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A NEW PURIFICATION PROCEDURE AND MOLECULAR PROPERTIES OF *PSEUDOMONAS* CYTOCHROME OXIDASE

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SUMMARY

A procedure has been developed for purification of the cytochrome oxidase from *Pseudomonas aeruginosa* (EC 1.9.3.2) using DEAE- and CM-cellulose chromatography, gel filtration and crystallization. The final preparation was found to be homogeneous according to ultracentrifugal and disc electrophoretic criteria. The crystalline preparation also exhibited nitrite reductase activity. The spectrum of the enzyme characterizes it as cytochrome *cd*. At 280 nm $E^{1\%}_{1\,cm}$ was 18.5 after dry weight analysis.

The molecular weight of the cytochrome oxidase was calculated to be 119000 based on a sedimentation coefficient $s^\circ_{20,w} = 7.36$ S, diffusion coefficient $D_{20,w} = 5.36 \cdot 10^{-7}$ cm$^2 \cdot$s^{-1} and partial specific volume of 0.72 ml/g. The iron content of the enzyme (0.166 %) indicates that this entity contains four iron atoms per molecule. Succinylation of the enzyme produced two probably identical subunits containing both hemes *c* and *d*, having a sedimentation coefficient $s^\circ_{20,w} = 4.30$ S and an approximate molecular weight of 65000. In dodecylsulphate–acrylamide gel electrophoresis the cytochrome oxidase also dissociates into two subunits with molecular weight of 63000.

INTRODUCTION

Cytochrome oxidase (*Pseudomonas* cytochrome *c*:oxygen oxidoreductase, EC 1.9.3.2) purified from *Pseudomonas aeruginosa* characterizes as cytochrome *cd*[1-3]. It also catalyzes the reaction, *Pseudomonas* cytochrome *c* (Fe^{2+}) + NO_2^- + H^+ = *Pseudomonas* cytochrome *c* (Fe^{3+}) + NO + OH^- (ref. 4). The nitrite reductase activity is evidently the physiological function of the enzyme[2].

The physicochemical properties of cytochrome oxidase have been studied with an approx. 70 % pure preparation[5]. The molecular weight, calculated from the values of sedimentation coefficient, diffusion coefficient and apparent partial specific volume, was approx. 90000. The crystalline preparation of cytochrome oxidase[6,7] was shown to have almost the same sedimentation coefficient as noncrystalline enzyme[8]. The molecular weight of the enzyme has been calculated as 67000 from its amino acid composition[9] and estimated as 85000 by gel filtration[10].

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In this communication we present a new purification procedure for *Pseudomonas* cytochrome oxidase, in which rivanol and ion-exchange resin treatment[6,7] have been replaced with DEAE-cellulose and CM-cellulose, respectively. In addition the results of studies upon the molecular properties of the crystalline preparation are presented.

MATERIALS AND METHODS

Pseudomonas cytochrome c_{551} was prepared by the method of Ambler[11]. The purity ratio of the preparation $(A^{red}_{551\ nm} - A^{red}_{570\ nm})/A_{280\ nm}$ was greater than unity. To make ferrocytochrome *c* the preparation was reduced with dithionite and the salts removed by elution through a column of Sephadex G-25 (Pharmacia), which was equilibrated with N_2-saturated activity assay buffer[12]. Catalase (C-100), horse heart cytochrome *c* (Type III) and crystalline bovine and egg albumin (Grade V) were purchased from Sigma, crystalline yeast alcohol dehydrogenase and glyceraldehyde-phosphate dehydrogenase (rabbit muscle) from Boehringer, Mannheim, and crystalline deoxyribonuclease from Nutritional Biochemicals. DEAE-cellulose was a commercial preparation (Whatman DE-11) and CM-cellulose was prepared according to Peterson and Sober[13]. Sodium dodecylsulphate (pract. Fluka) was crystallized from ethanol[14]. Dialysis tubing was purchased from Arthur Thomas.

Organism

Pseudomonas, strain No. 8 was a gift from Professor N. Kaplan. It was identified as *P. aeruginosa* after Stanier *et al.*[15].

Growth of the organism

The nitrate medium of Lenhoff and Kaplan[16] was supplemented by 1 g KH_2PO_4, 1.1 g Na_2HPO_4, 10 mg $FeCl_3 \cdot 6H_2O$ and 5 mg $CuSO_4 \cdot 5H_2O$ per l. Growth of the organism was carried out in a 400-l pilot plant fermentor (Getingeverken, Sweden) using 300 l of medium which was inoculated with 9 l of a culture grown for 24 h with stirring. The mass cultivation was carried out at 30 °C under three different conditions. These were with stirring, without stirring and aerating with a mixture of 5 % O_2 and 95 % N_2. The cytochrome oxidase activity of the bacteria was found to be independent of the growth conditions used. The highest yield of 2.2 g (dry wt) cells per l was obtained when the culture was either stirred or aerated with the gas mixture. The cultivation was stopped after 18–24 h when the pH of the medium had risen above 8. The cells were harvested in a De Laval separator and stored as a frozen paste at −16 °C.

Activity assays

The aerobic oxidation of reduced *Pseudomonas* cytochrome *c* by cytochrome oxidase was followed at 551 nm with a Beckman DK-1A recording spectrophotometer. Cuvettes contained approx. 6.5 μM ferrocytochrome *c* and 0.05 M sodium acetate (pH 5.3). The reaction was started by adding 1–20 μl enzyme solution. The final volume was 2.0 ml. Initial linear reaction rates were taken.

The nitrite reductase activity was measured under argon in Thunberg cuvettes[4,7].

Determination of protein

Protein concentrations were measured with the method of Lowry *et al.*[17], using bovine serum albumin (Finnish Red Cross) as a standard. The protein concentrations of the pure oxidase solutions were always measured spectrophotometrically at 280 nm, using $E^{1\,\%}_{1\,cm} = 18.5$, which was calculated on the basis of the dry weight determinations of the cytochrome oxidase samples.

Polyacrylamide gel electrophoresis

The electrophoretic apparatus described by Davis[18] was used. Analytical disc electrophoresis was performed using the standard 7.5 % (w/v) acrylamide gel with Tris–glycine electrode buffer (pH 8.3)[19]. The molecular weights in dodecylsulphate–polyacrylamide gel electrophoresis were determined according to Weber and Osborn[20]. The acrylamide concentration was 7.5 % (w/v). The excess Coomassie Brilliant Blue stain was washed from the gels with the destaining solution.

Iron analysis

Before iron analysis the crystallized cytochrome oxidase was treated with an iron chelator solution[21]. The chelators were removed and the sample was changed to 0.01 M NH_4HCO_3 with the gel filtration technique. The iron content of the samples was determined by the method of Cameron[22] using Mohr's salt (pro analysis, Merck) as an iron standard. The dry weights of the samples were determined by drying to constant weight at 105 °C. The samples were lyophilized before drying.

Succinylation

The cytochrome oxidase dissolved in 0.1 M sodium phosphate (pH 7.0) was treated with succinic anhydride (purum, Fluka) by the method of Hass[23].

Ultracentrifugal experiments

Sedimentation and diffusion coefficients were determined on a Beckman Analytical Ultracentrifuge (Spinco Model E) fitted with schlieren optics. Sedimentation–velocity runs were performed at 59780 or 52640 rev./min and diffusion runs at 8225 rev./min. In diffusion studies a synthetic boundary cell of the capillary type was used. Photographs were taken at 4- or 8-min intervals on Kodak Metallographic plates and with a Wratten 77-A filter.

Diffusion coefficients were calculated according to the "height–area" method, using a plot according to the relationship $(A/H)^2 = 4\,k^2\pi\,D\,(t-t_0)$, where A is the area under the gradient curve and the base line at the time t from the moment (t_0) when the boundary was formed, H the height of the curve, k the magnification factor along the base line and D the diffusion coefficient. The sedimentation and diffusion coefficients were corrected for the differences between density and viscosity of the medium and those of pure water at 20 °C[24]. The results are given in Fick units ($F = 10^{-7}\,cm^2 \cdot s^{-1}$).

Partial specific volume

The partial specific volume of the cytochrome oxidase was determined by the density gradient technique of Linderstrøm-Lang and Lanz[25]. The gradient was prepared[26] from Varsol (145/200, Esso) and bromobenzene, both were saturated with

4.5 % (w/v) KBr water solution. Standard sucrose solutions[27] and the enzyme solutions of different concentrations were dropped into the gradient with 1 μl siliconized capillaries. The partial specific volume was calculated according to Ulrich *et al.*[28].

Preparation of cell extracts

Pseudomonas cells were ground with glass beads according to Phillips *et al.*[29]. 200 g bacteria, 500 ml glass beads (0.2 mm in diameter) and 900 ml potassium phosphate buffer (pH 7.3, I 0.5)[30] were vigorously stirred in an ice bath. Simultaneously the mixture was pumped with a peristaltic pump through a silicon tube (inside diameter 5 mm) at the rate of 40 l/h. The disruption time was 15 min. The glass beads were separated from the mixture with an extra coarse sintered filter.

An alternative method used to prepare a crude extract with the same specific activity was to extract an acetone-dried powder of the cells[31]. 100 g of acetone-dried cells equivalent to 400 g of fresh bacteria was stirred to 950 ml of the extraction buffer for 3 h at 5 °C. Because the mixture was very viscous approximately 2 mg of DNAase was added. Cell-free extracts were prepared by centrifugation at 25000 $\times$ g for 60 min.

RESULTS AND DISCUSSION

Purification of Pseudomonas cytochrome oxidase

The procedures were carried out at 5 °C. A typical purification procedure has been summarized in Table I.

The crude extract was dialyzed against 0.02 M potassium phosphate (pH 6.9). The nucleic acids were then removed from the preparation with DEAE-cellulose column chromatography. When the DEAE-cellulose had been equilibrated with 0.02 M potassium phosphate buffer (pH 6.9) the cytochrome oxidase did not bind to the exchanger. The column size 3 cm $\times$ 30 cm is suitable when the extract is from 200 g of bacteria. The brownish red eluate was pooled. If the eluate was opaque and the ratio $A_{280\ nm}/A_{260\ nm}$ lower than unity the chromatography was repeated.

The pH of the DEAE-cellulose column eluate was adjusted to 6.4 and the solution was diluted with an equal volume of water. This solution was pumped (approx. 75 ml/h) to a CM-cellulose column (2 cm $\times$ 15 cm) which was equilibrated with 0.01 M potassium phosphate (pH 6.4). The cytochrome oxidase adsorbed to the top of the column whilst cytochrome *c* was not adsorbed. The column was washed with 0.01 M potassium phosphate (pH 6.7) until the eluate was colourless. The cytochrome oxidase was eluted with 0.04 M potassium phosphate (pH 6.9). The first deeply green fractions were pooled and concentrated by ultrafiltration (Diaflo UM-10 membrane, Amicon).

The concentrated enzyme solution (volume not exceeding 10 ml) was put on a Sephadex G-100 column (2.5 cm $\times$ 95 cm) which was equilibrated with 0.01 M potassium phosphate (pH 6.4). The elution curve of the gel filtration is shown in Fig. 1. The elution profile of the same size Sephadex G-200 column is similar. The green fractions comprising the main peak were pooled and concentrated with a short CM-cellulose column equilibrated with the above-mentioned buffer. The elution was carried out with 0.04 M potassium phosphate (pH 6.9).

TABLE I

SUMMARY OF RESULTS OBTAINED IN THE COURSE OF ONE PURIFICATION OF CYTOCHROME OXIDASE

The values shown are for 208 g of *P. aeruginosa* cells. A unit is 1 μmole cytochrome c_{551} reduced per min.

Enzyme fraction	*Total vol. (ml)*	*Total activity (units)*	*Total protein (mg)*	*Specific activity (units/mg protein)*	*Purification (-fold)*	*Yield (%)*	$A_{411\ nm}/A_{280\ nm}$	$A_{280\ nm}/A_{260\ nm}$
Extract	950	247	5900	0.04	1	100	0.12	0.60
DEAE-cellulose chromatography eluate	1076	162	1830	0.09	2.1	66	0.53	0.99
CM-cellulose chromatography eluate	7.6	93	66	1.40	33	38	0.78	1.47
Sephadex G-100 eluate	42	53	21	2.52	60	21	1.00	1.23
Crystallized	0.8	35	10.9	3.22	77	14	1.18	1.39
Recrystallized	0.5	30	7.8	3.78	90	12	1.18	1.42

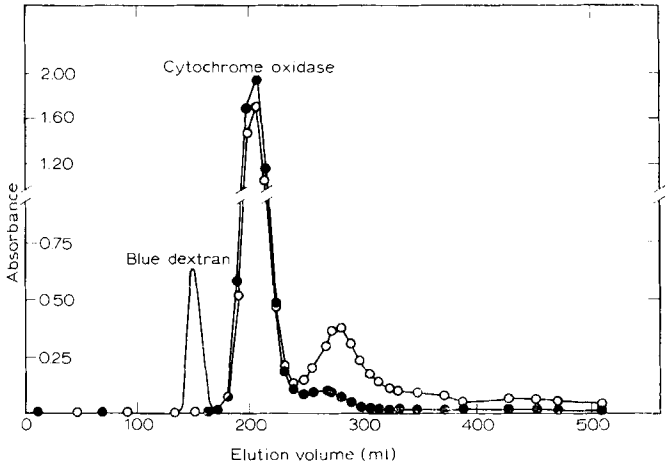

Fig. 1. Sephadex G-100 gel filtration of *Pseudomonas* cytochrome oxidase. The column (2.5 cm × 95 cm) was equilibrated with 0.01 M potassium phosphate buffer (pH 6.5). The volume of the sample was 7.5 ml containing 65 mg protein. ○—○, protein concentration expressed as absorbance at 280 nm; ●—●, absorbance at 411 nm. The Blue Dextran (Pharmacia AB) was run separately.

The concentrated cytochrome oxidase was crystallized from an $(NH_4)_2SO_4$ solution[6] using 0.01 M potassium phosphate (pH 6.7) as dissolving buffer.

Properties of crystalline cytochrome oxidase

The shape of the crystals are shown in Fig. 2. The form is similar to those observed by Yamanaka *et al.*[32]. Disc electrophoresis showed the recrystallized preparation to be homogeneous (Fig. 3). The absorption spectrum of the preparation was similar to that of Yamanaka and Okunuki[33]. The ratio $A_{411\,nm}/A_{280\,nm}$ equal to 1.2 is in agreement with those of Yamanaka and Okunuki[6] and Newton[10]. The nitrite reductase activity of the preparation ($A_{411\,nm}/A_{280\,nm} = 1.16$) was 139 moles cytochrome $c \cdot min^{-1} \cdot (119000\ g\ cytochrome\ oxidase)^{-1}$ whereas cytochrome activity was 107 moles cytochrome $c \cdot min^{-1} \cdot (119000\ g\ cytochrome\ oxidase)^{-1}$ in 0.05 M sodium phosphate buffer (pH 6.5) at 23 °C. It should be observed that the pH optimum for the cytochrome oxidase reaction is 5.3 (Table I).

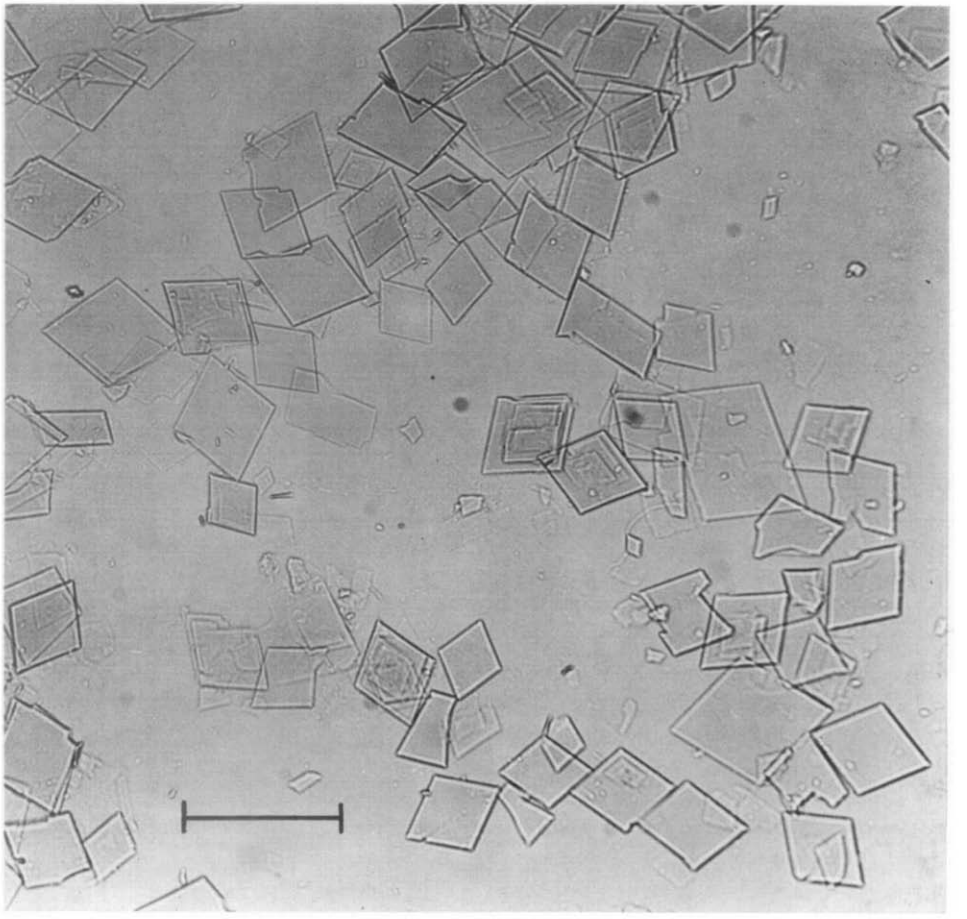

Fig. 2. Crystals of *Pseudomonas* cytochrome oxidase after recrystallization. A scale superimposed on the photograph indicates a length of 0.1 mm.

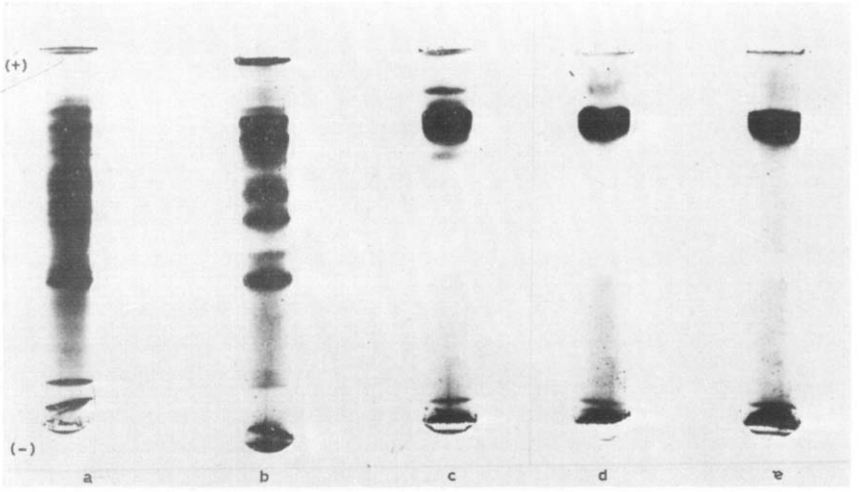

Fig. 3. Disc electrophoretic patterns of various fractions during the purification procedure of *Pseudomonas* cytochrome oxidase on 7.5% acrylamide gel at pH 8.3. (a) Dialyzed extract (70 μg protein). (b) Eluate from DEAE-cellulose column (70 μg protein). (c) Eluate from CM-cellulose column (44 μg protein). (d) Crystallized (27 μg protein). (e) Recrystallized (31 μg protein). The lowest band is bromphenol blue.

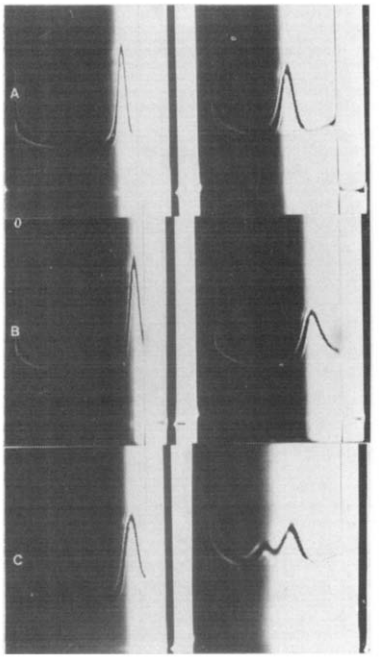

Fig. 4. Sedimentation velocity patterns of native succinylated *Pseudomonas* cytochrome oxidase. Sedimentation proceeded from right to left. The solvent was 0.1 M sodium phosphate buffer (pH 7.0) and temperature 20 °C. (A) Crystallized enzyme, concentration 5 mg/ml ($A_{411\ nm}/A_{280\ nm}$ = 1.1). (B) Same enzyme preparation after succinylation. Photographs were taken 16 and 48 min after the speed reached 59780 rev./min. The $s_{20,w}$ in Experiment A was 6.55 S and for B $s_{20,w}$ = 4.10 S. (C) Crystallized enzyme, concn 6 mg/ml ($A_{411\ nm}/A_{280\ nm}$ = 1.1), after partial succinylation. Photographs were taken 21 and 85 min after the speed reached 59780 rev./min. The lighter component had $s_{20,w}$ = 4.2 S and the other one $s_{20,w}$ = 6.9 S.

Sedimentation analyses

The crystalline cytochrome oxidase preparations with purity ratio $A_{411\,nm}/A_{280\,nm}$ = 1.1–1.2 dialyzed against 0.1 M sodium phosphate buffer (pH 7.0) gave only one single sedimentation boundary in the analytical centrifuge (Fig. 4A). The sedimentation coefficients determined at different protein concentrations are plotted in Fig. 5. The data are represented by the equation $s_{20,w} = (7.36 - 0.091\ c)$ S where c is the concentration of the enzyme in mg/ml.

Horio *et al.*[5] reported a value of $s_{20,w}$ equal to 5.8 S with noncrystalline cytochrome oxidase and noticed that the value was essentially independent of protein concentration between 0.5 and 1.0 % (w/v). Kijimoto[8] obtained $s_{20,w}$ = 5.88 S at the protein concentration of 0.96 % (w/v) with recrystallized cytochrome oxidase. At the present time we are not able to explain the high sedimentation coefficient obtained by ourselves ($s°_{20,w}$ = 7.36 S) compared to that of the other workers. It has, however, to be pointed out that the purification procedure presented in this communication differs from those of Horio *et al.*[5] and Kijimoto[7].

Diffusion coefficient

Diffusion coefficients were determined in 0.1 M sodium phosphate (pH 7.0) with crystalline preparations ($A_{411\,nm}/A_{280\,nm}$ = 1.1) at protein concentrations of 4.6–5.6 mg/ml. Three experiments gave a mean value of $D_{20,w}$ = 5.36 F. A representative plot using the data from one experiment is shown in Fig. 6. Horio *et al.*[5] obtained the value of 5.8 F with a preparation of purity ratio $A_{411\,nm}/A_{280\,nm}$ = 0.8.

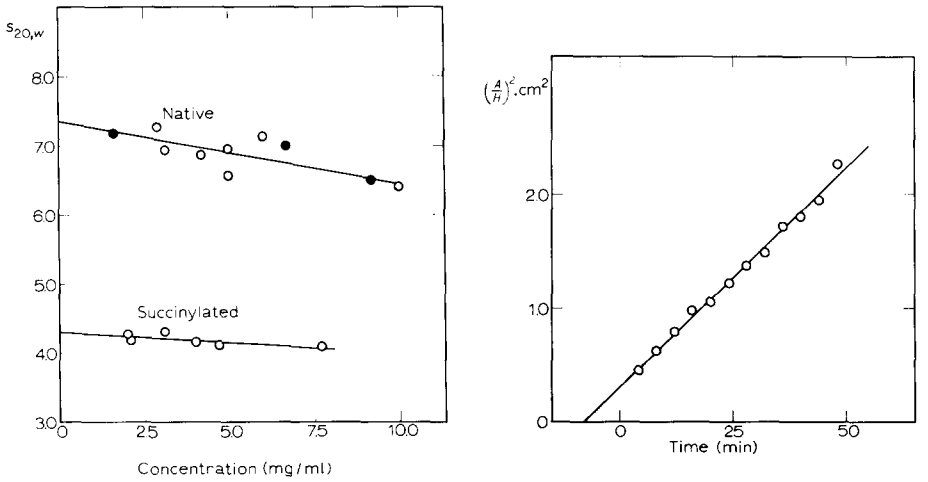

Fig. 5. Concentration dependence of the sedimentation coefficients of native and succinylated *Pseudomonas* cytochrome oxidase in 0.1 M sodium phosphate buffer (pH 7.0). ○, rotor speed 59720 rev./min; ●, rotor speed 52640 rev./min. The lines drawn are least squares fit to the experimental points.

Fig. 6. A plot according to the equation of the "height-area" method for the determination of the diffusion coefficient of *Pseudomonas* cytochrome oxidase. Time measured in minutes from the end of boundary formation. Protein concentration was 4.6 mg/ml in 0.1 M sodium phosphate buffer (pH 7.0). The rotor speed was 8225 rev./min. The line drawn corresponds to $D_{20,w}$ = 5.43 F.

Partial specific volume

The cytochrome oxidase ($A_{411\,nm}/A_{280\,nm}$ = 1.2) was dialyzed against 0.1 M sodium phosphate (pH 7.0) and the density at four protein concentrations was determined by the density-gradient technique. The calculated partial specific volume from the plot of the density against the enzyme concentration (Fig. 7) was 0.72 ml/g. The $\bar{v}$ value of Horio *et al.*[5] was 0.73 ml/g with a noncrystalline preparation ($A_{411\,nm}/A_{280\,nm}$ = 0.8).

Molecular weight

The molecular weight of the crystalline cytochrome oxidase calculated from the sedimentation coefficient, diffusion coefficient and partial specific volume using Svedberg's formula[24]

$$M = \frac{RT\, s^{\circ}_{20,w}}{D_{20,w}(1 - \bar{v}\rho)}$$

was found to be 119000. Horio *et al.*[5] calculated the molecular weight from their data to be approx. 90000. An estimated molecular weight equal to 85000 has been published by Newton[10].

The iron determinations for three crystalline cytochrome oxidase preparations ($A_{411\,nm}/A_{280\,nm}$ = 1.1) gave a mean value equal to 0.166 % (w/w). This corresponds to a molecular weight of 67000 for two iron atoms per molecule. In confirmation of this, the molecular weight of the cytochrome oxidase was found to be 63000 (7 determinations) in dodecylsulphate–polyacrylamide gel electrophoresis (Fig. 8). The

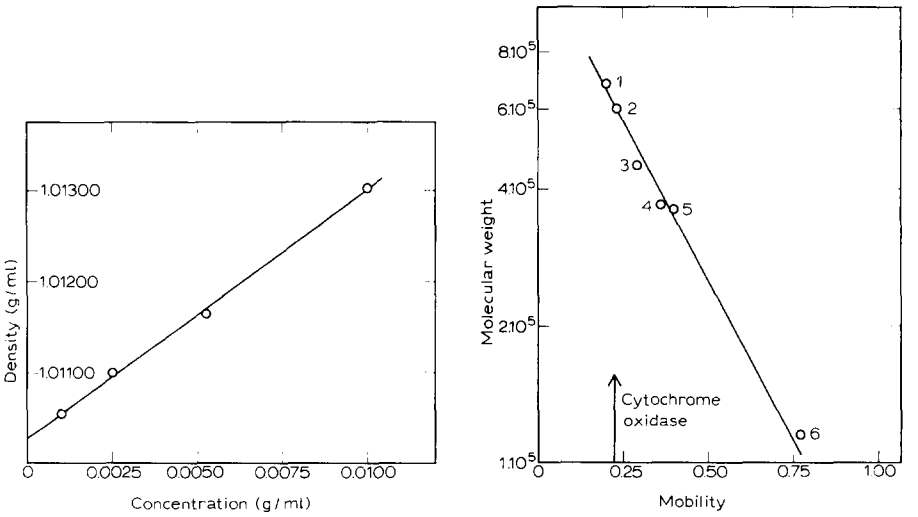

Fig. 7. A plot of density against *Pseudomonas* cytochrome oxidase concentration in 0.1 M sodium phosphate buffer (pH 7.0), for determination of the partial specific volume. From this plot the value 0.72 mg/ml is obtained. Each density value represents the mean density of five drops.

Fig. 8. Polyacrylamide gel electrophoresis in 0.1 % sodium dodecylsulphate. Standard proteins and their molecular weights[21] were: (1) bovine serum albumin (68000), (2) catalase (60000), (3) ovalbumin (45000), (4) yeast alcohol dehydrogenase (37000), (5) glyceraldehyde-3-phosphate dehydrogenase (36000), (6) cytochrome *c* (11700). *Pseudomonas* cytochrome oxidase has molecular weight of 63000 as judged by its mobility.

absence of β-mercaptoethanol from the dodecylsulphate dissociation procedure[20] did not change the molecular weight. This supports the view of Nagata *et al.*[9] that the cytochrome oxidase has no S–S bonds. Okunuki's[9] group has calculated the molecular weight of cytochrome oxidase from the amino acid composition. Their value 67000 is in agreement with our data. Also their iron determination supports the view that the molecular weight is under 70000. Kijimoto[8] has found that cytochrome oxidase dissociates in 1 % dodecylsulphate into subunits of 30000–40000 and 100000–150000.

Molecular weight of succinylated cytochrome oxidase

The spectrum of succinylated cytochrome oxidase was found to be similar to that of the crystalline enzyme, indicating that the modified enzyme contains hemes *c* and *d*. The sedimentation velocity of succinylated cytochrome oxidase differs from that of the native enzyme as shown in Fig. 4B. Fig. 4C illustrates a partially succinylated preparation containing the native enzyme and the succinylated subunit. The value of $s^\circ_{20,w}$ was found to be 4.30 S (Fig. 5). The molecular weight of succinylated cytochrome oxidase calculated from Svedberg's formula is 69000, using the diffusion and partial specific volume of the native enzyme. It is evident that succinylation unfolds the protein and so lowers the value of the apparent specific volume[23]. This might still lower the molecular weight of the succinylated cytochrome oxidase. A molecular weight of 68000 was obtained for the succinylated enzyme by dodecylsulphate–acrylamide gel electrophoresis. The succinyl residues are calculated to increase the molecular weight by about 4000, based on the amino acid composition[9].

Summary of molecular weight data

The molecular weight of the native crystalline cytochrome oxidase was 119000. In this entity the cytochrome oxidase contains four iron atoms per molecule. The native cytochrome oxidase dissociates after succinylation or in dodecylsulphate into subunits which contain two iron atoms per molecule. The molecular weight of the subunits are around 65000 (Table II) and they contain both hemes *c* and *d*.

TABLE II

SUMMARY OF MOLECULAR WEIGHT DATA FOR *Pseudomonas* CYTOCHROME OXIDASE SUBUNIT

Method	*Molecular weight found*
Dodecylsulphate gel electrophoresis	63 000 *
Iron analysis	67 000 * and <70 000 **
Amino acid analysis	67 000 **
Sedimentation velocity of succinylated enzyme	<69 000 *

* The present work.
** Nagata *et al.*[9].

REFERENCES

1 T. Horio, T. Higashi, H. Matsubara, K. Kusai, M. Nakai and K. Okunuki, *Biochim. Biophys. Acta*, 29 (1958) 297.
2 T. Yamanaka, *Annu. Rep. Sci. Works, Osaka Univ.*, 11 (1963) 77.
3 K. Okunuki, in M. Florkin and E. H. Stotz, *Comprehensive Biochemistry*, Vol. 14, Elsevier, Amsterdam, 1966, p. 232.

4 T. Yamanaka, A. Ota and K. Okunuki, *Biochim. Biophys. Acta*, 53 (1961) 294.
5 T. Horio, T. Higashi, T. Yamanaka, H. Matsubara and K. Okunuki, *J. Biol. Chem.*, 236 (1961) 944.
6 T. Yamanaka and K. Okunuki, *Biochim. Biophys. Acta*, 67 (1963) 379.
7 S. Kijimoto, *Annu. Rep. Biol. Works, Fac. Sci., Osaka Univ.*, 16 (1968) 1.
8 S. Kijimoto, *Annu. Rep. Biol. Works, Fac. Sci., Osaka Univ.*, 16 (1968) 19.
9 Y. Nagata, T. Yamanaka and K. Okunuki, *Biochim. Biophys. Acta*, 221 (1970) 668.
10 N. Newton, *Biochim. Biophys. Acta*, 185 (1969) 316.
11 R. P. Ambler, *Biochem. J.*, 89 (1963) 341.
12 T. Yonetani and G. S. Ray, *J. Biol. Chem.*, 240 (1965) 3392.
13 E. A. Peterson and H. A. Sober, *J. Am. Chem. Soc.*, 78 (1956) 751.
14 R. R. Burgess, *J. Biol. Chem.*, 244 (1969) 6168.
15 R. Y. Stanier, N. Palleroni and M. Doudoroff, *J. Gen. Microbiol.*, 43 (1966) 159.
16 H. Lenhoff and N. O. Kaplan, *J. Biol. Chem.*, 220 (1958) 967.
17 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
18 B. J. Davis, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
19 J. M. Brewer and R. B. Ashworth, *J. Chem. Educ.*, 46 (1969) 41.
20 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406.
21 H. Drucker, E. B. Trousil, L. L. Campbell, G. H. Barlow and E. Margoliash, *Biochemistry*, 9 (1970) 1515.
22 B. F. Cameron, *Anal. Biochem.*, 11 (1965) 164.
23 L. F. Hass, *Biochemistry*, 3 (1964) 535.
24 T. Svedberg and K. O. Pedersen, *The Ultracentrifuge*, Oxford University Press, London, 1940.
25 K. Linderstrøm-Lang and H. Lanz, *C. R. Lab. Carlsberg, Ser. Chim.*, 21 (1938) 315.
26 G. L. Miller and J. McGurren Gasek, *Anal. Biochem.*, 1 (1960) 78.
27 *International Critical Tables*, Vol. 2, McGraw-Hill, New York, 1927, p. 343.
28 D. L. Ulrich, D. W. Kupke and J. W. Beams, *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 349.
29 J. W. Phillips, C. Lamanna and M. F. Mallette, *Appl. Microbiol.*, 13 (1965) 460.
30 W. C. Boyd, *J. Biol. Chem.*, 240 (1965) 4097.
31 J. C. Gunsalus, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol 1, Academic Press, New York, 1955, p. 51.
32 T. Yamanaka, S. Kijimoto, K. Okunuki and K. Kusai, *Nature*, 194 (1962) 759.
33 T. Yamanaka and K. Okunuki, *Biochim. Biophys. Acta*, 67 (1963) 394.