Copeland, R. A., Smith, P. A., & Chan, S. I. (1987) *Biochemistry 26*, 7311-7316.
Gelles, J., & Chan, S. I. (1985) *Biochemistry 24*, 3693-3972.
Gelles, J., Blair, D. F., & Chan, S. I. (1986) *Biochim. Biophys. Acta 853*, 205-236.
Gray, H. B., & Solmon, E. I. (1986) in *Copper Proteins* (Spiro, T. G., Ed.) Vol. 3, pp 1-39, Wiley, New York.
Hartzell, C. R., & Beinert, H. (1974) *Biochim. Biophys. Acta 368*, 318-338.
Hill, B. C., & Robinson, N. C. (1986) *J. Biol. Chem. 261*, 15356-15359.
Hoffman, B. M., Roberts, J. E., Swanson, M., Speck, S. H., & Margoliash, E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1452-1456.
Li, P. M., Gelles, J., Chan, S. I., Sullivan, R. J., & Scott, R. A. (1987) *Biochemistry 26*, 2091-2095.
Martin, C. T., Scholes, C. P., & Chan, S. I. (1988) *J. Biol. Chem. 263*, 8420-8429.
Müller, M., Thelen, M., O'Shea, P., & Azzi, A. (1986) *Methods Enzymol. 126*, 78-87.
Nilsson, T., Gelles, J., Li, P. M., & Chan, S. I. (1988a) *Biochemistry 27*, 296-301.
Nilsson, T., Copeland, R. A., Smith, P. A., & Chan, S. I. (1988b) *Biochemistry*, (in press).
Peisach, J., & Blumberg, W. E. (1974) *Arch. Biochem. Biophys. 165*, 691-708.
Prochaska, L. J., & Fink, P. S. (1987) *J. Bioenerg. Biomembr. 19(2)*, 143-167.
Rigell, C. W., & Friere, E. (1987) *Biochemistry 26*, 4366-4371.
Scholes, C. P., & Malmström, B. G. (1986) *FEBS Lett. 198*, 125-129.
Smith, L. (1955) *Methods Biochem. Anal. 2*, 427-434.
Sone, N., & Nicholls, P. (1984) *Biochemistry 23*, 6550-6554.
Sone, N., Ogura, T., & Kitigawa, T. (1986) *Biochim. Biophys. Acta 850*, 139-145.
Stevens, T. H., Martin, C. T., Wang, H., Brudvig, G. W., Scholes, C. P., & Chan, S. I. (1982) *J. Biol. Chem. 257*, 12106-12133.
Wikström, M., & Casey, R. P. (1985) *J. Inorg. Biochem. 23*, 327-334.
Wikström, M., Krab, K., & Saraste, M. (1981) *Cytochrome Oxidase: A Synthesis*, Academic, New York.
Wyman, J. (1979) *Accad. Naz. Lincei, Ser. VIII 64*, 409-415.

Preparation of a One-Subunit Cytochrome Oxidase from *Paracoccus denitrificans*: Spectral Analysis and Enzymatic Activity†

Michele Müller, Beatrice Schläpfer, and Angelo Azzi*
Institut für Biochemie und Molekularbiologie der Universität Bern, Bühlstrasse 28, CH-3012 Bern, Switzerland
Received February 11, 1988; Revised Manuscript Received April 26, 1988

ABSTRACT: Cytochrome *c* oxidase was isolated from *Paracoccus denitrificans* as a two-subunit enzyme. Chymotrypsin-catalyzed proteolysis reduced the molecular weight of each subunit by about 8000. The spectral properties of this preparation, as well as its K_m for cytochrome *c* (1.7 μM), remained unchanged with respect to the native enzyme. V_{max} was reduced by about 55% when assayed in Triton X-100 or in Triton X-100 supplemented with asolectin. Following further proteolysis by *Staphylococcus aureus V8* protease, subunit I remained unchanged as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, whereas subunit II was split into small peptides. These were removed by ion-exchange high-performance liquid chromatography. The one-subunit enzyme had an apparent molecular weight of 43 000. The reduction of molecular weight was also confirmed by the diminution of the ultraviolet/Soret absorption ratio. This value was 1.8-2.1 for the native enzyme and 1.3-1.5 for the one-subunit enzyme. The spectral properties (including the spectrum CO reduced minus reduced) were not modified by the proteolytic treatment, indicating that cytochromes *a* and a_3 were present in equal amounts. The lack of spectral alteration and the known close association of the copper B atom with cytochrome a_3 suggest that copper B is also contained within the one-subunit enzyme. The K_m of the one-subunit oxidase was similar to that of the two-subunit enzyme; V_{max} was decreased by about 50%. The activity of the one-subunit oxidase had a salt-dependent maximum at 30 mM KCl, almost identical with that of the undigested enzyme, and was inhibited by micromolar concentrations of KCN.

In the large family of cytochrome *c* oxidases the bacterial enzymes have been studied with increasing frequency during the last 10 years, mainly because of their structural analogy with respect to the eukaryotic type (Fee et al., 1986; Ludwig, 1987). Although they contain only 1-3 subunits relative to the 9-13 present in eukaryotic cells, their enzymatic and spectral properties are almost identical. The cytochrome *c* oxidase of *Paracoccus denitrificans* was isolated as two-subunit enzyme (Ludwig & Schatz, 1980), and its main physicochemical characteristics have been analyzed (Ludwig et al., 1982; Solioz et al., 1982; Nalecz et al., 1985; Bolli et al., 1986). The minimum requirement for cytochrome *c* oxidase activity seems to be a two-subunit structure. The only known exception is *Thermus thermophilus* (Yoshida et al., 1984), which was isolated in an active form consisting of a single subunit. The gene sequences of *P. denitrificans* subunits I-III have been recently published (Steinrücke et al., 1987; Raitio et al., 1987). Despite the fact that a gene for subunit III was identified,

† This work was supported by the Swiss National Science Foundation (Grant 3.525-086).
* To whom correspondence should be addressed.

0006-2960/88/0427-7546$01.50/0

conclusive evidence for the existence of its translation product is still missing. The primary structures of subunits I and II derived from their DNA sequences were homologous to those already known from other organisms. The active centers of the enzyme (two hemes *a* and two copper atoms) have been located within these two subunits (Winter et al., 1980; Corbley & Azzi, 1984). On the basis of its sequence, subunit II is thought to contain one copper atom (CuA) (Steffens & Buse, 1979) and one heme (Wikström et al., 1985). Indirect evidence that the heme component is not present arises from a broad survey of subunit II oxidase sequences, which indicate that not enough evolutionarily conserved ligands are available in this subunit to coordinate even a single heme iron. This conclusion would promote subunit I to the role of host for three active centers, namely, the two hemes and one copper (Holm et al., 1987; Müller et al., 1987). To further complicate the issue of the oxidase metal centers and their location, Steffens et al. (1987) found that an additional copper atom is present in the *P. denitrificans* enzyme. In the present study we show that by a sequential two-step proteolytic digestion the two-subunit enzyme of *P. denitrificans* can be reduced to a one-subunit enzyme that exhibits spectral properties undistinguishable from the original oxidase and retains significant enzymatic activity.

Experimental Procedures

Cell Growth and Enzyme Preparation. *P. denitrificans* (strain ATCC 13543) cells were grown on a succinate medium in a fermentor of 10-L working volume as described by Ludwig (1986). Two or three cultures were pooled, and the enzyme was purified from the pool according to Ludwig (1986).

Chymotryptic Digestion of the Enzyme. The enzyme stock solution (160 μM cytochrome *a*) was diluted to 23 μM in a medium composed of 25 mM Tris-HCl[1] and 0.5% LM, pH 8.3. Chymotrypsin [8 mg/mL, dissolved in the same buffer without LM and treated with 0.5 mM *N*-(*p*-tosyl)-L-lysine chloromethyl ketone] was added to the diluted enzyme solution at a weight ratio of 1:1. The mixture was incubated for 60 min in the dark at 20 °C, and the digestion was stopped by the addition of 1 mM phenylmethanesulfonyl fluoride. The solution was then applied to a Whatmann DE-52 cellulose column equilibrated with the digestion buffer. The enzyme was retained by the column, whereas chymotrypsin and the various proteolytic fragments produced were washed out. The enzyme was eluted with 0.5 M Tris-HCl and 0.5% LM, pH 7.8.

Purification of Staphylococcus aureus V8 Protease. About 1 mg of *S. aureus V8* protease material (Worthinton) was dissolved in 20 mM sodium phosphate and 2 mM EDTA pH 7.2, and applied to a Baker PEI–silica HPLC column (4.6 × 250 mm). The mixture was eluted by a 0.5 M sodium phosphate and 2 mM EDTA, pH 7.2, gradient (1 mL/min). The gradient was linear (0–100%; 15 min), and the main peak eluted at 8.3 min. The fractions were concentrated by ammonium sulfate precipitation and frozen in small aliquots at −20 °C.

Digestion with S. aureus V8 Protease. The protein eluted from the DE-52 column was applied onto a Sephadex G-25 column equilibrated with 10 mM sodium phosphate, 2 mM EDTA, and 0.1 % LM, pH 7.8, and eluted with the same buffer. The cytochrome *a* concentration was about half of that used for chymotrypsin proteolysis. The treatment was started by the addition of the protease (1 mg/mL) to a final concentration of 50 units/mL. After 20-min incubation at 20 °C in the dark the reaction was stopped by the addition of 1 mM diisopropyl fluorophosphate. Forty minutes later the mixture was further chromatographed on a Sephadex G-100 column equilibrated with the same buffer used for the previous Sephadex column or absorbed on a DE-52 column and eluted with 0.5 M Tris-HCl, 2 mM EDTA, and 0.1% LM, pH 7.8.

High-Performance Liquid Chromatography. Further purification of the enzyme was achieved by separating the fragments of subunit II from subunit I after treatment with *S. aureus V8* protease by loading the mixture on a Baker Bond PEI wide-bore column (4.6 × 250 mm) equilibrated with 20 mM Hepes and 0.1% Triton X-100 R, pH 7.2. The one-subunit enzyme was eluted by a linear salt gradient, 0–1 M NaCl, in the same buffer at 1.5 mL/min. The chromatography was controlled by a Varian 5000 liquid chromatograph. Detection of the eluted components was made by using a Hewlett-Packard HP 8541A diode-array spectrophotometer equipped with an HPLC flow cell. The enzyme-containing fractions were pooled and concentrated by ammonium sulfate precipitation (50% saturation). After centrifugation, the green floating layer was collected, resuspended in 20 mM Hepes and 0.1% LM, pH 7.2, and passed through a Sephadex G-25 column equilibrated with the same buffer.

Labeling with Eosin Maleimide. EMA (Molecular Probes, Eugene, OR) was dissolved in 100 mM sodium phosphate, pH 7.2, at a concentration of 10 mg/mL, diluted with water at 1 mg/mL, and frozen in small aliquots. Labeling was generally carried out with an EMA molar excess of 5–10:1 with respect to the cytochrome *c* oxidase, either in a buffer composed of 10 mM sodium phosphate and 0.1% LM, pH 7.2, or in the sample buffer for SDS gel electrophoresis. The mixture was incubated for 30 min at room temperature in the dark before electrophoresis. Densitographic analysis of the labeled protein after SDS gel electrophoresis was performed at 529–600 nm by using a special attachment for the Aminco DW-2a spectrophotometer (Broger et al., 1979). Before staining, the EMA fluorescence in the slab gel was photographed through UV and red filters on Polaroid type 667 film, by use of a UV transilluminator.

Other Methods. The concentration of cytochrome *a* was calculated from spectra (dithionite reduced minus air oxidized) taken with an Aminco DW-2a or a Hewlett-Packard HP 8541A diode-array spectrophotometer by using an extinction coefficient $\epsilon_{605-630nm}$ = 11.7 cm^{-1} mM^{-1} (Ludwig & Schatz, 1980). Ferrocytochrome *c* was prepared by reduction with dithionite and purified by filtration on Sephadex G-25. Cytochrome *c* oxidase activity was measured spectrophotometrically at 550–540 nm and calculated by using $\epsilon_{550-540nm}$ = 19.4 cm^{-1} mM^{-1}. Protein was determined according to Gornall et al. (1949), Lowry et al. (1951), or Smith et al. (1985), using bovine serum albumin as standard. SDS–polyacrylamide gel electrophoresis was performed according to the slightly modified version of the procedure of Laemmli (1970) described by Müller and Azzi (1986), without the addition of already polymerized 0.5% polyacrylamide. Slab gels were stained with Coomassie blue or by silver according to the procedure of Bio-Rad.

Results

Cysteinyl Residue Content of Subunits I and II Determined by Labeling with Eosin 5-Maleimide. Labeling experiments with EMA show that under native conditions (Figure 1A, lane

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EMA, eosin 5-maleimide; EPR, electron paramagnetic resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; LM, lauryl maltoside; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

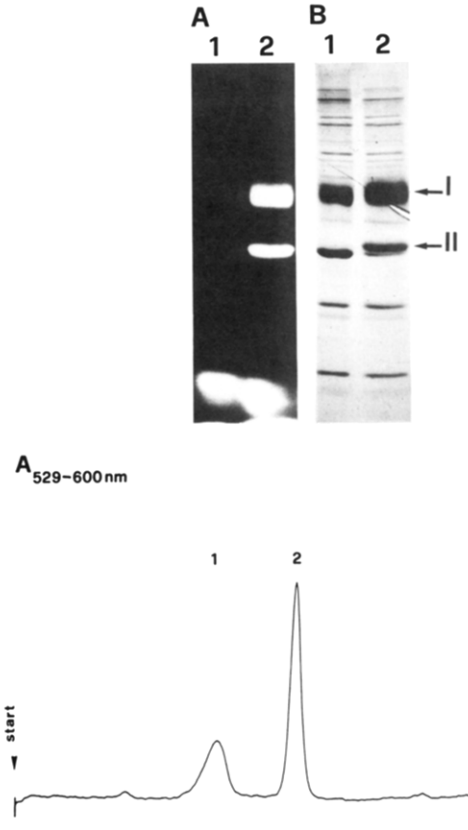

FIGURE 1: SDS–polyacrylamide gel electrophoresis of purified cytochrome *c* oxidase after labeling with EMA. (A) Fluorograph of the gel before staining with Coomassie blue (B). Lane 1: Labeling of the enzyme was performed under native conditions in a buffer containing lauryl maltoside. Lane 2: In this case the protein was first dissolved in gel electrophoresis sample buffer containing SDS. At the level of subunits I and II of the enzyme, no EMA fluorescence was detectable under native conditions, whereas after denaturation of the complex with SDS, labeling of both subunits was observed. Each lane contained about 1 nmol of enzyme. (C) Densitometric trace of lane 2 measured immediately after electrophoresis without any fixation. EMA absorbance was recorded at the wavelength pair 529–600 nm. Numbers indicate the subunits.

1) no modification of cysteinyl residues in either subunit was detected. After denaturation of the enzyme with SDS it was observed that both subunits were labeled (Figure 1A, lane 2). Since the EMA fluorescence intensity is not directly proportional to the amount of labeled residues, the EMA absorption in the gel at 529–600 nm was recorded (Figure 1C). The calculation of the separate absorption integrals of the two peaks revealed that subunit II contained double the amount of EMA with respect to subunit I. Similar analysis using equimolar amounts of beef heart cytochrome *c* oxidase revealed analogous results (data not shown). Comparison based on the sequence results of the bovine enzyme (subunit I has one cysteine and subunit II two) suggests that the *P. denitrificans* enzyme may have similar amount of EMA-reactive cysteines.

Chymotryptic Digestion of the Enzyme. Treatment of *P. denitrificans* cytochrome *c* oxidase with chymotrypsin under nondenaturing conditions resulted in the partial degradation of both subunits (Figure 2). An apparent molecular weight diminution of about 8000 was estimated for both subunits, and the cleavage position was after the cysteinyl residues (as shown by EMA labeling; Figure 2A). The chymotrypsin-treated enzyme was separated from the protease by ion-exchange chromatography on Whatmann DE-52 cellulose. Spectral analysis of the enzyme preparation did not show any visible difference compared with the undigested enzyme (data not shown). The loss of protein during proteolysis was reflected

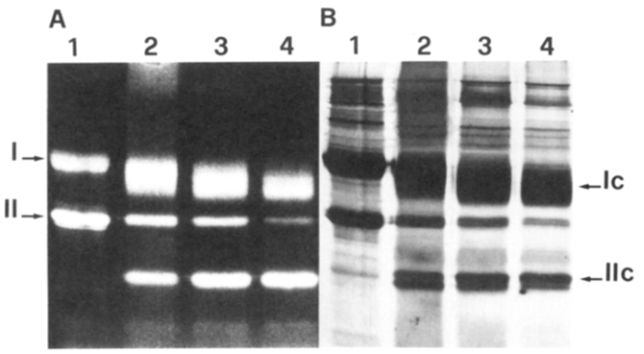

FIGURE 2: SDS–polyacrylamide gel electrophoresis of cytochrome *c* oxidase digested by chymotrypsin. (A) Fluorograph of the gel before staining with silver (B). Lane 1: Undigested enzyme. Lanes 2, 3, and 4: Kinetics of the chymotryptic digestion of cytochrome *c* oxidase 5, 10, and 20 min, respectively, after the addition of chymotrypsin. Conditions as under Experimental Procedures. Each lane contained about 100 pmol of enzyme. The native enzyme, composed of subunits I and II, was digested to a complex still having both subunits (Ic and IIc) but with reduced molecular weight. Both subunits were cleaved at positions following the cysteinyl residues, as shown by EMA labeling and fluorescence.

in the diminution of the 280/424-nm absorption ratio, which diminished from 1.8–2.1 for the intact enzyme to 1.6–1.7 for the chymotrypsin-treated enzyme (values determined in 0.1% dodecyl maltoside). Isoelectrofocusing of this preparation in 1% agarose cylindrical gels with Triton X-100 as detergent showed that both preparations (undigested and chymotrypsin treated) still had the same isoelectric point (p*I* = 6–7). SDS–polyacrylamide gel electrophoresis of the focused bands showed the same subunit composition as that of the nonfocused samples (data not shown). Manual Edman degradation of the N-terminus of the chymotrypsin-treated subunit II showed the following amino acid sequence: threonine, histidine, and asparagine. This sequence was found to correspond to positions 73–75, thus identifying phenylalanine 72 as the chymotrypsin cleavage point. This amino acid is located between the two hydrophobic transmembrane segments of the protein. It is also probable that digestion of the protein occurred at the C-terminal side without proceeding further than cysteines 216 and 220, because these residues were still labeled by EMA in the digested protein (Figure 2A).

Limited Proteolysis with S. aureus V8 Protease. S. aureus V8 protease cleaves proteins at the carboxyl terminus of aspartyl and glutamyl residues (Drapeau, 1976). Treatment of the chymotrypsin-treated enzyme with this protease selectively cleaved subunit II without modifying subunit I. Figure 3A shows an experiment in which the digestion was detected by EMA fluorescence; the fluorescence associated with subunit II (lane 1) was degraded to peptides of low molecular weight (lane 2, bottom). The same experiment is also shown in Figure 3B where the digestion was followed by protein silver staining; lane 3 shows clearly the degradation of subunit II to smaller peptides. The removal of these small fragments was achieved by gel filtration on a Sephadex G-100 or by ion-exchange chromatography on a HPLC poly(ethylenimine)–silica column (Figure 3B, lane 4). This preparation was spectrally characterized (reduced minus oxidized spectra, Figure 4A, and CO reduced minus reduced spectra, Figure 4B) in the visible region. The spectra shown in Figure 4 did not differ from those of the native enzyme. The absorption ratio 280/424 nm diminished further to a value of 1.3–1.5. This would be expected if subunit II were selectively digested. In the one-subunit enzyme the two hemes were present in equimolar amount as calculated from the spectra (Vanneste, 1966). The presence of an unmodified CO difference spectrum suggests that the

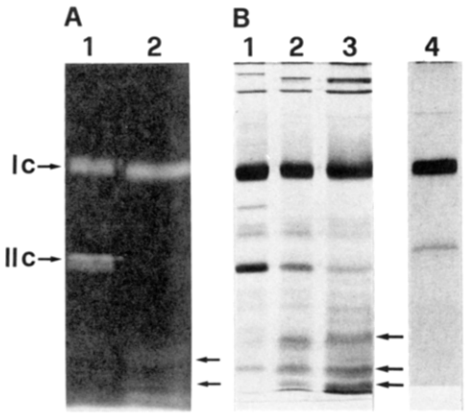

FIGURE 3: SDS–polyacrylamide gel electrophoresis of chymotrypsin-treated cytochrome *c* oxidase digested by *S. aureus V8* protease. (A) Fluorograph of a *S. aureus V8* protease digested enzyme previously labeled with EMA. Lane 1: Chymotrypsin-treated enzyme. Lane 2: Chymotrypsin-treated enzyme treated with *S. aureus V8* protease. (B) Silver-stained gels. Lane 1: Chymotrypsin-treated enzyme. Lanes 2 and 3: Digestion of subunit IIc by *S. aureus V8* protease producing small peptides of it (arrows). Lane 4: One-subunit enzyme after HPLC ion-exchange chromatography; the band was migrating at the level of ovalbumin, which has an apparent molecular weight of 43 000.

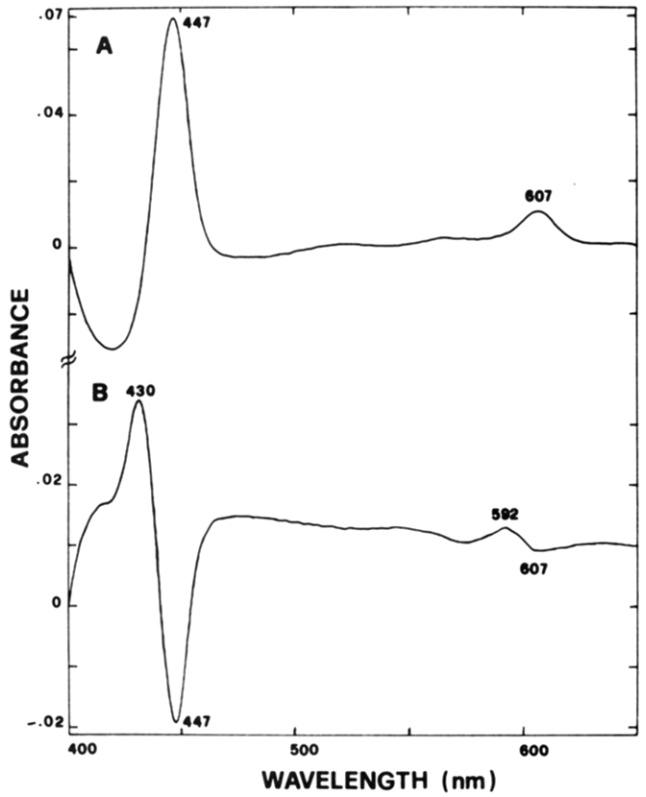

FIGURE 4: Spectral analysis of *P. denitrificans* cytochrome *c* oxidase after digestion with chymotrypsin and *S. aureus V8* protease. (A) Dithionite-reduced minus air-oxidized differential spectrum. (B) Dithionite-reduced (plus CO) minus dithionite-reduced differential spectrum.

binuclear center of cytochrome a_3, where the iron and copper B atoms are located, is intact. Further evidence to support this point comes from the sensitivity to KCN inhibition, which amounted to 70% at 1 μM KCN.

Kinetic Analysis of the Digested Preparations. Figure 5 illustrates the kinetic behavior of the three different enzyme preparations: the undigested, the chymotrypsin-treated, and the one-subunit preparation. In Figure 5A the enzymatic activity was measured in presence of 0.1% Triton X-100 whereas in Figure 5B the assay medium was supplemented

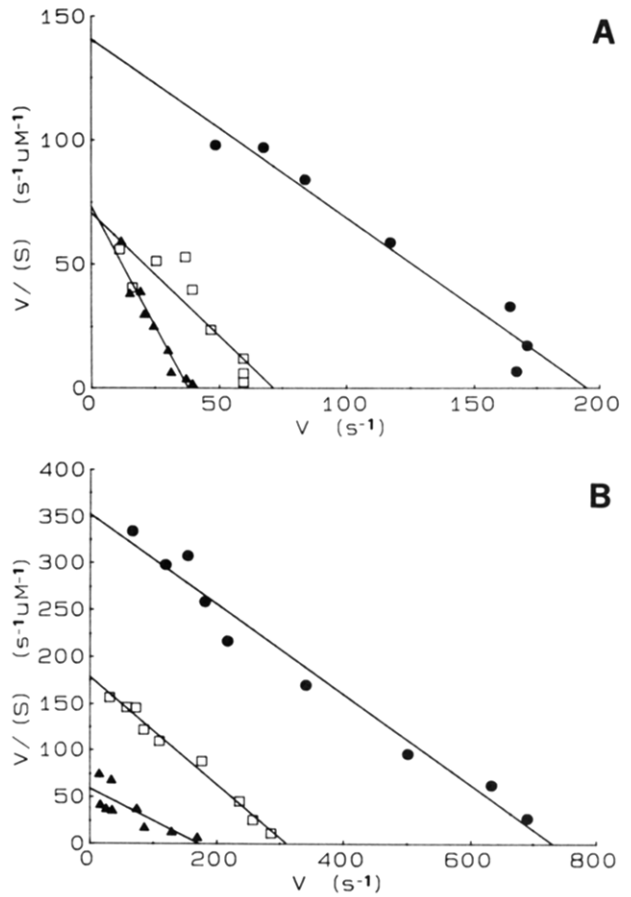

FIGURE 5: Eadie–Hofstee plots of cytochrome *c* oxidase activity measured spectrophotometrically. Ferrocytochrome *c* concentrations were between 0.3 and 25 μM. The molecular activity (V) is expressed as moles of cytochrome *c* oxidized per second per mole of cytochrome aa_3. (A) Cytochrome *c* oxidase activity measured in a medium composed of 10 mM Tris-HCl, 30 mM KCl, and 0.1% Triton X-100, pH 7.4. (B) Cytochrome *c* oxidase activity measured in the same buffer as in (A) supplemented with 2 mg/mL sonicated asolectin. The enzymes were preincubated with asolectin containing buffer before measurement. (●) Undigested enzyme; (□) chymotrypsin-treated enzyme; (▲) *S. aureus V8* protease treated enzyme.

Table I: Kinetic Parameters of the Cytochrome *c* Oxidase Activity of the Undigested and Chymotrypsin and *S. aureus V8* Protease Treated Enzyme Preparations[a]

	0.1% Triton X-100		0.1% Triton X-100 + asolectin (2 mg/mL)	
	K_m (μM)	V_{max} (s^{-1})	K_m (μM)	V_{max} (s^{-1})
undigested enzyme	1.4	196	2.1	731
chymotrypsin-treated enzyme	1.3	85	1.7	310
S. aureus V8 protease treated enzyme	0.5	38	2.8	168

[a] The composition of the measuring medium is given in the legend to Figure 5.

with asolectin lipid (2 mg/mL). The kinetics of the oxidase activity with horse heart ferrocytochrome *c* as substrate for all three preparations under both conditions showed linear Eadie–Hofstee plots. Table I summarizes the kinetic parameters of the different preparations and conditions. For the chymotrypsin-treated enzyme K_m values remained essentially unchanged in Triton X-100 as well in Triton X-100 supplemented with asolectin with respect to the undigested enzyme; V_{max} was about 42–43% of the original activity. Concerning the one-subunit preparation there was a further V_{max} diminution of 65% and 55% in Triton X-100 and in asolectin-sup-

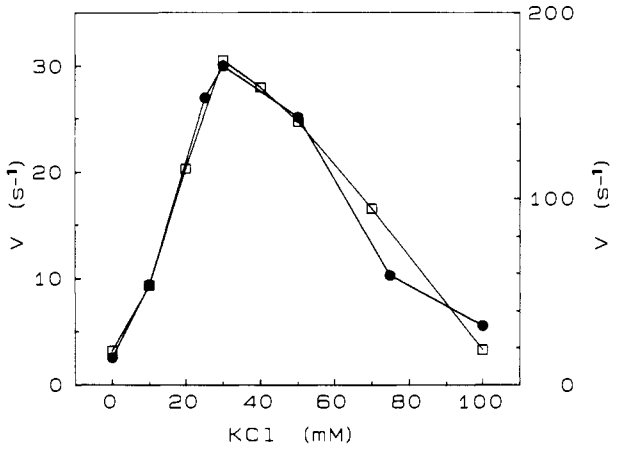

FIGURE 6: KCl concentration dependence of the cytochrome *c* oxidase activity. The activity was measured in a medium as in Figure 5 but containing the indicated amount of KCl. Ferrocytochrome *c* concentration was 10 μM. (●) Undigested enzyme (right *y* axis); (□) *S. aureus V8* protease treated enzyme (left *y* axis).

plemented Triton X-100, respectively, as compared with the chymotrypsin-treated enzyme. In both measuring media small but insignificant changes were observed in the K_m for cytochrome *c*. This suggests that cytochrome *c* in the intact enzyme interacts with subunit I as well as with subunit II (Bisson et al., 1977), as shown by cross-linking and chemical labeling experiments. Evidence further supporting this conclusion is shown in Figure 6: the salt dependence of the activity of the one-subunit enzyme is almost identical with that of the undigested enzyme, with an optimum at 30 mM KCl. The data of activity reported here were obtained with preparations in which the amount of residual subunit II was individually estimated to be lower than 5%. The reduction of V_{max} of the *S. aureus V8* protease treated enzyme with respect to the chymotrypsin-treated enzyme was of about 50% (see above and Table I). Therefore, this activity cannot be explained by the residual amount of subunit II.

DISCUSSION

Reactivity of Cysteine Residues in Subunits I and II. According to the published sequence data, subunit II of *P. denitrificans* oxidase contains four cysteines (Raitio et al., 1987). Using EMA as reagent, we were able to label only two of these which, in analogy with the beef heart enzyme, should be those believed to complex CuA (Müller & Azzi, 1985). The other two cysteines, which did not react with EMA even after exposure of the enzyme to SDS, were possibly modified, for instance, through posttranslational esterification with fatty acids. These two cysteines (51 and 57) are supposed to be located in a hydrophobic transmembranous segment. The same explanation could also be applied to subunit I where, out of a total of three, only one cysteine was labeled by EMA. Nevertheless, EMA labeling appears to be a useful tool to follow the proteolytic degradation of the *P. denitrificans* oxidase.

Digestion of the Enzyme. In this study the sequential proteolytic treatment of *P. denitrificans* cytochrome *c* oxidase, first by chymotrypsin and then by *S. aureus V8* protease, resulted in the formation of a relative stable one-subunit enzyme with a reduced apparent molecular weight of 43 000. It should be mentioned here that the two proteases applied alone or in reversed order were unable to produce the result described for their sequential combination. Chymotrypsin digestion was complete after 60 min, and further incubation overnight under the same conditions was ineffective. The long incubation time and the large amount of protease used in this experiment suggest that the chymotrypsin-sensitive sites are largely inaccessable. Optimal specificity and reproducibility of the peptide fragmentation was achieved when dodecyl maltoside was used to disperse the enzyme. The use of *S. aureus V8* protease with a protein exhibiting biological activity was not free of problems: by itself, this protease has a long inhibition time (40 min), and the preparation is contaminated with other proteases. Nevertheless, it was possible to selectively digest subunit II and produce a one-subunit cytochrome *c* oxidase. The rationale behind this experiment is that subunit II contains a large number of acidic amino acid residues (13 aspartates and 13 glutamates), for which *S. aureus V8* protease is highly specific.

Partial Characterization of the One-Subunit Enzyme. One of the most important findings of this work is that the removal of subunit II did not change the visible spectral characteristics of the remaining oxidase. This indicates that both hemes are located in subunit I of the enzyme. Moreover, both cytochromes *a* and a_3 must be a single polypeptide, as previously described for the two cytochromes *b* of the mitochondrial respiratory chain (Widger et al., 1984). One copper atom (CuB) forms the binuclear center with the heme iron in cytochrome a_3; therefore, it must be present in subunit I. If this were not the case and CuB had been removed by the proteolytic treatment, a spectral change would be expected in the one-subunit enzyme and it would be unable to reduce oxygen to water. The results obtained with *T. thermophilus* oxidase (which has only one subunit, homologous to subunit I of other oxidases and containing two coppers and two heme irons) together with the present results are very suggestive of a general oxidase model in which all catalytic metal centers are associated with subunit I. Yoshida et al. (1984) also found the EPR-detectable copper atom (CuA) in their *T. thermophilus* oxidase preparation. By analogy, the assignment of CuA in *P. denitrificans* oxidase could be shifted from subunit II to subunit I. Indeed, we were able to detect the optical signal of CuA at 820 nm in one-subunit preparation (M. Müller and A. Azzi, unpublished result). The findings of Steffens et al. (1987) suggesting that there are three instead of two copper atoms per oxidase monomer are not inconsistent with the picture emerging from our study. The question of the role of subunit II in the catalysis of the reaction remains open. The kinetic analysis of the reaction showed that the K_m for cytochrome *c* was almost unchanged whereas the V_{max} was diminished by about 75% after digestion of subunit II. Also, the salt dependence of the reaction velocity was unchanged. It seems, therefore, that the electron-transfer reaction of cytochrome *c* occurred with high affinity with subunit I and that subunit II may control the detachment of cytochrome *c* from its interaction site (k_{off}). It should be added that, until now, chemical labeling experiments indicated that the binding site for cytochrome *c* was mainly restricted to subunit II (Bisson et al., 1977; Millet et al., 1983; Kadenbach & Stroh, 1984). In all these studies, however, subunit I appears to be shielded against chemical modification by cytochrome *c*, similarly to subunit II. The latter was previously favored as a binding site because of the absolute magnitude of the shielding effect. Such a result is apparently caused by the presence of a number of aspartyl and glutamyl residues, which are not conserved in *P. denitrificans* (Steinrücke et al., 1987). The problem of the role of the metal center in subunit II requires some further discussion. Work of Chan and collaborators (Stevens et al., 1982) has shown that the ligands (two S and two N, O) present in subunit II complex CuA. This conclusion would be hard to reconcile with the results obtained with the one-subunit *T.*

thermophilus oxidase (Yoshida et al., 1984). Assuming that the spectral and enzymatic properties of the wheat oxidase are not different from those of other homologous enzymes, the involvement of subunit II in binding CuA in this enzyme would be inconsistent with the fact that one of the putative CuA ligands in subunit II, a cysteine, was found to be replaced by an arginine (Bonen et al., 1984).

ACKNOWLEDGMENTS

We are thankful to Dr. D. A. Deranleau, Theodor Kocher Institut Bern, for critical reading of the manuscript.

Registry No. KCN, 151-50-8; cytochrome *c* oxidase, 9001-16-5; cytochrome *c*, 9007-43-6.

REFERENCES

Bisson, R., Gutweniger, H., Montecucco, C., Colonna, R., Zanotti, A., & Azzi, A. (1977) *FEBS Lett. 81*, 147–150.

Bolli, R., Nalecz, K. A., & Azzi, A. (1986) *J. Bioenerg. Biomembr. 18*, 277–284.

Bonen, L., Boer, P. H., & Gray, M. W. (1984) *EMBO J. 3*, 2531–2536.

Broger, C., Allemann, P., & Azzi, A. (1979) *J. Appl. Biochem. 129*, 517–521.

Corbley, M., & Azzi, A. (1984) *Eur. J. Biochem. 139*, 535–540.

Drapeau, G. R. (1976) *Methods Enzymol. 45*, 471.

Fee, J. A., Kuila, D., Mather, M. W., & Yoshida, T. (1986) *Biochim. Biophys. Acta 853*, 153–185.

Gornall, A. G., Bardawill, C. J., & David, M. M. (1949) *J. Biol. Chem. 177*, 751–766.

Holm, L., Saraste, M., & Wikström, M. (1987) *EMBO J. 6*, 2819–2823.

Kadenbach, B., & Stroh, A. (1984) *FEBS Lett. 173*, 374–380.

Laemmli, U. K. (1970) *Nature (London) 277*, 680–685.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Ludwig, B. (1986) *Methods Enzymol. 126*, 153–159.

Ludwig, B. (1987) *FEMS Microbiol. Rev. 46*, 41–56.

Ludwig, B., & Schatz, G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 196–200.

Ludwig, B., Grabo, M., Gregor, I., Lustig, A., Regenass, M., & Rosenbusch, J. P. (1982) *J. Biol. Chem. 257*, 5576–5578.

Millet, F., deJong, C., Paulson, L., & Capaldi, R. A. (1983) *Biochemistry 22*, 546–552.

Müller, M., & Azzi, A. (1985) *FEBS Lett. 184*, 110–114.

Müller, M., & Azzi, A. (1986) in *Membrane Proteins* (Azzi, A., Masotti, L., & Vecli, A., Eds.) pp 109–118, Springer-Verlag, Berlin.

Müller, M., Labonia, N., Schläpfer, B., & Azzi, A. (1987) in *Cytochrome Systems: Molecular Biology and Bioenergetics* (Papa, S., Chance, B., & Ernster, L., Eds.) pp 239–246, Plenum, New York.

Nalecz, K. A., Bolli, R., Ludwig, B., & Azzi, A. (1985) *Biochim. Biophys. Acta 808*, 259–272.

Raitio, M., Jalli, T., & Saraste, M. (1987) *EMBO J. 6*, 2825–2833.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. K., Provenzano, M. D., Fujimoto, E. K., Goeke, M. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76–85.

Solioz, M., Carafoli, E., & Ludwig, B. (1982) *J. Biol. Chem. 257*, 1579–1582.

Steffens, G., & Buse, G. (1979) *Hoppe*-Seyler's *Z. Physiol. Chem. 360*, 613–619.

Steffens, G. C. M., Biewald, R., & Buse, G. (1987) *Eur. J. Biochem. 164*, 295–300.

Steinrücke, P., Steffens, G. C. M., Panskus, G., Buse, G., & Ludwig, B. (1987) *Eur. J. Biochem. 167*, 431–439.

Vanneste, W. H. (1966) *Biochemistry 5*, 838–848.

Widger, W. R., Cramer, W. A., Herrmann, R. G., & Trebst, A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 674–678.

Wikström, M., Saraste, M., & Pentillä, T. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N., Ed.) Vol. 4, pp 111–148, Plenum, New York.

Winter, D. B., Bruynickx, W. J., Foulke, F. G., Grinich, N. P., & Mason, H. S. (1980) *J. Biol. Chem. 255*, 11408–11414.

Yoshida, T., Lorence, R. M., Choc, M. G., Tarr, G. E., Findling, K. L., & Fee, J. A. (1984) *J. Biol. Chem. 259*, 112–123.