Biochimica et Biophysica Acta, 333 (1974) 446–459

BBA 46708

CHARACTERIZATION OF A MUTANT OF *SCHIZOSACCHAROMYCES POMBE* LACKING CYTOCHROME *b*-566

W. BANDLOW[a, b], K. WOLF[a], F. KAUDEWITZ[a] and E. C. SLATER[b]

[a] *Institut für Genetik der Universität München, D-8 München 19, Maria-Ward-Str. 1a (Germany) and* [b] *Laboratory of Biochemistry, B. C. P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, Amsterdam (The Netherlands)*

(Received July 26th, 1973)
(Revised manuscript received November 29th, 1973)

SUMMARY

1. A chromosomal respiration-deficient mutant of the petite-negative yeast *Schizosaccharomyces pombe* was isolated. Its mitochondria show respiration rates of about 7% of the wild-type respiration with NADH and succinate as substrate, and 45% with ascorbate in the presence of tetramethyl-*p*-phenylenediamine. Oxidation of NADH and succinate is insensitive to antimycin and cyanide and that of ascorbate is much less sensitive to cyanide than the wild type.

2. The amounts of cytochromes c_1 and aa_3 are similar in the mutant and wild type. Cytochrome *b*-566 could not be detected in low-temperature spectra after reduction with various substrates or dithionite. A *b*-558 is, however, present.

3. The *b*-cytochromes in the mutant are not reduced by NADH or succinate during the steady state even after addition of ubiquinone-1. QH_2-3 : cytochrome *c* reductase activity is very low and succinate oxidation is highly stimulated by phenazine methosulphate.

4. Antimycin does not bind to either oxidized or reduced mitochondrial particles of the mutant.

5. In contrast to the *b*-cytochromes of the wild type, *b*-558 in the mutant reacts with CO.

6. Cytochromes aa_3, *c* and c_1 are partly reduced in aerated submitochondrial particles isolated from the mutant and the EPR signal of Cu (II), measured at 35 °K, is detectable only after the addition of ferricyanide. In the mutant, a signal with a trough at $g = 2.01$ is found, in addition to the signal at $g = 1.98$ found in the wild type.

7. The ATPase activity of particles isolated from the mutant is much lower than in the wild type but is still inhibited by oligomycin.

Abbreviations: PMS, phenazine methosulphate; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

INTRODUCTION

Respiration-deficient mutants may be very helpful in the study of the function and the biogenesis of the respiratory-chain complexes. However, no biochemically well-defined mutant of the bc_1 segment, with a specific deficiency of a single cytochrome component or other function, is known. This may be due to a very tight regulation of the synthesis or incorporation into the membrane of components of this segment, resulting in a variety of pleiotropic effects. An exception may be represented by the antimycin-resistant mutants of *Candida utilis* described by Butow and Zeydel [1] and by Grimmelikhuijzen and Slater [2] which, however, are still capable of growing on non-fermentable substrates.

The mutant described here also shows pleiotropic deficiencies. However, it appears to be specifically lacking in cytochrome *b*-566.

MATERIALS AND METHODS

Mutant UV 119 was derived from the adenine-requiring strain of *Schizosaccharomyces pombe* 50 $h^- ad_7$ after ultraviolet irradiation as described by Wolf et al. [3]. It was selected by triphenyltetrazolium chloride as described by Bachofen et al. [4]. Cells were grown in the presence of 1.5% glucose and 0.5% yeast extract with aeration and harvested 12–24 h after all glucose was consumed. Mitochondrial particles were prepared as described by Mahler et al. [5], but with 0.25 M mannitol instead of sucrose in the isolation buffer. Protein was determined by the method of Cleland and Slater [6].

RESULTS

Enzymatic properties of the respiratory chain of wild type and mutant

The petite-negative yeast *S. pombe* [7] was used for the study of respiration-deficient mutants because it avoids the difficulties that may arise from rho-minus mutation during the culture. This may well complicate results obtained with respiration-deficient mutants of *Saccharomyces cerevisiae*.

Respiration-deficient mutants were induced by ultraviolet light and selected by plating the cell suspension on agar plates containing 3% glucose in the presence of 30 mg/l triphenyltetrazolium chloride [4]. All mutants tested bear single-gene mutations that are inherited chromosomally as could be shown either by random spore- or by tetrad analysis [3]. The mutants were first screened for their enzymatic activity of the various segments of the respiratory chain. Results obtained with one of these mutants, UV 119, will be described.

This strain grows in a medium supplemented with 3% glucose at about 30% of the wild-type rate, while it does not grow on glycerol. The viability after reaching stationary phase is close to 100%. The respiratory rate of whole cells, with glucose as substrate, is 1.7% of that of the wild type. Back mutants were obtained neither spontaneously nor by treatment with ultraviolet light, diethyl sulphate, ICR 170 or methylmethane sulphonate. The result of a random spore analysis after crossing with wild-type strain 88 $h^+ his_2$ is: respiration competent, 332; respiration deficient, 321; ade^+, 325; ade^-, 328; his^+, 330; his^-, 323. From the 2 : 2 segregation of all

TABLE I

ENZYMATIC ACTIVITIES OF VARIOUS SEGMENTS OF THE RESPIRATORY CHAIN OF MITOCHONDRIAL PARTICLES FROM THE WILD-TYPE AND THE RESPIRATION-DEFICIENT MUTANT UV 119

The activities are given as percentage of that of the wild-type 972 h^-. Enzymatic assay methods were the same as summarized in ref. 8. Ubiquinone: cytochrome *c* oxidoreductase was measured according to Rieske et al. [9].

Segment of respiratory chain	Relative activity
NADH : cytochrome *c* oxidoreductase	7
NADH : Q-2 oxidoreductase	60
Succinate : Q-2 oxidoreductase	51
Succinate : cytochrome *c* oxidoreductase	
in absence of phenazine methosulphate	3
in presence of phenazine methosulphate	47
QH_2-3 : cytochrome *c* oxidoreductase	6
Cytochrome *c* oxidase	37

markers tested, it is concluded that respiration deficiency in UV 119 is inherited nuclearly.

In Table I, it is shown that the QH_2–cytochrome *c* reductase activity is very much diminished in the mutant, whereas the activities of the succinate : Q-2 and NADH : Q-2 oxidoreductase and cytochrome *c* oxidase segments are less affected. The reduction of cytochrome *c* by succinate is greatly stimulated by by-passing the bc_1 segment by addition of phenazine methosulphate. These data indicate that the main block is localized in this segment.

Respiration experiments shown in Figs 1A and 1B strengthen this view. The trace given in Fig. 1B indicates that the mutant respires at only a very low rate with NADH (or succinate) as substrate. The addition of Q-1 causes no stimulation making it unlikely that the mutant is ubiquinone-deficient. The low rate of O_2 uptake with NADH or succinate is not affected by antimycin but is stimulated greatly by the addition of ascorbate plus TMPD or by PMS. Addition of cytochrome *c* causes stimulation by 10% with NADH as substrate in the wild type whereas it has no effect on the mutant either with NADH or ascorbate plus TMPD as substrate (not shown).

Spectral properties and kinetics of reduction

The spectra of the wild type are remarkable in several respects. The α band of cytochrome aa_3 is found at a higher wavelength than with *S. cerevisiae* or rat liver, viz. at 606 nm at 77 °K and 610 nm at room temperature. The cytochrome *b*-562 absorption band (558 nm at 77 °K) is only found in succinate-reduced particles frozen at 77 °K before anaerobiosis is reached. After anaerobiosis or reduction with succinate in the presence of antimycin (Fig. 2, Curve C) or with $Na_2S_2O_4$ (Curves A and B), the peak is found at about 560 nm. This is probably a composite of the α-bands of *b*-562 (α-peak at 558 nm) and *b*-566 (α-peak at 561.5 nm). A peak at 556 nm, corresponding to *b*-558 in mammalian mitochondria, is also seen in Spectrum B from which cytochromes *c*, c_1 and aa_3 are eliminated by adding ascorbate and TMPD

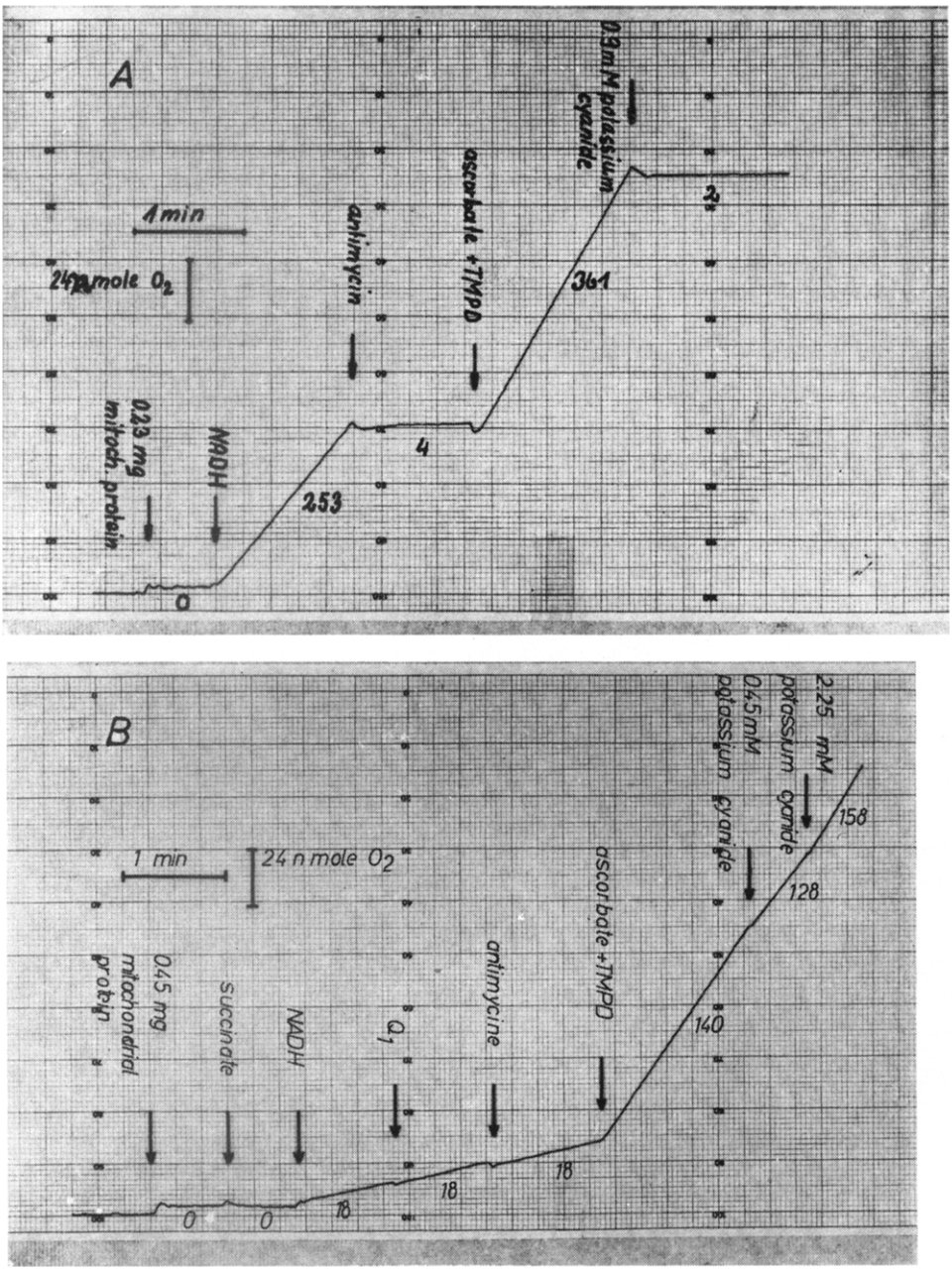

Fig. 1. Traces of O_2 consumption of mitochondrial particles from the wild type and of mutant UV 119 in the presence of various substrates and inhibitors. O_2 uptake was measured at 25 °C in a closed vessel equipped with a Clark-type electrode. The final concentrations used were 2.5 mM NADH, 13 mM succinate, 13 mM ascorbate, 48 μM TMPD, 16 μM PMS, 0.7 μg antimycin/ml in a buffer containing 100 mM potassium phosphate (pH 7.4). The final concentration of KCN is given. The numbers on the trace give nmoles of O_2 consumed per min/mg protein. A shows an O_2 consumption trace of wild-type mitochondrial particles, B of mutant UV 119.

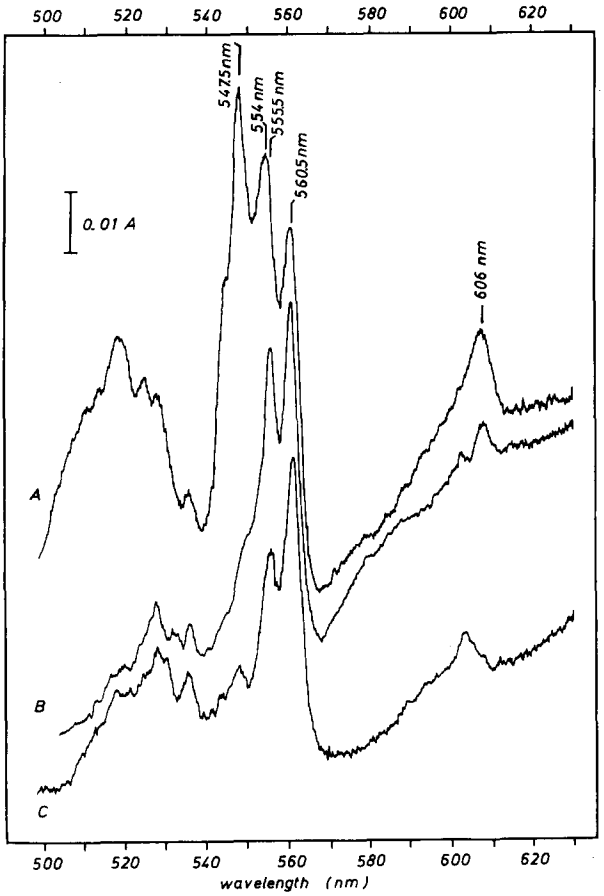

Fig. 2. Spectra of mitochondrial particles from wild type measured at 77 °K, obtained after reduction of mitochondrial particles with various substrates. Only the α- and β-regions are shown. 4.2 mg mitochondrial protein was used per cuvette, suspended in 0.25 M mannitol, 0.02 M Tris–HCl buffer and 1 mM EDTA at pH 7.4. Substrate and mediator concentrations were as described under Fig. 1. The light path was 3 mm. A shows the spectrum of dithionite minus oxidized (0.2 mM $K_3Fe(CN)_6$); B, dithionite minus (ascorbate+TMPD+PMS); C, succinate+antimycin minus oxidized.

to the reference cuvette. This absorption band (Spectrum B) is assigned to a *b*-cytochrome for two reasons: first, because it is reduced by NADH or succinate in the presence of antimycin and, secondly, because it is not reduced by ascorbate plus TMPD. The more intense band at about 554 nm in Spectrum A is due to interference of cytochrome *b*-558 by cytochrome c_1. Cytochrome *c* may have been partially lost in both the wild type and the mutant during the preparation of mitochondrial particles.

The spectra of mutant UV 119 (Fig. 3) show clearly the absence of an absorption band of cytochrome *b*-566 absorbing at 561.5 nm at 77 °K in the wild type. Cytochromes *b*-558 and *b*-562 are present as indicated by their absorption peaks at 556 and 558 nm, respectively, at 77 °K in spectrum B of Fig. 3. Cytochrome *b*-566

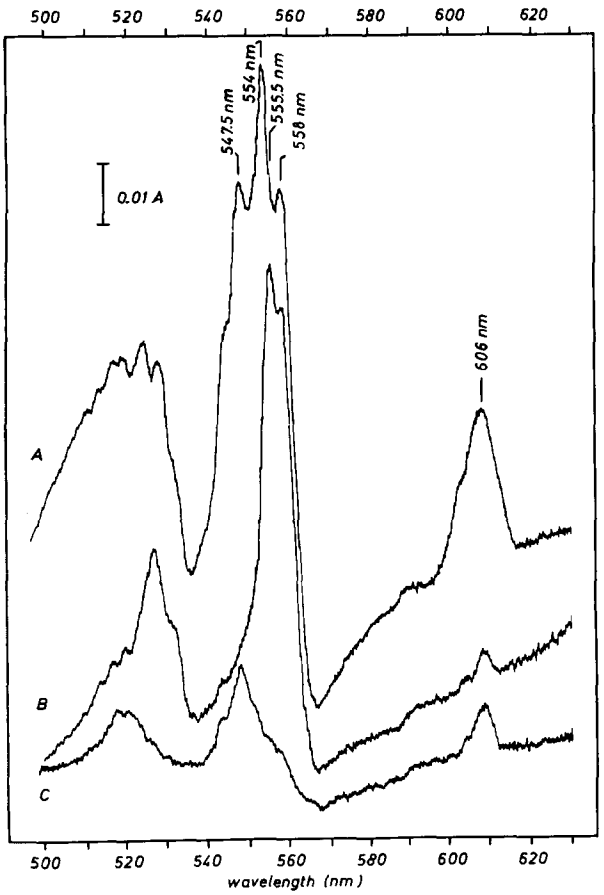

Fig. 3. Spectra of mitochondrial particles from mutant obtained as described under Fig. 2. In the presence of substrates, samples were preincubated in a syringe for 20 min under exclusion of O_2. 6.4 mg protein was used per cuvette. Same conditions as described in Fig. 2. A, difference spectrum dithionite reduced minus oxidized; B, dithionite minus (ascorbate+TMPD+PMS); C, succinate-reduced+antimycin minus oxidized.

TABLE II

CYTOCHROME CONTENT OF WILD TYPE AND MUTANT MITOCHONDRIA AND SUB-MITOCHONDRIAL PARTICLES

Submitochondrial particles were prepared as described earlier [8]. Cytochrome content, calculated from the absorbance change after reduction by dithionite for the wavelength pairs given in the table, according to Berden and Slater [10], is given as pmoles/mg protein.

	972 h⁻		UV 119	
	Mitochondria	Sub-mitochondrial particles	Mitochondria	Sub-mitochondrial particles
Cytochrome c_1 (553–540 nm)	—	620	—	640
Cytochrome b (563–575 nm)	540	980	330	560
Cytochrome aa_3 (608–590 nm)	360	590	420	710

is also missing in whole cells reduced by dithionite, the reference being oxidized by 30 mM H_2O_2.

According to spectra taken at room temperature, the concentration of cytochrome *b* (calculated from $\Delta A_{563-575\ nm}$) in the particles prepared from the mutant was 60% that of particles from wild type, whereas the concentration of cytochrome aa_3 (calculated from $\Delta A_{608-590\ nm}$) was about 15% higher. The actual values are listed in Table II.

Figs 4A and 4B show reduction kinetics for wild-type and mutant particles measured at the wavelength pair 563–575 nm. The cytochromes *b* in the mutant are not reduced by NADH even in the presence of antimycin. There is no anaerobic state reached with NADH or succinate, even after 30 min with 3.8 mg mitochondrial protein. Addition of PMS, however, leads to anaerobiosis within half a minute, and results in an almost quantitative reduction of the *b*-cytochromes. Dithionite reduces a residual 10% of cytochrome *b*.

Dual-wavelength spectra with reference to 575 nm were taken under the same conditions as described for the cytochrome *b* reduction kinetics. In this experiment, the sequence of additions was, however, NADH, PMS, antimycin, dithionite. Anaerobiosis was reached 20 s after PMS had been added. Spectrum A in Fig. 5 indicates that only cytochromes *c* and, to a lesser extent, c_1 are reduced by NADH. PMS in

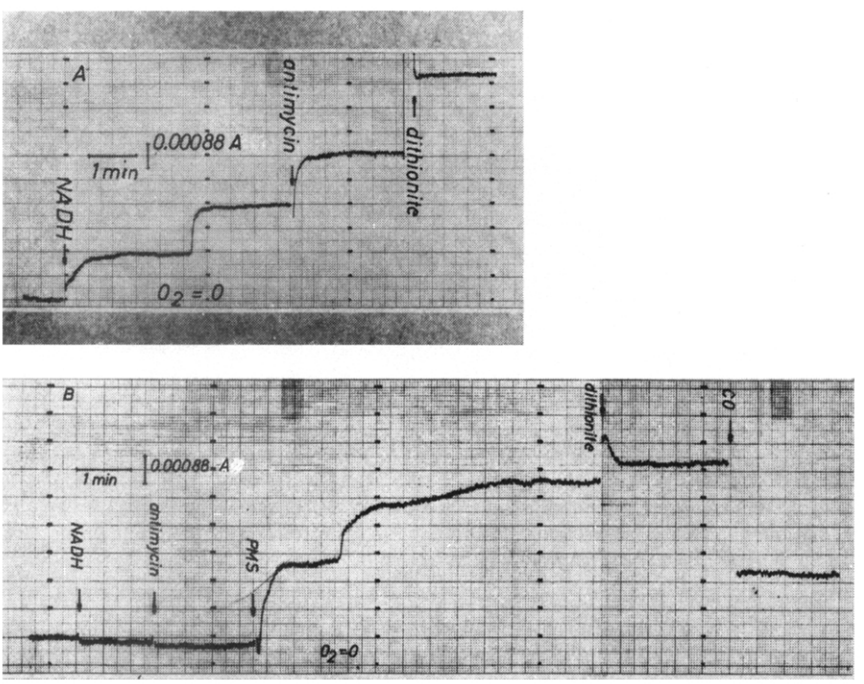

Fig. 4. Reduction kinetics of *b*-cytochromes. The particles were suspended in 0.25 M mannitol, 0.02 M Tris–HCl buffer, 1 mM EDTA at pH 7.4. The final concentration of NADH was 1.6 mM, of phenazine methosulphate 16 μM, and of antimycin 0.25 μg/ml. Kinetics were measured at room temperature using the wavelength pair 563–575 nm. A shows the trace of the wild type (2.4 mg protein) and B of mutant UV 119 (1.9 mg protein).

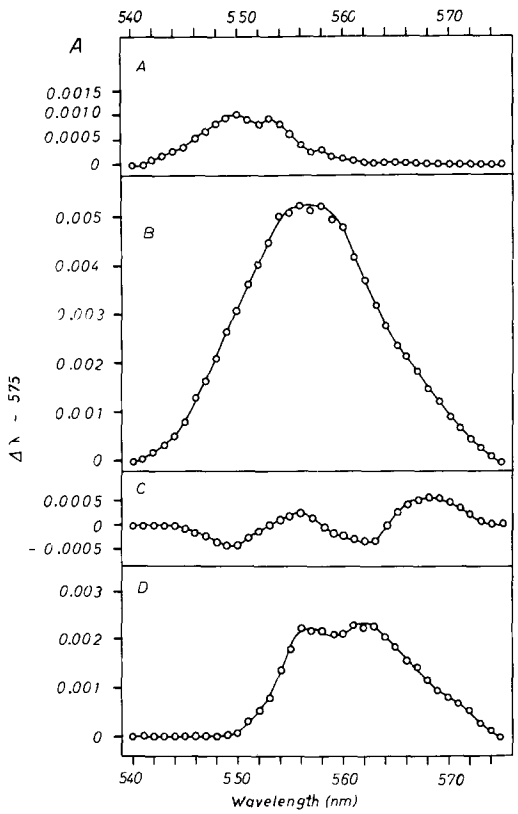

Fig. 5. Difference spectra at room temperature obtained from measurements at different wavelengths with 575 nm as reference wavelength for particles from mutant UV 119. Other conditions were as described in the legend of Fig. 4, but the sequence of additions was NADH, PMS, antimycin and dithionite. A shows a spectrum NADH minus oxidized; B, NADH+PMS minus NADH; C, NADH+PMS+antimycin minus NADH+PMS; and D, NADH+PMS+antimycin+dithionite minus NADH+PMS+antimycin.

the presence of NADH reduces the bulk of the cytochromes *b*, c_1 and *c*. The peak of the absorption band is at 557 nm and there is no evidence for the presence of an absorption band of cytochrome *b*-566 (Spectrum B). Addition of antimycin to particles reduced by NADH plus PMS causes a red shift of the *b*-cytochrome absorption band with an isosbestic point at 564 nm (cf. Trace C). Trace D shows that some cytochrome *b*-562 and *b*-558 is still reducible after addition of dithionite.

Reaction of mitochondrial particles with antimycin and CO

In order to get a deeper insight into the structural changes which might have occurred in the *b*-cytochromes as a result of the mutation, binding studies with antimycin [10, 11] and CO were carried out. Figs 6A and 6B show the binding studies with antimycin. In the wild type there is a strong binding to aerobic mitochondrial particles of about the same order as in beef heart. In mutant UV 119, there is no detectable quenching by aerobic mitochondrial particles of the fluorescence of antimycin in the presence of 6 mg/ml bovine serum albumin, showing that

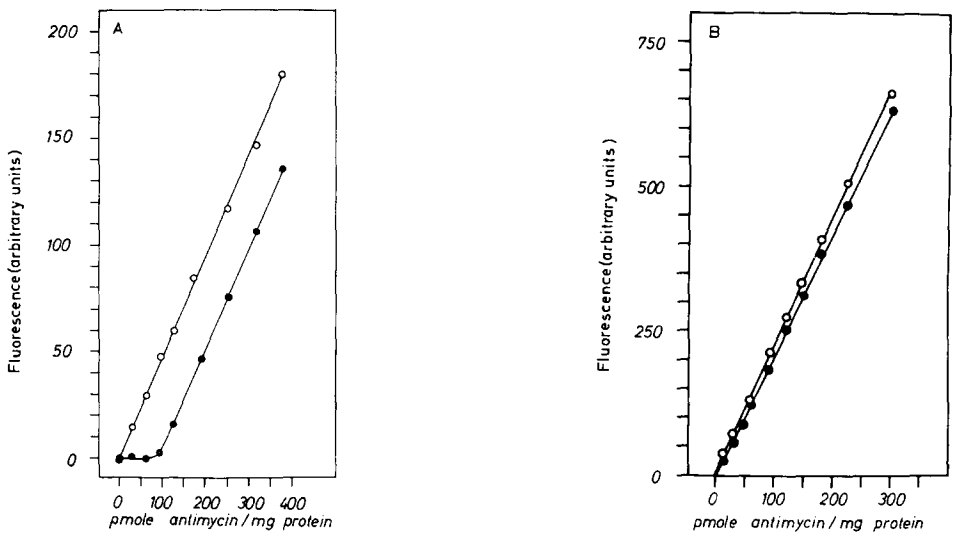

Fig. 6. Binding of antimycin to oxidized wild-type and mutant mitochondrial particles. The mitochondrial suspension was incubated in a buffer containing 20 mM Tris–HCl buffer, 1 mM EDTA at pH 7.4 and 0.6 % bovine serum albumin with increasing amounts of antimycin in methanol (●). After 15 min at room temperature, the suspension was centrifuged and the fluorescence of the antimycin–albumin complex measured as described by Berden and Slater [11]. The methanol concentration was kept constant in all samples. The controls received antimycin added to the particle supernatant after centrifugation (○). A, wild type (9.8 mg protein per sample); B, mutant UV 119 (20.6 mg protein per sample).

the binding of antimycin to oxidized mutant particles is less than that to serum albumin. There is also no evidence for strong binding of antimycin to mutant particles reduced by succinate in the presence of PMS and cyanide, or by $Na_2S_2O_4$ (not shown).

As shown in Fig. 7 (cf. Fig. 4B), the *b*-cytochromes of the mutant react with CO. It can be seen that the shoulder of cytochrome *b*-558 (at about 554 nm at 77 °K) disappears as well as the peak at 525 nm. The spectrum of the wild type is not affected by CO in the α, β region. This indicates that the structure of the *b*-cytochromes is altered in such a way that the heme groups, especially that of cytochrome *b*-558, become accessible for CO.

Cytochrome oxidase

The spectral properties of this enzyme, after reduction by dithionite (or ascorbate plus TMPD, not shown) are similar in wild type and mutant (see Fig. 3A and Table II). However, the results given in Fig. 1B and Table I have already indicated that cytochrome oxidase is also, but to a lesser extent, affected by the mutation (which is a single-gene mutation). The rate of ascorbate oxidation is only about 45% of the wild type and is scarcely affected by cyanide. Moreover, cytochromes aa_3 and *c* are not fully oxidized in aerated mutant particles, as can be shown by low-temperature spectra taken after the addition of ferricyanide to the reference cuvette. This indicates a lack of reactivity with O_2 of the cytochrome aa_3. This finding seems to be parallelled by the presence of a cyanide-insensitive shunt for ascorbate oxida-

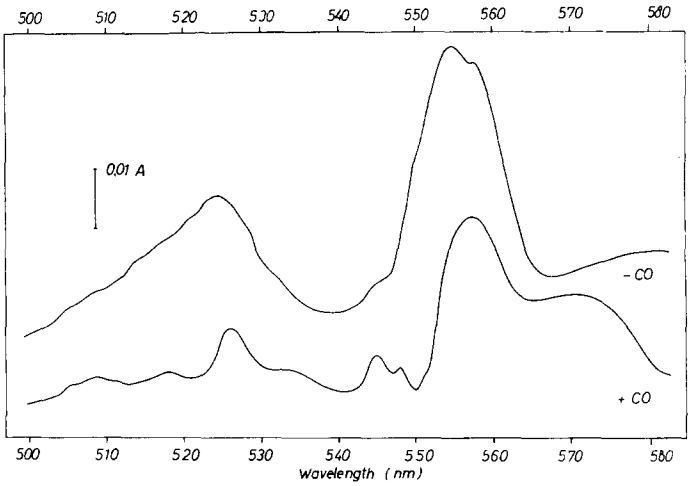

Fig. 7. Effect of CO on absorption spectra of mutant UV 119 at 77 °K. Conditions were the same as described in the legend of Fig. 2. Difference spectra $Na_2S_2O_4$ minus ascorbate+phenazine methosulphate+TMPD are shown. In the experiment indicated the reduced sample was bubbled with CO for 1 min. (The peaks of cytochrome *c* at 548 and 544 nm [12] in this spectrum are attributed to incomplete reduction of this cytochrome in the reference cuvette.)

tion, which suggests a block in or after cytochrome a_3 (leading to a higher steady-state reduction level for aa_3 than in the wild type, as evidenced from reduction kinetics with ascorbate+TMPD using the wavelength pair 608–590 nm, not shown). This shunt is, however, not, or only to a minor degree, accessible for NADH or succinate.

Electron paramagnetic resonance studies

The incomplete oxidation of cytochrome aa_3 in mutant particles in the presence of O_2 is also reflected by electron-spin resonance studies at 35 °K. No copper signal was detectable in the EPR spectrum unless ferricyanide (0.3 mM) was added. Ferricyanide had no effect on the intensity of the copper signal in wild-type particles.

In Fig. 8, Curve A shows that in the wild type the signal is found in oxidized particles at $g = 1.98$ (trough), the normal value of cytochrome *c* oxidase. The same signal is found in particles from the mutant UV 119. However, a second broad signal emerges with a trough at $g = 2.01$ (Fig. 8, Trace B). This is also found in other mutants with cyanide-insensitive ascorbate oxidation (Bandlow, W., unpublished) and may reflect structural changes, caused by the absence of the cyanide-sensitive site, in the environment of the copper atom that is not detectable in the EPR spectrum of the wild type.

Oligomycin-sensitive ATPase and oxidative phosphorylation

To see if the lowered electron flow through the *bc*, segment and cytochrome *c* oxidase is also reflected by an alteration of the oxidative phosphorylation machinery, an experiment was run with whole cells in order to check indirectly respiratory control in wild-typeand mutant cells by means of the action of uncoupler after inhibition of the mitochondrial adenine nucleotide-translocating system by bongkrekic acid. In an Oxygraph experiment, wild-type or mutant cells, deprived of endogenous substrates

by extensive aeration, were suspended in a buffer containing 50 mM citrate and 50 mM phosphate buffer (pH 4.3). After measuring the rate of oxygen uptake due to consumption of endogenous substrates, 20 mM glucose, 20–40 μM bongkrekic acid and 20 μM FCCP were sequentially added and the rates of O_2 consumption measured over periods of 2–4 min each. The residual respiration of mutant cells with glucose was 1.7% of the wild-type rate. It was inhibited by bongkrekic acid by 29% and FCCP stimulated the respiration of the inhibited cells by a factor of 3–6. For wild-type cells, the corresponding inhibition was 42% and the stimulation factor 2.4. The latter could be increased to 3.5 after preincubation of the cells with bongkrekic acid for 5 min in the absence of substrate.

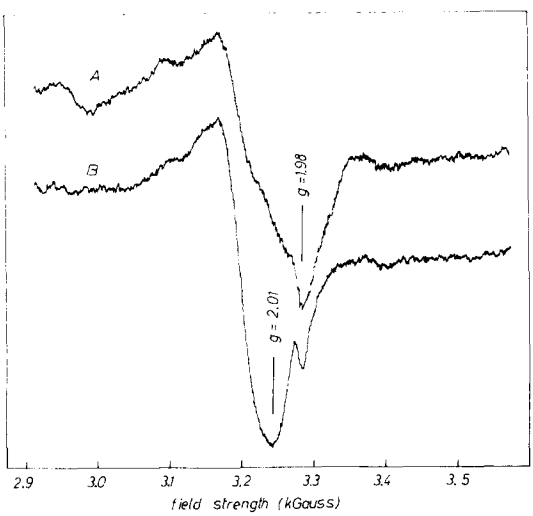

Fig. 8. Electron paramagnetic resonance spectrum at 35 °K of wild-type and mutant submitochondrial particles in the presence of O_2. A, wild-type particles, 40.2 mg protein/ml; B, particles of UV 119, after oxidation with 0.3 mM ferricyanide, 28.0 mg protein/ml. In both cases, a power of 4 mW was used, with modulation amplitude 12.5, frequency, 9.111 GHz. The same gain was used in each case.

To test the presence of oxidative phosphorylation more reliably, Mg^{2+}-dependent ATPase was tested in particles from the wild type and mutant by measuring the H^+ production during the hydrolysis of ATP [13] with 0.1 M oxalic acid as internal standard. The incubation medium consisted of 50 mM KCl, 100 mM sucrose, 10 mM Tris–HCl buffer, 5 mM $MgCl_2$, 3 mM ATP, 0.5 mM EDTA at a final pH of 9.0. As calculated from the rates after an incubation time of 1 min, the activity was 2.7 μmoles H^+/min per mg protein in the wild type and 0.2 μmole H^+/min per mg protein in the mutant. The ATPase activities were equally sensitive to oligomycin, half inhibition obtained with 3.7 μg oligomycin per mg protein in the wild type, and 3.3 μg oligomycin in the mutant. However, it cannot be excluded that mutant ATPase is partially solubilized during the preparation of mitochondrial particles.

DISCUSSION

Quite a variety of respiration-deficient mutants of yeast has been described in the literature (see, for instance [3, 7, 14–21]). Like the mutant described here, some of them are reported to be deficient in the bc_1 segment of the respiratory chain [3, 16–19]. However, spectroscopic evidence for the loss of cytochrome *b* or c_1 has been obtained only with whole cells after reduction with dithionite, which give a poor resolution of the absorption bands of these cytochromes due to the interference with the strong cytochrome *c* absorption, and which only show the presence or absence of absorption bands in the *b* or *c* region. The loss of distinct bands of the cytochromes *b* is difficult to detect and no conclusion about enzymatic reducibility of these cytochromes, and the binding of CO or antimycin can be drawn. Cytochrome aa_3 is spectrally quite normal in these mutants [3, 15–18]. For one mutant, ascorbate oxidation has been reported to be the same as the wild-type activity. Oxidative phosphorylation was reported [18] to be present and sensitive to oligomycin in those mutants in which only cytochrome *a* or *b* was lost due to the mutation. Since, however, cyanide sensitivity of the oxidation of reduced cytochrome *c* or ascorbate plus TMPD and the reduction kinetics and the reduction behaviour of the Cu(II) of cytochrome oxidase have not been checked, pleiotropic effects may have been present.

In mutants of *S. pombe* that show diminished QH_2–cytochrome *c* reductase activity as characterized so far in this laboratory, cytochrome oxidase is always less sensitive to cyanide, the EPR signal of oxidized particles is altered and the Mg^{2+}-dependent, oligomycin-sensitive ATPase is affected (Bandlow, W., unpublished) (cf. also ref. 21). It is likely that these pleiotropic deficiencies are caused by secondary regulatory effects, the mechanism of which is not known at present. Since the *b*-cytochromes are not reducible by substrates in the absence of mediators, are CO reactive, and antimycin is not bound by mutant particles, the primary deficiency in mutant UV 119 can be localized in the bc_1 segment, but the precise localization within the segment cannot yet be made. The absence of cytochrome *b*-566, but presence of *b*-558, suggests that these cytochromes are not a single cytochrome with a dual absorption band, as has been suggested [22, 23], but are structurally or environmentally different *b*-cytochromes. However, it cannot be excluded that the *b*-558 found in the mutant is a different cytochrome from that found in the wild type. It is doubtful, if the loss of cytochrome *b*-566 is the reason for the low respiratory activity of mitochondrial particles with NADH or succinate as substrate, since this cytochrome is probably not obligatory for electron transport from substrate to O_2 in particles of the wild type. It is also probably not the reason for the inability of the mutant to grow on non-fermentable substrate, since an oligomycin-resistant mutant of *Schizosaccharomyces pombe* has been found that also possesses no cytochrome *b*-566 but is still capable of growing on non-fermentable substrate [24].

It is possible that one sub-unit, common to all *b*-cytochromes (cf. [25]), may be altered in mutant UV 119, the alteration resulting in a structural change of all the *b*-cytochromes, so that they cannot receive electrons from ubiquinone, and the heme groups are rendered accessible for CO. This effect may also cause the non-integration of functional *b*-566 into the membrane and the non-binding of antimycin to the bc_1 segment. A similar structural change seems to occur during the isolation of

cytochrome *b* from beef heart, since isolated cytochrome *b* does not bind antimycin and reacts with CO [10].

The functional deficiency of the *b*-cytochromes may cause further regulatory effects, rendering the cytochrome oxidase activity less sensitive to cyanide, lowering the reactivity of cytochrome a_3 with O_2 and lowering the activity of oligomycin-sensitive ATPase. It cannot be decided, however, whether the regulation is on the level of integration of the complexes into the membrane or on the level of the transcription of the protein sub-units of the respiratory chain and oxidative phosphorylation.

ACKNOWLEDGEMENTS

The authors are indebted to Dr I.-Y. Lee for taking the EPR spectra at 35 °K. FCCP was kindly provided by Dr P. G. Heytler and bongkrekic acid by Dr W. G. Lijmbach. W. B. is grateful for the support of this work by the Deutsche Forschungsgemeinschaft. This work was supported by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

REFERENCES

1 Butow, R. A. and Zeydel, M. (1968) J. Biol. Chem. 243, 2545–2549
2 Grimmelikhuijzen, C. J. P. and Slater, E. C. (1973) Biochim. Biophys. Acta 305, 67–79
3 Wolf, K., Sebald-Althaus, M., Schweyen, R. J. and Kaudewitz, F. (1971) Mol. Gen. Gen. 110, 101–109
4 Bachofen, V., Schweyen, R. J., Wolf, K. and Kaudewitz, F. (1972) Z. Naturforsch. 27b, 252–256
5 Mahler, H. R., Mackler, B., Grandchamp, S. and Slonimski, P. P. (1964) Biochemistry 3, 668–677
6 Cleland, K. W. and Slater, E. C. (1953) Biochem. J. 53, 547–556
7 Heslot, H., Louis, C. and Goffeau, A. (1970) J. Bacteriol. 104, 482–491
8 Bandlow, W. (1972) Biochim. Biophys. Acta 282, 105–122
9 Rieske, J. S. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds), Vol. 10, pp. 239–245, Academic Press, New York
10 Berden, J. A. and Slater, E. C. (1970) Biochim. Biophys. Acta 216, 237–249
11 Berden, J. A. and Slater, E. C. (1972) Biochim. Biophys. Acta 256, 199–215
12 Claisse, M. L. (1969) Antonie van Leeuwenhoek J. Microbiol. Serol. Suppl. Yeast Symp. 35, 21–22
13 Chance, B. and Nishimura, M. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds), Vol. 10, pp. 641–650, Academic Press, New York
14 Sherman, F. and Slonimski, P. P. (1964) Biochim. Biophys. Acta 90, 1–15
15 Sherman, F. (1964) Genetics 49, 39–48
16 Reilly, C. and Sherman, F. (1965) Biochim. Biophys. Acta 95, 640–651
17 Lachowicz, T. M., Kotylak, Z., Kołodyński, J. and Sniegocka, Z. (1969) Arch. Immunol. Ther. Exp. 17, 72–85
18 Šubík, J., Kužela, Š., Kolarov, J., Kováč, L. and Lachowicz, T. M. (1970) Biochim. Biophys. Acta 205, 513–519
19 Šubík, J., Kováč, L. and Kolarov, J. (1972) Biochim. Biophys. Acta 283, 146–154
20 Wakabashi, K., Takahashi, T., Kawai, K. and Hirosawa, K. (1972) J. Biochem. Tokyo 72, 411–416
21 Goffeau, A., Colson, A. M., Landry, Y. and Foury, F. (1972) Biochem. Biophys. Res. Commun. 48, 1448–1454

22 Sato, N., Wilson, D. F. and Chance, B. (1971) Biochim. Biophys. Acta 253, 88–97
23 Sato, N., Ohnishi, T. and Chance, B. (1972) Biochim. Biophys. Acta 275, 288–297
24 Bandlow, W. in The Biogeneses of the Mitochondrion, Proc. 8th Bari Symp., 1973, Academic Press, New York, in the press
25 Berden, J. A. (1972) Site II of the Respiratory Chain. Studies on the Cytochromes *b* and c_1 in Mitochondria and Sub-mitochondrial Fragments. Ph.D. Thesis, University of Amsterdam, Amsterdam, Gerja, Waarland