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Haem O can replace haem A in the active site of cytochrome *c* oxidase from thermophilic bacterium PS3

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Thermophilic bacterium PS3 cultured under slightly air-limited conditions showed a mitochondrion-like cytochrome pattern similar to that in vigorously aerated cells, but an *o*-type cytochrome replaced cytochrome a_3 as the CO-binding centre. Cytochrome *cao*-type oxidase was purified from the cell membranes by almost the same procedure as used for cytochrome caa_3. The turnover number of cytochrome *cao* was higher than that of cytochrome caa_3, but the K_m's of the two enzymes for cytochrome *c* and O_2 were almost the same. Gel electrophoresis in the presence of sodium dodecyl sulfate gave bands of four subunits at the identical positions both for cytochrome *cao* and cytochrome caa_3. Cytochrome *cao* contained a novel kind of haem in addition to haems C and A. This novel haem is likely to be haem O, very recently found as the chromophore of the cytochrome *bo* complex in *Escherichia coli*. These data suggest that cytochrome *cao* is an alternative form of cytochrome *c* oxidase (cytochrome caa_3), in which the cytochrome a_3 centre of the enzyme is replaced with cytochrome *o*.

Cytochrome *c* oxidase; Haem O; Oxygen-binding site; Effect of aeration; Thermophilic bacterium PS3

1. INTRODUCTION

A variety of terminal oxidases have been identified in aerobic and facultative aerobic bacteria [1–3]. We reported that the Gram-positive, spore-forming thermophilic bacterium PS3 shows different cytochrome patterns depending on the aeration conditions [4]. Cytochrome caa_3-type oxidase with 4 subunits has been prepared from highly-aerated PS3 cells, and shows the CO difference spectrum of cytochrome a_3 [5,6]. A terminal oxidase named 'cytochrome *o*' with 3 subunits (60, 30 and 16 kDa) has been obtained from cells cultured under air-limited conditions [7]. The PS3 cells grown under slightly air-limited conditions show the characteristic CO-difference spectrum of cytochrome *o*, although their reduced-form spectrum is very similar to that of highly-aerated cells.

We found that the pigment giving the characteristic CO-difference spectrum was co-fractionated with cytochrome *a* on DEAE-Fractogel chromatography. Here we report the purification and properties of cytochrome *cao* from thermophilic bacterium PS3 cultured under slightly air-limited conditions, in which the cells contain a normal level of cytochrome *a*, but the CO-difference spectrum showed the presence of cytochrome *o* instead of cytochrome a_3. We found that cytochrome *cao* has probably the same subunit proteins as cytochrome caa_3, and that a novel haem is the chromophore in cytochrome *o*. Very recent work has showed that the chromophores of *Escherichia coli* cytochrome *bo* complex are not protohaems [8]. The suggested structure of the novel haem (named haem O) is a haem A-like molecule with a hydroxyethylfarnesyl side chain, but with no formyl group [8]. Several characteristics of the novel haem in PS3 cytochrome *cao* were in accordance with those of haem O. It has also been shown that subunits in the *E. coli* cytochrome *bo* are homologous with those of the cytochrome aa_3-type oxidase of mitochondria and bacteria [9–11].

Abbreviations: MOPS, 4-morpholinepropane sulfonic acid; TMPD, *N,N,N',N'*-tetramethyl *p*-phenylenediamine

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2. MATERIALS AND METHODS

The thermophilic bacterium PS3 was cultured in medium containing 0.8% polypeptone, 0.4% yeast extract and 0.3% NaCl at 66–69°C under slightly air-limited conditions by decreasing the flow rate of air. These conditions allow the cells to grow linearly with time at a rate of 0.1–0.15 absorbance units at 650 nm per hour, and the cells were harvested when they showed absorbance 0.7–0.9 at 650 nm. These cells contained almost the same levels of cytochrome *a* as highly-aerated cells, but contained CO-reactive *o*-type cytochrome(s) instead of cytochrome a_3. Membranes were prepared from these cells, and cytochrome *cao* was purified from the membranes by the same method as used to purify cytochrome caa_3 (cytochrome *c* oxidase) from cells cultured with vigorous aeration [12]. The last purification

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step (hydroxyapatite column chromatography) was, however, replaced by chromatography on a small Q-Sepharose column (1 × 3 cm) with a solution of 1.5% octyl glucoside, 25 mM NaP_i buffer (pH 7.2) and 50 mM NaCl for elution.

Proteoliposomes containing PS3 cytochrome *cao* were prepared by the freeze-thaw sonication, and H^+-pumping activity was followed with a pH meter [13].

TMPD and cytochrome *c* oxidase activities were measured with a pH meter (Beckman 4500) with ascorbate (10 mM) as the final electron donor in medium containing 25 mM KCl, 2.5 mM $MgSO_4$ and 1 mM K-MOPS (ph 6.5) at 32°C as described previously [14]. Oxidase activity was monitored with an oxygen electrode (YSI no. 4001) in a semi-closed cell. The K_m for oxygen was determined by recording traces of oxygen uptake in the medium with a very low concentration of oxygen using a high-sensitivity strip chart recorder (Yokokawa model 3066).

The contents of protohaem and haem C were determined from the pyridine haemochrome spectra (reduced minus oxidized). Values of $E_{604-630\,nm}$ = 25 $mM^{-1} \cdot cm^{-1}$ and $E_{550-535\,nm}$ = 24 $mM^{-1} \cdot cm^{-1}$ for haem A and haem C, respectively [15] were used for the calculation. To stimulate formation of pyridine haemochrome and minimize haem degradation, we added sodium dodecylsulfate (1%), and used relatively low concentrations of pyridine (10%) and NaOH (0.07 M). A small amount of $Na_2S_2O_4$ was used for reduction. Copper was determined colorimetrically with bathocuproin sulfate [16]. Haem compounds were separated by reverse-phase high-pressure liquid chromatography using a Rechrosorb RP-18 column (Cica-Merck).

E. coli cytochrome *bo* complex [17] was kindly donated by Prof. Anraku and Dr Mogi of the University of Tokyo. Yeast cytochrome *c* from *Saccaromyces cerevisiae* (type VIII) was purchased from Sigma (St. Louis, MO), DEAE-Fractogel (Toyopearl) from Toso Co. (Tokyo), and Q-Sepharose from Pharmacia (Uppsala), and low molecular weight protein standards were from Bio-Rad (Richmond, CA).

3. RESULTS

3.1. *Purification of cytochrome* cao *oxidase*

The reduced minus oxidized difference spectrum of the membrane fraction from slightly air-limited cells was almost the same as that of vigorously aerated cells (see Fig. 2 of ref. [4]), which resembles that of mitochondria. However, the CO-difference spectrum of the same sample showed peaks at 417, 536 and 568 nm and a trough at 432 nm (see below). This indicates that the CO-binding centre is an *o*-type cytochrome in slightly air-limited cells. The fact that there was scarcely any peak in the 592 nm region indicated that the cells contained little, if any, cytochrome a_3 which is present in vigorously-aerated cells [5].

We purified the CO-reactive pigment from the membranes of cells cultured under slightly air-limited conditions by the procedure used previously to purify cytochrome caa_3 [12]. Most of the CO-reactive pigment was co-purified with cytochrome *c* and cytochrome *a*. Almost all (over 90%) TMPD oxidase activity was also recovered in the same fractions. A very small amount of the activity was also found in the fraction of cytochrome b_{558} (which we formerly called 'cytochrome *o*-type oxidase' [7]). In a typical purification experiment, the final yields of the CO-reactive pigment and cytochrome *a* were 27% and 33%, respectively. Thus we obtained cytochrome *cao*-type oxidase instead of the

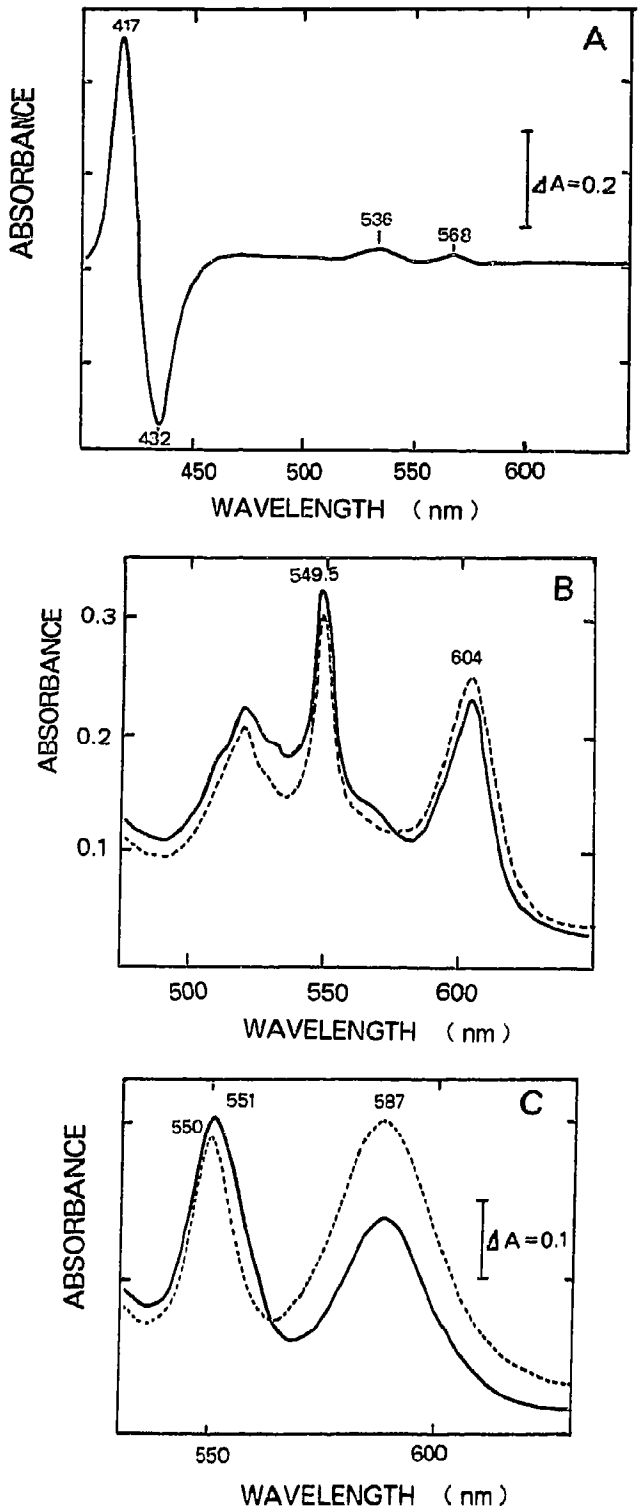

Fig. 1. Absorption spectra of cytochrome *cao*. A. CO-difference spectrum. The cytochrome *cao* preparation (1.1 mg protein/ml) was reduced with ascorbate and TMPD, and the sample cuvette was bubbled with CO gas for 1 min. B. Absolute spectra of the reduced forms of cytochrome *cao* (——) and cytochrome caa_3 (– – –). The protein concentrations used were 1.3 mg protein/ml for cytochrome *cao*, and 1.1 mg/ml for cytochrome caa_3. C. Redox difference spectra of pyridine haemochrome of cytochrome *cao* (——) and cytochrome caa_3 (– – –). The amounts of sample used were 0.13 mg of cytochrome *cao* and 0.16 mg of cytochrome caa_3.

caa_3-type from the cells cultured under slightly air-limited conditions.

3.2. *Spectral properties*

Fig. 1A shows the CO-difference spectrum of the purified cytochrome *cao* preparation. The spectrum was similar to that of the *E. coli* cytochrome *bo* complex [17,18]. Fig. 1B shows the spectrum of the reduced form of cytochrome *cao* (——) in comparison with that of cytochrome caa_3 (---). In the cytochrome *cao* spectrum, the band at 604 nm was a little lower, and there was a tiny shoulder around 563 nm. The major difference in the spectra seems to result from superimposition of a broad peak from 530 to 570 nm present in the cytochrome *cao* spectrum. The superimposed spectrum is reminiscent of the high spin-type deoxyhemoglobin spectrum having a broad peak at 555 nm [18].

Next we prepared pyridine ferrohaemochromes to obtain more information about the haem species and their amounts. Fig. 1C shows the reduced minus oxidized pyridine ferrohaemochrome spectra of the cytochrome *cao* (——) and cytochrome caa_3 (---) preparations. Cytochrome *cao* showed peaks at 587 and 551 nm, while cytochrome caa_3 showed peaks at 587 and 549.5 nm. Moreover the peak of cytochrome *cao* at 587 nm due to the pyridine ferrohaemochrome of haem A was lower than that of cytochrome caa_3. Pyridine haemochromes of haem C and protohaem are known to have α-bands at 549.5 and 557 nm [15]. So the peak shift of the haem C spectrum to 551 nm is probably caused by the presence of another haem (probably not protohaem) absorbing in this region. In fact the HCl-acetone extract of cytochrome *cao* showed peaks at 552 and 587 nm upon addition of NaOH and pyridine (see Fig. 3).

3.3. *The presence of a novel haem*

Haems extracted with HCl-acetone are analyzed by reverse-phase chromatography after extraction with ethylacetate and washing with water. The chromatographic analysis of haems from cytochromes *cao* (Fig. 2A) and caa_3 (Fig. 2B) shows that most of the extractable haem from cytochrome caa_3 is haem A, while those from cytochrome *cao* contained both haem A and the novel haem O, since haem A from the beef heart cytochrome aa_3 and haem O from the *E. coli* cytochrome *bo* gave peaks eluting at 30.5 and 35 min, respectively (not shown).

Fig. 3 shows pyridine ferro-haemochrome spectra of the first peak at 30.5 min (A) and the second peak at 35 min (B) obtained by the reversed-phase chromatography of the haem fraction extracted from the PS3 cytochrome *cao*. The former showed a peak at 587 nm, indicating that this was haem A, while the latter showed a peak at 552.5 nm. This position of the absorption band is almost identical with that reported for haem O of the *E. coli* cytochrome *bo* [19]. The dotted line in the figure shows the pyridine haemochrome of HCl-acetone fraction before the chromatography. The Soret peak of haem O was at 394 nm, while those of haem A and protohaem were at 406 and 398 nm, respectively. Since the extinction coefficients of Soret peaks of these compounds are almost the same, the

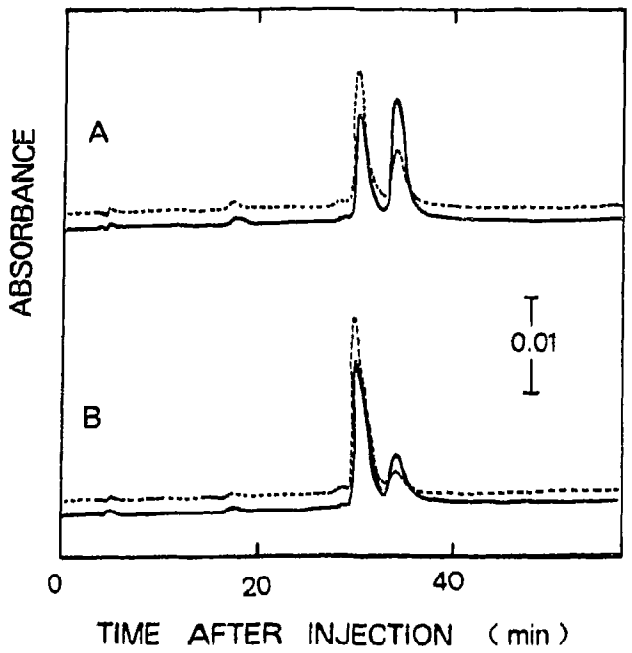

Fig. 2. Elution profiles of haem compounds in reverse-phase chromatography. The solvent was acetonitrile containing trifluoroacetic acid (0.05%), and a gradient elution (50-100%) was performed. Flow rate was 0.8 ml/min. (——), absorbance at 395 nm; (– – –), absorbance at 406 nm. A. Cytochrome *cao* preparation. B. Cytochrome caa_3 preparation.

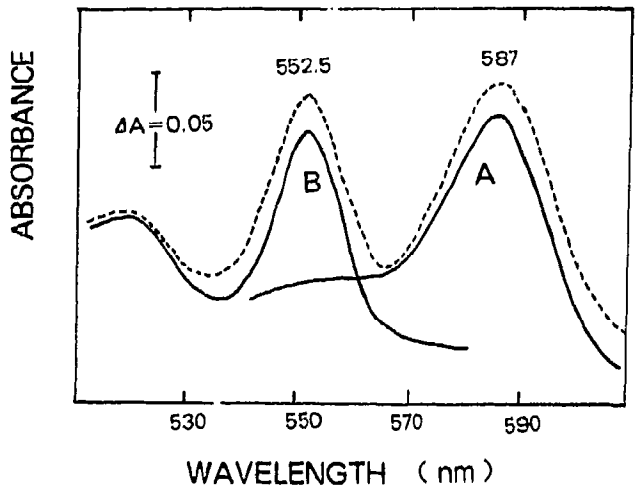

Fig. 3. Absorption spectra of pyridine ferrohaemochrome of haem A (A) and haem O (B). The haems were isolated by reverse-phase chromatography as in Fig. 2, and pyridine haemochromes were prepared as described in section 2. The dotted line in the figure shows the haem fraction before chromatography.

HCl–acetone-extractable fraction of PS3 cytochrome *cao* seemed to contain haem A and haem O almost at an equimolar ratio.

3.4. *Subunit composition*

Fig. 4 shows results on the subunit composition of cytochrome *cao*. The profiles of three different preparations of cytochrome *cao* on gel electrophoresis in the presence of dodecyl sulfate (lanes 2–4) were the same as that of cytochrome caa_3 (lane 1), whose subunits have been reported to be 56, 38, 22 and 12 kDa [5,6]. The molecular masses of the subunits of cytochrome caa_3 deduced from their DNA sequences support these values, except for that of the very hydrophobic subunit I, which is deduced to be about 68 kDa [11]. Thus the molecular masses of PS3 cytochromes caa_3 and *cao* are probably both approx. 140 kDa. Using this value, the data in Fig. 1C suggest that one cytochrome *cao* containes one haem C, one haem A and one novel haem (haem O), while the cytochrome caa_3 molecule contains one haem C and two haem A's, in accordance with the previous report [5]. The copper/haem C ratio in cytochrome *cao* was 2.1–2.5 as in cytochrome caa_3.

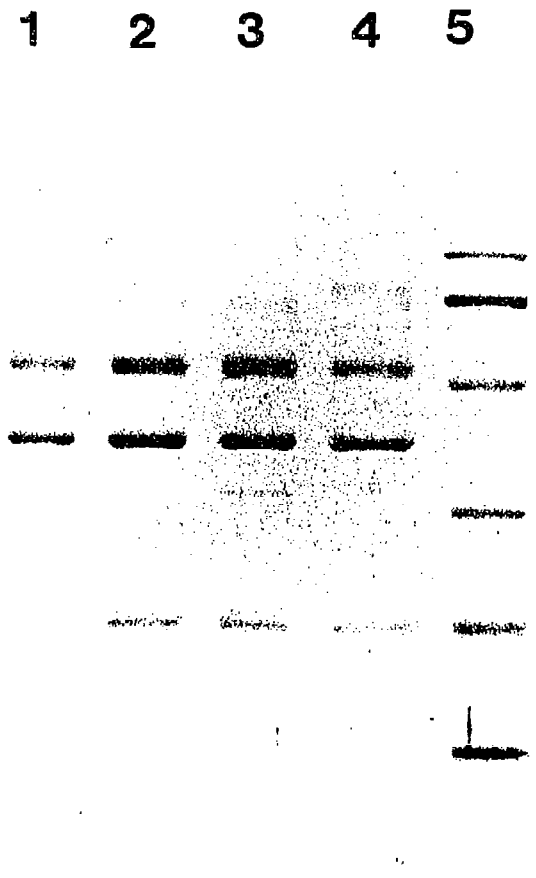

Fig. 4. Subunit profiles of cytochrome *cao* and cytochrome caa_3. Gel electrophoresis with sodium dodecyl sulfate was carried out in the presence of 6 M urea [27]. The gels were stained with Coomassie brilliant blue R-250. Lane 1, cytochrome caa_3; lane 2–4, cytochrome *cao* (3–5 μg); lane 5, marker proteins (phosphorylase b, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme).

Table I

Kinetic constants of cytochrome *cao* and cytochroma caa_3. The oxidase activity was measured with a pH meter in the presence of 10 mM ascorbate, and the K_m for O_2 was measured with an oxygen electrode.

	K_m (μM) for cyt. *c*		V_{max} (s^{-1}) for cyt. *c*	V (s^{-1}) for 0.1 mM TMPD
		O_2		
cytochrome *cao*	5.5	0.5	164	122
cytochrome caa_3	5.7	0.6	85	56

3.5. *Catalytic activities*

PS3 cytochrome *cao* oxidized yeast cytochrome *c* and TMPD actively. As summarized in Table I, the K_m's of cytochrome *cao* for yeast cytochrome *c* and O_2 are almost the same as those of cytochrome caa_3. On the other hand, the V_{max} value of cytochrome *cao* for cytochrome *c* and the oxidation rate of 0.1 mM TMPD are both about twofold higher than those of cytochrome caa_3. Results with two other preparations of cytochrome *cao* were similar. Cytochrome *cao* reduced molecular oxygen to water with ferrocytochrome *c*, because 4 mol of ferrocytochrome *c* was oxidized for reduction of 1 mol of O_2. Cytochrome *c* oxidation by cytochrome *cao* was also coupled to H^+ pumping similar to that by cytochrome caa_3 (N. Sone, to be published).

4. DISCUSSION

The present data showed that cytochrome *cao* from the cells cultured under air-limited conditions contained a novel haem in addition to haems A and C. The novel haem seems to be a haem O due to its chromatographic and spectral properties (Figs. 2 and 3). Haem O found in the *E. coli* cytochrome *bo* complex is tentatively assigned to be a haem A-like molecule having a 17-carbon hydroxy-ethylfarnesyl side chain, but with a methyl group replacing the formyl group [8]. It is also noteworthy that the substrate specificity (Table I) and the subunit pattern (Fig. 4) of cytochrome *cao* were almost the same as those of cytochrome caa_3 and that both enzymes could be purified by the same method. Thus, PS3 cytochrome oxidase apoprotein seemed to bind the novel haem (haem O) instead of haem A at the O_2-binding active site, when the cells were cultured under slightly air-limited conditions. Purification and properties of the cytochrome *cao* (*aco*)-type oxidase from alkalophilic *Bacillus* YN-2000 were reported recently [20]. Although this enzyme was reported to

contain protohaem, the pyridine haemochrome spectrum suggests that this haem is really haem O as in the PS3 enzyme.

The contents of cytochromes acting as the terminal oxidase in PS3 depend on the aeration conditions: PS3 produces cytochrome caa_3 under sufficiently aerated conditions [5], cytochrome *cao* under slightly air-limited conditions (the present work), and cytochrome b_{558} under air-limited conditions [7]. Cytochrome *o* (with cytochrome a_3) has been found spectrophotometrically with CO in several Gram-positive bacteria such as *B. subtilis* [21,22], *Staphylococcus aureus* [23] and *Mycobacterium phlei* [24]. The demostration of cytochrome *o* in two additional *Bacillus* species, namely in PS3 (present work) and the alkalophile [20] as a component of cytochrome *cao*, suggests that in all these bacteria cytochrome *o* is haem O at the O_2-binding site. It has also been reported that amino-acid sequences of respective subunits of *E. coli* cytochrome *bo* are quite similar to those of mitochondrial and bacterial cytochrome aa_3 [9–11]. Like cytochrome caa_3, PS3 cytochrome *cao* pumped H^+ coupled to the electron transfer. Thus different haem species at the O_2 binding site do not affect the catalytic activity much, although importance of formyl group of haem A has been suggested [25].

Cytochrome b_{558} which we called 'cytochrome *o*-type oxidase' is a different protein. The enzyme has three subunits (60, 30 and 16 kDa), and shows appreciable oxidase activity but poorly reacts with CO [7]. Moreover, the haem component of this enzyme was analyzed to be protohaem (Sone, unpublished observation). Therefore, we now refer to this third oxidase as cytochrome b_{558} instead of 'cytochrome *o*-type oxidase'. A similar cytochrome *b*-type oxidase has been found in *Rhodobacter capsulata* [26]. Cytochrome b_{558} from air-limited PS3 cells did not pump H^+, but mediated electrogenic electron transfer from cytochrome c_{551} and molecular oxygen [7].

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REFERENCES

[1] Poole, R.K. (1988) in: Bacterial Energy Transduction (C. Anthony, ed.) pp. 231–316, Academic Press, London.
[2] Anraku, Y. (1988) Annu. Rev. Biochem. 57, 101–132.
[3] Sone, N. (1990) in: The Bacteria (Vol. 12), Bacterial Energetics (T.A. Krulwich, ed.) pp. 1–32, Academic Press, New York.
[4] Sone, N., Kagawa, Y. and Orii, Y (1983) J. Biochem. 93, 1329–1336.
[5] Sone, N. and Yanagita, Y. (1982) Biochim. Biophys. Acta 682, 216–226.
[6] Sone, N., Shimada, S., Ohmori, T., Souma, Y., Gonda, M., and Ishizuka, M. (1990) FEBS Lett. 262, 249–252.
[7] Sone, N., Kutoh, E. and Sato, K. (1990) J. Biochem. 107, 597–602.
[8] Puustinen, A. and Wikström, M. (1991) Proc. Natl. Acad. Sci. USA, 88, 6122–6126.
[9] Chepuri, V., Lemieux, L., Au, D.C.-T. and Gennis, R.B. (1990) J. Biol. Chem. 265, 11185–11192.
[10] Saraste, M., Raitio, M., Jalli, T., Lemieux, I., Chepuri, V. and Gennis, R.B. (1988) Ann. N.Y. Acad. Sci. 550, 314–324.
[11] Ishizuka, M., Machida, K., Shimada, S., Mogi, A., Tsuchiya, T., Ohmori, T., Souma, Y., Gonda, M. and Sone, N. (1990) J. Biochem. 108, 866–873.
[12] Sone, N (1986) Methods Enzymol. 126, 145–153.
[13] Sone, N., Yanagita, Y. (1984) J. Biol. Chem. 259, 1405–1408.
[14] Nicholls, P. and Sone, N. (1984) Biochim. Biophys. Acta, 767, 240–247.
[15] Berry, E.A., and Trumpower, B.L. (1987) Anal. Biochem. 161, 1–15.
[16] Tsuzuki, T., Orii, Y. and Okunuki, K. (1967) J. Biochem. 62, 37–45.
[17] Kita, K., Konishi, K. and Anraku, Y. (1982) J. Biol. Chem. 259, 3368–3374.
[18] Antonini, E., Brunori, M. (1971) in: Hemoglobin and Myoglobin in Their Reactions with Ligands, p. 19, Elsevier, Amsterdam.
[19] Puustinen, A., Finel, M., Haltia, T., Gennis, R.B. and Wikström, M. (1991) Biochemistry 30, 3936–3942.
[20] Qureshi, M.H., Yumoto, I., Fujiwara, T., Fukumori, Y. and Yamanaka, T. (1990) J. Biochem. 107, 480–485.
[21] Castor, L.N. and Chance, B. (1959) J. Biol. Chem. 234, 1587–1592.
[22] Edwards, C., Beer, S., Siviram, A. and Chance, B. (1981) FEBS Lett. 128, 205–207.
[23] Tabor, H.W. and Morrison, M. (1964) Arch. Biochem. Biophys. 105, 367–379.
[24] Revsin, B., Marquez, E.D. and Brodie, A.F. (1970) Arch. Biochem. Biophys. 139, 114–120.
[25] Babcock, G.T. and Callahan, P.M. 91983) Biochemistry 22, 2314–2319.
[26] Hüdig, H. and Drews, G. (1984) Biochim. Biophys. Acta 765, 171–177.
[27] Merle, P. and Kadenbach, B. (1980) Eur. J. Biochem. 105, 499–507.