The Reaction of Cytochrome Oxidase with Oxygen in the Fission Yeast *Schizosaccharomyces pombe* 972h⁻

STUDIES AT SUBZERO TEMPERATURES AND MEASUREMENT OF APPARENT OXYGEN AFFINITY

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1. Cytochrome *a*₃ in whole-cell suspensions of the fission yeast *Schizosaccharomyces pombe* reacted in the reduced form with CO to give a photodissociable CO complex with absorption maxima at 429, 543 and 591 nm in CO-liganded reduced-minus-reduced difference spectra. 2. Other CO-bound haemoproteins, cytochromes *P*-420 and *P*-450, were not photodissociated under the conditions employed. 3. Measurements of the rates of re-association of CO with cytochrome *a*₃ after flash photolysis over the temperature range from -101 to -109°C gave a value for *E*_{act.} of 28.6 kJ/mol. 4. Between -94 and -106°C, O₂ reacted with cytochrome oxidase in intact cells to give an oxygenated intermediate (compound A). 5. At -70°C compound A was converted into a second spectrally distinct intermediate (compound B). 6. Electron transport, indicated by the oxidation of cytochromes *a* + *a*₃ and cytochrome *c*, did not occur until the temperature was raised to -50°C. 7. At room temperature cytochrome oxidase was oxidized to 50% of its steady-state concentration by 0.35 μM-O₂.

The reaction of cytochrome oxidase with O₂ involves the formation of spectroscopically distinct complexes, both at room temperature (Orii & Okunuki, 1963; Lemberg & Mansley, 1966; Wainio, 1965; Wharton & Gibson, 1968; Greenwood *et al.*, 1974) and at sub-zero temperatures (Erecinska & Chance, 1972; Chance *et al.*, 1975*a,b*, 1977). The functional significance of several of the oxygenated forms of cytochrome oxidase has been questioned, but two of the intermediates of O₂ reduction, compounds A and B, have been shown to be able to oxidize cytochrome *c* (Chance *et al.*, 1978*a*). The detection and characterization of compounds A and B was made possible by the observation that ligand exchange between the CO complex of reduced cytochrome oxidase and O₂ does not occur at a significant rate in the dark at -15 to -35°C (Chance *et al.*, 1975*c*). Aeration of the CO complex of cytochrome oxidase is thus possible in the presence of relatively low concentrations of non-aqueous solvents in the liquid state before flash photolysis at temperatures (down to -120°C) at which oxygenated

intermediates have long half-lives. Although low-temperature studies of the cytochrome oxidase-O₂ reaction have been extensively pursued only with suspensions containing isolated mitochondria and with solubilized enzyme preparations (Chance *et al.*, 1978*b*), these methods are also applicable to intact cells. The present paper describes the characterization of O₂ intermediates formed during the cytochrome oxidase reaction in whole-cell suspensions of the fission yeast *Schizosaccharomyces pombe*. We have also employed the sensitive O₂ assay developed by Schindler (1967) and Oshino *et al.* (1972) to measure the apparent O₂ affinity of the respiratory chain.

Materials and Methods

Maintenance, growth and harvesting of the organisms

S. pombe 972h⁻ was maintained on a defined medium solidified with 1.5% (w/v) agar and grown with forced aeration in the same defined medium containing 1% glucose, exactly as described previously (Poole *et al.*, 1973). Harvesting of cultures was at 4°C by acceleration to 6000 rev./min followed by immediate deceleration in the 6 × 250 ml rotor of a Sorvall RC2-B centrifuge. Organisms were washed once by resuspension in 50 mM-potassium phosphate buffer (pH 7.2) and then repeating the

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Fig. 1. Difference spectra of intact cells of *S. pombe*

Cells harvested from an exponential culture were washed and resuspended in a buffer that contained 0.25 M-mannitol and 50 mM-potassium phosphate (pH 7.4). The baseline (oxidized minus oxidized) showed no discernible features over the wavelength range shown. Curve (a) was obtained after reduction of the contents of the sample cuvette with 1% glucose for 5 min and oxidation of the reference cuvette with 0.1% H_2O_2 before immersion in liquid N_2 ; recording of the spectrum was at 77 K. In curve (c) both sample and reference cuvettes contained suspensions of intact cells reduced with 1% glucose for 5 min; the spectrum was recorded at room temperature; curve (b) is the difference spectrum obtained after bubbling the contents of the sample cuvette in (c) with CO for 2 min. In (a), the pathlength was 2 mm and the spectral bandwidth 2 nm. In (b) and (c), the pathlength was 10 mm and the spectral bandwidth 4 nm. Cell concentrations were 3.0×10^9 cells/ml in (a) and 1.2×10^9 cells/ml in (b) and (c). The numbers on the curves are wavelengths.

harvesting procedure. *Photobacterium phosphoreum* was grown in a medium modified from that described by Green & McElroy (1957), which contained (per litre): peptone (1 g); glycerol (3.8 g); NaCl (30 g); Na_2HPO_4 (5.3 g); KH_2PO_4 (2.1 g); $(\text{NH}_4)_2\text{HPO}_4$ (0.5 g); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 g). After inoculation

with a loopful of cells from solidified medium and growth for 8 h at 30°C with vigorous aeration, the culture was harvested by centrifugation at 8000g for 10 min with the above centrifuge. After two washes in 3% (w/v) NaCl containing 0.1 M-potassium phosphate buffer (pH 7.0) the cells were resuspended

in a medium containing 0.5M-mannitol, 0.1M-KCl and 0.01M-potassium phosphate (pH7.0) to a final concentration of approx. 100mg (wet wt. of cells)/ml of medium.

Cytochrome spectra

Difference spectra at room temperature and at the temperature of liquid N₂ were obtained by using a split-beam spectrophotometer (Yang & Legallais, 1954) fitted with the attachment described by Chance (1957).

Low-temperature flash photolysis

The technique employed was that of Chance *et al.*

(1975c). Washed whole-cell suspensions of *S. pombe* containing 30% (v/v) ethylene glycol were incubated at room temperature (21°C) with 10mM-glucose in 4mm-light-path cuvettes for 5min. CO was bubbled through the anaerobic suspension for 2min at room temperature after the surface of the cuvette contents had been sprayed with a trace of anti-foam (Dow Corning Silicone Antifoam A; Midland, MI, U.S.A.); the saturated suspension contained 1.2mM-CO. A further 2min incubation at room temperature preceded the first step in the chilling procedure, in which the cuvette was immersed in a cooling bath containing ethanol and solid CO₂ at -20°C. After equilibration in the dark, oxygenation of the CO-

Fig. 2. Repetitive wavelength scanning of the reaction of cytochrome oxidase with CO at -101°C

The spectrum of a suspension of CO-liganded glucose-reduced cells to which O₂ had not been added was scanned and stored in the digital memory of a dual-wavelength spectrophotometer. The reference wavelength was 575 nm. Subsequent scans are difference spectra with the stored spectrum subtracted. The broken line is the CO-reduced minus CO-reduced baseline recorded before initiation of the reaction at -101°C. Eleven spectra (1→11) were recorded after photolysis at intervals of 90s. Absorbance increments and the wavelengths (in nm) of distinctive features of the spectra are shown.

inhibited suspension was by 20 up-and-down strokes of a stirrer; this procedure gives $200 \mu\text{M-O}_2$ in the suspension (Chance *et al.*, 1975c). The cuvette was then transferred to a bath containing ethanol and solid CO_2 at -80°C in the dark. Spectrophotometric analysis was performed in a dual-wavelength-scanning spectrophotometer (Chance *et al.*, 1972); baseline corrections (spectra of CO-ligated anaerobic cell suspensions) were stored in a digital memory. The cuvette was cooled by a stream of cold N_2 gas to the required temperature [which was monitored and maintained by a thermocouple adjacent to the sample (Chance *et al.*, 1975c)] before flash photolysis (six flashes of a 20J xenon flash lamp). Negligible photodissociation was produced by the monitoring

light beam before photolysis or during subsequent spectral scanning of the cuvette contents.

O₂-affinity measurements

These involved measurements of steady-state oxidation-reduction levels of cytochromes *c* and *a+a₃* by dual-wavelength spectrophotometry and simultaneous recording of the bioluminescence of *P. phosphoreum* as described by Oshino *et al.* (1972). Wavelength pairs were 550–540 nm (cytochrome *c*) and 445–450 nm (cytochrome *a₃+a*); light emission from the bacterium was measured at 475 nm, and the O_2 tension in the suspensions was controlled by altering the argon/ O_2 ratio in the gas phase. Redox states of cytochromes are expressed relative to the

Fig. 3. Photodissociation of the compound formed in the reaction of cytochrome oxidase with CO at -89°C . Spectral measurements were made as described in the legend to Fig. 2. The three scans are indicated by 1, 2 and 3. During the third scan after flash photolysis, and at the times indicated on the traces, a further flash was given and the spectrum following recovery of the photomultiplier was recorded. The broken line is the CO-reduced minus CO-reduced baseline before photolysis. Absorbance increments and the wavelengths (in nm) of distinctive features of the spectra are shown.

steady-state redox concentrations under a gas phase of O_2 .

Results

Cytochromes in whole-cell suspensions

Fig. 1(a) shows the reduced-minus-oxidized difference spectrum at 77 K after reduction of the sample suspension with 1% glucose and oxidation of the reference suspension by addition of 0.1% H_2O_2 . Absorption maxima due to cytochrome $a+a_3$ are evident at 600 nm and at about 444 nm; cytochrome c gives an α -band at 548 nm and a β -absorption maximum at 519 nm. Two b -type cyto-

chromes are shown (α -bands at 560 and 554 nm; γ -band at 425 nm). CO-difference spectra (glucose-reduced plus CO minus glucose-reduced; Fig. 1b) at room temperature revealed the presence of cytochrome a_3 (absorption maximum in the CO-ligated state at 430 and 593 nm, absorption minimum at 445 nm). Absorption maxima at 419 and 453 nm have been assigned to cytochromes $P-420$ and $P-450$ respectively (Poole *et al.*, 1974).

Photodissociation experiments at sub-zero temperatures; CO binding

A suspension of yeast allowed to respire to anaerobiosis in the presence of 10 mM-glucose was

Fig. 4. Reaction of cytochrome oxidase with O_2 at $-102^\circ C$

The spectrum of a suspension of CO-ligated glucose-reduced cells into which O_2 had been stirred at $-25^\circ C$ was scanned at $-102^\circ C$ and the spectrum stored in the digital memory of a dual-wavelength spectrophotometer. The reference wavelength was 575 nm. Subsequent scans are difference spectra with the stored spectrum subtracted. The broken line is the CO-reduced minus CO-reduced baseline recorded before initiation of the reaction by flash photolysis. Twelve spectra (1→12) were subsequently recorded at intervals of 90 s. Absorbance increments and the wavelengths (in nm) of distinctive features of the spectra are shown.

Fig. 5. *Non-photodissociability of the compound formed between cytochrome oxidase and O_2 at $-89^\circ C$*
Spectral measurements were made as described in the legend to Fig. 4. During the third scan, at each of the points indicated on the traces (at approx. 625 and 465 nm) a further flash was given, and the spectrum following recovery of the photomultiplier was recorded. The broken line is the CO-reduced minus CO-reduced baseline before photolysis. Absorbance increments and wavelengths (in nm) of distinctive features are shown.

Fig. 6. *Reaction between cytochrome oxidase and O_2 observed in the α -region at $-70^\circ C$*

The methods used and the presentation of results is identical with that of Fig. 4, except that the reaction was initiated and observed at $-70^\circ C$.

scanning spectrophotometer. The baseline (broken) of Fig. 2 represents the difference spectrum (CO-reduced minus CO-reduced) of the suspension scanned at $-101^\circ C$ with the absorption at 575 nm as reference. The stored spectrum was automatically subtracted from all subsequent scans. A total of six flashes was required to give maximal dissociation of the haem-liganded CO. The traces shown represent successive spectrophotometric scans at 90 s intervals after the final flash. Maxima at 444 and 611 nm were due to the production of reduced cytochrome a_3 by photodissociation of the cytochrome a_3 -CO complex (absorption minima at 429, 543 and 591 nm). Progressive reassociation of CO with cytochrome a_3 in the dark leads to decreasing absorption maxima at 444 and 611 nm concomitantly with decreasing absorption minima at 429 and 591 nm. In this experiment no evidence was obtained for any photodissociable CO-binding pigment other than cytochrome a_3 ; a similar result was obtained with cell suspensions that had been bubbled with CO for 30 s, or for 10 min. The photoreversibility of the reassociation of CO with cytochrome a_3 at $-89^\circ C$ is shown in Fig. 3. The experimental procedures were similar to those of the last experiment, but, after following CO reassociation (scans 1 and 2), single flashes were given before the third scan of the α -absorption region and of the Soret region of the

bubbled with CO for 2 min at room temperature. The spectrum of this preparation was scanned and stored in the digital memory of a dual-wavelength-

Fig. 7. Redox states of cytochromes in intact cells of *S. pombe* depending on the O_2 concentration

The reaction mixture contained in a final volume of 30 ml, 0.2 ml of the *Photobacterium* suspension (see the Materials and Methods section), 3.06×10^9 cells of *S. pombe*, 10 mM-glucose, 0.45 M-mannitol, 0.09 M-KCl and 9 mM-potassium phosphate, pH 7.0. At the points indicated by the arrows, O_2 concentrations in the gas flow were changed.

spectrum. Increased absorption at 611 minus 591 nm and 444 minus 427 nm in the third scan indicated reversal of CO binding. Measurements of the rates of reaction of cytochrome a_3 with CO (at 444–462 nm) over the temperature range -101 to -109.5°C allowed the construction of an Arrhenius plot, which indicated a value for $E_{act.}$ of 28.6 kJ/mol for the reaction.

Reaction of cytochrome oxidase with O_2 at subzero temperatures

Spectral changes after photodissociation of CO from the cytochrome a_3 -CO complex in the presence of $200 \mu\text{M-O}_2$ at -102°C are shown in Fig. 4. The spectrum obtained immediately after flash photolysis differed in several respects from that obtained in the absence of O_2 (Fig. 2): (a) the α -absorption minimum at 587 nm with shoulder at 592 nm replaced a single minimum at 591 nm; (b) the α -absorption maximum occurred at 600–602 nm instead of at 611 nm; (c) a second absorption minimum was evident at 607 nm after photolysis in the presence of O_2 ; (d) the spectral maximum centred at 445 nm was symmetrical, whereas that at 444 nm obtained in the absence of O_2 was skewed to the shorter wavelengths; (e) the absorbance change at 437–429 nm compared with that at 445–437 nm was greater after photolysis in the presence of O_2 than in its absence. These features persisted in successive scans during ligand binding

and were reproducible in 14 separate experiments over the temperature range -94 to -105°C . That these distinctive spectral characteristics were due to an O_2 compound of cytochrome a_3 was confirmed by the non-photodissociability of the complex (Fig. 5). Similar results were also obtained at -105.8°C and at -94°C .

When flash photolysis of CO-liganded cytochrome a_3 was performed at -70°C in the presence of $200 \mu\text{M-O}_2$, further transient changes in absorption spectra were recorded (Fig. 6). Twelve scans at 90 s intervals revealed that, at this temperature, the formation of the O_2 compound previously detected at -102°C preceded a distinct shift in the position of α -absorption minima to give an asymmetric trough at 604 nm, which became more pronounced in successive spectra. No oxidation of cytochrome c was detected at -70°C (results not shown).

At higher temperatures electron transport was observed after flash photolysis in the presence of O_2 . Oxidation of cytochrome $a+a_3$ (at 593 and 602 nm) and of cytochrome c (at 548 nm) was evident in successive scans in the α -absorption regions of the spectra at temperatures higher than -50°C .

Measurement of the apparent affinity of cytochrome oxidase for O_2

The intensity of luminescence of *P. phosphoreum* is maximal at O_2 concentrations greater than $10 \mu\text{M}$,

Fig. 8. Relationships between the redox states of cytochrome oxidase (a) and cytochrome c (b) and the O_2 concentrations. Results obtained in four separate experiments similar to that shown in Fig. 7 were replotted as a function of O_2 concentration. Open and closed symbols represent results obtained during oxygenation and deoxygenation respectively; symbols of the same form are results from the same experiment.

and is dependent on O_2 tension below that concentration (Oshino *et al.*, 1972). Typical traces for the simultaneous measurements of bioluminescence of *P. phosphoreum* and cytochrome absorption of *S. pombe* at different steady-state O_2 concentrations are shown in Fig. 7. Under anaerobic conditions, photoemission from the bacterium was undetectable and cytochromes c and $a+a_3$ were fully reduced. Stepwise increases in the O_2 tension of the gas phase gave incremental increases in bioluminescence and in steady-state levels of oxidation of the cytochromes. When further increases in O_2 concentration gave no further oxidation of cytochromes, the proportion of O_2 in the gas phase was decreased in a stepwise manner until eventually anaerobiosis was again attained. When the changes in redox levels of the cytochromes were plotted as a function of O_2 concentration [obtained from a calibration curve relating intensity of bioluminescence to O_2 concentration (Oshino *et al.*, 1972)] the relationship shown in Fig. 8 was obtained. The curves indicate that the O_2 concentration oxidizing cytochrome $a+a_3$ halfway to the steady-state level was $0.35 \mu M$; at this point the extent of oxidation of cytochrome c was only 25% of its steady-state redox level under a gas phase of O_2 .

Discussion

Photodissociation of the CO-bound haemoproteins of intact cell suspensions of *S. pombe* revealed the presence of one species, cytochrome a_3 ; thus it was possible to study the reaction with O_2 of cytochrome oxidase without interference from alternative terminal oxidases (see Lloyd, 1974; Degn *et al.*, 1978) or cytochromes P-420 or P-450 (Poole *et al.*, 1973). Reassociation of CO with reduced cytochrome a_3 occurred after photolysis in the absence of O_2 even at temperatures as low as $-110^\circ C$. Yonetani *et al.* (1973) have shown that mammalian cytochrome oxidase-CO is photolysed irreversibly at $-196^\circ C$ and that the mid-point temperature for recombination is $-93^\circ C$. Protohaem-CO also shows spectral changes on photolysis that suggest photodissociation followed by reassociation at temperatures down to $-253^\circ C$ (Alberding *et al.*, 1976), but Yoshida & Orii (1977) suggest that the processes responsible for the spectral changes may involve photoexcitation rather than photodissociation. In view of the previous claims for the presence of cytochrome o in yeast (see Lloyd, 1974), it is interesting that we were unable to detect any photodissociable b-type cytochrome-CO complex in *S. pombe*, although identical experimental conditions have been used to study cytochrome o in *Escherichia coli* (Poole *et al.*, 1979).

The redox centre of cytochrome oxidase consists of cytochromes a and a_3 and two copper ions, closely associated with their respective haems. All four components may lie within a distance of approx. 1 nm. The reaction of reduced cytochrome oxidase with O_2 at low temperatures proceeds by way of at least two intermediates (Chance *et al.*, 1975a,b):

In the temperature range -105.8 to $-89^\circ C$ reaction of *S. pombe* cytochrome a_3 with O_2 gave an intermediate with spectral characteristics similar to those described for compound A in bovine heart mitochondria (Chance *et al.*, 1977). At a higher temperature ($-70^\circ C$), compound A was unstable and was transformed into a second spectrally distinct intermediate ($t_{1/2}$ 3 min) which may be compound B. Electron transport was observed (by the oxidation of cytochromes $a+a_3$ and c) only when the temperature was raised to $-50^\circ C$. The temperature-dependence of the velocities of these changes in intact cells was therefore similar to those previously reported for mitochondria from mammalian sources (Chance *et al.*, 1977) and from *Candida utilis* (Chance *et al.*, 1978c).

The apparent affinity for O_2 of the terminal oxidase of the respiratory chain of *S. pombe* at room temperature ($22^\circ C$) is high; however, the value of $0.35 \mu M$ O_2 , which gave 50% oxidation of cytochrome

$a+a_3$ of intact cells of this organism, is not as low as that for *Saccharomyces cerevisiae* cytochrome a_3 ($0.03\ \mu\text{M}$) determined by the same procedure (Oshino *et al.*, 1972). Schindler (1964) has determined the apparent K_m for O_2 in baker's yeast using the *Photobacterium* system, and gives a value of $0.05\ \mu\text{M}$; higher values of 0.65 and $1.5\ \mu\text{M}$ have been obtained by polarographic methods of O_2 assay (Longmuir, 1954; H. Degn, personal communication). Chance *et al.* (1975a) have previously discussed the apparently contradictory observations that, whereas at -100°C O_2 appears to be highly dissociated from cytochrome a_3 , at 23°C the apparent affinity can be less than $1\ \mu\text{M}$. At the higher temperature, rapid electron transport reducing O_2 to a number of different intermediates, creates a trap that leads to an almost irreversible utilization of all available molecules of O_2 . The intracellular mitochondria of the intact yeast cell evidently have a high apparent affinity for O_2 ; it has been suggested that in hepatocytes and in neurons mitochondria have a lowered O_2 affinity compared with the isolated organelles (Jones, 1978; Jobsis & Rosenthal, 1978).

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