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# Cytochrome *c* Oxidase of *Euglena gracilis*: Purification, Characterization, and Identification of Mitochondrially Synthesized Subunits[1]

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## Abstract

Cytochrome *c* oxidase was purified from mitochondria of *Euglena gracilis* and separated into 15 different polypeptide subunits by polyacrylamide gel electrophoresis. All 15 subunits copurify through various purification procedures, and the subunit composition of the isolated enzyme is identical to that of the immunoprecipitated one. Therefore, the 15 protein subunits represent integral components of the *Euglena* oxidase. In an *in vitro* protein-synthesizing system using isolated mitochondria, polypeptides 1–3 were radioactive labeled in the presence of [$^{35}$S]methionine. This further identifies these polypeptides with the three largest subunits of cytochrome *c* oxidse encoded by mitochondrial DNA in other eukaryotic organisms. By subtraction, the other 12 subunits can be assigned to nuclear genes. The isolated *Euglena* oxidase was highly active with *Euglena* cytochrome $c_{558}$ and has monophasic kinetics. Using horse cytochrome $c_{550}$ as a substrate, activity of the isolated oxidase was rather low. These findings correlate with the oxidase activity of mitochondrial membranes. Again, reactivity was low with cytochrome $c_{550}$ and 35-fold higher with the *Euglena* cytochrome $c_{558}$. The data show that the cytochrome *c* oxidase of the protist *Euglena* is different from other eukaryotic cytochrome *c* oxidases in number and size of subunits, and also with regard to kinetic properties and substrate specificity.

**Key Words:** Cyrochrome *c* oxidase; kinetics; subunit composition; mitochondrially synthesized polypeptides; *Euglena gracilis*.

## Introduction

Cytochrome *c* oxidase [or ferrocytochrome *c*: $O_2$ oxidoreductase (EC 1.9.3.1)] in fungi, mammalia, and higher plants use mitochondrial cytochrome *c* as

[1] Abbreviations: kDa, kilodalton; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TN, turnover number.

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electron donors that form a very homogeneous class with respect to their optical absorption (Lemberg and Barrett, 1973). Protozoan cytochromes *c* have certain unusual features that distinguish them from other mitochondrial cytochromes *c*. The mitochondrial cytochrome $c_{558}$ isolated from the green flagellate *Euglena gracilis* is markedly different from most other mitochondrial cytochromes *c* with respect to optical absorption and primary structure (Pettigrew, 1979). The reactivity of various protozoan cytochromes *c* (including *Euglena* cytochrome $c_{558}$) with mammalian (bovine) cytochrome *c* oxidase was studied by Ferguson-Miller *et al.* (1976) and Errede (1976), who reported that, at high concentrations, all attained the same maximal velocity as mammalian and yeast cytochromes *c*. The *Euglena* cytochrome $c_{558}$, however, exhibited much lower affinity to the mammalian oxidase relative to the mammalian and fungal cytochromes *c*. The reverse experiment, in which an isolated protozoan cytochrome oxidase is tested for reactivity with mammalian cytochrome $c_{550}$, still has not been attempted. However, by the use of mitochondrial preparations from *Euglena* cells, some hints have been obtained. When horse cytochrome *c* was added to depleted *Euglena* mitochondria, no activity (Collins *et al.*, 1975; Pettigrew, 1979) or very low activity (Calvayrac *et al.*, 1978) was detected, whereas *Euglena* cytochrome $c_{558}$ restored normal respiration. All of these observations together suggest that they are not only caused by variation in cytochrome *c* molecules, but may also reflect fundamental differences between protozoan and mammalian cytochrome *c* oxidases.

In the work described in this article, *Euglena* cytochrome *c* oxidase for the first time was purified and characterized. The enzyme was isolated from cells of a streptomycin-bleached strain of *E. gracilis*. The structural and kinetic properties of the enzyme were analyzed and compared with those of mammalian oxidase. The capability of isolated *Euglena* mitochondria to synthesize proteins *in vitro* (Brönstrup and Hachtel, 1986) was used to determine the mitochondrially synthesized subunits of the *Euglena* cytochrome *c* oxidase.

## Materials and Methods

### *Cell Culture and Preparation of Mitochondria*

The colorless, apoplastidial mutant K2 of *Euglena gracilis* Klebs var. *saccharophila* (strain 1224-5/9, Sammlung von Algenkulturen Göttingen, FRG) had been induced by streptomycin. Cells were grown as described previously (Brönstrup and Hachtel, 1986). Mitochondria were prepared by a modification of the method described by Buetow and Buchanan (1964). Algal

cells were suspended as a thick slurry in isolation buffer [0.3 M sucrose, 50 mM $KH_2PO_4$, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.5 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.4, supplemented with 0.5% bovine serum albumin], and broken at 4°C by mortaring together with glass beads ($\phi$ = 0.3 mm).

### *Isolation of Cytochrome c Oxidase*

Cytochrome *c* oxidase was isolated from *Euglena* mitochondria according to a procedure described by Kadenbach *et al.* (1986) for mammalian mitochondria. Mitochondria were extracted with Triton X-114 (0.75 mg/mg protein). Subsequently, the oxidase was solubilized by the use of Triton X-100 (5% vol/vol in 200 mM potassium phosphate, pH 7.2). The mixture was centrifuged at 250,000 *g* for 30 min. The green supernatant was chromatographed on a DEAE–Sephacel (Pharmacia) column. The green fractions were combined, and cytochrome *c* oxidase was precipitated with ammonium sulfate (21–38% saturation) in the presence of 1% sodium cholate. If not stated otherwise, this preparation was used for further analyses. Polypeptide subunits of the cytochrome *c* oxidase were isolated by a procedure described by George-Nascimento and Poyton (1981).

### *Measurement of Cytochrome c Oxidase Activity*

Oxygen consumption was measured polarographically according to the method described by Kuhn-Nentwig and Kadenbach (1986) over a cytochrome $c_{558}$ concentration range of 0.07–12 $\mu$M in 25 mM Tris–acetate, pH 7.8, 0.05% laurylmaltoside, 25 mM potassium ascorbate, 0.7 mM tetramethyl-*p*-phenylenediamine (TMPD), and 200 pmol cytochrome *c* oxidase in a total volume of 2.0 ml. The following extinction coefficients were used to calculate concentrations from the difference spectra (dithionite minus air oxidized): cytochrome *c* oxidase (cytochrome $aa_3$), $\Delta\varepsilon$ (603–630 nm) = 24 $mM^{-1}\,cm^{-1}$; and cytochrome $c_{558}$, $\Delta\varepsilon$ (558–542 nm) = 19.1 $mM^{-1}\,cm^{-1}$. Oxidase activity was also measured spectrophotometrically. The assay system consisted of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) adjusted to pH 7.5 with KOH, 0.1% Triton X-100, 0.15–40 $\mu$M ferrocytochrome $c_{550}$ (horse heart, type VI, Sigma), and 100–200 nM cytochrome $aa_3$. At variance to oxygen consumption, the spectrophotometrically determined oxidation of cytochrome *c* was not stimulated by the use of laurylmaltoside instead of Triton X-100.

### *Labeling Experiments*

To obtain mitochondria capable of protein synthesis *in vitro*, the pellicle of cells grown in a vitamin $B_{12}$-deficient medium was digested with 0.5%

trypsin under strictly controlled conditions before rupturing the cells and isolating the organelles. The mitochondria were labeled *in vitro* with [$^{35}$S] methionine (1200 Ci mmol$^{-1}$; Amersham) as described previously (Brönstrup and Hachtel, 1986).

### *Electrophoresis and Fluorography*

Polyacrylamide gel electrophoresis (PAGE) was performed on a vertical slab gel electrophoresis apparatus according to the method described by either Kadenbach *et al.* (1983) or Chua (1980). In the system described by Chua, sodium dodecyl sulfate (SDS) was replaced by lithium dodecyl sulfate. Gels were stained with Coomassie blue R250 and prepared for fluorography as described (Brönstrup and Hachtel, 1986).

### *Immunological Procedures*

Antisera against *Euglena* holocytochrome *c* oxidase were raised in rabbits by following conventional procedures (Harboe and Ingild, 1973). The enzyme had been suspended in water before immunization.

The immunoglobulin G (IgG) fraction was partially purified by ammoniumsulfate precipitation and chromatography on a protein A–Sepharose CL-4B (Pharmacia) column. Immunoprecipitation was obtained both from mitochondrial membranes and purified enzyme. Starting with mitochondrial membranes (2–3 mg protein), cytochrome *c* oxidase was solubilized by the use of Triton X-100. For immunoprecipitation, 3–4 mg of antibodies were added to the enzyme in a final volume of 1 ml phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, and 1.5 mM $KH_2PO_4$, pH 7.2). For immunoblot detection of cytochrome *c* oxidase subunits after SDS–PAGE, proteins were transferred from slab gels to nitrocellulose (Schleicher & Schüll) (Towbin *et al.*, 1979). After incubation with oxidase antibodies, the washed nitrocellulose sheets were incubated with protein A–peroxidase (Boehringer Mannheim) and subsequently with 4-chloro-1-naphthol and $H_2O_2$.

Antibodies against the cytochrome *c* oxidase from *Neurospora crassa* (Werner, 1974) were used to prepare an affinity matrix. Antibodies (1.7 mg) and EDC [*N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide] (40 mg) were conjugated to 1 g AH–Sepharose 4B (Pharmacia) essentially according to the manufacturer's instructions. *Euglena* cytochrome *c* oxidase (0.74 mg protein) prepared as described above was loaded on the affinity column. After binding, the enzyme was washed out with 0.15 M PBS buffer, pH 7.2, containing 0.1% Triton X-100. Oxidase was eluted by 0.1 M glycine-HCl, pH 3.0, and the fractions were neutralized with 2 M Tris immediately after collection. Green fractions containing the enzyme were again concentrated by precipitation with ammonium sulfate (21–38% saturation) in the presence of 1% sodium cholate.

## Results

### *Polypeptide Composition of Euglena Cytochrome c Oxidase*

The purified oxidase was in fact spectroscopically pure (data not shown). From the difference spectra (dithionite minus air oxidized), a heme *a* content of 5.8 nmol/mg enzyme protein was calculated corresponding to a 24-fold concentration. The Soret-band/α-band ratios of the oxidized and the dithionite-reduced oxidase as well as the Soret $(Fe^{2+})/(Fe^{3+})$ and the $\alpha(Fe^{2+})/(Fe^{3+})$ ratios are within the ranges given by Lemberg and Barrett (1973) for oxidase preparations free of other cytochromes.

The polypeptide composition of the isolated *Euglena* cytochrome *c* oxidase was analyzed by two different PAGE systems and compared with the rat liver enzyme (Fig. 1). In the system according to Kadenbach *et al.* (1983), 15 polypeptides with apparent molecular weights between 6 and 42 kDa were

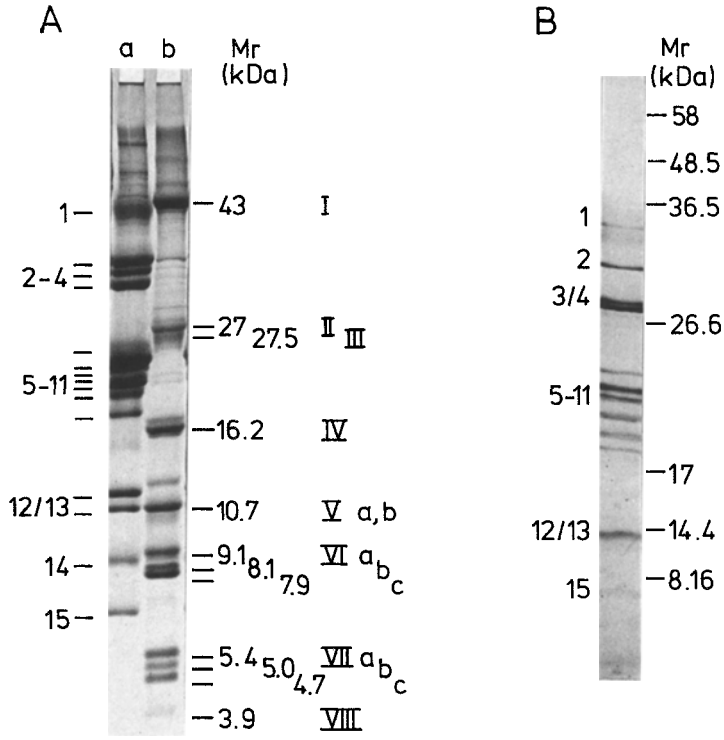


**Fig. 1.** Polypeptide pattern of isolated *Euglena* and rat liver cytochrome *c* oxidase obtained by electrophoresis. (A) SDS–PAGE according to Kadenbach *et al.* (1983); 18.75% acrylamide, 6 M urea: (a) *Euglena gracilis* and (b) rat liver. (B) Lithium dodecyl sulfate PAGE according to Chua (1980); 15–20% acrylamide. Designation and apparent molecular weight (Mr) of the subunits of the rat liver enzyme according to Merle and Kadenbach (1980).

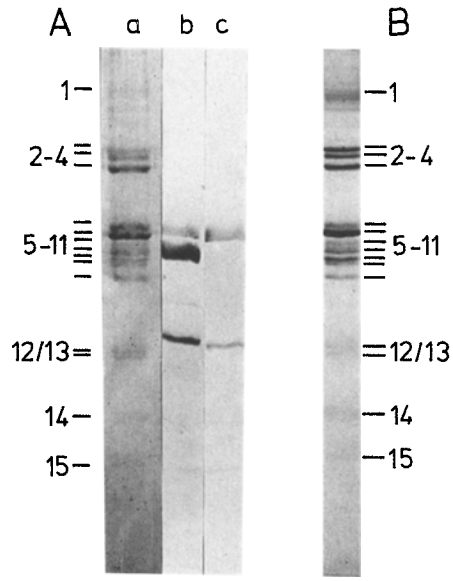


**Fig. 2.** Purification of *Euglena* cytochrome *c* oxidase by affinity chromatography using polyclonal antibodies against *Neurospora crassa* oxidase. (A) *Euglena* oxidase purified by DEAE-Sephacel chromatography was separated by SDS-PAGE and transferred onto nitrocellulose. Strip a was stained with Ponceau S. Strips b and c were incubated with *N. crassa* antibodies. Strip b subsequently was incubated with protein A-peroxidase and then stained with chloronaphthol and $H_2O_2$. Strip c was not incubated with protein A-peroxidase prior to staining. (B) SDS-PAGE of *Euglena* cytochrome *c* oxidase purified by DEAE-Sephacel chromatography and affinity chromatography on a matrix of *N. crassa* antibodies conjugated to AH-Sepharose CL-4B.

observed. Assuming a 1 : 1 stoichiometric ratio, we calculated a molecular weight of 320 kDa for the holoenzyme. The PAGE system according to Chua (1980) resolved only 14 polypeptides. A two-dimensional gel (not shown) revealed comigration of polypeptides 12 and 13 in Chua's system. In further analyses (George-Nascimento and Poyton, 1981), water-soluble polypeptides and hydrophobic polypeptides were separated. Polypeptides 2 and 6 were identified as water-soluble proteins whereas all other polypeptides proved to be hydrophobic. Polypeptide 1 was not present in gels of ethanol-precipitated enzyme. This observation is indicative for strong hydrophobicity of this polypeptide (Rubin and Tzagoloff, 1973a).

An alternative procedure to prepare the enzyme was also attempted. The solubilized oxidase was enriched first by ammonium sulfte precipitation (55–65% saturation) and then further purified by gel filtration using Sephadex G-100 (Pharmacia) as described by George-Nascimento and Poyton (1981). The heme *a* content of oxidase-containing fractions was ~6 nmol/mg

protein. Electrophoresis revealed identical polypeptide compositions of the enzyme purified by DEAE–Sephacel chromatography and the oxidase purified by Sephadex G-100 gel filtration (electropherograms not shown).

Additional evidence for the subunit composition of *Euglena* cytochrome *c* oxidase comes from affinity chromatography. Polyclonal antibodies against the cytochrome *c* oxidase of *Neurospora crassa* (Werner, 1974) were available by courtesy of Prof. Werner. We were able to demonstrate a highly specific cross-reaction between these antibodies and subunit 8 and a cross-reaction of low specificity with polypeptide 12 of the *Euglena* oxidase (Fig. 2A). The *Neurospora* antibodies then were conjugated to AH–Sepharose CL-4B. *Euglena* cytochrome *c* oxidase was first purified by DEAE–Sephacel chromatography and subsequently applied to the affinity matrix. Electrophoretic analysis of the eluted enzyme could not detect any remarkable change in the polypeptide composition by this additional step (Fig. 2B).

### *Identification of Mitochondrially Synthesized Subunits*

In a previous article, we reported on the *in vitro* synthesis of mitochondrially localized polypeptides in isolated *Euglena* mitochondria (Brönstrup

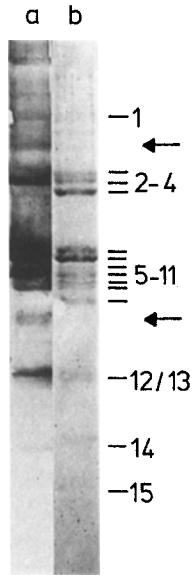


**Fig. 3.** Immunodetection of *Euglena* cytochrome *c* oxidase polypeptides by polyclonal antibodies against the *Euglena* holoenzyme. The oxidase was separated by SDS-PAGE and transferred to nitrocellulose. (Lane a) Immunodetection by peroxidase staining. (Lane b) Reversible Ponceau S staining before the incubation with the antibodies. Protein bands only visible after immunodetection are indicated by arrows.

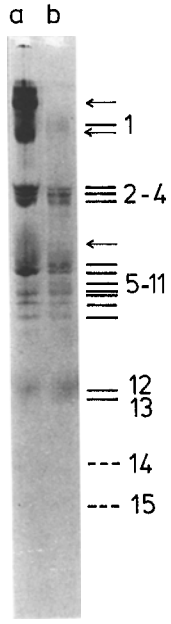


**Fig. 4.** Polypeptide composition after SDS–PAGE of *Euglena* cytochrome *c* oxidase prepared by immunoprecipitation with antibodies against *Euglena* holoenzyme (lane a) as compared with cytochrome *c* oxidase purified by DEAE–Sephacel chromatography (lane b) (6 nmol heme *a*/mg protein). The IgG bands in lane a are indicated by arrows.

and Hachtel, 1986). In order to identify subunits of the *Euglena* cytochrome *c* oxidase among these products of mitochondrial protein synthesis, we prepared polyclonal antibodies against the holoenzyme. As shown by immunoblot analysis, the antisera contained antibodies against most, but not all, oxidase subunit polypeptides (Fig. 3). Antibodies against polypeptides 2, 4, and 15 were not detectable. These subunits possibly had not been exposed to the surface of the water-suspended holoenzyme used in the immunization procedure. Polypeptides 5, 6, and 12 exhibited endogenous peroxidase activity. However, the immunostaining of these bands was drastically stimulated in the presence of antibodies. The antibodies almost completely inhibited cytochrome *c* oxidase activity at a ratio of 50 $\mu$g IgG/$\mu$g mitochondrial protein.

For immunoprecipitation, mitochondria isolated by the trypsin method were extracted by Triton X-100. The solubilized proteins were subjected to immunoprecipitation with the antibodies against the *Euglena* holoenzyme. In SDS–PAGE, the polypeptide pattern of the immunoprecipitated cytochrome *c* oxidase corresponds well to the subunit composition of the enzyme purified by DEAE–Sephacel chromatography (Fig. 4). The antibodies then were used for the immunodetection of cytochrome *c* oxidase subunits labeled in isolated

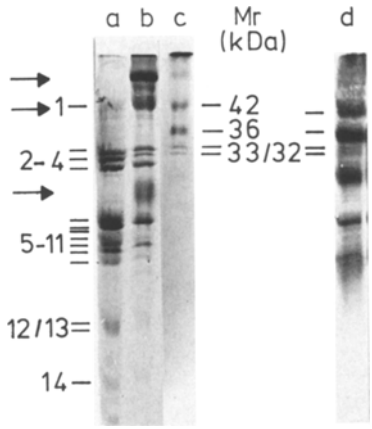


**Fig. 5.** Identification of subunits of the *Euglena* cytochrome *c* oxidase as products of protein synthesis in isolated mitochondria by immunoprecipitation, SDS–PAGE, and fluorography: (a) purified cytochrome *c* oxidase (6 nmol heme a/mg protein) (Coomassie blue stain), (b) immunoprecipitated cytochrome *c* oxidase from isolated mitochondria labeled *in vitro* with [$^{35}$S] methionine (stained), (c) fluorograph of lane b, and (d) total of radiolabeled products of protein synthesis in isolated mitochondria (fluorograph). Apparent molecular weights are given in kDa. The IgG bands in lane b are indicated by arrows.

mitochondria. After *in vitro* incorporation of radioactive methionine by the isolated mitochondria, immunoprecipitates were obtained as described above and analyzed by SDS–PAGE and fluorography (Fig. 5). In the fluorograph of the immunoprecipitate (Fig. 5, lane c) there are three prominent radioactive bands that correspond to subunits 1, 2, and 3 (42 kDa, 33 kDa, and 32 kDa, respectively) of the cytochrome *c* oxidase. A polypeptide with a molecular weight of 36 kDa is labeled, but was never found in stained oxidase gels. A labeled 36-kDa protein was also detected in the supernatant from the immunoprecipitation. On the unproven assumption of the identity of these two 36-kDa polypeptides, one might assume that they represent a precursor of one of the mitochondrially synthesized subunits. A fifth radioactive band in the high molecular weight range might be due to nonspecific adsorption of radioactive material by the IgG molecules banding at this position. Alternatively, it might represent aggregates composed of a number of smaller labeled compounds. The immunoprecipitated subunits 1–3 and the 36-kDa polypeptide are easily identified among the total of radiolabeled products of protein synthesis in isolated *Euglena* mitochondria (Fig. 5, lane d) whereas the labeled compound of high molecular weight is missing.

### *Kinetic Properties*

In a first set of experiments, cytochrome *c* oxidase activity of both isolated mitochondria and the purified enzyme was studied by measuring

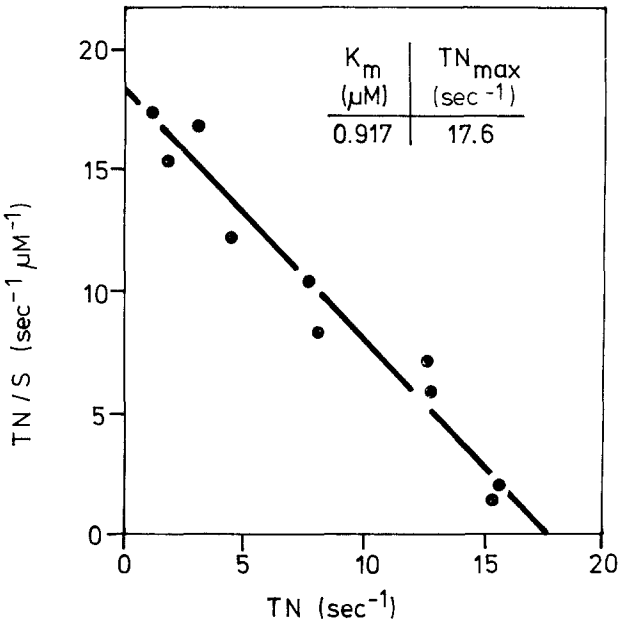


**Fig. 6.** Kinetics of cytochrome $c_{558}$ oxidation by isolated *Euglena* cytochrome *c* oxidase. The activities of an enzyme sample (100 nM cytochrome $aa_3$) were measured polarographically as molecular turnover number (TN) (mol cytochrome $c \times \sec^{-1} \times$ mol cytochrome $aa_3^{-1}$) in the presence of ascorbate and *N*, *N*, *N'*, *N'*-tetramethyl-*p*-phenylenediamine (TMPD) at various cytochrome *c* concentrations (S) between 0.07 μM and 12 μM. Cytochrome $c_{558}$ was purified from *Euglena* cells as described by Calvayrac *et al.* (1978). The correlation coefficient of the graph is 0.977.

oxygen consumption. With bovine cytochrome $c_{550}$ as a substrate, we were unable to detect oxidase activity. This correlates with the rather low activities observed in the photometric assay (see below). Therefore, cytochrome $c_{558}$ was purified from *Euglena* mitochondria according to the method described by Calvayrac *et al.* (1978). With this cytochrome as substrate a turnover number (TN) of 208 mol cytochrome $c \times \sec^{-1} \times \text{mol}^{-1}$ cytochrome $aa_3$ was found when the oxidase activity of total mitochondria was determined after solubilization in laurylmaltoside (0.05%). The activity of the purified cytochrome *c* oxidase was studied as a function of cytochrome $c_{558}$ concentration in the presence of TMPD and ascorbate. As can be seen from the Eadie-Hofstee plot of the data (Fig. 6), the *Euglena* cytochrome has monophasic kinetics with an apparent $K_m$ of 0.97 μM and a TN of 17.6 $\sec^{-1}$ at infinite cytochrome *c* concentrations.

The kinetic properties of *Euglena* cytochrome *c* oxidase were also measured spectrophotometrically using bovine cytochrome $c_{550}$ as substrate. With mitochondrial membranes, initial TNs of $\sim 6\,\sec^{-1}$ were obtained under

conditions of substrate saturation. Cytochrome *c* oxidase was completely inhibited in the presence of 0.01% KCN. The activity of the purified cytochrome *c* oxidase was measured at various cytochrome *c* concentrations at the determined pH optimum (= 7.5). Biphasic curves were obtained in an Eadie–Hofstee plot of the data (not shown). For the phase of higher affinity (phase 1), we obtained $K_m$ values of 1.2 $\mu$M and $V_{max}$ values corresponding to a TN of 0.2 $sec^{-1}$. Obviously, the $K_m$ of phase 1 is not significantly different from the $K_m$ of the monophasic kinetics obtained with cytochrome $c_{558}$, though the exogenous substrate transfers electrons to the oxidase much less efficiently.

## Discussion

### *Subunit Composition and Site of Subunit Synthesis*

One of the most intricate aspects of cytochrome *c* oxidase is that of the subunit composition of the enzyme. The cytochrome *c* oxidase complex of various mammalian tissues could be separated into 13 different polypeptide components (Kadenbach *et al.*, 1983). In the yeast *Saccharomyces cerevisiae*, the enzyme contains nine subunits (Power *et al.*, 1984) whereas 10 subunits have been found for the oxidase from the yeast *Candida parapsilosis* (Camougrand *et al.*, 1987). Cytochrome *c* oxidase from the slime mold *Dictyostelium discoideum* consists of six subunits (Bisson *et al.*, 1985). At variance with these findings, the *Euglena* enzyme is composed of 15 different polypeptides. On the basis of its polypeptide composition, the *Euglena* enzyme appears to be the most complicated eukaryotic oxidase isolated so far. The comparatively high molecular weight of 320 kDa may explain the low heme *a* content of the purified *Euglena* enzyme (6 nmol/mg protein) relative to other purified cytochrome *c* oxidases. The theoretically expected value, based on a ratio of 2 mol heme *a* per 1 mol oxidase (Steffens *et al.*, 1987) would be 6.24 nmol heme *a*/mg protein. However, an exact heme *a* content is difficult to determine due to possible inaccuracies in the Lowry protein determination method (Kadenbach *et al.*, 1986).

The possibility that some of the 15 described subunits may represent impurities can be excluded for the following reasons. First, all 15 subunits were copurified by a number of procedures including ammonium sulfate fractionation, ion-exchange chromatography on DEAE–Sephacel in the presence of Triton X-100, gel filtration on Sephadex G-100 in the presence of cholate, and affinity chromatography. Second, the immunoprecipitated enzyme shows the same subunit composition as the purified enzyme (Fig. 4). The absorption spectra (data not shown) do not reveal a contamination with *b*- and *c*-type cytochromes. The presence of other redox enzymes could be possibly found by metal analysis of the isolated oxidase complex.

An argument against the 15-subunit composition would be the occurrence of proteolytic breakdown products in the enzyme preparation. However, no loss of any polypeptide in the subunit pattern was observed when protease inhibitors (1 mM PMSF plus 0.05% benzamidine chloride) were present during the entire isolation procedure. Furthermore, almost identical polypeptide patterns were found in enzyme preparations isolated within 24 h or over several days. According to these criteria, only one polypeptide band located between polypeptides 11 and 12 is probably due to proteolytic degradation or might be a contaminant polypeptide and, therefore, was not included in the numbering. Whether some of the bands could represent proteolytic degradation products could be definitely shown by testing cross-reactivity of monospecific antibodies against the individual polypeptides.

The elucidation of the function of the various subunits requires the knowledge of their stoichiometry in the enzyme complex. Staining intensity of the Coomassie-blue stained bands was determined by scanning the absorbance at 546 nm and measuring the peak areas (data not shown). The peak area/molecular weight ratios seem to indicate 1 : 1 stoichiometry of polypeptides 2–4 and 6–13. The other subunits, in particular the polypeptides 1, 14, and 15, are found in lesser amounts. An explanation might be that these subunits are weakly stained due to their hydrophobic nature. This deserves to be analyzed by total amino acid analysis of hydrolyzed bands.

In all eukaryotic cytochrome *c* oxidases studied so far, the subunits I, II, and III are encoded by the mitochondrial genome (Rubin and Tzagoloff, 1973b; Mason and Schatz, 1973). Using antibodies against subunits I, II, or III, respectively, of the cytochrome *c* oxidase of *Neurospora crassa* (Werner, 1974), we were not able to identify the mitochondrially encoded subunits among the 15 polypeptides that constitute the *Euglena* oxidase (not shown). Our data obtained from the *in vitro* approach identify the three largest polypeptides (1–3) with the three largest subunits encoded by mitochondrial DNA in other eukaryotic organisms. *Euglena* polypeptide 1 (42 kDa) probably corresponds to subunit I in other eukaryotes because of its similar molecular weight and extreme hydrophobicity (Rubin and Tzagoloff, 1973a). The molecular weight of *Euglena* polypeptide 2 (33 kDa) differs only slightly from the molecular weights of subunits II of other oxidases. Subunits III of other eukaryotes are of lower molecular weight than polypeptide 3 (32 kDa) of *Euglena*. In *Rhodopseudomonas sphaeroides*, however, a subunit III of even higher molecular weight (37,000) has been found (Azzi and Gennis, 1986).

The overall differences in the number of subunits are accounted for by different numbers of nuclear-encoded subunits. These are suggested to have regulatory functions, and their number and size might reflect the regulatory adaptations of organisms to environment (Kadenbach and Merle, 1981). Euglenoid flagellates offer potential for further investigation of this hypothesis.

They represent a collection of green phototrophs, as well as osmotrophs and phagotrophs without chloroplasts, that are all closely related to one another (Leedale, 1967). The green *Euglena* itself is a facultative phototroph that is capable of utilizing a variety of organic compounds as the sole source of carbon and energy for growth (Schiff and Schwartzbach, 1983) and exhibits a peculiar respiratory metabolism (U. Kümmel and K. Brinkmann, unpublished results).

### *Kinetic Properties*

Large differences in the activity of isolated *Euglena* cytochrome *c* oxidase with different substrates have been demonstrated in the present study. As compared with horse cytochrome $c_{550}$, a drastic stimulation occurred using *Euglena* cytochrome $c_{558}$. The values of maximal velocity obtained with the purified enzyme (TN = $17.6\,sec^{-1}$), however, are much lower than those measured with solubilized mitochondrial membranes (TN = $208\,sec^{-1}$). If the latter value is applicable to the membrane-bound enzyme, turnover numbers might largely depend on the lipid environment of the oxidase since lipids are known to affect strongly the activity of cytochrome *c* oxidases (Caughy *et al.*, 1976; Vik *et al.*, 1981).

An important aspect of cytochrome *c* oxidase involves the number of catalytically active sites on the oxidase protein of different affinities for the mitochondrial cytochrome *c*. The biphasic kinetics of the oxidase, as obtained in Eadie–Hofstee plots, were interpreted either by two catalytic binding sites of yeast and mammalian enzymes (Ferguson-Miller *et al.*, 1976, 1978; Veerman *et al.*, 1980), or by one catalytic and one regulatory binding site for cytochrome *c* (Speck *et al.*, 1984), whereas Büge and Kadenbach (1986) suggested the existence of only one binding site per oxidase monomer. The latter authors assumed that increasing concentrations of cytochrome *c*, like other polyvalent ions, can decrease $K_m$ for ferrocytochrome *c*, probably by means of conformational changes in the binding domain. They consider the higher apparent $K_m$ of the mammalian oxidases to be the more physiological since it predominates at physiological ionic strength. The monophasic kinetics obtained with cytochrome $c_{558}$ in the polarographic assay indicate the presence of only one catalytically active site on the *Euglena* cytochrome *c* oxidase protein. The apparent $K_m = 1\,\mu M$ is in the range of the Michaelis constants detected for the so-called "low-affinity binding site" of most other eukaryotic cytochrome *c* oxidases (Ferguson-Miller *et al.*, 1976; Bisson *et al.*, 1985; Kadenbach *et al.*, 1986; Camougrand *et al.*, 1987). Similar data were reported for the amoeba cytochrome *c* oxidase of *Dictyostelium discoideum*. The endogenous cytochrome *c* has, in constrast to the various eukaryotic substrates tested, monophasic kinetics with an apparent $K_m$ ($1.26\,\mu M$)

comparable to that found for the *Euglena* oxidase with its endogenous substrate (Bisson *et al.*, 1985).

In *Euglena*, not only the cytochrome *c* oxidase, as demonstrated by the data of this study, but also the cytochrome *c*, differ markedly from the corresponding molecules in yeast and eukaryotes. Ferguson-Miller *et al.* (1976) examined the reactions of different cytochromes *c* with beef heart oxidase and found for the *Euglena* cytochrome *c* an apparent $K_m$ value (0.4 $\mu$M) that was 10 times that obtained for either the horse or the yeast cytochromes. They could explain these differences in terms of changes in primary structure of cytochromes *c*. Of particular relevance are variations in amino acid residues that have been implicated in the interaction between cytochrome *c* and the oxidase (Lemberg and Barrett, 1973; Ferguson-Miller *et al.*, 1976; Pettigrew, 1979). It must be emphasized that any detailed examination of the functional concomitants of evolutionary changes in structure will have to consider the complete matrix of possible interactions of cytochrome *c* with the oxidases of a large number of species. On the basis of the available data, we speculate that the divergent evolution of cytochromes *c* in *Protozoa* in general and in the *Euglenophytes* in particular has triggered a coevolution of cytochrome *c* oxidase with substrate affinities and reactivities divergent from those of other eukaryotes. Noteworthy, in trypanosomes, which are believed to be phylogenetically linked with the euglenoid flagellates (Kivic and Walne, 1984; Corliss, 1987), a number of amino acids so far thought to be crucial for the functioning of cytochrome *c* oxidase subunit II are missing. Among these, six of the 11 conserved acidic residues postulated to function as a high-affinity binding site for cytochrome *c* are absent from trypanosome sequences (Benne, 1985).

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