The Mitochondrial Cytochromes of an Unusual Budding Yeast, *Sterigmatomyces halophilus*: Spectral Characterization Exploiting Fourth-order Finite Difference Analysis

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Low-temperature difference spectra of gradient-purified mitochondria from *Sterigmatomyces halophilus* revealed the presence of *a*-, *b*- and *c*-type cytochromes, spectrally similar to those of other yeasts. Fourth-order finite difference analysis resolved the broad α -band of *b*- and *c*-type cytochromes into eight peaks. Absorption maxima at about 539, 543.5 and 547.5 nm were attributed to one, or perhaps two, cytochrome(s) *c* that are loosely bound to the mitochondrial membrane. Cytochrome *c*₁ was identified at 550.5 to 551.5 nm. Maxima at about 554, 556, 559 and 562 nm were attributed to three or four distinct *b*-type cytochromes on the basis of their differential reduction by NADH, dithionite, or ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine in the absence or presence of antimycin. Difference spectra in the presence of CO or cyanide indicated the presence of cytochromes *a* (600 nm) and *a*₃ (608 nm). Finite difference analysis of the cytochrome *c* oxidase peak centred at 600 nm revealed two components; the major component at 600 nm was identified as cytochrome *a* and the minor component at 605 nm as cytochrome *a*₃. A further CO-binding haemoprotein was tentatively identified as cytochrome *o*.

INTRODUCTION

Yeasts provided the first experimental material for difference spectrophotometry by dual-beam methods (for a survey of early work, see Chance *et al.*, 1978). Subsequently, the mitochondrial cytochromes of yeast have been extensively studied (for a review, see Lloyd, 1974), but certain aspects remain controversial. Sato *et al.* (1972) attributed four α -absorption bands (558, 561.5, 563 and 565 nm) in difference spectra of *Candida utilis* to three *b*-type cytochromes, cytochrome *b*_T having a double α -band at 565 and 558 nm. On the basis of the response of the latter bands to antimycin, however, Grimmlikhuijzen *et al.* (1975) suggested that these bands belong to two separate cytochromes. Furthermore, an unequivocal assignment of cytochromes *a* and *a*₃ to the classical α -band of their composite absorption spectrum has not been achieved, in spite of many attempts (for references, see Kuschmitz & Hess, 1975, and Chance *et al.*, 1978).

Sterigmatomyces halophilus is an unusual budding yeast, in which daughter cells or buds are formed remote from the mother cell on fine projections called sterigma (Fell, 1966). Since the sterigma are readily disrupted by mild mechanical methods (I. Salmon, A. Ajmeri & R. K. Poole, unpublished results), this organism offers unique advantages for the study of temporal and spatial aspects of development during the cell cycle, and the distribution of newly synthesized components between mother and bud (Salmon & Poole, 1978*a*). As part of this programme, we have studied the mitochondrial cytochromes of the organism and report here on their spectral characterization. We have exploited, for the first time in

yeasts, fourth-order finite difference analysis† of low-temperature difference spectra to resolve the components present (Butler & Hopkins, 1970*a*; Butler, 1979). The markedly enhanced resolution has enabled us to identify two or three *c*-type cytochromes and multiple *b*-type cytochromes, and to demonstrate directly the presence and relative contributions of two components (*a* and *a*₃) in the α -absorption band of cytochrome *c* oxidase. Parts of this work have been briefly reported previously (Salmon & Poole, 1978*b*).

METHODS

Organism, maintenance and growth. *Sterigmatomyces halophilus* CBS 4609 was obtained from the Centraal-bureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands. It was maintained by routine subculturing on a medium that contained (per l distilled water): 30 g NaCl, 5 g casein hydrolysate, 5 g yeast extract, 3 g bacteriological peptone, 40 g glucose, 15 g agar, 2 mg *meso*-inositol, 400 μ g thiamin.HCl, 400 μ g calcium pantothenate, 400 μ g nicotinic acid, 400 μ g pyridoxine.HCl, 200 μ g *p*-aminobenzoic acid, 200 μ g riboflavin, 2 μ g D-biotin, 2 μ g folic acid.

Batch cultures were grown in the defined medium of Barnett & Ingram (1955) but the quantity of KH_2PO_4 was increased to 10.5 g l^{-1} and NaCl (30 g l^{-1}) and K_2HPO_4 (4 g l^{-1}) were added. Starter cultures were prepared by inoculating a loopful of the organism from the solid maintenance medium into 25 ml of the defined medium in a 250 ml Erlenmeyer flask and shaking for 4 d at 200 rev. min^{-1} and 25 °C. A portion of this culture (10 ml) was then used to inoculate 5 l of medium in a 5 l Quickfit fermenter to give an initial cell count of 5×10^5 to 10×10^5 cells ml^{-1} . The vessel was sparged with air at 5 l min^{-1} and magnetically stirred at 300 rev. min^{-1} and 25 °C.

Harvesting and disruption of organisms. After about 50 h growth, when the culture was in the 'deceleration phase' of growth (the period between exponential and stationary phases) and the cell count was 5×10^7 to 10×10^7 cells ml^{-1} , cells were collected by passing the culture at 1 l min^{-1} through an MSE continuous action rotor fitted with a high efficiency insert, operating at 15000 rev. min^{-1} at 4 °C in an MSE High Speed 18 centrifuge. Cells were harvested from the resultant concentrated suspension and washed once with disruption buffer (0.6 M-sorbitol, 1 mM-EDTA, 0.1 % bovine serum albumin, pH 7.0) by acceleration to 10000 rev. min^{-1} in the 6×250 ml rotor of the same centrifuge, followed by immediate deceleration.

The disruption procedure was based on that of Lang *et al.* (1977). Washed cells (20 to 30 g wet wt) were resuspended in disruption buffer (0.4 g wet wt ml^{-1}) and poured into a 1 l stoppered glass bottle. Cold glass beads (0.4 mm diam.; washed with acid and then disruption buffer) were added to give 3 to 4 g ml^{-1} . The bottle was shaken through an amplitude of about 0.5 m at a frequency of 2 Hz. Periods of 30 s shaking were alternated with 30 s cooling until the bottle had been shaken for a total of 4 min. The suspension was decanted from the beads, and intact cells and debris were removed by acceleration to 12000 rev. min^{-1} in the 8×50 ml rotor of the MSE 18 centrifuge and immediate deceleration. The supernatant was retained and the pellet was recycled once through the disruption procedure. The second broken cell suspension was decanted, combined with several washings from the beads and centrifuged as above to yield a supernatant that was pooled with the first.

Preparation of the mitochondrial fraction. The combined cell-free extracts were centrifuged in the 8×50 ml rotor of an MSE Superspeed 65 centrifuge at 21000 rev. min^{-1} for 20 min (38000 g; r_{av} , 7.4 cm). The pellets were washed once by resuspension in a small volume of disruption buffer and centrifugation as above, and then resuspended in an equal volume of disruption buffer by 2 strokes of a loose-fitting hand-held homogenizer. The suspension was layered on a 10 to 45 % (w/v) gradient of Urografin 370 (a mixture of sodium diatrizoate and meglumine diatrizoate). The 36 ml gradient was linear with respect to volume and also contained 1 mM-EDTA, pH 7.0. It was centrifuged at 40000 rev. min^{-1} (140000 g; r_{av} , 7.4 cm) in the 8×50 ml angle rotor of the MSE 65 centrifuge for 60 min. The brown mitochondrial layer was removed from the centre of the gradient (median density about 1.23 g ml^{-1}), diluted with 2 vol. disruption buffer, centrifuged at 21000 rev. min^{-1} for 20 min in the same rotor and centrifuge, and finally resuspended in a small volume of buffer. All operations were at 4 °C.

Analytical methods. Difference spectra (Chance, 1957) were obtained at 77 K using a Pye Unicam SP1700 spectrophotometer, fitted with an accessory constructed in this Department. This consisted of a Dewar flask, of which the lower 3 cm was silvered (Day-Impex, Station Road, Earls Colne, Essex), and which was positioned close to the photomultiplier. A brass cuvette holder, the lower end of which was immersed in

† The algorithm described and used by Butler & Hopkins (1970*a*, *b*) and employed in this study does not strictly yield a fourth derivative as stated by these authors, but rather a type of fourth-order finite difference spectrum (Shipp, 1972). The latter description is used in this paper.

liquid nitrogen in the Dewar flask, held two small (0.5 ml capacity, 2 mm path length) Perspex cuvettes about 5 cm from the photomultiplier. Suspensions of cells or mitochondria were pipetted into the cuvettes which were then frozen by immersion in liquid nitrogen and inserted into the cuvette holder. Condensation on the Dewar flask was eliminated by blowing a stream of air over its outer surfaces.

Protein was determined by Lowry's method, with bovine serum albumin as standard and with appropriate corrections for interference by the disruption buffer.

Numerical analysis of spectra. The analogue output from the spectrophotometer to the chart recorder was interrogated by means of a digital voltmeter (Solartron LM 1420.2) under the control of a data logger unit (Solartron Schlumberger) which transferred absorbance values to a Facit paper tape punch (Solartron Schlumberger 3245) at 0.2 nm intervals. To improve the signal-to-noise ratio, six successive scans were summed for each spectrum. No baseline corrections were made.

The paper tape was analysed by a Modus Modular One computer (Computer Technology, Hants.) programmed to prepare finite order difference spectra by using the algorithm of Butler & Hopkins (1970b). The fourth-order finite difference spectrum was plotted on an X-Y recorder under computer control.

Noise levels in the method were estimated by computing a fourth-order finite difference spectrum of two opaque cuvettes or two cuvettes each containing fully reduced cell or mitochondrial suspensions. The peak-to-peak noise levels are indicated on the Figures by the vertical bars at either end of the finite difference spectra. Wavelengths are quoted to the nearest 0.5 nm to indicate reproducibility but a precision of 1 nm is a more realistic estimate of that obtainable with this instrumentation.

Chemicals. Casein hydrolysate and bacteriological peptone were from Oxoid, yeast extract from Difco, antimycin A from Boehringer, and Urografin 370 (76%) from Schering Chemicals, Burgess Hill, West Sussex, RH15 9NE. Bovine serum albumin was from Armour Pharmaceuticals Co., Eastbourne, Sussex. All other chemicals were of analytical grade.

RESULTS

Cytochrome components of difference spectra

Difference spectra ($\text{Na}_2\text{S}_2\text{O}_4$ -reduced minus oxidized) of mitochondrial particles recorded at room temperature (results not shown) revealed the presence of cytochrome components similar to those of other yeasts (Lloyd, 1974). Broad absorption maxima were observed at 432 and 445 nm in the Soret region (γ -bands of $b + c$ -type and a -type cytochromes, respectively), at about 530 nm (β -bands of $b + c$ -type cytochromes), at 563 nm (α -bands of $b + c$ -type cytochromes) and at 604 nm (α -band of cytochromes $a + a_3$). At 77 K, these maxima were displaced by 2 to 3 nm to shorter wavelengths (Fig. 1a). Additional absorption maxima, not clearly visible at room temperature, were observed at 536 and 552 nm. It was evident that the major absorption bands were asymmetrical and probably contained greater detail than was immediately resolvable in the spectra.

Addition of 10 mM-ascorbate plus 1 mM- N,N,N',N' -tetramethyl- p -phenylenediamine to an oxidized mitochondrial fraction resulted in reduction of a - and c -type cytochromes. Prominent absorption maxima were observed at 416, 447, 520, 553, 560 and 602 nm (Fig. 1b). Peaks at 429 and 530 nm were not well-resolved and a shoulder was evident at about 550 nm.

Addition of NADH to an aerobic mitochondrial suspension in the presence of 70 μM -antimycin A resulted in reduction of b -type cytochromes and enabled their spectral contributions to be assessed (Fig. 1c). Maxima were observed at 429, 508, 528, 536 and 560 nm. The less prominent peaks at 417 (cytochrome c) and 600 nm (cytochromes $a + a_3$) probably reflected electron transport past the antimycin 'block' and reduction of c - and a -type cytochromes. Shoulders were also visible at 440 to 450 nm (cytochromes $a + a_3$) and 554 (c -type cytochrome) but their true intensities were obscured by the neighbouring intense peaks. A broad absorption maximum centred at 473 to 476 nm was observed in all three spectra of Fig. 1; it has not been identified.

Resolution of reduced minus oxidized difference spectra by fourth-order finite difference analysis

Figure 2(a) shows the α - and β -regions of the NADH-reduced minus oxidized difference spectrum of mitochondrial particles. The fourth-order finite difference spectrum (Fig. 2b)

Fig. 1. Difference spectra of gradient-purified mitochondria from *S. halophilus*. The contents of the reference cuvette were oxidized by aeration while the contents of the sample cuvettes were reduced by adding either (a) a few grains of $\text{Na}_2\text{S}_2\text{O}_4$, (b) 10 mM-ascorbate plus 1 mM-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine or (c) 3 mM-NADH after the addition of 70 μM -antimycin A. Spectra were scanned at 2.0 nm s^{-1} with a spectral bandwidth of 0.8 nm. The mitochondrial suspension contained $8.1 \text{ mg protein ml}^{-1}$.

Fig. 2. Numerical analysis of difference spectra of gradient-purified mitochondria from *S. halophilus*. Spectra (a) and (c) are each the sum of six spectra obtained by oxidizing the contents of a reference cuvette by aeration and reducing the contents of the sample cuvette with either (a) 3 mM-NADH or (c) $\text{Na}_2\text{S}_2\text{O}_4$. Spectra (b) and (d) are the fourth-order finite difference analyses of spectra (a) and (c), respectively, and were computed using differencing intervals of 1.8, 1.4, 1.2 and 1.0 nm. Spectra (a) and (c) were scanned at 0.2 nm s^{-1} with a spectral bandwidth of 0.8 nm. The mitochondrial suspension contained $8.1 \text{ mg protein ml}^{-1}$.

revealed considerable complexity. The most prominent peaks in the spectrum of Fig. 2(a) at 550.5 and 558.5 nm were intensified in the finite difference spectrum and were observed at identical or similar wavelengths, 550.5 and 559.5 nm, respectively.

At least four further bands were resolved in the α -region by this analysis, at 544, 547.5, 556.5 and 562.5 nm. Their presence in Fig. 2(a) was suggested only by shoulders on the broad absorption band.

Resolution in the β -region (500 to 540 nm) was poor. Four of the bands resolved in the fourth-order difference spectra (536, 528, 516.5 and 509 nm) were of wavelengths close to those at which distinct components were visible in the spectrum of Fig. 2(a). Further peaks were at 539, 531, 524, 519 and 513 nm; their intensities were greater than the estimated noise levels. Detailed analysis of the β -region was not attempted. Purified cytochrome *c* (Lemberg & Barrett, 1973; I. Salmon & R. K. Poole, unpublished results) and *c*₁ (Yu *et al.*, 1972) each exhibit at least six peaks in this region. Spectral separation of individual cytochromes in *Neurospora crassa* also reveals extreme complexity in this region of the spectrum (von Jagow *et al.*, 1973).

Reduction of mitochondrial particles with $\text{Na}_2\text{S}_2\text{O}_4$ (Fig. 2c), rather than NADH, resulted in the appearance of absorption peaks in the fourth-order finite difference spectrum (Fig. 2d) at wavelengths identical, or similar (within 1 nm), to those in Fig. 2(b). The only

Fig. 3. Numerical analysis of difference spectra of gradient-purified mitochondria from *S. halophilus*. Spectra (a) and (c) are each the sum of six spectra obtained by oxidizing the contents of a reference cuvette by aeration and reducing the contents of the sample cuvette with either (a) 3 mM-NADH in the presence of 70 μ M-antimycin A or (c) 10 mM-ascorbate plus 1 mM-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Spectra (b) and (d) are the fourth-order finite difference analyses of spectra (a) and (c), respectively, and were computed using differencing intervals of 1.8, 1.4, 1.2 and 1.0 nm. Spectra (a) and (c) were scanned at 0.2 nm s⁻¹ with a spectral bandwidth of 0.8 nm. The mitochondrial suspension contained 8.1 mg protein ml⁻¹. The slightly different positions of the absorption maxima labelled in (a) and (c) compared with those in Fig. 1(b, c) are the result of the 10-fold decrease in scan speed and the reduction in signal-to-noise ratio (obtained by summing spectra) in the above Figure.

qualitative distinction between these two spectra was the appearance of a distinct peak at 554 nm following reduction with Na₂S₂O₄.

The difference spectrum of mitochondrial particles reduced by NADH in the presence of antimycin (Fig. 3a) and its numerical analysis (Fig. 3b) were characterized by the decreased intensity of certain peaks prominent in Figs 2(b) and (d), namely those at 544, 547.5, 550.5 and 556.5 nm. Those bands remaining prominent at 553.5, 559 and 562.5 nm may be attributed to *b*-type cytochromes. The component at 556 nm was reducible by NADH or Na₂S₂O₄ (Fig. 2), but was not seen after reduction by NADH in the presence of antimycin. In mitochondria reduced by ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (Fig. 3c) the prominent bands resolved by fourth-order finite difference analysis (Fig. 3d) were at 543.5, 547.5, 551.5 and 559 nm. A peak at 556 nm was well-resolved but its true intensity was probably masked by the large peaks at 551.5 and 559 nm. Surprisingly, the

Fig. 4

Fig. 5

Fig. 4. Difference spectrum of intact cells of *S. halophilus* and its numerical analysis. Difference spectra were obtained by oxidizing the contents of the reference cuvette with 4.5 mM- H_2O_2 and reducing those of the sample cuvette with 22 mM-glucose for 2 min. (a) is the sum of eight such spectra and (b) is its fourth-order finite difference analysis, computed using differencing intervals of 2.2, 1.8, 1.6 and 1.4 nm. Spectra were scanned at 0.2 nm s^{-1} with a spectral bandwidth of 1.0 nm. The cell suspension contained 15 mg total protein ml^{-1} .

Fig. 5. Difference spectrum of cytochrome *c* oxidase in gradient-purified mitochondria from *S. halophilus* and its numerical analysis. The contents of the sample cuvette were reduced with 3 mM-NADH and those of the reference cuvette were oxidized by aeration. Spectra were scanned at 0.2 nm s^{-1} with a spectral bandwidth of 1.0 nm (a). Six spectra were summed and the fourth-order finite difference spectrum (b) was computed using differencing intervals of 3.0, 2.6, 2.4 and 2.2 nm. The mitochondrial suspension contained 8.1 mg protein ml^{-1} .

latter peak was prominent in particles reduced either by ascorbate or by NADH in the presence of antimycin. A component absorbing at 562 nm and reducible by ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine was noted in *Acanthamoeba castellanii* by Edwards *et al.* (1977). Bands in the β -region that are prominent in Fig. 3(d) but not in Fig. 3(b) were at 510 and 513 nm.

The reduced minus oxidized difference spectrum of intact cells (Fig. 4a) in the region 535 to 580 nm showed that the ratio of *c* type cytochromes (absorption maximum 548 nm) to *b*-type cytochromes (absorption maximum 558 nm) was greater than in mitochondrial fractions. This presumably reflected the loss of loosely bound cytochrome *c* during cell breakage or fractionation. The fourth-order finite difference spectrum (Fig. 4b) revealed components at 536, 540, 544, 548, 552, 557 and 560 nm. Components absorbing at 554 and 562.5 in mitochondrial particles (Fig. 2) were not observed, probably due to the poorer signal-to-noise ratio of spectra of intact cells and the larger differencing intervals used. Also, the increased peak intensity at 548 nm may have obscured the 550.5 nm component.

The α -absorption band of cytochromes *a* + *a*₃ is shown in Fig. 5(a). Fourth-order finite difference analysis (Fig. 5b) showed that the band was split into two components with maxima at 600 and 605 nm. The true magnitude of the 605 nm component was probably masked by the trough associated with the major 600 nm component. The unfavourable noise in

Fig. 6

Fig. 7

Fig. 6. CO difference spectra of gradient-purified mitochondria of *S. halophilus*. The contents of both cuvettes were reduced with $\text{Na}_2\text{S}_2\text{O}_4$. The recorded baseline (not shown) showed no irregularities. The sample cuvette was sparged with CO for 30 s and spectra were recorded (a) at room temperature and (b) at 77 K. Both spectra were scanned at 2 nm s^{-1} with a spectral bandwidth of 0.8 nm. The mitochondrial suspension contained $9.6 \text{ mg protein ml}^{-1}$.

Fig. 7. Effects of cyanide on difference spectra of gradient-purified mitochondria from *S. halophilus*. (a) The contents of both cuvettes were reduced with $\text{Na}_2\text{S}_2\text{O}_4$ and then 5 mM-KCN was added to the sample cuvette. (b) The contents of both cuvettes were oxidized by aeration and KCN was added to both. The sample cuvette was then reduced with $\text{Na}_2\text{S}_2\text{O}_4$. (c) The sample cuvette was aerated; 5 mM-KCN was then added, followed by $\text{Na}_2\text{S}_2\text{O}_4$. The contents of the reference cuvette were reduced with $\text{Na}_2\text{S}_2\text{O}_4$. All spectra were scanned at 2 nm s^{-1} with a spectral bandwidth of 1.0 nm. The mitochondrial suspension contained $8.1 \text{ mg protein ml}^{-1}$. In comparing (b) with Fig. 5(a), note that the latter is a sum of six spectra.

this region was due largely to inadequacies in the photomultiplier and precluded more detailed analysis. Our previous attempts (Salmon & Poole, 1978b) to resolve the 600 nm band were frustrated by insufficient cell material and inferior signal-to-noise ratios. A further minor component at 647 nm has not been identified. There is no filter change in the spectrophotometer at this wavelength and the band has been observed in two separate experiments.

CO difference spectra

A mitochondrial suspension was reduced by $\text{Na}_2\text{S}_2\text{O}_4$ and divided into two portions; one was bubbled with a slow stream of CO for 30 s. The difference spectrum (CO-reduced minus reduced) recorded at room temperature exhibited prominent minima at 448, 561 and about

610 nm (Fig. 6*a*). At 77 K (Fig. 6*b*) peaks at 590 and 545 nm and minima at 446/451 and 608 nm were attributed to the presence of cytochrome a_3 and its ferrous-CO complex (Castor & Chance, 1955). A maximum at about 430 nm, due to the a_3^{2+} -CO complex, was probably obscured by the CO-reactive haemoproteins absorbing at about 420 nm; numerical analyses of these spectra were not attempted. Other spectral features have not been rigorously identified, but the broad maximum at 420 to 422 nm was probably a composite band arising from the presence of various CO-binding haemoproteins ('P-420'). The troughs at about 560 and 527 nm, which were not observed in similar CN^- difference spectra (Fig. 7*a*), may indicate the presence of a CO-binding *b*-type cytochrome spectrally similar to cytochrome *o* (Daniel, 1970); there is currently no evidence for this component being a terminal oxidase in *Sterigmatomyces halophilus*, however. Although yeast haemoglobin exhibits similar spectral properties in CO difference spectra (Oshino *et al.*, 1973), we could find no evidence for its 595 nm peak in reduced minus oxidized spectra (Figs 1 and 5).

Difference spectra in the presence of cyanide

Reaction of KCN with reduced cytochrome a_3 resulted in an absorption minimum at 608 nm in the difference spectrum of Fig. 7(*a*) and a broad maximum at 590 nm, corresponding to the ferrous cytochrome a_3 - CN^- complex (Yonetani, 1960). The corresponding CO difference spectrum (Fig. 6*b*) was similar in this region. For the difference spectrum in Fig. 7(*b*), the contents of both cuvettes were oxidized by aeration in the presence of KCN, and then the contents of the sample cuvette were reduced with dithionite and rapidly frozen. Since cytochrome a_3 is not readily reduced when complexed with CN^- , it is effectively excluded from the spectrum. Cytochrome *a*, however, does not react with CN^- and its reduced minus oxidized spectrum, together with that of other non- CN^- reacting components, was obtained. A peak at 601 nm was thus attributed to cytochrome *a*. In Fig. 7(*c*), both cuvettes initially contained aerated mitochondria. KCN was added to the sample cuvette only and then $\text{Na}_2\text{S}_2\text{O}_4$ was added to both cuvettes before they were rapidly frozen. This spectrum shows the CN^- -reacting haemoprotein (a_3) as a minimum at about 610 nm in an oxidized minus reduced difference spectrum. In the 500 to 550 nm region, the spectrum suggests that the contents of the two cuvettes were reduced to slightly different extents.

DISCUSSION

The narrow peaks obtained when absorption spectra are submitted to fourth-order finite difference analysis allow the resolution of individual components that overlap in composite spectra. Such finite difference spectra have the advantage that the peaks observed have absorption maxima at wavelengths often, but not always, identical with the constituent bands (Butler & Hopkins, 1970*a*). Good resolution is generally achieved by the fourth derivation (Talsky & Mayring, 1978). High-order finite difference spectra have the additional advantage that peak positions may be reliably determined in the presence of significant baseline drift. Such drifts (together with broad absorption bands) are eliminated because the numerical analysis discriminates strongly in favour of narrower bands. The band intensity in the fourth-order finite difference spectrum is inversely proportional to the fourth power of the bandwidth. We recognize that the detectability of absorption bands in the fourth derivative spectra is limited by the signal-to-noise ratio in the original data and we have taken two steps to improve this. First, the sum of at least six replicate spectra is analysed; the signal-to-noise ratio is thus improved by a factor of $(6/\sqrt{6}) = 2.45$. Secondly, the noise-reducing properties of the algorithm were exploited, the four differencing intervals being similar but not identical (Butler & Hopkins, 1970*b*). Caution must be exercised with regard to the possible generation of spurious peaks. For example, artefactual bands may arise when two bands with the same wavelength maximum but different band widths are super-

Table 1. *Absorption maxima observed in difference spectra at 77 K of gradient-purified mitochondria from S. halophilus*

Difference spectra (reduced, as indicated, minus oxidized) were subjected to fourth-order finite difference analysis. Values given for the absorption maxima observed were obtained from spectra of one mitochondrial preparation, although similar maxima (generally within 0.5 nm) were observed in 12 analyses. Maxima observable in the α -region only are shown; those in parentheses were minor components.

Absorption maxima (nm)				Tentative identification and comments
NADH	Dithionite	NADH + Antimycin	Ascorbate + TMPD*	
539.0	540.0	ND	(539.5)	<i>c</i> -type cytochrome, probably distinct from <i>c</i> below
544.0	544.0	(542.0)	543.5	α_2 band of cytochrome <i>c</i>
547.5	547.5	ND	547.5	α_1 band of cytochrome <i>c</i>
550.5	550.5	(550.5)	551.5	cytochrome <i>c</i> ₁
(554.0)	554.0	553.5	ND	<i>b</i> -type cytochrome, reducible by NADH only in the presence of antimycin
556.5	556.5	(556.0)	556.0	<i>b</i> -type cytochrome?, but not reducible by NADH + antimycin. Possibly associated with <i>b</i> ₅₅₉
559.5	559.5	559.0	559.0	<i>b</i> -type cytochrome. Includes CO-binding, but not CN ⁻ -binding, component
562.5	562.5	562.5	563.0	<i>b</i> ₆₆₂
600	NT	NT	NT	<i>a</i>
(605)	NT	NT	NT	<i>a</i> ₃

ND, Not detectable, or peak intensity within noise levels; NT, not tested.

* TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

imposed (Morrey, 1968). Surprisingly, the applications of such numerical analyses to cytochrome absorption spectra have remained few, but are likely to increase with the advent of on-line derivative facilities in microprocessor-controlled spectrophotometers such as the Hitachi 557 (Perkin Elmer) and on-line analogue computing accessories (Talsky & Mayring, 1978).

A comparison of the α -bands of *a*-, *b*- and *c*-type cytochromes observed in four different kinds of difference spectra (Table 1) shows the reproducibility of the wavelengths at which maxima are observed. Similar peaks have been observed in a total of 100 derivative spectra obtained with 90 samples of intact cells and four preparations of mitochondrial particles. For each band, the standard deviation of the observed wavelength maximum was between 0.6 and 0.8 nm. Such variations can be accounted for by sampling errors, errors in the measured wavelengths and small shifts introduced by variations in the relative intensities of the neighbouring peaks. In our spectra, the observed maxima occur at similar wavelengths even when the intensity of adjacent bands is dramatically changed (see Figs 2 to 4).

The tentative assignment of cytochrome types to the ten peaks in the α -region is based, in part, on comparison with the results of other workers and, in part, on the response of the bands to the addition of inhibitors and various reductants. Maxima at about 547.5 and 543.5 nm are attributed to the split (α_1 and α_2) bands of a cytochrome *c* on the basis of the similarities between the observed absorption maxima and those of cytochrome *c* in other systems (Lemberg & Barrett, 1973). The ratio of intensities of these two remains between 1.5 and 2.0 in both mitochondrial particles (reduced by Na₂S₂O₄, NADH or ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine) and in intact cells. The maximum at about 539.5 nm is small, but consistently observed, and appears to bear no reproducible stoichiometry with either the 543.5 or 547.5 nm peaks, thus suggesting that it represents an additional cytochrome *c*.

The band at 550.5 to 551.5 nm (552 nm in intact cells) is attributed to cytochrome c_1 on the basis of its similarity to the absorption maxima reported by other workers in a variety of cell types (e.g. 551.5 nm, von Jagow *et al.*, 1973; 553.2 nm, Shipp, 1971) including yeast (552 nm, Claisse & Pajot, 1974). Unlike cytochrome c , it is not substantially lost from mitochondrial particles.

The remaining four peaks in the broad band extending from 540 to 565 nm are probably due to b -type cytochromes. Evidence for multiple b -type cytochromes in other yeasts has come from kinetic, potentiometric and spectroscopic measurements (Mitsushima *et al.*, 1977; Grimmlikhuijzen *et al.*, 1975; Sato *et al.*, 1972; Poole *et al.*, 1974). Only in the case of avian and mammalian mitochondria has this multiplicity been confirmed by fourth-order finite difference analysis (Shipp, 1971; Davis *et al.*, 1972). Three of the putative b -type cytochromes in the present study have absorption maxima at similar wavelengths to the b -type cytochromes observed in purified succinate:cytochrome c reductase (555, 559 and 562 nm) at 77 K (Yu *et al.*, 1972). The component seen in our spectra at 553.5 to 554.5 is reducible by NADH only in the presence of antimycin, whereas the other components are extensively reduced by NADH or $\text{Na}_2\text{S}_2\text{O}_4$. The peak at 559 nm is relatively unaffected by the nature of the reductant or the presence of antimycin. At least part of the absorption at this wavelength is due to a CO-binding (but not CN^- -binding) cytochrome similar to the 'cytochrome o ' described in other yeasts by Mok *et al.* (1969). Cytochrome b_{562} is reduced extensively by NADH (with or without antimycin) and only slightly by ascorbate plus N,N,N',N' -tetramethyl- p -phenylenediamine.

Adding antimycin to mitochondrial particles from *Sterigmatomyces halophilus* (Fig. 3*b*) causes no measurable shifts in the positions of the putative b peaks resolved by numerical analysis. However, in agreement with previous reports on other yeasts (Sato *et al.*, 1972; Grimmlikhuijzen *et al.*, 1975), a small shift to the red [from 558 and 558.5 (Fig. 2*a, c*) to 559 nm (Fig. 3*a*)] is observed in the unanalysed spectra. Numerical analysis of the spectra shows that antimycin causes a change in the relative intensity of two resolved bands (556 and 559.5 nm; the former is barely detectable in Fig. 3*b*) presumably explaining the apparent shift in the original spectra.

Using the classical inhibitory ligands of cytochrome a_3 , CN^- and CO, we find the absorption of this component to be at 608 nm (Figs 6*b*, 7*a*, 7*c*), close to, but not identical with, the minor component at 605 nm seen by numerical analysis of reduced minus oxidized spectra (Fig. 5). Such a discrepancy can readily be attributed (Butler & Hopkins, 1970*a*) to interference with the true position of the minor (605 nm) component by the predominant peak (identified as cytochrome a) at 600 nm. Attempts to assess the contributions of a and a_3 to the composite α -absorption in other yeasts have given conflicting results. Conclusions similar to ours have been reached for *Candida utilis* by Chance *et al.* (1978) on kinetic and spectral grounds, although in this organism the bands of a (598 nm) and a_3 (609 nm) are well-resolved without recourse to numerical analysis of the spectrum. In contrast, the higher wavelength component (604 nm) in *Saccharomyces cerevisiae* has been equated with cytochrome a and the lower wavelength component (600 nm) with a_3 (Kuschmitz & Hess, 1975).

We consider that fourth-order finite difference analysis provides a powerful and useful technique to aid in the resolution of complex spectra (Butler, 1979). This is illustrated by a comparison of the detailed information obtained by this method (Table 1) with the information obtainable by mere inspection of the low-temperature spectra shown in the Figures. The resolution of individual components also enables more accurate quantification of peak heights than could be achieved by estimating the intensities of components that are seen only as 'shoulders' in the original spectra (I. Salmon & R. K. Poole, unpublished results). We are currently exploiting these advantages in a study of cytochrome synthesis during the cell cycle of *Sterigmatomyces halophilus*.

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