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Pseudomonas Cytochrome *c* Peroxidase

II. Localization of Cytochrome *c* Peroxidase in *Pseudomonas fluorescens*

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The distribution of cytochrome *c* peroxidase among cell fractions obtained from *Pseudomonas fluorescens* has been studied. When the cells were broken mechanically in an extrusion press, by grinding with alumina powder or with glass beads in a peristaltic pump, about 30–59 % of cytochrome *c* peroxidase was found in the cell debris and membrane fraction. When the cells were treated with organic solvents (acetone and butanol), 93–73 % of cytochrome *c* peroxidase could be extracted from the cells. When the cell walls were lysed with lysozyme and a pure membrane fraction was isolated, about 90 % of cytochrome *c* peroxidase and 85 % of cytochrome *c* oxidase was found in the membrane fraction. The isolated membranes were disrupted further by ultrasonic treatment and the subfractions obtained were centrifuged in sucrose gradients. Several subfractions were separated at 25 000 *g* in low sucrose gradients. Cytochrome *c* peroxidase as well as cytochrome *c* oxidase were present in most of the subfractions. The results are discussed.

Lenhoff and Kaplan [1,2] first discovered the presence of cytochrome *c* peroxidase (cytochrome c:H_2O_2 oxidoreductase, EC 1.11.1.5) in *Pseudomonas fluorescens*. They showed that it plays an active part in the respiratory chain of the bacterium at low oxygen tensions.[3] The distribution of cytochrome *c* peroxidase (CCP) in the cells of *P. fluorescens* has not been studied. The difficulties in bringing CCP into solution for purification purposes suggested that it is located in the particle fraction of the cell.[4] CCP has been localized in the cytoplasmic membrane of anaerobically grown yeast and in the mitochondria of aerobically grown yeast.[5–7] In this publication the results of studies on the distribution of CCP in cells of *P. fluorescens* are described. The results indicate that CCP is located in the membrane fraction of the cell.

MATERIALS AND METHODS

Pseudomonas fluorescens, strain No. 8, was provided by Professor N. Kaplan. This strain has been used in previous studies on CCP.

Cultivation of P. fluorescens. The bacteria were cultivated in 4—8 l volumes of citrate-nitrate medium saturated with a gas mixture containing 5 % oxygen and 95 % nitrogen as described previously.[4] The cells were harvested at the end of the logarithmic growth phase and stored as a frozen paste until subjected to mechanical and chemical treatments or as a suspension at 4°C before enzymatic lysis.

Mechanical rupture of the cells. The cells were broken mechanically with an extrusion press (X-Press, AB Biox, Nacka, Sweden) according to Edebo,[8] with alumina powder according to Lenhoff and Kaplan [2] and with glass beads in a peristaltic pump (Multifix M 80, Alf. Schwinnherr, Schwäbisch Gmünd, West Germany) according to Phillips *et al.*[9] The cells were suspended in 0.1 M sodium phosphate buffer of pH 7.5 before these treatments. Three passages through the extrusion press were necessary to break all the cells. Alumina powder was centrifuged down at 2000 *g* for 5 min at 4°C and the cell debris and particle fractions were collected after centrifugation at 25 000 *g* for 30 min at 4°C.

Treatment of the cells with organic solvents. The cells were treated with butanol according to Morton [10] and with acetone as described previously.[4] The cells were treated with butanol and extracted by dialyzing against 0.05 M sodium phosphate buffer of pH 7.5 overnight at 4°C, after which the cell debris was centrifuged at 30 000 *g* for 1 h. After acetone treatment the cells were extracted with the mentioned buffer by mixing for 1 h at 4°C and centrifuged at 25 000 *g* for 30 min at 4°C.

Enzymatic lysis of the cells was performed with lysozyme (Nutritional Biochemicals Corporation) in the presence of ethylenediaminetetraacetic acid (EDTA) by the method of Repaske [11] as modified by Burrous and Wood.[12] An average incubation time of 5 min was sufficient to lyse all the cells as judged from the decrease in turbidity of the cell suspension. A fraction containing membranes and DNA was isolated by the method of Burrous and Wood.[12] The fraction was suspended in 0.01 M $MgCl_2$, and DNA hydrolyzed with desoxyribonuclease (DN-100 from Sigma Chemicals Co.). Further disruption of the membranes was performed for 5 min with a cooled 9 kc Raytheon (model S 102 A) ultrasonic oscillator.

Ultracentrifugations in sucrose gradients were performed in a Spinco L 50 preparative ultracentrifuge using rotors 30 and 50. The sucrose gradients were prepared manually from four sucrose solutions according to Brakke.[13]

CCP activity was measured with 2,6-dichlorobenzenoneindo-3′-chlorophenol as substrate according to Lenhoff and Kaplan [2] and with *P. fluorescens* cytochrome *c*(-551) as substrate as previously described.[4]

Cytochrome c oxidase activity was measured in 0.02 M sodium phosphate buffer of pH 6.0 as described previously.[4]

Cytochrome c(-551) of P. fluorescens was prepared from acetone-dried cells by the method of Ambler.[14]

Spectrophotometry. Absorption spectra were measured with a recording Perkin-Elmer model 137 UV spectrophotometer.

Protein determinations were carried out by the method of Lowry *et al.*[15] Serum albumin from the Finnish Red Cross was used as standard.

All chemicals were of analytical grade.

RESULTS

Mechanical methods of breaking the cells. The distribution of the CCP activity between the sediment and the supernatant obtained at 25 000 *g* after the mechanical treatments of the cells is shown in Table 1. A considerable amount of CCP (30 – 59 %) remained in the sediment in every method. The best recovery of the activity per gram of bacteria was obtained with the extrusion press. The grinding of bacteria with alumina powder was found to be an inefficient way of breaking the cells. After 10 min grinding, a large percentage

Table 1. Distribution of cytochrome *c* peroxidase between the sediment containing cell debris and large membrane particles and the supernatant containing cytoplasm and small membrane particles. The cells were suspended in 0.1 M sodium phosphate buffer of pH 7.5, broken as described in the text and centrifuged at 25 000 *g* for 30 min at 4°C. The sediment was suspended in the same buffer for activity and protein measurements. The CCP activity was measured with 2,6-dichlorobenzenoneindo-3′-chlorophenol as substrate.

Cell disruption technique	Amount of cells used g (wet weight)	Activity				Protein				Specific activity (units/mg protein)	
		Sediment		Supernatant		Sediment		Supernatant			
		Total activity (units)	%	Total activity (units)	%	Total protein (mg)	%	Total protein (mg)	%	Sediment	Supernatant
Extrusion press [a]	2.0	8750	51	8450	49	87	67	43	33	101	197
Grinding with alumina powder	0.9	2530	59	1740	41	8	27	22	73	316	79
Grinding with glass beads in a peristaltic pump	2.0	3340	30	7780	70	36	23	123	77	93	63

[a] The same distribution of CCP was obtained also with *P. fluorescens* ferrocytochrome *c* as substrate.

Table 2. The distribution of cytochrome *c* peroxidase between the extract and the cell debris when the cells were treated with organic solvents before extraction. The cells were treated with butanol and acetone, extracted with 0.05 M sodium phosphate buffer of pH 7.5 and centrifuged at 30 000 *g* for 1 h at 4°C after the treatment with butanol, and at 25 000 *g* for 30 min at 4°C after the treatment with acetone as described in the text. The cell debris was suspended in the same buffer for activity and protein measurements. The CCP activity was measured with 2,6-dichlorobenzenoneindo-3′-chlorophenol as substrate.

Treatment	Amount of cells used g (wet weight)	Activity				Protein				Specific activity (units/mg protein)	
		Cell debris		Extract		Cell debris		Extract			
		Total activity (units)	%	Total activity (units)	%	Total protein (mg)	%	Total protein (mg)	%	Cell debris	Extract
Butanol	1.8	547	27	1480	73	84	74	30	26	7	49
Acetone [a]	1.8	1200	7	15000	93	—	—	50	—	—	300

[a] The same distribution of CCP was obtained also with *P. fluorescens* ferrocytochrome *c* as substrate.

of intact cells was left in the sediment. Cell rupture was complete with the other two mechanical techniques as revealed by microscopic examination of the sediments after the treatments. The highest recoveries of CCP and protein in the supernatant were obtained by grinding the cells with glass beads in a peristaltic pump, but even using this technique 30 % of CCP remained in the cells.

Treatment of the cells with organic solvents. The bulk of CCP was extracted when cells were treated with butanol or acetone before the extraction with a buffer (Table 2). The loss of the CCP activity is considerable in the treatment with butanol, at least when carried out as done here. Acetone treatment of the cells was the most efficient way of getting CCP into solution as revealed by the recovery of enzyme per gram of bacteria and the specific activity of enzyme in the extract as compared with the other methods used. The extraction of CCP from acetone-treated cells was equally efficient with distilled water as with the buffer used in the distribution experiments.

Enzymatic lysis of the cells. When the pure membrane fraction was isolated from the fresh cells and the membranes were disrupted with the ultrasonic oscillator for activity measurements, 86 – 91 % of CCP and 72 – 85 % of cytochrome *c* oxidase were found in the membrane fraction (Table 3). Frequently, more of the cytochrome *c* oxidase activity than of the CCP activity passed into the discarded wash supernatant during the isolation of spheroplasts and into the cytoplasmic fraction. A higher percentage of both enzymes in aged cells went into the discarded wash supernatant and cytoplasm. The percentages of CCP in the discarded supernatant, cytoplasm, and membrane fraction were 37, 18, and 45 %, respectively, when the cells were 14 days old.

Table 3. The distribution of cytochrome *c* peroxidase and cytochrome *c* oxidase between cytoplasm and membranes when the cell walls were lysed enzymatically with lysozyme in the presence of EDTA. The fresh cells were treated with lysozyme and the membrane fraction was isolated and freed from DNA as described in the text. The membranes were further disrupted by ultrasonic oscillation for activity measurements. Both activities were measured with *P. fluorescens* ferrocytochrome *c* as substrate.

	Activity					
	Discarded supernatant from the isolation of the intact spheroplasts		Cytoplasm		Membranes	
	Total activity (units)	%	Total activity (units)	%	Total activity (units)	%
Cytochrome *c* peroxidase	5.2	3	9.1	6	138.6	91
Cytochrome *c* oxidase	1.7	1	23.1	14	138.6	85

Ultracentrifugations in sucrose gradients. The particles obtained from the isolated membranes by ultrasonic oscillation were studied first by centrifuging at 100 000 – 140 000 g for 1 – 4 h in different sucrose gradients between 0 and 80 % (w/v) but all the particles sedimented in those conditions. When the centrifugal force was reduced to 25 000 g and centrifugation was carried out for 1 h in sucrose gradients between 5 and 40 % (w/v), several fractions were obtained. A typical example of the distribution of the particles is shown in

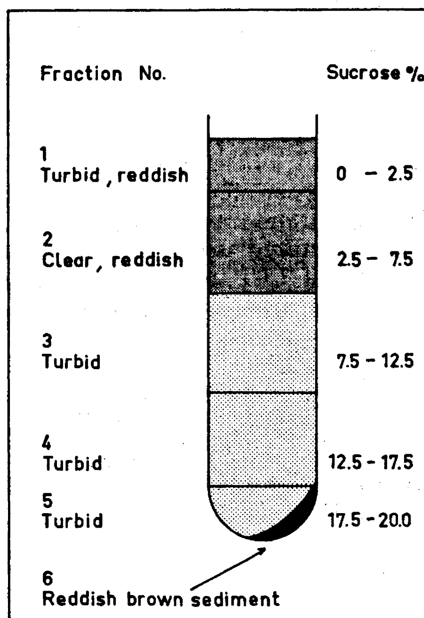

Fig. 1. Distribution in 5–20 % (w/v) sucrose gradient at 25 000 g of the subfractions obtained from isolated membranes of *P. fluorescens* by ultrasonic disruption. The sucrose concentrations were estimated from the linear concentration gradient.

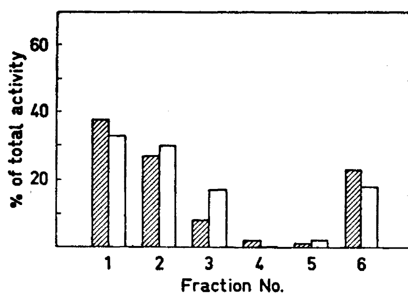

Fig. 2. Distribution in 5–20 % (w/v) sucrose gradient at 25 000 g of cytochrome c peroxidase (dark columns) and cytochrome c oxidase (light columns) in subfractions obtained from isolated membranes of *P. fluorescens* by ultrasonic disruption.

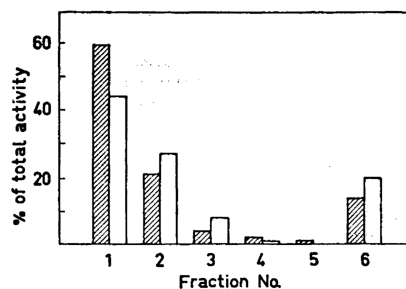

Fig. 3. Distribution in 10–40 % sucrose gradient at 25 000 g of cytochrome c peroxidase (dark columns) and cytochrome c oxidase (light columns) in subfractions obtained from isolated membranes of *P. fluorescens* by ultrasonic disruption.

Fig. 1. Similar fractionations were obtained in different gradients at this centrifugation speed. CCP and cytochrome *c* oxidase activities were found in most of the subfractions (Figs. 2 and 3). The fractions containing considerable amounts of both enzymes were coloured and gave distinct absorption spectra of hemoproteins with the Soret band in the region of 410 – 420 nm and, in the reduced form, α-bands at about 550 nm.

DISCUSSION

The results obtained indicate that the CCP of *P. fluorescens* is located in the membrane fraction of the cell. After centrifugation following mechanical disintegration of the cells, about half of the CCP activity was retained in the sediment which contains the cell walls and the large membrane particles and about half in the supernatant which contains besides cell fluid also smaller membrane particles.[16] The treatment with organic solvents releases the membrane-bound enzymes as observed after the treatment of the cells with butanol and acetone; most of CCP went into solution. When the cell walls were lysed enzymatically and the pure membrane fraction was isolated, CCP and cytochrome *c* oxidase were almost quantitatively present in the membranes.

No particles of special size containing CCP were isolated in the ultracentrifugations in the sucrose gradients. CCP was distributed among most of them. Obviously, the 9 kc ultrasonic oscillator breaks down the membranes to relatively large particles which are all sedimented at 100 000 – 140 000 *g*. At lower speeds, distribution of the particles in the sucrose gradients occurred. The particle fractions containing CCP also contained other respiratory chain components such as cytochrome *c* oxidase and cytochromes as revealed by activity measurements or absorption spectra. The results do not contradict the observation that membranous organelles of special size could not generally be isolated from broken bacteria.[16]

Yeast CCP was first considered to be a soluble enzyme.[17,18] Later it was definitely established that CCP is of mitochondrial origin in aerobically grown yeast, and that it can be easily released from mitochondria by mechanical disruption.[5–7] CCP of *P. fluorescens* could not be easily extracted from the membranes, whereas cytochrome *c* oxidase was less firmly bound as shown in this work. CCP has been found in anaerobically grown yeast in the cytoplasmic membrane and "promitochondria"[5] and in the vesicles of the cytoplasmic membrane.[6] The particulate fraction obtained from anaerobically grown yeast was not homogeneous when subjected to differential centrifugation.[6]

REFERENCES

1. Lenhoff, H. M. and Kaplan, N. O. *Nature* **172** (1953) 730.
2. Lenhoff, H. M. and Kaplan, N. O. *J. Biol. Chem.* **220** (1956) 967.
3. Lenhoff, H. M., Nicholas, D. J. D. and Kaplan, N. O. *J. Biol. Chem.* **220** (1956) 983.
4. Ellfolk, N. and Soininen, R. *Acta Chem. Scand.* **24** (1970) 2126.

5. Avers, C. J. *J. Bacteriol.* **94** (1967) 1225.
6. Kawaguchi, K., Ishidate, K. and Tagawa, K. *J. Biochem. (Tokyo)* **66** (1969) 21.
7. Yonetani, T. and Ohnishi, T. *J. Biol. Chem.* **241** (1966) 2983.
8. Edebo, L. *J. Biochem. Microbiol. Technol. Eng.* **2** (1960) 453.
9. Phillips, J. W., LaManna, C. and Mallette, M. F. *Appl. Microbiol.* **13** (1965) 460.
10. Morton, R. K. *Nature* **166** (1950) 1092.
11. Repaske, R. *Biochim. Biophys. Acta* **22** (1956) 189.
12. Burrous, S. E. and Wood, W. A. *J. Bacteriol.* **84** (1962) 364.
13. Brakke, M. K. *Arch. Biochem. Biophys.* **45** (1953) 275.
14. Ambler, R. P. *Biochem. J.* **89** (1963) 341.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
16. Tissiérs, A. *Hematin Enzymes, A Symposium of the International Union of Biochemistry*, Canberra 1959, Vol. I, p. 218, Eds., J. E. Falk, R. Lemberg and R. K. Morton, Pergamon, Oxford 1961.
17. Chantrenne, H. *Biochim. Biophys. Acta* **18** (1955) 58.
18. Lindemayer, A. and Smith, L. *Biochim. Biophys. Acta* **93** (1964) 445.

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