Interaction of Cytochrome *c* with Cytochrome *c* Oxidase Studied by Monoclonal Antibodies and a Protein Modifying Reagent†

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ABSTRACT: C/57 black mice were immunized with beef heart cytochrome *c* oxidase, generating 48 hybrid cell lines that secrete antibodies against the different subunits of the enzyme. Immunoblot analysis showed reactions with 7 of the 13 subunits. Among the monoclonal antibodies produced, only those to subunit II gave significant inhibition; these inhibited the enzyme activity completely and prevented cytochrome *c* binding to the enzyme. Epitope mapping studies indicate that a peptide including residues 200–227 reacts with the antibody, suggesting that the C-terminus of the protein is essential for the binding of this antibody. The carboxyl modifying reagent 1-ethyl-3-[3-(trimethylammonio)propyl]carbodiimide (ETC) was chosen to investigate further the relationship between antibody and cytochrome *c* binding domains. ETC caused 50% inhibition of the enzyme activity with a first-order time during the first 20 min; a slower reaction over 3 h resulted in 90% inhibition. Cytochrome *c* binding to the oxidase was inhibited to a similar extent as cytochrome *c* oxidation, and protection against both effects was afforded by the presence of cytochrome *c* during ETC modification. Anion-exchange of FPLC of the modified forms of cytochrome oxidase revealed extensive inhomogeneity, indicating random derivatization of a number of different carboxyls even during the first-order reaction, and precluding identification of carboxyl residues related to a specific phase of the reaction. Cytochrome *c* and the subunit II-specific antibody protected against radioactive labeling of subunit II by ETC in the presence of [^{14}C]glycine ethyl ester, demonstrating that the antibody and cytochrome *c* occupy significant and overlapping areas on the subunit II surface. These results, along with activity and binding studies, strongly support the concept that subunit II provides the primary electron-transfer site for cytochrome *c* on cytochrome oxidase. Since more than one cytochrome *c* binding site is blocked by a single antibody binding to subunit II, it is suggested that two sites are in close proximity.

Cytochrome *c* oxidase [ferrocytochrome *c*:oxygen oxidoreductase (EC 1.9.3.1)] is an intrinsic protein of the inner mitochondrial membrane containing tightly bound phospholipids, heme, and copper centers, and up to 13 nonidentical polypeptides. The 3 largest polypeptides (I–III) are synthesized in the mitochondria while the 10 smaller peptides (IV–XIII) are encoded by the nuclear genome and are transported to the mitochondria posttranslationally.

Subunits I and II appear to contain all the redox centers of the enzyme (Winter et al., 1980; Suarez et al., 1984; Wikstrom et al., 1985). Subunit III has been implicated in proton translocation by several investigators (Casey et al., 1979, 1980; Prochaska et al., 1981), but its role appears to be facilitative rather than essential (Thompson et al., 1985; Puettner et al., 1985; Sarti et al., 1985; Finel & Wikstrom 1986; Gregory & Ferguson-Miller, 1987; Haltia et al., 1991). The function of the smaller subunits (IV–XIII) is not yet known; however, there is evidence that some may be important for tissue-specific regulation of the enzyme function (Merle & Kadenbach, 1982). Another important functional question is the number, location, and role of cytochrome *c* binding sites on the oxidase. Previous cytochrome *c* binding experiments revealed two dissociation constants for cytochrome *c* similar to the two K_m values determined from kinetic analysis (Ferguson-Miller et al., 1976; Garber & Margoliash, 1990), suggesting that the two kinetic phases could be due to two distinct sites active in conversion of substrate into product (Ferguson-Miller et al., 1976; Nicholls et al., 1980). Alternatively, biphasic kinetics could be explained by other mechanisms, including a single binding site that is altered by different conformations of the enzyme (Brzezinski & Malmstrom, 1986) or negative cooperativity between monomers in a dimer (Nalecz et al., 1983).

The location of the cytochrome *c* binding site(s) has been investigated using chemical cross-linking (Briggs & Capaldi, 1978; Erecinska, 1977), photoaffinity-labeled derivatives of cytochrome *c* (Bisson et al., 1980, 1982a), and carboxyl modifying reagents (Seiter et al., 1979; Bisson & Montecucco, 1982; Millett et al., 1982, 1983; Kadenbach & Stroh, 1984). Results of these studies indicate that the high-affinity binding site is located in subunit II, with some of the nuclear subunits constituting part of the interaction domain (Bisson & Montecucco, 1982; Kadenbach & Stroh, 1984). In contrast, the results of Müller et al. (1988) suggest that the K_m for cytochrome *c* is unaltered after proteolytic digestion of subunit II in *Paracoccus denitrificans* cytochrome oxidase. The location and significance of the low-affinity site have yet to be determined. Earlier studies by Vik et al. (1981) suggested that phospholipids could be involved in the binding at the low-affinity site. However, enzyme preparations with less than 1 mol of phospholipid per mole of enzyme show unaltered biphasic kinetics (Gregory, 1988).

In this study, we show that a monoclonal antibody to subunit II of cytochrome *c* oxidase completely inhibits the electron-transfer activity of the enzyme and blocks cytochrome *c* binding. Definition of the minimal epitope of the antibody and studies with the carboxyl modifying reagent 1-ethyl-3-

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[3-(trimethylammonio)propyl]carbodiimide (ETC)[1] provide further insight into the physical location of the cytochrome c binding domains.

MATERIALS AND METHODS

Materials. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) hydrochloride was purchased from Sigma (St. Louis, MO). [^{14}C]Glycine ethyl ester hydrochloride (46.5 mCi/mmol) was from New England Nuclear Research Products (Mount Prospect, IL). Beef heart cytochrome c oxidase was purified according to the method of Suarez et al. (1984). When necessary, the enzyme was further purified by ion-exchange fast protein liquid chromatography (FPLC) on a mono-Q column, using the detergent lauryl maltoside (5 mM) to disperse the enzyme.

Production of Monoclonal Antibodies. Female C/57 black mice (7 weeks old) were injected intraperitoneally with 100 μg each of purified beef heart oxidase emulsified in RIBI adjuvant system. (RIBI Immunochem.) A second injection of 25 μg of enzyme in RIBI adjuvant was given 4 weeks later. The mice were bled from the tail 1 week after the second injection, and the serum antibody response was tested by ELISA (see below). The two mice with the highest response were boosted each with 25 μg of the enzyme, 3 weeks after the second injection. The mice were killed 4 days after the last injection, and the spleens were removed for fusion. Hybridoma production was done essentially as described by De Witt et al. (1982).

Separation of Monomers and Dimers of Cytochrome Oxidase. Size-exclusion HPLC was carried out using a combination of a Zorbax GF-250 column (25 × 0.94 cm) and a GF-450 column of the same dimensions connected in series. The HPLC setup was composed of a Water's Model 600 multisolvent delivery system, a Model 712 Water's intellegent sample processor, and a Spectroflow Model 813 UV detector. All conditions of chromatography were those of Hakvoort et al. (1985), except that 1 mM lauryl maltoside in the high ionic strength buffer (100 mM Tris–acetate, 1 mM EDTA, and 300 mM NaCl, pH 7.5) was used in all cases. The flow rate was 0.8 mL/min.

Tryptic and V8 Digestion. Subunit II was purified from beef heart cytochrome oxidase by reverse-phase HPLC using a Vydac C_4 column as described by Robinson et al. (1989). The subunit II-containing fraction was dried under nitrogen, and the peptide (0.8–1 mg) was dissolved in 600 μL of the digestion buffer which was composed of 100 mM ammonium bicarbonate, 1 mM calcium chloride, and 0.2% SDS, pH 8.2. To 500 μL of the subunit II solution was added 5 μL (12.5 μg) of TPCK-treated trypsin, and the mixture was incubated at 37 °C for 1–3 h in a rocking shaker. The digestion was stopped by incubating the digest in a boiling water bath for 5 min. V8 protease digestion was done at an enzyme to subunit II ratio of 1:100 w/w. The digestion was done at 37 °C for 1–4 h.

Carboxypeptidase Digestion. This was done essentially as described by Klemm (1984) with minor modifications. Purified subunit II (1 mg of protein) was dissolved in 500 μL of 50 mM citrate buffer, pH 6.5, containing 0.1% SDS. Digestion was started by the addition of 20 μg of yeast carboxypeptidase. At different times, 50-μL aliquots were withdrawn, immediately mixed with 10 μL of glacial acetic acid to stop the digestion, and analyzed by SDS–PAGE.

Cleavage of Cysteine Residues. Subunit II was chemically cleaved following the method of Nefsky and Bretscher (1989). The HPLC-purified protein was dissolved in 100 mM Tris-HCl, pH 8.0, containing 8 M urea, 5 mM dithiothreitol, and 0.1% SDS. The modified protein was dialyzed against a 100-fold excess of 50% acetic acid for 2 h and recovered by drying under nitrogen gas. The protein was dissolved in the cleavage buffer (2 M glycylglycine, 100 mM sodium borate, and 8 M urea, pH 9.0). The cleavage reaction was started by the addition of potassium cyanide (1 mM final concentration) and was allowed to go for 12 h at 37 °C. The cleavage product was diluted 20-fold with a 0.05% solution of SDS and concentrated using a concentration device with a cutoff size of 3 kDa (Centricon-3). This treatment removed most of the urea prior to HPLC purification on a Vydac C_4 column for mass spectrometry.

N-Terminal Sequencing. The tryptic peptides were separated by reverse-phase HPLC using a Vydac C_8 column. N-Terminal sequencing was done on a Model 477 pulsed-liquid protein peptide sequencer, with an on-line Model 120 PTH-amino acid analyzer (Applied Biosystems).

Cytochrome c Binding. Excess cytochrome c was separated from the oxidase-bound cytochrome c by gel filtration chromatography as described by Ferguson-Miller et al. (1976). Unless otherwise indicated, beef heart cytochrome oxidase (10 μM aa_3) was incubated for 15 min with 30 μM ferricytochrome c in a total volume of 150 μL made by the addition of 25 mM Tris–acetate buffer, pH 7.9, containing 1 mM lauryl maltoside. Fifty microliters of this mixture was applied to a Sephadex G-75 column (12 × 0.7 cm) previously equilibrated with the same buffer containing 25 μM ferricytochrome c. Fractions (0.5 mL each) were analyzed spectrally for cytochrome c (A_{550}) and cytochrome oxidase (A_{605}) content. Preliminary experiments were conducted to determine the contribution of cytochrome c to the absorbance of the oxidase at 605 nm and the contribution of the oxidase to the absorbance at 550 nm under the conditions of the experiment, and suitable corrections were applied. Following ETC modification (see below), the oxidase solution, originally in 10 mM sodium phosphate, pH 7.0, was diluted with an equal volume of 25 mM Tris–acetate buffer, pH 7.9, and applied to a Centricon-30 membrane concentrator. The resulting enzyme (usually around 50 μM aa_3) was used for cytochrome c binding studies. The effect of the antibody on cytochrome c binding was studied by incubating the enzyme with subunit II antibody, or with control antibody at a 1:2 molar ratio. Under such conditions, over 95% of the enzyme was bound by antibody as determined by immunoprecipitation. After 1-h incubation at room temperature, the enzyme–antibody mixture was made 30 μM in cytochrome c and incubated on ice for 15 min before chromatography. Nonspecific binding of cytochrome c to the antibody was corrected for by doing the same experiment in the presence of the antibody but no oxidase.

Synthesis of ETC. Three hundred milligrams of EDC-HCl was dissolved in 3 mL of 40% potassium carbonate. The resulting solution was immediately transferred to a separatory funnel and mixed with 3 mL of anhydrous diethyl ether. The ether layer, which contains the dissolved EDC, was mixed with dry magnesium sulfate to remove any traces of moisture. The magnesium sulfate was removed by centrifugation and the supernatant transferred to a round-bottom flask to which

[1] Abbreviations: BSA, bovine serum albumin; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbant assay; ETC, 1-ethyl-3-[3-(trimethylammonio)propyl]carbodiimide; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

250 μL of methyl iodide was added. The reaction was left to proceed overnight in the sealed flask with stirring. The resulting ETC precipitate was dried under nitrogen gas and stored in a sealed vial at –20 °C until used.

Reaction of ETC with Cytochrome Oxidase. Unless otherise indicated, the enzyme (10 μM aa_3) and ETC (2 mM) were incubated at room temperature in 10 mM sodium phosphate buffer, pH 7, containing 2 mM lauryl maltoside. ^{14}C labeling of the enzyme subunits was done in the presence of 0.5 mM [^{14}C]glycine ethyl ester as described by Kadenbach and Stroh (1984). The reaction was stopped by the addition of ammonium acetate to 0.1 M final concentration.

Protection of Oxidase from ETC Modification by Antibodies. Cytochrome *c* oxidase (3 μM aa_3) was incubated for 2 h with 400 μL of antibody (control or subunit II-specific) conjugated to Affi-Gel. The mixture was centrifuged, and the pellet was washed 3 times (once with 0.8 mL and twice with 0.5 mL) with 10 mM sodium phosphate, pH 7.0, containing 2 mM lauryl maltoside. The pellet (which contained oxidase bound to the antibody) was resuspended in 0.5 mL of buffer and treated with 10 μL of ETC (4 mM final concentration) plus [^{14}C]glycine ethyl ester for 2 h. The reaction was stopped by the addition of ammonium acetate, and the mixture was centrifuged. The pellet was resuspended in 0.5 mL of buffer, and the pH of the solution was brought to below 3 by addition of hydrochloric acid. After a 5-min incubation to remove the bound oxidase from the Affi-Gel–antibody conjugate, the mixture was centrifuged, and the supernatant was carefully collected and treated with an equal volume of 10% trichloroacetic acid to precipitate the proteins. The precipitate was rinsed with 25 mM Tris-HCl buffer, pH 7.2, containing 5% sucrose and finally dissolved in the electrophoresis sample buffer.

SDS–PAGE and Western Blotting. Beef heart cytochrome *c* oxidase was precipitated with 5% trichloroacetic acid and redissolved in sample buffer, pH 6.8, containing 4% SDS. Electrophoresis was carried out essentially as described by Kadenbach et al. (1983), using 1% methylenebis(acrylamide) in the stock acrylamide monomer solution. Proteins were transferred to nitrocellulose or Immobilon membranes at 60 V and a maximum current of 150 mA for 2 h. The transfer buffer was composed of 12.5 mM Tris, 96 mM glycine, and 20% methanol, pH 8.2. No SDS was added to the transfer buffer, but the filter paper on the cathodal side of the gel was equilibrated in a solution of 0.5% SDS in transfer buffer prior to the assembly of the transfer sandwich. Subsequent blocking and probing with antibody were carried out by standard procedures.

Analysis of the Radioactive Peptides. Following electrophoresis, the gels containing the radioactive peptides were subjected to fluorography as described by Bonner and Laskey, (1974). The dried gel was exposed to a Kodak X-OMAT AR film at –70 °C for 36–48 h.

Miscellaneous. Cytochrome *c* oxidase activity was assayed polarographically as described by Thompson and Ferguson-Miller (1983). Immunoprecipitation of native cytochrome oxidase was done using a 50% suspension of *Staphylococcus aureus* cells coated with rabbit anti-mouse IgG. Protein concentrations were measured by the bicinchoninic acid method (Smith et al., 1985). Antibodies were purified from serum-free cell supernatant on a protein A column as described by Ey et al. (1978). Enzyme-linked immunosorbant assay (ELISA) was done as described by Finney et al. (1984) using an enzyme solution of 20 μg/L to coat the 96-well plate.

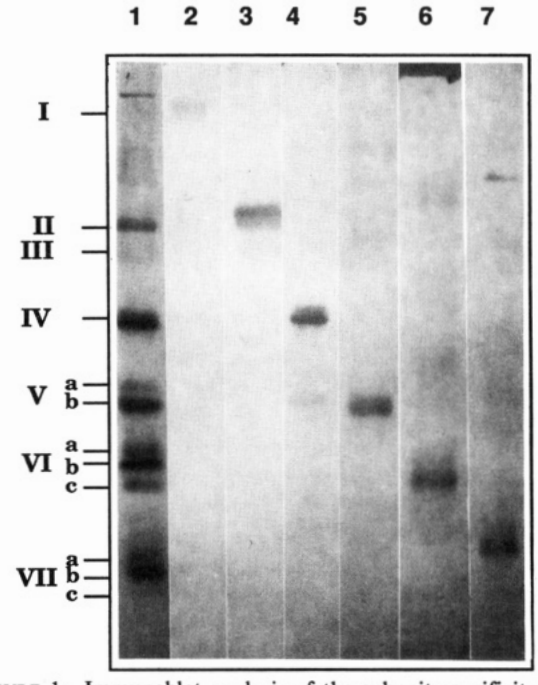

FIGURE 1: Immunoblot analysis of the subunit specificity of the antibodies. Beef heart oxidase (20 μg/lane) was subjected to SDS–PAGE, transferred to nitrocellulose, and probed with the antibodies to subunit I (lane 2), subunit II (lane 3), subunit IV (lane 4), subunit Vb (lane 5), subunit VIc (lane 6), and subunit VIIa (lane 7). Lane 1 shows a Western blot of the enzyme subunits after amidoblack staining. Lanes 2–7 were probed with alkaline phosphatase conjugated goat anti-mouse antibody and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

Generation and Characterization of the Monoclonal Antibodies. Our initial attempts to generate monoclonal antibodies against cytochrome oxidase using Freunds adjuvant, SDS, and urea to disperse the enzyme were not successful. When the enzyme was dispersed in lauryl maltoside and emulsified with the RIBI adjuvant, we isolated from a single fusion 48 hybrid cell lines that produced antibodies to the enzyme. The immunogenicity of the enzyme subunits varied considerably, with subunits IV and II being the most antigenic peptides. Thus, of the 48 antibodies produced, 22 were against subunit IV, 11 were against subunit II, and the rest were scattered among the other subunits. None of the antibodies reacted with subunit II.

The experiments described in this paper were conducted using a subunit II-specific antibody, E_2. Another antibody to subunit II, C_2, gave the same results in all the experiments where it was tested. Neither antibody gave a strong reaction with cytochrome oxidase subunit II of other species. Antibody B_3 against subunit IV was used as a positive control since it immunoprecipitates the native enzyme without affecting its activity. As a negative control, an antibody against rat brain hexokinase (HK) was used (a generous gift from Allan Smith, Department of Biochemistry, Michigan State University). All cell lines used in this study were cloned by limiting dilution (Galfre & Milstein, 1981). Antibodies C_2, B_3, and HK are of the IgG_{2a} subclass, while antibody E_2 is an IgG_{2b}.

Figure 1 is an immunoblot analysis demonstrating the specificity of the antibodies. The appearance of two bands at the subunit IV position is consistent with earlier reports that the lower band is a proteolytic product of the subunit (Merle et al., 1981).

Effect of Subunit II-Specific Monoclonal Antibodies on Cytochrome Oxidase. Despite quantitative immunoprecip-

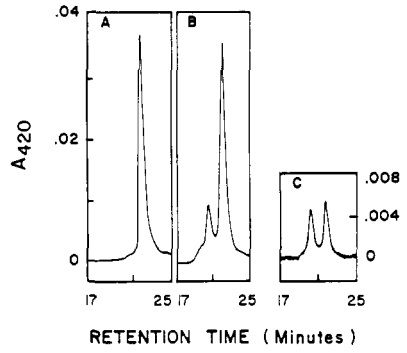

FIGURE 2: HPLC separation of monomers and dimers of beef heart cytochrome oxidase. The separation was done using a combination of a Zorbax G-250 column (25 × .94 cm) connected in series with a G-450 column of the same dimensions. The buffer was composed of 100 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, and 1 mM lauryl maltoside, pH 7.5. In panels A and B, 20 μL containing 0.39 nmol of oxidase from two different preparations was injected to the system. In panel C, the dimer peak from panel B was reinjected. The flow rate was 0.8 mL/min. The molecular mass (in daltons) standards used for calibration of the column are as follows: thyroglobulin (669 × 10^3), ferritin (440 × 10^3), catalase (232 × 10^3), aldolase (158 × 10^3), ovalbumin (44 × 10^3), myoglobin (17.2 × 10^3), and cytochrome c (12.4 × 10^3).

itation (86–95%), initial studies indicated that the subunit II antibody could only inhibit 65% of the enzyme activity. The remaining enzyme activity exhibited normal biphasic kinetics, suggesting the presence of a fully active rather than an altered form of the enzyme. A possible explanation of these results was that some of the enzyme might be present in the dimer form which could be completely immunoprecipitated, but not fully inhibited, by a single antibody binding. The presence of oligomeric forms in our enzyme preparation was examined by size-exclusion HPLC. As shown in Figure 2, the enzyme could be separated into two forms with apparent molecular masses of 550 and 300 kDa, corresponding to the dimeric and monomeric species, respectively (Figure 2B). The content of dimer was estimated at 25%, which would account for much of the discrepancy between binding and inhibition.

An important piece of evidence for the involvement of dimer in the incomplete inhibition would be the demonstration that monomeric cytochrome c oxidase could be completely inhibited by the antibody while the dimer could only be 50% inhibited. The HPLC profile of one beef heart preparation that is predominantly in the monomer form is shown in Figure 2A. The subunit II antibody inhibited 90% of that enzyme activity. The remaining 10% of the activity was insensitive to immunoprecipitation or inhibition, suggesting the presence of altered forms of the enzyme other than dimer. Further purification of the enzyme by FPLC resulted in a 95% inhibitable form. Attempts to purify the dimer form to show that it could be completely immunoprecipitated but only 50% inhibited were not successful since the isolated dimer rapidly reequilibrates between the two forms (Figure 2C).

Since a number of lines of evidence indicate that subunit II provides a binding site for cytochrome c, inhibition of enzyme activity by the subunit II-specific antibody seemed likely to be a result of hindered access of cytochrome c to its electron-transfer site on the oxidase. To test this hypothesis, the effect of the subunit II antibody on cytochrome c binding to the oxidase was examined by gel filtration chromatography using a Sephadex G-75 column. Under these conditions (see Materials and Methods), the native enzyme bound 1.4 mol of cytochrome c/mol of oxidase when incubated in the presence of a control antibody and 0 mol of cytochrome c/mol of oxidase when the subunit II-specific antibody was present. This result supports the idea that the antibody is inhibiting oxidase activity by preventing cytochrome c binding to a site (or sites) on subunit II that is (are) active in electron transfer. Although a second binding site was not completely saturated in these experiments, the results also suggest that two binding sites for cytochrome c are blocked by a single antibody. A complete Scatchard analysis of cytochrome c binding to the native enzyme showed that saturation of a second binding site requires >50 μM cytochrome c (data not shown). This level of cytochrome c could not be used for the antibody experiments because of high nonspecific binding of cytochrome c to the antibody.

To further clarify the relationship between antibody and cytochrome c binding domains, we designed experiments to define the antibody epitope and to compare it with the cytochrome c interaction site on subunit II.

Definition of the Antibody Epitope by Peptide Mapping. Subunit II was purified and dissected by proteolytic and chemical methods, separated into fragments, and probed with the antibody. The identity of the fragments that reacted with the antibody was determined by N-terminal sequencing and mass spectrometry.

The use of reverse-phase HPLC to purify subunit II (Robinson et al., 1989) proved to be the most convenient method for getting sufficient amounts of the protein in a pure form that is readily soluble in the digestion buffer. Tryptic digestion of subunit II resulted in two immunogenic peptides with molecular masses of 15 and 10 kDa. Both peptides were purified and subjected to N-terminal sequencing. The 15-kDa peptide gave an N-terminal sequence of Thr-Met-Gly-His-Gln, and the 10-kDa peptide gave Leu-Leu-Glu-Val-Asp. Similar experiments with V8 protease indicated that another 10-kDa peptide with the sequence Asp-Gln-Arg-Val-Val reacted with the antibody. These peptides, which are assumed from their apparent molecular weights to include the C-terminus, are indicated in Figure 3.

In an attempt to narrow down the epitope, cysteine-specific chemical cleavage of subunit II was performed. Figure 4 (lane a) shows the resulting peptides as resolved by SDS–PAGE. Two peptides were produced in addition to uncleaved subunit II: one of 22–23 kDa and another with a molecular mass of less than 5 kDa. Only the small molecular weight peptide (and native subunit II) was recognized by the antibody, as shown in the immunoblot (lane b). The exact mass of this peptide was determined by fast atom bombardment mass spectrometry to be 3.2 kDa. This peptide can be identified unequivocally as corresponding to residues 200–227, indicating that the minimum epitope is in the C-terminus of the protein. In a further attempt to refine the epitope, subunit II was subjected to carboxypeptidase digestion. It was found that removal of about 1.5 kDa from the C-terminus resulted in loss of reactivity with the antibody (data not shown), suggesting an even smaller minimal epitope strongly dependent on the last 12 residues of the C-terminus.

Despite the strong inhibitory effect of the antibody on cytochrome c binding, the minimal epitope does not include any of the carboxyls that have been implicated in cytochrome c binding (Millett et al., 1983). However, recent studies by Paterson et al. (1990) show that the actual area covered by an antibody in the native protein can be much larger than a minimal epitope.

To define the native epitope, analysis of protection against chemical modification is an appropriate method. In the case of cytochrome oxidase, the carboxyl modifying agent ETC appeared to be a suitable reagent for this purpose, since

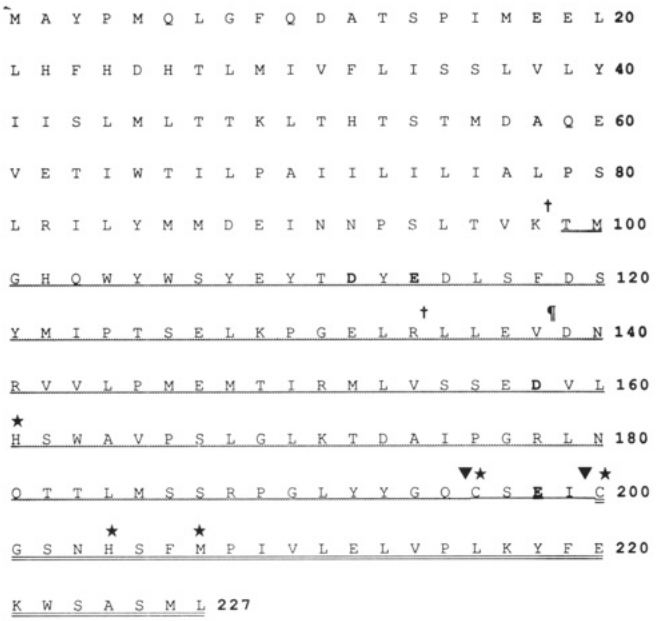

FIGURE 3: Immunogenic peptides of subunit II of beef heart cytochrome *c* oxidase. The cleavage sites of trypsin (†), V^8 protease (¶), and cysteine-specific chemical reaction (▼) are indicated on the complete amino acid sequence. The shortest peptide obtained from enzymatic digestion that reacted with the antibody is underlined, while the minimum immunogenic peptide that resulted from chemical cleavage is double-underlined. Also shown in boldface letters are the carboxyls that may be involved in cytochrome *c* binding. The starred residues have been suggested to be ligands for the Cu_A center. Peptides 99–227 (15 kDa) and 135–227 (10 kDa) resulted from tryptic cleavage. Peptide 138–227 (10 kDa) resulted from V8 protease digestion while peptides 196–227 and 200–227 (3.2 kDa) were produced by chemical cleavage.

previous studies showed that it inhibited oxidase activity by reaction with subunit II and that cytochrome *c* could protect against both modification and inhibition (Bisson & Montecucco, 1982; Millett et al., 1982; 1983; Kadenbach & Stroh, 1984).

Characterization of the ETC Modification Reaction and Products. Before protection experiments were attempted, a careful examination of the modification conditions was performed. It was found that 50% inhibition of the enzyme activity occurred in 20 min with a first-order time course while a slower reaction took nearly 150 min to cause maximal inactivation of the enzyme (Figure 5). Inhibition of activity was accompanied by a parallel reduction in cytochrome *c* binding stoichiometry. The presence of cytochrome *c* protected against both of these effects (Table I),demonstrating that the ETC-modified carboxyl groups were involved in cytochrome *c* binding and electron transfer. Analysis of the kinetics of the partially inhibited enzyme (20-min reaction) indicated that the high-affinity phase of the biphasic kinetic plot was more strongly inhibited than the low-affinity phase. However, repeated kinetic analyses revealed that this differential inhibition was not consistently observed with different enzyme preparations. Furthermore, examination of the ETC-treated enzyme by ion-exchange FPLC revealed a variety of modified forms even after a short exposure time. As shown in Figure 6, there was some physical inhomogeneity present in the original enzyme (panel A), but this was increased in the partially modified (panel B) and the completely modified (panel C) enzyme, showing multiple forms with different enzymatic activity. Attempts to eliminate the inhomogeneity by subjecting the enzyme to FPLC and using the leading peak for ETC modification produced a more homogeneous control, but the modified enzyme again showed multiple peaks (not

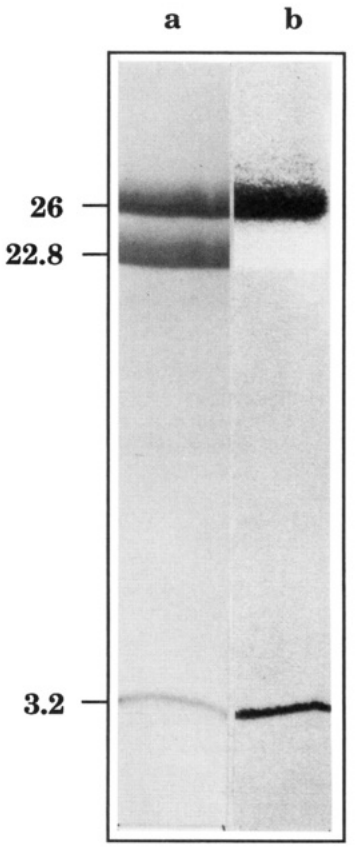

FIGURE 4: SDS–PAGE and immunoblot analysis of the cysteine cleavage products of subunit II. Purified subunit II was dissolved in 600 μL of modification buffer (100 mM Tris, 4 M urea, and 0.1% SDS, pH 8.0) containing 5 mM dithiothreitol. Modification of the cysteine residues was done for 15 min at room temperature, by the addition of 15 mM 5,5′-dithiobis(2-nitrobenzoic acid). The excess reagent was removed by dialysis against 50% acetic acid, and the protein was recovered by lyophilization. The cleavage reaction was done for 12 h at 37 °C, in glycylglycine/borate buffer, pH 9.0, and was initiated by the addition of potassium cyanide to a final concentration of 1 mM. Lane a shows the Coomassie blue-stained gel of the cleavage product while the immunoblot (onto diazotized aminophenyl thioether paper) with the subunit II antibody is shown in lane b.

shown). The time course of ETC modification was indistinguishable before and after FPLC purification (Figure 5), indicating that whatever the source of apparent inhomogeneity of the native enzyme it did not appreciably affect the accessibility of carboxyls. It would appear that a number of carboxyls are equally rapidly and randomly labeled even during the first 20 min of reaction with the apparently first-order time course. Therefore, identification of specific carboxyls that might be related to the high- or low-affinity reactions of cytochrome *c* appeared to be unfeasible.

Protection against ETC Labeling by Cytochrome c and Antibody. Fluorographic studies using the whole enzyme show that a similar degree of protection against ETC modification of subunit II was afforded by both antibody and cytochrome *c* while antibodies to subunit IV and hexokinase did not have this effect. Figure 7 shows a broad radioactive band in the region of subunit II which represents several forms of this peptide with different electrophoretic mobility due to different degrees of labeling with ETC in the presence of [^{14}C]glycine ethyl ester (see Materials and Methods). As previously observed, the electrophoretic mobility of subunit II increases with increased labeling (Millett et al., 1982). In the presence of control antibody (lane 2), there is no change in the labeling pattern. In the presence of the subunit II-specific antibody

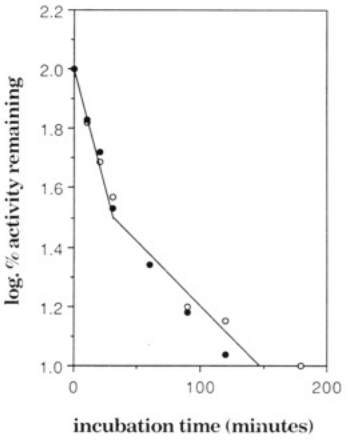

FIGURE 5: Time course of the inactivation of cytochrome oxidase by ETC. Beef heart cytochrome *c* oxidase (10 μM aa_3) was incubated with 2 mM ETC in 10 mM sodium phosphate buffer pH 7.0, containing 2 mM lauryl maltoside. At the indicated times, aliquots of 45 μL were withdrawn, and the reaction was immediately stopped by the addition of ammonium acetate to a final concentration of 100 mM. Enzyme activity was assayed polarographically in 50 mM potassium phosphate buffer, pH 6.5. Other conditions of the assay are as described under Materials and Methods. The open circles represent the enzyme that has been subjected to anion-exchange FPLC prior to ETC modification while the solid circles represent the enzyme that did not receive this treatment.

Table I: Effect of ETC Modification on the Enzymic Activity and Cytochrome *c* Binding to Cytochrome *c* Oxidase[a]

	cyt *c* binding		activity	
experimental conditions	ratio	%	TN (s^{-1})	%
control	2.0	100	220	100
ETC (20 min)	1.2	60	145	65
ETC (3 h)	0.8	40	100	45
ETC (20 min) + cytochrome *c*	1.8	90	ND	ND[b]

[a] The enzyme activity and cytochrome *c* binding were measured in 25 mM Tris–acetate buffer, pH 7.9, as described under Materials and Methods. [b] Activity in 50 mM KH_2PO_4, pH 6.5, was 82% of control.

(Figure 7, lane 3), the radioactive labeling is much reduced compared to lanes 1 and 2 although a small amount of the fast migrating, heavily labeled form of subunit II was still produced. In the presence of cytochrome *c* (Figure 7, lane 4), only a small amount of lightly labeled species, that migrated at the original position of subunit II, was seen. This difference in protective effect could be due to the fact that a small portion of the enzyme is totally unprotected in the presence of antibody because it is uncomplexed, while the majority of the enzyme is completely protected by essentially irreversible antibody binding. In the case of cytochrome *c*, a more complete but less stable complex is formed; thus, the reagent would get access to all enzyme molecules but only to a limited extent.

DISCUSSION

The fact that a monoclonal antibody to subunit II completely inhibits cytochrome *c* oxidase activity and blocks cytochrome *c* binding to the enzyme confirms earlier results which showed that subunit II contains a cytochrome *c* interaction domain (Briggs & Capaldi, 1978; Bisson et al., 1980, 1982b; Bisson & Montecucco, 1982; Millett et al., 1983). Our data provide further evidence that the cytochrome *c* interaction with subunit II is in fact the major electron-transfer site.

Despite the strong inhibitory effect of the antibody on cytochrome *c* binding and oxidation, its binding epitope defined by peptide mapping does not appear to include any of the carboxyls implicated in cytochrome *c* binding by other studies

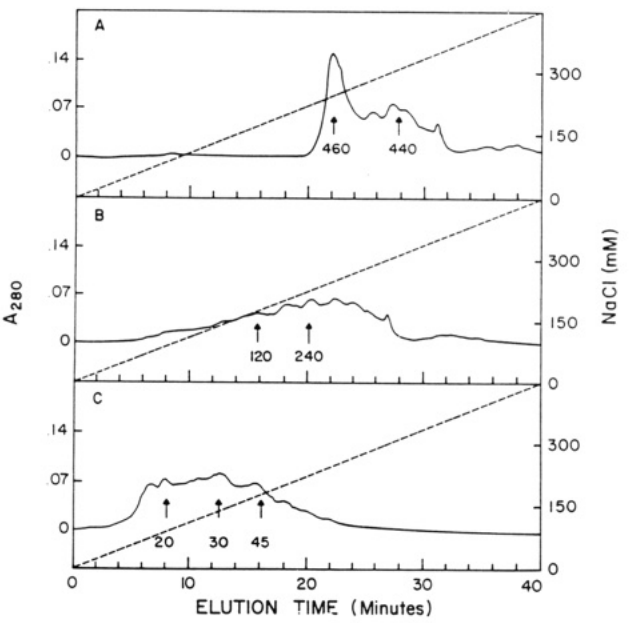

FIGURE 6: FPLC profiles of native and ETC-modified enzyme. Beef heart cytochrome *c* oxidase (10 μM aa_3) was unmodified (panel A) or treated with 2 mM ETC for 20 min (profile B) or 3 h (profile C). In each case, 400 μL of the enzyme solution was equilibrated with 20 mM sodium phosphate buffer, pH 7.0, containing 2 mM lauryl maltoside using a Centricon-30 concentration device. The enzyme was then loaded on a mono-Q 5/5 column previously equilibrated with the same buffer. The collected fractions were analyzed spectrally for oxidase content and polarographically for enzyme activity. The numbers under the arrows denote the turnover number of the enzyme from the fractions eluted at the indicated times.

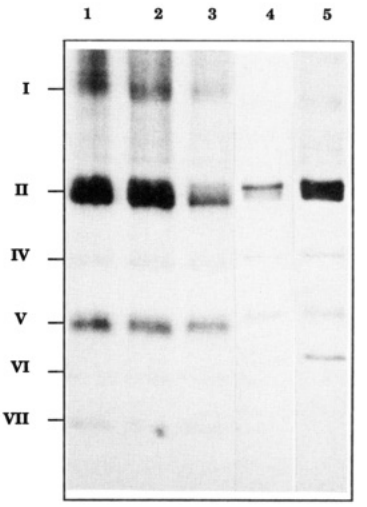

FIGURE 7: Protection by antibody and cytochrome *c* against ETC modification of subunit II. In lanes 1–3, the enzyme (2.5 μM aa_3) was incubated with antibody to hexokinase (1), to subunit IV (2), or to subunit II (3). In each case, the enzyme–antibody complex was treated with 4 mM ETC in the presence of 0.5 mM [^{14}C]glycine ethyl ester. The proteins were precipitated by the addition of trichloroacetic acid and subjected to SDS–PAGE followed by fluorography. In lanes 4 and 5, the enzyme (2 μM aa_3) was incubated in the presence (4) or absence (5) of cytochrome *c* for 2 h. ETC (4 mM) was added for 2 h in the presence of 0.5 mM [^{14}C]glycine ethyl ester. Control antibody (to hexokinase) was also present in this experiment. The enzyme was recovered by centrifuging out the cells, precipitated by the addition of an equal volume of 10% trichloroacetic acid and redissolved in electrophoresis sample buffer.

(Bisson et al., 1982a; Millett et al., 1983). However, the area covered by an antibody is likely to be much larger than the minimal epitope indicated by mapping with denatured peptides. Indeed, antigen/antibody interfaces of 600 Å^2 (Amit et al., 1986) and 750 Å^2 (Paterson et al., 1990) have been

recently defined by X-ray crystallography and two-dimensional nuclear magnetic resonance analysis. In this study, the subunit II antibody afforded a similar level of protection against ETC modification of subunit II as did cytochrome *c*, which is also likely to occupy an area of at least 400 Å^2 (Ferguson-Miller et al., 1978; Rieder & Bossard, 1978). Thus, the ability of the antibody to block cytochrome *c* binding and to prevent ETC modification of subunit II is consistent with the likely size of its interaction domain on the native protein. It is highly likely that the carboxyl residues previously implicated in cytochrome *c* binding are included in the antibody binding domain on the native enzyme even though direct epitope mapping failed to indicate that. A more precise result could be obtained using Fab fragments of the subunit II antibody, but this approach was not attempted due to the need for large quantities of the pure Fab fragments to conduct this type of protection experiment.

We attempted to define the epitope of the antibody on the native protein using the carboxyl modifying reagent ETC. However, the ETC reaction gave multiple species, indicating random modification of a large number of available carboxyls even during a short first-order reaction. The combination of inhomogeneity in the native enzyme and numerous modified forms made it unlikely that singly modified species could be obtained for definitive structure/function analysis. These results emphasize the complexities of dealing with membrane proteins such as cytochrome *c* oxidase which exist in different stages of aggregation and with different degrees of association with phospholipids and detergent (Hartzell et al., 1988).

Whether the binding of one (Antalis & Palmer, 1982; Brzezinski & Malmstrom, 1986) or two (Ferguson-Miller et al., 1976; Errede & Kamen, 1978; Nicholls et al., 1980; Speck et al., 1984; Sinjorgo, et al., 1986) cytochrome *c* molecules on the oxidase is necessary for the biphasic kinetics remains to be clarified. Our data show that a monoclonal antibody to subunit II completely inhibits the enzyme activity and blocks cytochrome *c* binding to the enzyme under conditions where 1.4 mol of cytochrome *c* is bound. These results suggest that there are two cytochrome *c* binding sites on the oxidase in sufficient proximity that a single antibody binding can block both sites. The data do not provide any evidence as to whether the two binding sites are actually involved in the electron-transfer activity.

One model for explaining the biphasic kinetics with only one cytochrome *c* binding site per oxidase monomer proposes that it involves negative cooperativity between monomers (Nalecz et al., 1983); hence, the biphasic behavior should only be seen in the dimer form. However, we observe that antibody inhibition is incomplete in the presence of dimer and that the uninhibited activity is biphasic, and yet the dimer is completely immunoprecipitated. From this we conclude that only one monomer within a dimer has antibody bound and that the other monomer still exhibits biphasic kinetics.

In conclusion, the complete block of activity by a monoclonal antibody to subunit II supports the contention that this peptide provides at least one active site for electron transfer from cytochrome *c* to cytochrome oxidase, and further suggests that a second site, whether it is active or regulatory, is in close proximity to the first.

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