	25 (3)	559.8 (0.9)	12.1 (2.0)	+33 (33)
	29 (1)	-166 (4)	30 (1)	562 (0.2)	12.0 (0.5)	-166 (8)
<i>a</i> + <i>a</i> ₃	33 (3)	+363 (18)	40 (10)	600 (4)	21 (6)	+340 (15)
	67 (3)	+202 (6)	60 (10)	603 (2)	17 (3)	+190 (10)

A combined analysis of the potentiometric and spectral data (Fig. 2) revealed that the high-potential component in the 537 to 575 nm region was a *c*-type cytochrome (λ_m = 550.9 nm; Table 1) whilst the absorption maxima of the other two components (559.8 and 562 nm) were appropriate for *b*-type cytochromes. In the 580 to 625 nm region, the two broad components were shown to be separated by only 3 nm (600 and 603 nm, respectively).

Combined potentiometric and spectral analysis at 77 K

The value of recording cytochrome spectra at 77 K is well documented (Chance, 1957; Estabrook, 1961; Wilson, 1967) and will not be reiterated here. The most dramatic effect of lowered temperature was the splitting of the 550.9 nm band seen at room temperature (Fig. 2; Table 1) into components at approximately 547 and 552 nm (Fig. 3; Table 2). A band at 547 nm or 548 nm is clearly visible in 77 K difference spectra (Poole, 1981) without recourse to deconvolution techniques. This band and the 552 nm band titrate with potentials similar to each other (Table 2) and to mitochondrial cytochrome *c* (Dutton & Wilson, 1974), making it likely that they arise from a single *c*-type cytochrome with a split α -band, although the possibility that there are at least two spectrally distinct, but potentiometrically similar, cytochromes *c* cannot be

Fig. 2. Three-dimensional plot of spectra of the cytochromes in membranes poised at different redox potentials at 298 K. The absorbance is plotted perpendicular to the plane determined by the wavelength and potential axes. The solid lines are spectra recorded at different redox potentials at 298 K as described in Methods. The reticular broken lines represent the best-fitting three-component solution for the *b*- and *c*-type cytochromes (530 to 575 nm) and the best two-component solution for the *a*-type cytochromes (575 to 625 nm). In this particular titration, the computed values for λ_m , w_λ , and $E_{m,\lambda}$, respectively, in the 530 to 575 nm region, were: 550.6 nm, 9.8 nm, +208 mV; 559.2 nm, 13.6 nm, +8 mV; 561.9 nm, 12.1 nm, -166 mV. The corresponding values for the 575 to 625 nm region were: 597.3 nm, 26.7 nm, +345 mV; 603.9 nm, 16.1 nm, +200 mV. Protein concentration was 4.8 mg ml⁻¹.

Fig. 3. Three-dimensional plot of spectra of the cytochromes in membranes poised at different redox potentials and then trapped and measured at 77 K. Presentation is as in Fig. 3. The solid lines are spectra recorded after trapping at 77 K samples from a 298 K redox titration as described in Methods. The reticular broken lines represent the best-fitting solution for five components; full details of the parameters are given in Table 2.

Table 2. *Sets of best-fitting parameters obtained from deconvolution of 77 K spectra of the α -bands of b- and c-type cytochromes in PS3 cytoplasmic membranes*

The parameters have the same meanings as in Table 1. A chi-square test for each set gave values of 98, 47 and 40 for four, five and six components, respectively. Components that are regarded as equivalent (in terms of λ_m and E_0) in the three solutions are shown on the same line.

Four-component fit				Five-component fit				Six-component fit			
Percentage area	λ_m (nm)	w_λ (nm)	$E_{m,7}$ (mV)	Percentage area	λ_m (nm)	w_λ (nm)	$E_{m,7}$ (mV)	Percentage area	λ_m (nm)	w_λ (nm)	$E_{m,7}$ (mV)
30	547.3	5.4	+226	27	547.2	5.2	+229	27	547.2	5.2	+230
14	552.1	4.7	+215	13	552.0	4.9	+221	13	552.0	4.9	+221
28	557.7	9.7	+102	27	556.9	9.3	+104	27	556.9	9.4	+105
29	558.4	11.4	-165								
				16	560.8	7.9	-130	17	560.3	8.4	-123
				17	554.4	13.9	-194	15	553.8	14.0	-197
								2	562.9	4.3	-350

discounted. The band widths are also almost identical. The cytochrome *c* that copurifies with cytochrome oxidase *aa*₃, and has λ_{max} of 550 nm at room temperature (Sone *et al.*, 1979; Sone & Yanagita, 1982) presumably contributes to, or is, the 547 nm component at 77 K.

The three solutions shown in Table 2 each reveal the presence of a component with an absorption maximum at 556.9 to 557.7, which is probably a *b*-type cytochrome. Additional components are seen at 558.4 nm in the four-component fit, at 560.8 and 554.4 nm in the five-component fit, and at 560.3, 553.8 and 562.9 nm in the six-component fit. An unequivocal decision between these solutions is problematic, although the fit of the experimental spectra to the predicted is little improved on increasing the number of components from five to six (as assessed in a chi-square test, Table 2).

The most important fact that argues against accepting the four-component model is its failure to reveal a peak at about 554 nm (Fig. 3; Table 2), in spite of the sharp trough at 553 to 554 nm consistently seen in CO + reduced or CN⁻ + reduced *minus* reduced difference spectra at 77 K (Poole, 1981; this paper, Fig. 6*d*). A shoulder is also visible at about 554 nm in NO difference spectra. The components at about 547, 552 and 557 nm in all three solutions also coincide with peaks seen in the spectra of Poole (1981). Further evidence against the four-component model is that the longest wavelength maximum is seen at 558.4 nm. At room temperature, 30% of the total absorbance area is due to a 562 nm component which would thus have to be shifted by almost 4 nm on lowering the temperature. This shift is somewhat greater than would normally be expected (2 to 3 nm; Salmon & Poole, 1980). The five-component model satisfies the existence of a peak at 552 to 554 nm and introduces a peak at 560.8 nm, which could correspond with the 562 nm peak at room temperature. In addition, a peak at 560.5 nm is seen in fourth-order finite difference analyses (see below). The essential difference between the five- and six-component solutions is the presence in the latter of a component of very low midpoint potential and narrow band width at about 563 nm.

Fourth-order finite difference and derivative analyses of spectra recorded at 77 K

By effectively diminishing the band width of individual components in a composite spectrum, fourth-order finite difference (e.g. Salmon & Poole, 1980; Scott & Poole, 1982) and derivative analyses allow increased resolution of the number of components present. As a sole approach to spectral deconvolution, these techniques should be used with caution (Morrey, 1968; Butler, 1979; Meister, 1982) because of the possibility of introducing artefactual components. However, when the spectrum for analysis is relatively noise-free, and differencing intervals are carefully chosen, such spectra should correspond with independently derived solutions to complex spectra. In addition, the fourth derivative analysis of the experimental data and of the deconvolution selected on potentiometric grounds should be comparable.

Figures 4 and 5 compare the numerical analyses of spectra of reduced membranes obtained using either the Butler & Hopkins (1970) algorithm or the method of Savitsky & Golay (1964).

Fig. 4. Reduced *minus* oxidized difference spectrum of *b*- and *c*-type cytochromes and its fourth-order finite difference analysis using the algorithm of Butler & Hopkins (1970). Spectrum (a) is the sum of four spectra recorded at 77 K under the following conditions: spectral band width, 0.9 nm; scan rate, 0.2 nm s⁻¹; data sampling, 2 s⁻¹; protein concentration, 7 mg ml⁻¹; path length, 2 mm. The differencing intervals used for numerical analyses were: (b) 2.5, 2.3, 2.2, 2.1 nm; (c) 1.8, 1.6, 1.5, 1.4 nm; (d) 1.4, 1.2, 1.1, 1.0 nm. Dashed lines represent zero absorbance in the numerical analyses.

Figure 4(a) shows the Na₂S₂O₄-reduced *minus* oxidized difference spectrum used for analysis; note the high signal-to-noise ratio of the summed spectra. The 547 nm peak is clearly resolved but at higher wavelengths individual maxima are not observed. At successively smaller differencing intervals (Fig. 4b to d), greater complexity is revealed. At the largest differencing intervals used (Fig. 4b), four of the six components of Table 2 are already seen. At smaller intervals (Fig. 4c) there is excellent correspondence in the number and positions of peaks with the six-component model. With the exception of the largest and broadest peak, the labelled absorption maxima in this analysis and those in the six-component fit of Table 2 vary only by 0 to 1.0 nm. The intensity of the 562 to 563 nm component in all the derivative analyses of Fig. 4 is presumably a consequence of its very narrow band width (Table 2), since the algorithm discriminates strongly in favour of such bands (Butler & Hopkins, 1970). The potentiometric analysis (Table 2) suggests that it contributes only 2% of the total spectral area. Similarly, the low intensity of the 554 to 555 nm component in Fig. 4(c), which is seen most clearly at the smallest intervals (Fig. 4d), probably results from the broadness of this band revealed in both the potentiometric analysis (Table 2) and the flash photolysis studies (Fig. 6, see later). In Fig. 4(d) the broad 545 nm band is split to reveal the 547 nm band (Table 2; Fig. 4a); the significance of this is unclear.

Fig. 5. Fourth derivative spectrum of membranes poised at -424 mV and then trapped at 77 K. Spectrum (a) (solid line) is the experimental spectrum recorded as described in Methods. Its fourth derivative is the solid line, curve (b). The five component curves of (b), one of which is labelled (c), are also shown. The dotted line seen on (a) at about 540 nm shows the simulated spectrum based on the resolved five components; in other regions of the spectrum, the simulated and actual spectra were superimposed. The dotted lines at either end of (b) show the fourth derivative of the simulated spectrum where this was not superimposed on the experimental spectrum. The absorbance maxima of the resolved components in (c) and (in parentheses) the percentage area and band width, respectively, are: 547.3 nm (35.6 , 5.75 nm), 551.6 nm (9.12 , 3.40 nm), 554.3 nm (16.25 , 5.01 nm), 559.2 nm (32.6 , 6.69 nm) and 563.4 nm (6.05 , 5.02 nm).

Figure 5 shows the fourth derivative analysis of Savitsky & Golay (1964). A five-component fit of the original spectrum is almost indistinguishable from the fit of the simulated spectrum constructed from the five presumptive components (Table 2). The components found correspond well with those in Fig. 4(c) and (d) although the 557.5 nm band is missing; this, like the 554 nm band, is rather broad (Table 2). Apart from minor differences (0.1 to 1.0 nm) in band positions, this analysis, like that in Fig. 4 is in good agreement with the six-component fit of Table 2.

CO-binding cytochromes and photodissociability

Bubbling a suspension of reduced membranes with CO for 2.5 min led to small decreases in the absorbances of *a*-, *b*- and *c*-type cytochromes (Fig. 6*a, b*). Note that the intensity of the cytochrome oxidase (aa_3) band at about 603 nm is diminished by only about 10% , in agreement with previous results (Poole, 1981). A simplistic interpretation is that cytochrome a_3 (by definition the ligand-binding component of the complex) makes only a small contribution to this region of the spectrum. Alternatively (see Wikstrom *et al.*, 1976), the slight decrease in intensity on ligand binding is due to a decrease in the extinction coefficient of cytochrome *a* as a result of haem-haem interaction. This interaction also explains the close similarity of the midpoint potentials of this band, reduction of one haem forcing the other into a high spin state with a drastically decreased affinity of the latter for an electron (decrease in E_m).

The difference spectrum (reduced *minus* CO + reduced, Fig. 6*c*) reveals more clearly ligand-binding cytochromes of the *c*- (547 nm), *b*- (428 and 555 nm) and *a*-type (443 and 602 nm). The photodissociation spectrum (Fig. 6*d*) also shows (as peaks) the positions of ferrocyclochromes that form photodissociable compounds with CO and (as troughs) the positions of these

Fig. 6. CO-binding cytochromes in membranes from PS3. Spectrum (a) is a reduced (with ascorbate + TMPD) *minus* oxidized (with ammonium persulphate) difference spectrum; in (b; dotted line) the reduced sample was then bubbled with CO for 2.5 min before recording the CO-reduced *minus* oxidized spectrum. The dashed line is an oxidized *minus* oxidized baseline. Spectrum (c) is a computed difference between the reduced and CO-reduced spectra used in (a) and (b). Spectra (a) to (c) were recorded at 173 K. Spectrum (d) was obtained by recording and storing the spectrum of an NADH-reduced plus CO sample (the dashed line is the CO-reduced *minus* CO-reduced baseline) and then photolysing the sample at 170 K with 25 flashes from a xenon lamp, to give the photolysed (=reduced) *minus* CO-reduced difference spectrum. The vertical bar represents the following ΔA (below and above about 500 nm respectively): (a), (b) 0.29 and 0.145; (c) 0.073 and 0.029; (d) 0.029 and 0.0145. The protein concentration in (a) to (c) was 1.52 mg ml⁻¹, and in (d), 3.2 mg ml⁻¹.

compounds. Such spectra identify cytochrome *a*₃ with absorption maxima in the reduced form at about 443 and 605 nm. The CO compound absorbs at 589 nm. These positions are close to those found for mitochondrial cytochrome *c* oxidase (Wikstrom *et al.*, 1976).

The troughs at 417, 535 and 567 nm are characteristic of the CO complex of cytochrome *o*. Identification of the α -peak of its reduced form with one of the components resolved in Table 2 is more problematic, however, since the positions of cytochrome *o* bands (555 to 565 nm; Lemberg & Barrett, 1973) and their midpoint potentials are extremely variable, e.g. from -30 mV for *Azotobacter vinelandii* (Yang, 1981) to +210 mV for *Paracoccus denitrificans* (Willison *et al.*, 1981). *Vitreoscilla* cytochrome *o* contains haems with different midpoint potentials (-122 and +118 mV; Tyree & Webster, 1978), further complicating analysis.

The peak at 547 nm in Fig. 6(d) suggests that the CO complex of a *c*-type cytochrome is photodissociable, although photochemical action spectra (Poole *et al.*, 1982) do not indicate an oxidase function for this component. Although CO-binding cytochromes *c* are widely distributed in bacteria (Iwasaki & Shidara, 1969; Tonge *et al.*, 1974), their function (or

functions) is unclear. Only a few, such as that in *Beneckea natriegens* (Weston & Knowles, 1973) are similar to the PS3 cytochrome in having a high midpoint potential and being membrane-bound.

Prolonged incubation (90 min) of reduced membranes in an atmosphere of CO (results not shown) led to the formation of a peak (in reduced *minus* reduced + CO spectra) at 554 nm, together with a new band at 563 nm; the latter could have arisen from CO binding to partially denatured cytochrome *b* (Ben-Gershom, 1961). Under these conditions, the intensity of the cytochrome *aa*₃, α -peak was again diminished by only approximately 10%.

Conclusions

We have adopted an integrated approach to study the complex membrane-bound cytochromes of aerobically grown PS3. The main advantages of the method (van Wielink *et al.*, 1982) are (a) the simultaneous characterization of each component with respect to its midpoint potential and spectral properties, and (b) the constancy in the characteristics of the components resolved as more components are introduced, major and minor peaks being resolved sequentially.

The most satisfactory interpretation of the spectral deconvolution at 77 K is that five *b*- and/or *c*-type cytochromes contribute to the α region. These are *c*-type cytochromes at (i) 547 nm (+229 mV) and (ii) 552 nm (+221 mV) and *b*-type cytochromes at (iii) 554 nm (−194 mV), (iv) 557 nm (+104 mV) and (v) 561 nm (−130 mV) (Table 2). The existence of these components is strongly supported by (a) fourth derivative and (b) fourth-order finite difference analyses. On the basis of the midpoint potentials, *b*_{557 nm} is tentatively identified as the CO-binding cytochrome oxidase *o*.

The α -band of the *a*-type cytochrome oxidase at 600 to 605 nm is due mostly to cytochrome *a* but is a composite of two potentiometrically distinct haems. Despite difficulties in explaining the ligand-binding properties of this oxidase, the important point is that, potentiometrically and spectrally, the *a*-type oxidase of this bacterium closely resembles the mitochondrial enzyme, fully justifying efforts to characterize further its relatively simple subunit composition and exploit this feature in studies of the mechanism of oxygen reduction and proton pumping.

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