

These two preparations are also reducible by sodium ascorbate under anaerobic conditions.

Spectral and Oxidation-Reduction Potentials. Table II summarizes the spectral bands, midpoint potential values, and contribution of the heme redox components to the $\Delta\epsilon$ (reduced minus oxidized) at the α -band absorption. Remarkably, the spectral band locations are virtually identical except for the Soret band in the oxidized procedure III preparation.^{14, 15} The midpoint potential values, $E^{0'}$, for each preparation indicate close agreement. For procedure III preparations, these $E^{0'}$ values are given for measurements made in the presence of 0.05 mol of cytochrome *c* per mole of cytochrome oxidase. In the absence of cytochrome *c*, this preparation exhibited equal $E^{0'}$ values at 280 mV. Finally, the contribution of the high- and low-potential hemes *a* to the extinction change at 604(605) nm upon reduction is summarized in Table II. Both the $\Delta\epsilon$ value and percentage change are listed, and it appears that either the method of $E^{0'}$ and $\Delta\epsilon$ determination or the preparations reflect these differences.

In summary, the cytochrome oxidase preparations reported herein greatly shorten the time of preparation and exposure to bile salts, yield higher redox component ratios per milligram of protein, and exhibit excellent enzymic activity for cytochrome *c* oxidation by molecular oxygen.

[11] Purification of Cytochrome Oxidase from *Neurospora crassa* and Other Sources

By HANNS WEISS and WALTER SEBALD

A chromatographic procedure¹ is described by means of which cytochrome oxidase has been purified from a variety of organisms including the fungus *Neurospora crassa*,^{2,3} the unicellular alga *Polytoma mirum*,⁴ the insect *Locusta migratoria*,⁵ the frog *Xenopus muelleri*,⁴ and the mammal *Rattus norvegicus*.⁴ This procedure can be used to equal effect for large-scale preparations, starting from grams of mitochondrial protein, or for small-scale preparations starting from milligrams. The cytochrome

¹ H. Weiss and T. Bücher, *Eur. J. Biochem.* **17**, 561 (1970).

² H. Weiss, W. Sebald, and T. Bücher, *Eur. J. Biochem.* **22**, 19 (1971).

³ W. Sebald, H. Weiss, and G. Jackl, *Eur. J. Biochem.* **30**, 413 (1972).

⁴ H. Weiss, unpublished results.

⁵ H. Weiss, B. Lorenz, and W. Kleinow, *FEBS Lett.* **25**, 49 (1972).

oxidase preparations from the different organisms are enzymically active. They show similar subunit compositions.

Purification

Principle

The purification procedure involves two steps.

Chromatography on Oleylpolymethacrylic Acid Resin. This chromatographic technique is described in more detail in this volume [23]. Mitochondrial membrane proteins are solubilized with deoxycholate and bound to the oleylpolymethacrylic acid resin in that the carboxylate and oleyl groups of the resin replace the detergent. Then the bulk of the proteins (including cytochromes *b* and *c*₁) is eluted by solutions containing deoxycholate, cholate, and salt. By this means, cytochrome oxidase remains bound to the resin. It is only released by a solution containing the nonionic detergent Tween 80 and high concentrations of salt.

Chromatography on DEAE-Cellulose. The crude cytochrome oxidase preparation is dialyzed in order to decrease the salt concentration and then bound to DEAE-cellulose. It is washed in order to remove the excess of Tween 80 and is eluted with a salt solution.

Procedure

A small-scale preparation of cytochrome oxidase from *Neurospora crassa* is treated as an example (Table II). The scale arbitrarily can be enlarged by increasing the amount of mitochondrial membrane protein, the column size, the volumes of the eluents, and the flow rate. For large-scale preparation the length of the column is relatively increased while the volume remains the same (e.g., 2 g of mitochondrial membrane proteins are applied on a 5.0 × 12 cm column containing 200 g of wet resin).

Chromatography on Oleylpolymethacrylic Acid Resin. The synthesis of the resin and the preparation and loading of the column is described in this volume [23].

Twenty milligrams of mitochondrial membrane proteins are solubilized in 1 ml of 0.3 M KCl, 20 mM Tris-acetate, pH 8, and 2% K deoxycholate and applied on a 0.8 × 5 cm column of 2 g of wet resin. Then the column is washed successively with the solutions 1-6 listed in Table I. Cytochrome oxidase, finally, is eluted with solution 7. It is collected in fractions of about 1 ml. The flow rate is 8 ml/hr: the temperature, 4°.

TABLE I
SOLUTIONS USED FOR THE PURIFICATION OF CYTOCHROME OXIDASE BY
CHROMATOGRAPHY ON OLEYLPOLYMETHACRYLIC ACID RESIN

Solution No.	Volume (ml)	Concentration					pH
		Tris-acetate (M)	K cholate ^a (%)	K deoxy-cholate ^a (%)	KCl (M)	Tween 80 ^b (%)	
1	7	0.5	—	—	—	—	6
2	20	0.5	—	—	—	—	8
3	40	0.5	0.75	0.5	—	—	8
4	40	0.5	0.75	0.5	1.5	—	8
5	20	0.2	0.75	0.5	3.0	—	8
6	10	0.2	—	—	3.0	—	8
7	20	0.2	—	—	3.0	0.5	8

^a Cholic acid, puriss., and deoxycholic acid, puriss. are from C. Roth, Karlsruhe, Germany.

^b From Serva, Heidelberg, Germany.

TABLE II
PURIFICATION OF CYTOCHROME OXIDASE FROM *Neurospora crassa*^a

Preparation	Volume	Cytochrome oxidase ^b		Radioactivity	
		Total (nmol)	Percent	Total (10 ⁻³ cpm)	Percent
Mitochondrial membrane ^c	1	6	100	1680	100
Cytochrome oxidase from oleylpolymermethacrylic acid resin	11	2	33	46	2.7
Cytochrome oxidase from DEAE-cellulose	0.3	1	17	16	1

^a The cells were radioactively labeled by *in vivo* incorporation of 50 μ Ci of [¹⁴C]leucine per gram of protein during the exponential growth phase. This led to a specific radioactivity of the mitochondrial membrane protein of 84,000 cpm/mg.

^b Estimated spectrophotometrically using the absorbance coefficient $\Delta E_{605\text{nm}}$ (reduced-oxidized) = 24 $\text{mM}^{-1}\text{cm}^{-1}$ [B. F. van Gelder, *Biochim. Biophys. Acta* **118**, 36 (1966)].

^c *Neurospora crassa* mitochondria are prepared as described by H. Weiss, G. von Jagow, M. Klingenberg, and T. Bücher, *Eur. J. Biochem.* **14**, 75 (1970) (see also this series, Vol. 55 [18]). Mitochondrial membranes are defined as 100,000 *g* sediment of *Neurospora crassa* mitochondria sonicated under cooling at a protein concentration of 5 mg/ml in 0.3 M KCl, 20 mM Tris-acetate, pH 8.

Chromatography on DEAE-Cellulose. The light-green fractions containing the crude cytochrome oxidase preparations are combined and dialyzed for 6–8 hr against iced water. They are then pumped at a flow rate of 20 ml/hr through a 0.4×0.8 cm DEAE-cellulose column (DEAE 23 SH, Serva, Heidelberg, Germany) previously equilibrated with 50 mM Tris-acetate pH 8. By this means, cytochrome oxidase is bound as a sharply limited green zone. (If this zone is diffuse or if cytochrome oxidase passes without being bound, the time of dialysis has to be prolonged. The DEAE-cellulose column should be of such a size that the green zone covers at least two-thirds of the column.) The column is washed with 10 ml of 50 mM Tris-acetate, pH 8, to remove the excess of Tween 80; cytochrome oxidase is then eluted as a dark green solution with 0.3 M KCl and 50 mM Tris-acetate, pH 8. The preparation can be stored at -20° for at least several weeks.

A purification is summarized in Table II.

Properties of Cytochrome Oxidase Purified from *Neurospora crassa*

Absorption Spectra. The absorption spectra of the air-oxidized and dithionite-reduced form of purified cytochrome oxidase are shown in Fig. 1. The ratio of the absorbances at 427 nm (oxidized) to 280 nm (oxidized) is 0.4. The specific heme content of the preparation amounts to 14–15 μmol per gram of protein.⁶ This result is obtained from protein determinations by quantitative amino acid analysis and from spectrophotometric

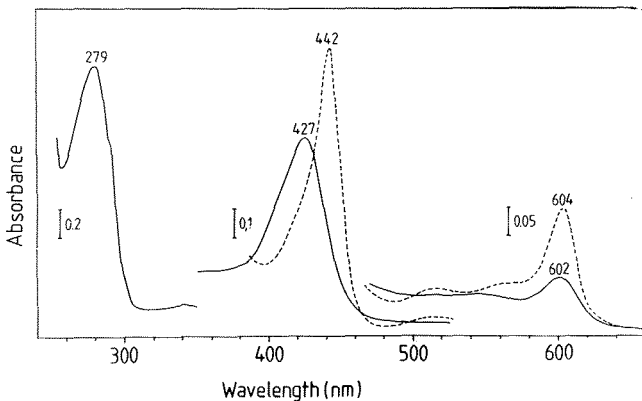


FIG. 1. Absorption spectra of cytochrome oxidase purified from *Neurospora crassa*. —, Air-oxidized form; - - -, dithionite-reduced form.

⁶ W. Sebald, W. Machleidt, and J. Otto, *Eur. J. Biochem.* **38**, 311 (1973).

TABLE III
MOLECULAR WEIGHT AND RELATIVE PROPORTION OF PROTEIN OF THE CYTOCHROME OXIDASE SUBUNITS FROM *Neurospora crassa*^a

Subunit No.	Apparent molecular weight in SDS	Leucine (% of total)	Leucine ($\mu\text{mol}/\text{mg}$ protein)	Protein (% of total)	Protein (g/mol cytochrome oxidase ^b)	Molar ratios	Coomassie Blue stain (% of total)
1	41,000	35.9	1.25	27.9	39,800	1.03	21.4
2	28,500	19.6	1.05	18.0	25,700	1.11	12.5
3	21,000	16.9	1.16	14.2	20,300	1.03	8.7
4	16,000	4.9	ND ^c	ND	ND	ND	11.7
5	14,000	8.0	0.70	11.1	16,000	0.90	15.3
6	11,500	7.7	0.84	8.2	11,700	0.98	12.3
7	10,000		0.42				
8	9,500	7.5	0.66	13.5	19,200	1.02	18.9

^a The relative proportions of leucine of any of the subunits to leucine of total cytochrome oxidase were evaluated from the ³H radioactivity band pattern obtained by sodium dodecyl sulfate (SDS) gel electrophoresis of [³H]leucine-labeled cytochrome oxidase [W. Sebald, W. Machleidt, and J. Otto, *Eur. J. Biochem.* **38**, 311 (1973)]. The leucine contents of the subunits were obtained from amino acid analysis (Table IV). The relative proportions of protein of the subunits to total cytochrome oxidase protein were calculated according to the formula:

$$\% \text{ protein/subunit} = \frac{\% [^3\text{H}] \text{leucine/subunit} \times \mu\text{mol of leucine/mg cytochrome oxidase}}{\mu\text{mol leucine/mg subunit}}$$

This protein quantitation cannot be performed on the basis of the relative proportion of Coomassie Blue, as the specific staining of the subunits 1-3 is only about one-half that of subunits 4-8.

^b Cytochrome oxidase is estimated spectrophotometrically using the absorbance coefficient ΔE_{605} (reduced-oxidized) = $24 \text{ mM}^{-1} \text{ cm}^{-1}$ [B. F. van Gelder, *Biochim. Biophys. Acta* **118**, 36 (1966)].

^c ND, Not determined.

TABLE IV
AMINO ACID COMPOSITION OF CYTOCHROME OXIDASE AND OF THE CYTOCHROME
OXIDASE SUBUNITS FROM *Neurospora crassa*^a

Amino acid	Cytochrome oxidase (mol %)	Subunit			
		1	2	3	4-8
Aspartic acid	7.86	6.45	8.55	7.21	8.32
Threonine	5.01	4.8	4.14	6.51	5.24
Serine	7.59	10.09	9.36	7.55	4.47
Glutamic acid	8.27	4.28	9.80	6.13	11.71
Proline	6.90	6.84	7.33	4.69	6.86
Glycine	8.30	10.20	6.81	9.56	7.80
Alanine	8.11	7.53	4.90	7.46	10.45
Valine	6.35	7.14	7.71	6.38	4.97
Methionine	2.83	1.27	2.05	1.28	4.91
Isoleucine	7.31	8.72	9.16	9.41	4.63
Leucine	10.65	13.37	11.68	12.59	7.47
Tyrosine	4.42	4.18	4.89	3.65	4.74
Phenylalanine	6.57	8.25	5.38	9.15	5.00
Lysine	3.21	1.51	2.50	1.24	5.61
Histidine	3.04	2.56	2.64	4.62	2.82
Arginine	3.54	2.81	3.07	2.56	4.97

^a Subunits 1, 2, and 3 were isolated by preparative dodecyl sulfate gel electrophoresis [W. Sebald, W. Machleidt, and J. Otto, *Eur. J. Biochem.* **38**, 311 (1973)]. The amino acid composition of the group of subunits 4-8 was calculated from the amino acid composition of subunits 1, 2, and 3 and of total cytochrome oxidase on the basis of the relative proportion of subunits to total cytochrome oxidase (Table III). The contents of cysteine and tryptophan were not determined.

heme determination using the molar absorbance coefficient ΔE_{605nm} (reduced-oxidized) $\times 12 \text{ mM}^{-1} \text{ cm}^{-1}$.⁷

Enzymic Activity. In a test system containing 0.1 M Tris-acetate pH 7.5, 0.5% Tween 80, and 7 μM ferrocytochrome C^8 (horse heart, Boehringer, Mannheim, Germany) at 20°, the purified cytochrome oxidase shows turnover rates of 2-5 mol of ferrocytochrome $c \times$ millimoles of cytochrome $aa_3^{-1} \times \text{min}^{-1}$. Sonicated mitochondrial membranes under the same test conditions show turnover rates of 5-8 mol of ferrocytochrome $c \times$ millimoles of cytochrome $aa_3^{-1} \times \text{min}^{-1}$.

⁷ B. F. van Gelder, *Biochim. Biophys. Acta* **118**, 36 (1966).

⁸ L. Smith and H. Conrad, *Arch. Biochem. Biophys.* **63**, 403 (1956).

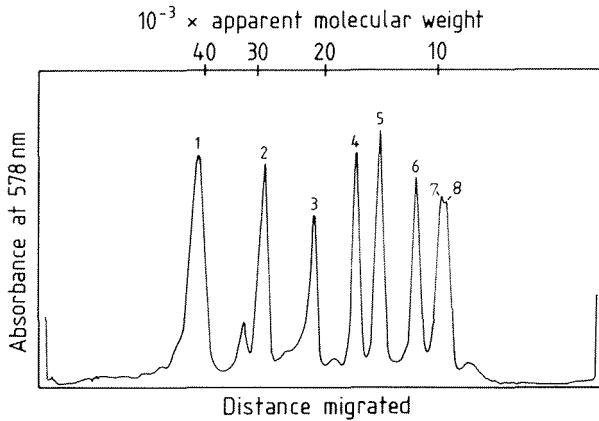


FIG. 2. Dodecyl sulfate gel electrophoresis of cytochrome oxidase purified from *Neurospora crassa*. The electrophoresis was performed on 15% polyacrylamide gels in 0.5% sodium dodecyl sulfate and 0.1 M Tris-acetate pH 8. The protein was stained with Coomassie Blue [W. Sebald, W. Machleidt, and J. Otto, *Eur. J. Biochem.* **38**, 311 (1973)].

Subunit Composition and Molecular Weight. The protein moiety of purified cytochrome oxidase is separated by dodecyl sulfate gel electrophoresis into 8 subunits^{3,6} (Fig. 2) with the molecular weights listed in Table III. The relative proportion of protein of these subunits to the total cytochrome oxidase protein is also given in Table III.

When these proportions of protein are divided by the apparent molecular weights, equimolar relations are obtained. Assuming that any of the subunits is present once, the minimum molecular weight 150,000 results for cytochrome oxidase.⁶ This is consistent with the minimum

TABLE V
WAVELENGTH POSITION OF THE ABSORPTION BANDS AND RATIOS OF ABSORBANCES AT 420–426 nm/280 nm OF CYTOCHROME OXIDASE PURIFIED FROM VARIOUS ORGANISMS

Organism	Wavelength position (nm)		Ratio of absorption at 280 nm/420–426 nm
	Oxidized form	Reduced form	
<i>Neurospora crassa</i>	603 427	605 443	0.4
<i>Polytoma mirum</i>	603 427	605 445	0.34
<i>Locusta migratoria</i>	599 420	601 441	0.31
<i>Xenopus muelleri</i>	600 420	602 441	0.31
<i>Rattus norvegicus</i>	600 421	601 441	0.33

TABLE VI
APPARENT MOLECULAR WEIGHTS OF THE SUBUNITS OF CYTOCHROME OXIDASE FROM
VARIOUS ORGANISMS

Organism	Apparent molecular weight $\times 10^{-3}$							
<i>Neurospora crassa</i>	41	28.5	21	16	14	11.5	10	9.5
<i>Polytoma mirum</i>	40	30	18	15	15	11.5		10.5
<i>Locusta migratoria</i>	38	24	19	14.5	12.5	10		8
<i>Xenopus muelleri</i>	42	24	22	16	14	12		9
<i>Rattus norvegicus</i>	40	22	20	15	12	10		8

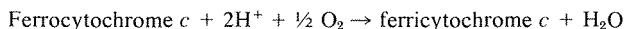
molecular weight per 2 mol of heme, namely 140,000, which is derived from the specific heme content of purified cytochrome oxidase.

Amino Acid Composition. With regard to the amino acid composition, cytochrome oxidase seems to be composed of two types of subunits: on the one hand, subunits 1, 2, and 3, which have a high content of unpolar amino acids, and, on the other hand, subunits 4–8, the amino acid compositions of which are more polar (Table IV).⁶ As described in this series, Vol. 56 [5], the subunits 1–3 are translated on mitochondrial ribosomes and are most probably coded for on mitochondrial DNA whereas subunits 4–8 are provided by extramitochondrial protein synthesis.

Cytochrome Oxidase Purified from Other Sources. By means of the chromatographic procedure described above, cytochrome oxidase has, in addition, been purified from mitochondria of *Polytoma mirum*,⁴ *Locusta migratoria*,⁵ *Xenopus muelleri*,⁴ and *Rattus norvegicus*.⁴ All preparations showed similar enzymic activity and absorption spectra. The wavelength positions of their main absorption bands are shown in Table V. Upon dodecyl sulfate gel electrophoresis, all preparations showed a similar subunit pattern (Table VI).

[12] Cytochrome Oxidase of *Saccharomyces cerevisiae*

By MERYL S. RUBIN and ALEXANDER TZAGOLOFF



Assay Method

Principle. The rate of oxidation of reduced cytochrome *c* is measured spectrophotometrically.¹

¹ L. Smith, in "Methods in Biochemical Analysis," Vol. II, p. 427. Wiley (Interscience), New York, 1955.