LBL-10187 c.2
Preprint

Lawrence Berkeley Laboratory
UNIVERSITY OF CALIFORNIA

ENERGY & ENVIRONMENT
DIVISION

Submitted to Archives of Biochemistry and Biophysics

EFFECT OF CROSSLINKING ON MITOCHONDRIAL CYTOCHROME c OXIDASE

Maurice Swanson and Lester Packer

December 1979

Prepared for the U.S. Department of Energy under Contract W-7405-ENG-48

DISCLAIMER

EFFECT OF CROSSLINKING ON MITOCHONDRIAL CYTOCHROME c OXIDASE

Maurice Swanson and Lester Packer

Membrane Bioenergetics Group

Lawrence Berkeley Laboratory

and Department of Physiology/Anatomy

University of California

Berkeley, California 94720

ABSTRACT

Purified and reconstituted cytochrome c oxidase and mitochondria were crosslinked with biimidates in the presence and absence of cytochrome c. These experiments indicate that oxidase subunit interactions are required for activity and that cytochrome c mobility may be required for electron transport activity.

Biimidate treatment of purified and reconstituted oxidase crosslinks all of the oxidase protomers except subunit I when ≥20% of the free amines are modified and inhibits steady state oxidase activity. Transient kinetics of ferrocytochrome c oxidation and ferricytochrome a reduction indicates inhibition of electron transfer from heme a to heme a_3. Crosslinking xidase molecules to form large aggregates displaying rotational correlation times ≥1 ms does not affect oxidase activity.

Crosslinking of mitochondria covalently binds the bc_1 and aa_3 complexes to cytochrome c, and inhibits steady-state oxidase activity considerably more than in the case of the purified oxidase. Addition of cytochrome c to the purified oxidase or to c-depleted mitoplasts increases inhibition slightly. Cytochrome c oligomers act as competitive inhibitors of native c, however, crosslinking of cytochrome c to c-depleted mitoplasts or purified oxidase (with dimethyl suberimidate or hetrobifunctional crosslinking reagents) results in a catalytically inactive complex.

Cytochrome c oxidase (ferrocytochrome c:oxygen oxidoreductase, EC 1.9.3.1) is a transmembrane and oligomeric enzymatic lipoprotein complex consisting of 6-10 polypeptides, two copper atoms, and two a-type hemes (1). The oxidase complex has been postulated to be a control point of electron transport (2). It is the only respiratory complex which reacts with both oxygen and with cytochrome c, the only water-soluble protein of the mitochondrial respiratory chain. Thus, information about motion within the oxidase complex and between it and other inner membrane components, particularly cytochrome c, might provide insight into how spatial relationships within and between respiratory complexes could modulate electron flow.

In previous studies from this laboratory, the role of polypeptide and lipid motions in electron transport has been investigated by chemical modification of mitochondrial proteins with imidates, reagents which react with primary amines to form cationic amidines. Such treatments inhibit ATPase and the NADH, succinate, and ascorbate-TMPD[1] oxidase activities of rat liver mitochondria to varying degrees (5,6). Crosslinking by the biimidate reagent dimethyl suberimidate (11 Å between reactive groups) is much more inhibitory than simple amidination by the monoimidate ethyl acetimidate. This has led to the conclusion that biimidates inhibit enzyme activity by limiting molecular motion.

Amidination of mitochondria may result in a variety of modifications including: 1) monofunctional reactions with any primary amine-containing lipids and/or proteins; 2) crosslinking of subunits within enzyme complexes or between identical proteins such as linking cytochrome c to itself; and 3) crosslinking between electron transport complexes or other membrane-associated matrix components such as crosslinking of cytochrome oxidase to cytochrome reductase. In this study, the structural effects of mono- and

biimidates on mitochondria and isolated cytochrome oxidase, and their consequences upon steady-state and transient kinetics of cytochrome oxidase-mediated electron transport, were investigated.

A preliminary account of a portion of this work has appeared (7).

EXPERIMENTAL PROCEDURE

Preparations - Beef heart mitochondria for the isolation of cytochrome c oxidase, kindly provided by Dr. T.P. Singer and H. Tisdale and/or Dr. D.E. Green, were purified by the method of Blair (8). For the study of imidate effects on intact beef heart mitochondria, small scale preparations were performed by the method of Smith (9). Rat liver mitochondria were isolated as described previously (10). Mitoplasts were isolated by rupturing the outer membrane in hypotonic salt (15 mM KCl), and cytochrome c-depleted mitoplasts were then prepared according to Jacobs and Sanadi (11).

For the isolation of phospholipids from both beef heart and rat liver mitochondria, a chloroform-methanol extraction was performed (12). Purified phospholipids were stored at -20° C for no longer than 24 hours prior to use.

For the study of intracomplex motion, cytochrome oxidase was isolated from either mitochondria or Keilin-Hartree particles by a variety of techniques (13-20). Table I shows the detergents and salts which are employed in each of these methods, and the lipid contents and purities obtained; values are within the range reported in the literature. Most experiments employed oxidase preparations obtained by the method of Capaldi and Hayashi (18), and from Keilin-Hartree particles by Van Buuren's procedure (15). Preparations were stored in liquid nitrogen as described (21).

Reagents - Phosphatidyl choline and diphosphatidyl glycerol, from Avanti Biochemicals, were >99% pure. Asolectin, containing 95% soy phosphatides, was from Associated Concentrates, and cholic and deoxycholic acids from Aldrich were recrystallized twice from ethanol. Tween 20 (polyoxyethylene srbitan monooleate), Triton X-100 and X-114, and cytochrome c (Type VI) were from Sigma Chemical Co. Dimethylsuberimidate dihydrochloride (DMS), dimethyladipimidate dihydrochloride (DMA), dithiobisproprionimidate dihydrochloride (DTBP),

methyl acetimidate (MA), methyl butyrimidate (MMBI), p-azidophenylacylbromide (APAB), and 4-fluoro-3-nitrophenyl azide (FNPA) were from Pierce Chemical Co. Gel filtrations resins employed were from Pharmacia Co., and electrophoresis reagents were from Bio-Rad. The spin label, 4-maleimido-2,2,6,6-tetramethyl-piperidinooxyl (MSL) was from Syva Associates.

Chemical-Modification - Stock imidate solutions were prepared by the method of Tinberg et al. (4) using either 0.133 M triethanolamine-0.33 M sucrose or 0.133 M KH_2PO_4-0.33 M sucrose for the mitochondrial preparations (both buffers gave similar results). For oxidase and cytochrome c samples, the same buffers were used omitting sucrose. DMA, DMS, and DTBP were used as 1-40 mM incubation solutions, and MA, MBI, and EA as 2-80 mM. Mitochondria (1-10 mg/ml), isolated oxidase (1-5 mg/ml), and cytochrome c (6.5 mg/ml) were incubated for 30 min. at 20° C. Immediately after the incubation time had expired, the oxidase and mitochondria preparations were either directly assayed for ascorbate-TMPD oxidase activity or dialyzed prior to assay in 50 mM KH_2PO_4, pH 7.4; these procedures led to identical results. Sodium acetate (3.0 M, pH 5.0) was added to hydrolyze excess reagent. Samples were exhaustively dialyzed against deionized water at 4° C prior to SDS gel electrophoresis.

Amidinated cytochrome c was prepared as above except the sodium acetate addition was omitted. Immediately after incubation, preparations were subjected to gel filtration through Sephadex G-75 Superfine in a 0.7 X 30 cm column equilibrated in 50 mM Tris-acetate, pH 7.5 (22).

Spin-labeling with the short-chain maleimide prove (MSL) was performed for 15 min at 20° C using 100 moles MSL per mole aa_3. The samples were then chromatographed in Sephadex G-25 in 50 mM KH_2PO_4, pH 8.5, in order to remove non-covalently bound MSL prior to imidate treatment.

Electrophoretic Analysis - Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out in a Bio-Rad Model 220 Dual Vertical Slab Cell at 50 mA per gel for 90-120 min. Gels were made according to the discontinuous Tris-glycine system of Laemmli (23) using 3% stacking and 12.5% separation gels (acrylamide/bisacrylamide stock, 30/0.8%) for the separation of mitochondrial and cytochrome c samples. For purified and reconstituted oxidase, 5% stacking and 13.5-16.0% gradient gels (acrylamide/bisacrylamide stock, 30/1.6%) were utilized to achieve resolution of smaller molecular weight subunits. Gradient gels were prepared at 4° C with pre-cooled solutions by means of a Buchler gradient former and peristaltic pump. The samples were suspended in a 1-3% SDS solubilization cocktail at 20° C and either not heated, heated at 37° C for 2 hours, or heated at 100° C for 2 minutes. Incubation at 100° C induced aggregation in control oxidase samples but not in mitochondria, while various concentrations of SDS and heating at 37° C did not affect the electrophoretic pattern. Mitochondrial preparations required heating in a solubilization cocktail composed of glycerol, SDS (1%), stacking buffer, mercaptoethanol, and a trace of bromophenol blue tracking dye in order to achieve acceptable resolution. Care was taken to avoid freezing samples at any step in the procedure since this led to irreversible aggregation. Gels were fixed and stained with coomassie blue as described (24) in a stirred Corning ACI electrophoresis chamber. Densitometric analyses (data not shown) of stained gels were performed at 545 nm using a linear transport accessory attached to a Gilford Model 240 spectrophotometer. Gels were calibrated for molecular weight using phosphorylase a, bovine serum albumin, ovalbumin, chymotrypsin A, myoglobin, lyzozyme, cytochrome c, and bovine trypsin inhibitor as standard proteins. Gels of all crosslinked samples were calibrated against appropriate controls

to estimate the amount of protein not entering the separation gel by weighing reproductions of densitometer tracings. The calculation of the oligomer 'spread', which is due to heterogeneous monofunctional attack and hydrolysis of the biimidates, was performed assuming molecular weights for the reacted imidates (DMS = 171.2, DMA = 143.2). For comparison studies, SDS-urea gel electrophoresis was performed according to Downer et al. (25) using a Bio-Rad Model 150A Tube Gel Electrophoresis Cell.

Activity Assays - Two assays were employed to measure cytochrome c oxidase steady-state activity. Polarographic assays were run in 4.0 ml of buffer at 25° C either by the method of Nicholls et al. (26) in the presence of 1% asolectin in 67 mM KH_2PO_4, 0.5% Tween 80, pH 7.4, using 12.5 mM potassium ascorbate, 0.5 mM TMPD, and 2.5 - 25 μM cytochrome c or by the method of Ferguson-Miller et al. (27) for the determination of the dissociation constant for high affinity binding using 25 mM Tris-acetate, pH 7.8 and 7 mM potassium ascorbate, 0.8 mM TMPD, 0.004 - 4 μM cytochrome c and 50 mM cytochrome aa3 (final concentration). In the latter case, monomeric ferro-cytochrome c was obtained by gel filtration (22).

Assays for the activity and orientation of oxidase incorporated into liposomes was performed at 20° C in 50 mM KH_2PO_4, pH 7.5 using 25 mM potassium ascorbate and 1 mg/ml cytochrome c (28). Uncoupling was achieved by adding 0.5 μg/ml valinomycin and 2.5 μM FCCP; orientation was determined by comparing rates of oxygen consumption in the absence and presence of 3% Tween 80 (w/v). The baseline in the absence of cytochrome c was determined in all cases and subtracted from the rates at various c concentrations.

Transient kinetics of ferrocytochrome c oxidation by cytochrome c oxidase were performed spectrophotometrically at 20° C as described (29) in 100 mM KH_2PO_4, pH 7.4 (or 25 mM Tris-acetate, pH 7.8) and 1%

Tween 80. An Aminco-Morrow Stopped Flow attachment, equipped with a 1 cm observation chamber (dead time = 4 msec), on an Aminco DW-2 spectrophotometer was used, and data were collected on a storage oscilloscope.

Analytical - Heme a concentration was determined from the reduced minus oxidized difference spectrum using a $\Delta\varepsilon_{605-630}$ = 13.1 $mM^{-1}cm^{-1}$ (30), and heme a_3 concentration was determined from the reduced versus reduced plus CO difference spectrum using a $\Delta\varepsilon_{428.5-445}$ = 148 $mM^{-1}cm^{-1}$ (30). Cytochrome c concentration was also determined spectrophotometrically using a $\Delta\varepsilon_{540-550}$ = 20 $mM^{-1}cm^{-1}$ (27).

Free amine groups were detected fluorometrically using a Perkin-Elmer MPF 44A fluorescence spectrophotometer following reactions with fluorescamine by a modification of Bohlen et al. (31) employing 1% SDS. Protein concentration was determined by the method of Lowry et al. (32) using defatted bovine serum albumin as a standard. A trichloroacetic acid precipitation modification of the procedure was used when solvent or salt interference was a problem. For analysis of mitochondrial and crude oxidase protein concentration, the rapid biuret procedure was employed (15).

Phospholipid content analysis was performed by a total phosphorous determination of an alkaline chloroform:methanol (2:1) extract and concentraton was expressed as mg/mg protein.

Sulfhydryl content was determined using 5,5'-dithiobis-(2-nitrobenzoic acid) in 0.1% SDS (33).

Midpoint potentials of cytochrome c were measured as described by (34,35) at 20° C. Osmotic sensitivity measurements (3) were performed by incubating mitochondria (0.5 mg - 1.0 mg protein/ml) in either 0.25 M sucrose or deionized water. The samples were allowed to equilibrate for 15 minutes at 25° C, and then 546 nm absorbance was recorded. An empirical index of osmotic sensitivity was taken as the ratio $(Ab_{546}\ (H_2O)/Ab_{546}$ (sucrose).

Liposome Preparation - Liposomes composed of partially pure asolectin, mitochondrial lipids, or purified lipids were prepared by the cholate dialysis procedure (36) or by a sonication procedure (37). The extent of enzyme incorporation into vesicles was assayed by the Ficoll gradient technique (28). Samples having oxidase unincorporated were not utilized.

EPR Spectra - A Varian E-109E X-band spectrometer was employed in the absorption mode for the first harmonic in phase. A 1.0 mm inside diameter glass capillary was used together with a quartz dewar. The temperature was controlled with a Varian E4540 Variable Temperature Controller. Spectra were recorded with a 100 kHz (1 gauss peak to peak amplitude) field modulation. Data were stored and integrated by a PDP 11/34 computer interfaced with the spectrometer.

RESULTS

Purified Cytochrome Oxidase : Crosslinking - To detect crosslinking during biimidate treatment of purified cytochrome oxidase, samples were subjected to SDS gel electrophoresis (Figure 1). Interpolypeptide crosslinking results in the disappearance of bands and the appearance of new bands of lower relative mobilities. Polypeptide aggregates exceeding 110,000 daltons are excluded from the separation gel. To eliminate the possibility that aggregation created the altered electrophoretic patterns seen in the imidate-treated samples, several solubilization methods were used (Experimental Procedures), but the gel patterns showed that extensive crosslinking only occurred with bifunctional imidates. Polypeptide I appears resistant to crosslinking, whereas disappearance of all of the other subunits coincided with the formation of a new band of MW 87,000. This might correspond to a crosslinked aggregate of polypeptides II-VII with an apparent MW of 85,000 $\pm$ 2,000. Treatment of cytochrome oxidase with 5-40 mM DTBP, a cleavable biimidate, results in electrophoretic patterns similar to those shown for 5-40 mM DMS in the absence of a disulfide reducing agent. Addition of mercaptoethanol results in uncrosslinked patterns similar to those shown for MA-treated samples. Unfortunately, DTBP strongly inhibits cytochrome oxidase at very low concentrations (~1 mM), because of H_2S, which is apparently generated during biimidate treatment. Sulfide has been shown to be a noncompetitive inhibitor towards oxygen with a K_i = 0.2 μM (38).

The gradient gels used to separate the oxidase protomers resolves not only the seven components and two contaminants reported by Downer et al. (25), but also higher molecular weight contaminants. These larger polypeptides,

which can be eliminated by Sepharose 4B chromatography, did not affect the crosslinking patterns significantly (data not shown). The number of primary amino groups modified is compared in Table II with the percent of total protein not entering the separation gel. The assay for crosslinking underestimates the amount of interpolypeptide coupling, since if two polypeptide VII's are coupled (MW = 4400), these crosslinked products will still enter the separation gel and be scored as uncrosslinked protein.

Table II

The electrophoretic profile of cytochrome oxidase is not markedly changed when a 1:1 mixture of cytochrome c and oxidase is treated with MA, but small differences are detected in the case of DMS. Subunits II and III stain less intensely at 5 mM DMS when the enzyme is incubated with cytochrome c (data not shown). The variations in staining intensity are small, however, and conclusions as to the cytochrome c binding site(s) on the oxidase complex cannot be drawn because of the high ionic strength at which imidate treatments are performed (27). Sephadex G-75 gel filtration of cytochrome oxidase, which had been incubated with cytochrome c and treated with 5 mM DMS, resulted in the elution of two peaks. The first peak contained all of the cytochrome oxidase and 0.2 - 0.4 cytochrome c molecules/ aa_3 unit, but this fraction did not possess ascorbate-TMPD oxidase activity in the absence of added cytochrome c.

Figure 2

Enzyme Activity - MA and DMS inhibit oxidase activity (Figure 2) although the bifunctional is much more effective at concentrations $\leq$10 mM. Table II compares the enzyme inactivation versus the percent separation gel excluded protein. At DMS concentrations $\geq$ 20 mM, the opacity of the isolated oxidase solutions increased. Sepharose 4B chromatography showed that intermolecular crosslinking occurred. However, increasing the concentration of enzyme in the incubation mixture enhances activity both in control and

20 mM DMS-treated oxidase (Figure 2). Although DMS inhibition of ferro- and ferricytochrome oxidase activity is identical, MA inhibition of the activity of the reduced form is ~15% greater than the oxidized form. The smaller MA inhibition appears to be due to the reaction of 5-10% more free amines in the reduced as compared with the oxidized enzyme. Figure 2 also demonstrates that the addition of cytochrome c to the enzyme during imidate treatment slightly affects the extent of activity inhibition. However, DMS inhibition in the presence of cytochrome c is only 5% more than in its absence, and the disparity between the enzyme activity at a particular degree of amine modification is constant in the two cases. Under pre-steady-state assay conditions, transient oxidation rates of ferrocytochrome c by control and crosslinked preparations (20% free amines modified) are identical with a second order rate constant of ~3 X $10^6 M^{-1} sec^{-1}$.

Imidate treatment of cytochrome oxidase reconstituted into lipid vesicles composed of phosphaticyl choline and diphosphatidyl glycerol resulted in activity inhibition similar to the detergent-solubilized enzyme. If partially pure asolectin or mitochondrial lipids were used for reconstition, higher imidate concentrations were required to obtain a particular degree of activity inhibition and crosslinking due to the presence of primary amine-containing lipids. It is interesting that crosslinking of reconstituted oxidase did not affect the respiratory control ratio displayed by any of these vesicle types (RCR = 3-5, depending on the preparation).

Spin Label Studies - To detect micro-environment alterations in the oxidase complex, a maleimide spin label which binds covalently to a group on the protein independent of imidoester reaction sites was chosen. This label reacts approximately 1000 times more readily with sulfhydryls at pH 7.0 than with lysines (39). It was found that 4-6 spin labels were

attached per aa_3 at pH 7.0. Spin labeling of cytochrome c oxidase with MSL results in an EPR spectrum displaying a strongly-immobilized and weakly-immobilized component (Figure 3). While at low imidate concentrations (10 mM for DMS or 20 mM for MA), the spectrum of spin-labeled oxidase is unaffected , at higher concentrations an increase in the weakly-immobilized signal is detectable. This weakly-immobilized signal increase appears to occur because of a detergent-like effect of imidoester hydrolysis products (40) since spectra obtained after dialysis show that the weakly immobilized signal has returned to the line height of the control for the MA-treated preparation. In agreement with the results of Tinberg et al. (6), the hydrolysis product of DMS, dimethylsuberate, was not inhibitory to ascorbate-TMPD oxidase activity at lower concentrations ($\leq$10 mM) but was at higher concentrations. This indicates that the inhibition of activity at DMS concentrations > 10 mM not only results from crosslinking, but also from other factors such as a possible detergent effect of dimethylsuberate. The strongly immobilized signal in the DMS-treated sample becomes much larger than the weakly-immobilized signal after dialysis. Oxidase activity of spin-labeled and imidoester-treated preparations were identical to samples not labeled.

Mitochondria:Crosslinking - At DMS concentrations $\geq$ 10 mM (Figure 4), some crosslinked material is unable to enter the stacking gel, which implies that the apparent molecular weight of these crosslinked aggregates exceeds 500,000 daltons. MA appeared to disrupt the electrophoretic profile of mitochondria more than in the case of the purified enzyme. Nevertheless, mitochondria treated with 200 mM MA show hyposmotic lysis behavior essentially identical to control preparations as detected by light scattering (Experimental Procedures) indicating that treatment with the monofunctional reagent

does not cause sufficient crosslinking to effect the osmotic properties of the inner membrane. Mitochondria treated with DMA or DMS concentrations $\geq$2 mM were osmotically insensitive. Mitochondria treated with the cleavable biimidate, DTBP, may be lysed only after the disulfide bond is cleaved by dithiotheitol. Crosslinking of oxidase polypeptides to other complexes is believed to have occurred since less than 1% of the electrophoretically resolvable material of whole mitochondria remains in the separation gel at high DMS concentrations. By crosslinking mitochondria at low biimidate concentration (5 mM) and subsequent detergent fractionation and Octyl-Sepharose 4B hydrophobic chromatography (41) it was not possible to purify cytochrome $\underline{aa}_3$ independent of cytochrome bc_1 or to entirely separate cytochrome $\underline{c}$ from cytochrome $\underline{aa}_3$.

Mitochondrial Enzyme Activity - MA and DMS inhibit ascorbate-TMPD oxidase activity in mitochondria (Table III, Figure 5). DMS-induced inhibition is linear up to 60% free amine modification while MA is much less inhibitory. The increased inhibition by MA at 50% free amine modification might result from introduction of the bulky N-alkyl acetamidine group onto protein and/or lipid sites, however, the larger monoimidate, methyl butyrimidate (MBI), increases the inhibition only 5-8% more than MA, while the inhibition pattern remains quire similar. Addition of cytochrome $\underline{c}$ during incubation of mitochondria with MA does not affect inhibition of the activity, but in the case of DMS, inhibition was significantly increased. Hypotonic lysis of the outer membrane and salt-induced removal of cytochrome $\underline{c}$ results in mitoplasts which are less inhibited by biimidate crosslinking whereas the decline of native mitochondrial oxidase activity is intermediate between mitochondria which are $\underline{c}$-supplemented and those which are $\underline{c}$-depleted.

Cytochrome c Crosslinking - Cytochrome c slightly increases DMS inactivation of cytochrome c oxidase. Since cytochrome c contains 19 lysine residues which constitutes 20% of the residues in its primary structure, c could be randomly crosslinked to various portions of the oxidase (or to the inner membrane surface) and inactivate it indirectly, or it could be immobilized at its active site. Cytochrome c was therefore treated with MA or DMS, and the relative abilities of these modified cytochrome c molecules to reduce unmodified purified cytochrome oxidase were assayed. DMS-treated cytochrome c was separated on Sephadex G-75 into monomer and oligomer fractions. SDS gel electrophoresis shows that separation of monomers from oligomers was complete, but some monomers are present in the oligomer fraction (Figure 6). It is noted that in MA- and DMS-treated samples band diffusiveness increases in these gels particularly around the dimer region. This may result from random alkylation of free amines; this has been used to calculate effective 'oligomeric regions' as is shown in Figure 6. Crosslinking at this cytochrome c concentration (0.5 mM) also results in some polymer formation (> decamer). The midpoint potentials ($E_{m7.0}$ = 285± 5 mV) of native, MA, and DMS-treated cytochrome c were unchanged.

A Lineweaver-Burk plot of ascorbate-TMPD oxidase activity (Figure 7) using control and imidoester-modified cytochrome c molecules as substrates, indicates that MA and control samples have nearly identical V_{max} and identical K_m values. The unseparated DMS-treated sample possessed a much lower V_{max} and higher K_m, but when the DMS-treated was separated by column chromatography, the monomer fraction which was recovered was only slightly inhibited while the oligomer fraction was very inhibited. One possibility is that oligomer formation was decreasing the effective concentration of ferrocytochrome c. This possibility appears to be eliminated

by the finding that oligomeric c is a partial competitive inhibitor or hative c with a K_i = 5.4 μM. Complete separation of oligomeric from monomeric cytochrome c was acheived at low ionic strength (FIgure 3). Eadie-Hofstee-Scatchard plots demonstrate that the K_d for the high affinity site is similar for native, MA, and $DMS_{monomeric}$-c (3×10^{-8} M), but much greater for $DMS_{polymeric}$-c (1.1×10^{-6} M).

Heterobifunctional Crosslinking Studies - Since cytochrome c covalently bound to its high affinity interaction site on cytochrome oxidase might inhibit electron transport between heme c and a, attempts were made to crosslink c to oxidase using the combination of methyl mercaptobutyr imidate (MMBI) and p-azidophenylacylbromide (APAB) (42). The advantage of this two-step crosslinking reaction is that disulfide exchange between MMBI-labeled cytochrome c SH groups and cysteine residues on the oxidase does not occur, and the crosslink is stable to disulfide reducing conditions. When modified cytochrome c and cytochrome oxidase are combined and chromatographed on a Sephadex G-75 column at low ionic strength, they elute together in the range 0.8 - 1.1:1 ($c:aa_3$). This complex is not cytochrome c stably bound to its high affinity interaction site (27), however, it is expected that a significant proportion of the total c population does remain bound at this site. After activation of the nitrene (42), this mixture was again chromatographed on Sephadex G-75 in 50 mM KH_2PO_4, pH 7.4. All of the cytochrome c which is not covalently bound should dissociate and elute later, however, a significant amount of the modified cytochrome c remained bound to the oxidase ($c:aa_3$ ratio in the first elution peak equals 0.5-0.6:1). Precipitation of the enzyme with ammonium sulfate (0.35 saturation) did not alter the concentration of cytochrome c with respect to the enzyme.

Margoliash and coworkers (27) have shown that cytochrome c bound to its

high affinity site on cytochrome oxidase can be reduced by ascorbate plus TMPD but not by ascorbate alone at low ionic strength in a buffer containing no competitive ions, such as phosphate. Polarographic tracings of experiments conducted in 25 mM Tris-cacodylate, pH 7.8, with ascorbate plus TMPD as reductants show that oxygen utilization is quite low, however, addition of native cytochrome c appreciably increases the rates in both buffers. This suggests that c is not bound to its high affinity interaction site. Similar results were obtained with another heterobifunctional reagent, 4-fluoro-3-nitrophenyl azide (FNPA), although modification of cytochrome c by this reagent significantly increases the K_d for the high affinity site (K_d 0.13×10^{-6} M). These samples were also subjected to SDS gel electrophoresis. The cytochrome c-oxidase complex showed decreased staining intensity of both subunits II and III.

DISCUSSION

Experiments using chemical crosslinking (3,4,5,6) and other techniques (21) indicate that long-range motion of cytochrome oxidase with respect to other components in the respiratory chain is not required for activity. Some degree of motion is, however, clearly essential for a fully functional enzyme, and for it to interact normally in the electron transport chain. The problem is to identify the degree to which such motion is required and the specific groups involved on the interacting proteins.

Interaction and Motion of Oxidase Polypeptide Subunits and the Role of Lipid - Crosslinking of purified oxidase inhibits heme *a* to a_3 electron transfer. Thus, under pre-steady conditions, transient ferrocytochrome *c* oxidation and ferricytochrome *a* reduction is similar for control and crosslinked preparations. Bonaventura et al. (43), who also investigated transient kinetics of ferrocytochorme *c* oxidation by control and crosslinked oxidase, arrived at similar conclusions. Furthermore, they have demonstrated that the rate of CO ligation to heme a_3 is identical for both the control and crosslinked enzyme.

Intermolecular crosslinking of oxidase with DMS did not appear to affect enzyme activity. Oxidase can be isolated as a 'membranous' patch (44,45) in which it is regularly ordered in a two-dimensional crystalline array (46,47). Highly purified oxidase preparations when reconstituted into lipid vesicles also display a tendency to aggregate (21), and there have been speculations (1) that the active enzymatic unit is a dimer of four hemes and four copper atoms. Hackenbrock and Hammon (48) have provided evidence by freeze-fracture electron microscopy that cytochrome oxidase may exist in an aggregated form within the mitochondrial inner membrane.

The experiments reported here indicate that intermolecular corsslinking of oxidase does not affect cytochrome c accessibility to the heme c-heme a electron transfer site. This implies that intermolecular corsslinking creates, or possibly stabilizes, regularly ordered cytochrome oxidase aggregates. Thus using saturation transfer EPR techniques, Swanson et al (21) have recently demonstrated that the rotational correlation time for oxidase reconstituted into lipid vesicles is insensitive to biimidate-induced intersubunit crosslinking. Oxidase could be reconstituted in a relatively immobile aggregated patch ($\tau_2 \geq 1$ ms), or in a highly mobile form ($\tau_2 = 40$ μs), but activity of the mobile and immobile oxidase complexes were identical ($V_{max} = 400$ electrons/s), and reconstituted vesicles displayed high respiratory control. Kunze and Junge (49) have shown by linear dichroic measurements that the cytochrome a_3 component of the oxidase complex displays no rotational motion $\leq$ 100 ms. Taken together, these results imply that cytochrome oxidase exists in an aggregated form in the inner mitochondrial membrane.

Our studies (21) also show that the fluidity of lipids adjacent to the oxidase depends on the rotational mobility of the protein. If oxidase is not aggregated when it is reconstituted into vesicles, lipid spin probes covalently bound to the protein do not display a strongly immobilized EPR spectral component, whereas if the protein displays little rotational motion a strongly immobilized lipid component is also observed. However, the activity of cytochrome oxidase with highly fluid and strongly immobilized lipid is identical. Hence, lipid fluidity is not required for electron transport through the oxidase complex.

Intercomplex Motion and Cytochrome c-Oxidase Interactions - Crosslinking of oxidase to cytochrome c reductase occurs concurrently with inhibition of

ascorbate-TMPD oxidase activity in mitochondria. The question which arises is whether crosslinking of the oxidase and reductase complexes to each other is, in part, responsible for the inhibition of enzyme activity or whether the results may be entirely explained by crosslinking of the oxidase subunits which we have shown to be inhibitory. Chemical modification results with mitochondria and isolated oxidase cannot be directly compared because of unavoidable differences in reagent accessibility and amine content. Assuming that the inner membrane contains 25-29% (50) of the total mitocondrial protein, and 20% of this is oxidase protein (1), then oxidase comprises 5% of the total mitochondrial protein. Since the lysine content of purified oxidase (4-5%) and mitochondria (6%), and the amine phospholipid content are similar, then at a specific percentage of free amine modification the inhibition of mitochondrial oxidase activity should be at least an order of magnitude less than purified oxidase if the two preparations are incubated wth imidates at the same heme a concentration. The assumptions are that oxidase polypeptides are not more accessible than the other complexes' polypeptides, and that cytochrome c accessibility remains similar in the two cases. Experiments reported here demonstrate that the inhibition of mitochondrial oxidase activity is considerably more than expected based on cmparative studies with the purified oxidase. This suggests that intercomplex crosslinkng also affects cytochrome oxidase activity.

Rendon et al. (51) have reported that rat liver mitochondria treated with DMS at 0° C retain respiratory control. Proton permeability, state IV succinate oxidase and adenine nucleotide translocase activities were not affected by crosslinking when assayed at 30° C, but ATPase and ADP-stimulated succinate oxidase activities were inhibited. We have demonstrated that when oxidase vesicles having high respiratory control are treated with DMS,

ascorbate-TMPD oxidase activity is inhibited but respiratory control is not affected. Hence, crosslinking minimally perturbs membrane integrity but inhibits enzyme activities differentially.

Addition of cytochrome c during biimidate treatment increases the extent of inactivation, but our results using heterobifunctionally-derivatized cytochrome c indicate that it is crosslinked at sites which do not interfere with the binding of native c. Formation of covalent complexes of cytochrome c oxidase results in very little endogenous electron transport.

Bisson et al. (52) have used FNPA to derivatize cytochrome c. This reagent suffers from the disadvantage that it changes positively-charged lysine residues to uncharged secondary amines, and elimination of positive charge near the oxidase high affinity binding domain on the c molecule is known to alter c-aa_3 binding interactions (27). These investigators also noted that FNPA-c-aa_3 complexes were incapable of electron transport in the absence of added native cytochrome c. Erecinska et al. (53) have used a 2,4-dinitro-5-fluorophenyl azide (DNPA)-cytochrome c derivative which displayed an apparent K_m for the reaction with cytochrome oxidase 5-6 fold greater than native c. The addition of these DNPA-c molecules to cytochrome c-depleted rat liver mitochondria, and light activation of the nitrene, resulted in a 1:1 (c:aa_3) complex. In contrast to our results and those of Bisson et al. (52), DNPA-c-aa_3 complexes were active. Moreover, electron transport rates were only slightly stimulated by added native c.

Recently, Rosen et al. (54) have crosslinked horse heart cytochrome c to isolated Rhodopseudomonas sphaeroides (R-26) reaction centers (RC) using DTBP. RC-DTBP-cytochrome c complexes displayed fast cytochrome oxidation kinetics even at high ionic strength. However, none of these crosslinking

studies (52,53,54) have involved examinations of the possible rotational and/or lateral mobility of covalently attached cytochrome c. Since all of these crosslinking reagents are ≈10 Å or greater in length, cytochrome c covalently linked to any enzyme complex may still move laterally over this distance, and of course, the rotational mobility of cytochrome c may be unaffected by crosslinking.

A Model of Cytochrome c-Cytochrome Oxidase Interactions in the Inner Mitochondrial Membrane - Margoliash and coworkers (27,54,55) have demonstrated by selective chemical modification and antibody studies that interaction domains on the cytochrome c molecule for the reductase and oxidase are essentially identical. Brown and Wuttrich (56) have shown that cytochrome c binds to phosphatidyl choline-diphosphatidyl glycerol vesicles by its 'backside' (near residue 60) and causes clustering of diphosphatidyl glycerol. Our studies indicate that covalent bonding of cytochrome c to oxidase can be obtained, but that this complex is incapable of electron transfer activity. These results indicate that cytochrome c rotational and/or lateral movement on the inner membrane surface may be required for electron shuttling. To expalin our results, we suggest that the ferricytochorme c dipole axis (57) preferentially orients toward its high affinity interaction site near heme c_1 on the reductase. When electron transfer results in the reduction of heme c, charge distribution of the high affinity interaction site changes and this results in dissociation of c from the reductase and rebinding to phospholipid sites. The c dipole axis reorients towards the high negative charge density on the oxidase interaction site and binding occurs; electron transfer then takes place between heme c and heme a.

ACKNOWLEDGMENT

We wish to thank Dr. Lars Chr. Petersen for collaboration during the early part of this work; and Dr. T.P. Singer and H. Tisdale; and Dr. D.E. Green for providing beef heart mitochondria used in these studies.

*This work was supported by the Department of Energy (Energy and Environment Division of the Lawrence Berkeley Laboratory) under contract No. W-7405-ENG-48.

The abbreviations employed are: TMPD, N,N,N',N'-tetramethyl phenylenediamine dihydrochloride; DMS, dimethyl suberimidate; EA, ethyl acetimidate; DMA, dimethyl adipimidate; DTBP, dithiobisproprionimidate dihydrochloride; MA, methyl acetimidate; MBI, methyl butyrimidate; MMBI, methyl mercaptobutyrimidate; APAB, p-azidophenylacylbromide; FNPA, 4-fluoro-3-nitrophenyl azide; MSL, 4-maleimido-2,2,6,6-tetramethyl piperidinooxyl; FCCP, carbonyl cyanide, p-trifluoromethoxyphenylhydrazone; aa_3, cytochrome c oxidase; DNPA, 2,4-dinitro-5-fluorophenyl azide.

REFERENCES

1. Capaldi, R.A. and Briggs, M. (1976) in "The Enzymes of Biological Membranes", (Martonosi, A., ed.) pp. 87-102, Plenum Press, New York.
2. Erecinska, M. and Wilson, D. (1978) Arch. Biochem. Biophys. 188, 1-14.
3. Tinberg, H.M. and Packer, L. (1976) in "The Enzymes of Biological Membranes" (Martonosi, A., ed.) pp. 171-198, Plenum Press, New York.
4. Tinberg, H.M. and Packer, L. (1979) Methods Enzymol. 56, 622-629.
5. Tinberg, H.M., Lee, C., and Packer, L. (1975) J. Supramol. Struct. 3, 275-283.
6. Tinberg, H.M., Nayudu, P.R.V., and Packer, L. (1976) Arch. Biochem. Biophys. 172, 734-740.
7. Swanson, M., Peterson, L. Chr., and Packer, L. (1977) in "Mechanisms of Oxidizing Enzymes" (Singer, T.P. and Ondarza, R.N., eds.) pp. 251-260, Elsevier/North-Holland, New York.
8. Blair, P. (1956) Methods. Enzymol. 10, 78-81.
9. Smith, A. (1965) Methods Enzymol. 10, 81-86.
10. Stancliff, R.C., Williams, M.A., Utsumi, K., and Packer, L. (1969) Arch. Biochem. Biophys. 131, 629-642.
11. Jacobs, E.E. and Sanadi, R. (1960) J. Biol. Chem. 290, 531-534.
12. Bligh, E.G. and Dyer, W.J. (1959) Can.J. Biochem. Physiol. 37, 911-920.
13. Yonetani, Y. (1965) Methods Enzymol. 10, 332-335.
14. Kuboyama, M., Yong, F.C. and King, T.E. (1972) J. Biol. Chem. 247, 6375-6383.
15. Van Buuren, K.J.H. (1972) Ph.D. Thesis, University of Amsterdam, Gerja, Waarland.
16. Fowler, L.R., Richardson, S.H., and Hatefi, Y. (1962) Biochim. Biophys. Acta 64, 170-173.

17. Wharton, D.C. and Tzagoloff, A. (1965) Methods Enzymol. 10, 245-250.

18. Capaldi, R.A. and Hayashi, H. (1972) FEBS Lett. 26, 261-263.

19. Errede, B., Kamen, M.D. and Hatefi, Y. (1978) Methods Enzymol. 53, 40-47.

20. Hartzell, C.R. and Beinert, H. (1974) Biochim. Biophys. Acta 368, 318-338.

21. Swanson, M.S., Quintanilha, A., and Thomas, D.D. (1979) J. Biol. Chem. submitted.

22. Brautigan, D.L., Ferguson-Miller, S., and Margoliash, E. (1978) Methods Enzymol. 53, 128-164.

23. Laemmli, U.K. (1970) Nature 227, 680-685.

24. Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.

25. Downer, N.W., Robinson, N.C. and Capaldi, R.A. (1976) Biochem. 15, 2930-2936.

26. Nicholls, P., Peterson, L.C., Miller, M., and Hanson, F.B. (1976) Biochim. Biophys. Acta 449, 188-196.

27. Ferguson-Miller, S., Brautigan, D.L. and Margoliash, E. (1978) J. Biol. Chem. 253, 149-159.

28. Carroll, R.C. and Racker, E. (1977) J. Biol. Chem. 252, 6981-6990.

29. Gibson, Q.H., Greenwood, C., Wharton, D.C., and Palmer, G. (1965) J. Biol. Chem. 240, 888-894.

30. Vanneste, W.H., Ysebaert-Vanneste, M., and Mason, H.S. (1974) J. Biol. Chem. 193, 265-275.

31. Bohlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213-221.

32. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

33. Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70-77.

34. Dutton, P.L. (1971) Biochim. Biophys. Acta 226, 63-80.

35. Cammack, R. and Palmer, J.M. (1977) Biochem. J. 166, 347-355.

36. Racker, E. (1972) J. Membrane Biol. 10, 221-235.

37. Hansen, F.B., Miller, M., and Nicholls, P. (1978) Biochim. Biophys. Acta 502, 385-399.

38. Petersen, L. Chr. (1977) Biochim. Biophys. Acta 460, 299-307.

39. Means, G.E., and Feeney, R.E. (1971) in "Chemical Modification of Proteins", Holden-Day, San Francisco.

40. Peters, K. and Richards, F. (1977) Ann. Rev. Biochem. 46, 523-551.

41. Rosen, S. (1978) Biochim. Biophys. Acta 523, 314-320.

42. Erecinska, M. (1977) Biochem. Biophys. Res. Commun. 76, 495-501.

43. Bonaventura, C., Bonaventura, J., Brunori, M. and Wilson, M. (1978) FEBS Lett. 85, 30-34.

44. Jacobs, E.E., Andrews, C., Cunningham, W., and Crane, F.L. (1966) Biochem. Biophys. Res. Commun. 25, 87095.

45. Sun, F.F., Prezbindowski, K.S., Crane, F.L., and Jacobs, E.E. (1968) Biochim. Biophys, Acta 153, 804-818.

46. Vanderkooi, G., Senior, A.E., Capaldi, R.A. and Hayashi, H. (1972) Biochim. Biophys. Acta 274, 38-48.

47. Henderson, R., Capaldi, R.A., and Leigh, J.S. (1977) J. Mol. Biol. 112, 631-648.

48. Hackenbrock, C.R. and Hammon, K.M. (1975) J. Biol. Chem. 250, 9185-9197.

49. Kunze, U. and Junge, W. (1977) FEBS Lett 80. 429-434.

50. Altman, P.L. and Katz, D.D. (1976) in "Cell Biology", Fed. Am. Soc. Exp. Biol., Bethesda, MD. pp. 144.

51. Rendon, A., Rott, R., and Avi-Dor, Y. (1979) IUB Symp. #93, Abstr. 69(A).

52. Bisson, R., Azzi, A., Gutweniger, H., Colonna, R., Montecucco, C., and Zanotti, A. (1978) J. Biol. Chem. 253, 1874-1880.

53. Erecinska, M., Vanderkooi, J.M., and Wilson, D. (1975) Arch. Biochem. Biophys. 171, 108-116.

54. Rosen, D., Okamura, M.Y., and Feher, G. (1979) Biophys. J. 25, 204a.

55. Speck, S.H., Ferguson-Miller, S., Osheroff, N., and Margoliash, E. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 155-159.

56. Osheroff, N., Jemmerson, R., Speck, S., Ferguson-Miller, S., and Margoliash, E. (1979) J. Biol. Chem., in press.

57. Brown, L.R. and Wüthrich, K. (1977) Biochim. Biophys. Acta 468, 389-410.

58. Koppenol, W.H., Broonland, C.A.J., and Braams, R. (1978) Biochim. Biophys, Acta 503, 499-508.

59. Eytan, G.D., Carroll, R.C., Schatz, G., and Racker, E. (1975) J. Biol. Chem. 250, 8598-8603.

60. Yu, C.A., Yu, L., and King, T.E. (1977) Biochem. Biophys. Res. Commun. 74, 670-676.

61. Freedman, J.A., Tracy, R.D., and Chan, S.H.P. (1979) J. Biol. Chem. 254, 4305-4308.

TABLE I

Beef Heart Cytochrome c Oxidase

Isolation Procedure	Detergents	Salts	Lipid Content mg/mg protein	Heme a Concentration nmoles/mg protein
Yonetani(13)[a]	Cholate	$(NH_4)_2SO_4$	0.15	8.6
1 preparation			0.10	8.1-8.5
Kuboyama et al(14)[a]	Cholate	$(NH_4)_2SO_4$	0.26	9.5
1 preparation	Tween 20		0.20	11.0
Van Buuren(15)[a]	DOC	KCl	0.26-0.31	8.6-11.7
3 preparations	Cholate	$(NH_4)_2SO_4$	0.20-0.30	10.0-12.0
Fowler et al(16)[b]	DOC	KCl	0.31-0.42	8.2-9.1
2 preparations		$(NH_4)_2SO_4$	0.30-0.40	8.4-8.7
Wharton & Tzagoloff (17)[b] 1 preparation	DOC	KCL	0.35	8.0
	Cholate	$(NH_4)_2SO_4$	0.30	7.7-8.5
Capaldi & Hayashi(18)[b]	DOC	KCl	0.15-0.32	8.0-10.1
5 preparations	Cholate	$(NH_4)_2SO_4$	0.17-0.25	9.4-10.6
Errede et al(19)[b]	DOC	KCl	0.35-0.42	9.0-10.5
3 preparations	Cholate	$(NH_4)_2SO_4$	0.30-0.40	10.5-10.9
Hartzell & Beinert (20)[b] 3 preparations	Triton	KCl	0.02-0.06	10.0-11.8
	Cholate	$(NH_4)_2SO_4$	0.02-0.05	13.0-14.0

Numbers in brackets refer to literature values.

[a] Starting preparations were: Keilin-Hartree particles or [b] beef heart mitochondria

TABLE II

Imidate Treatment of Oxidized Cytochrome c + Purified Oxidase

Imidate	Concentration	Primary Amino Group Decrease	Separation Gel Excluded Protein	Oxidase Activity Remaining
	(mM)	(%)	(%)	(%)
MA	10	12	<1	99
	80	66	<1	55
MBI	10	30	<1	100
	80	54	<1	47
DMS	5	20	5	62
	40	64	23	11

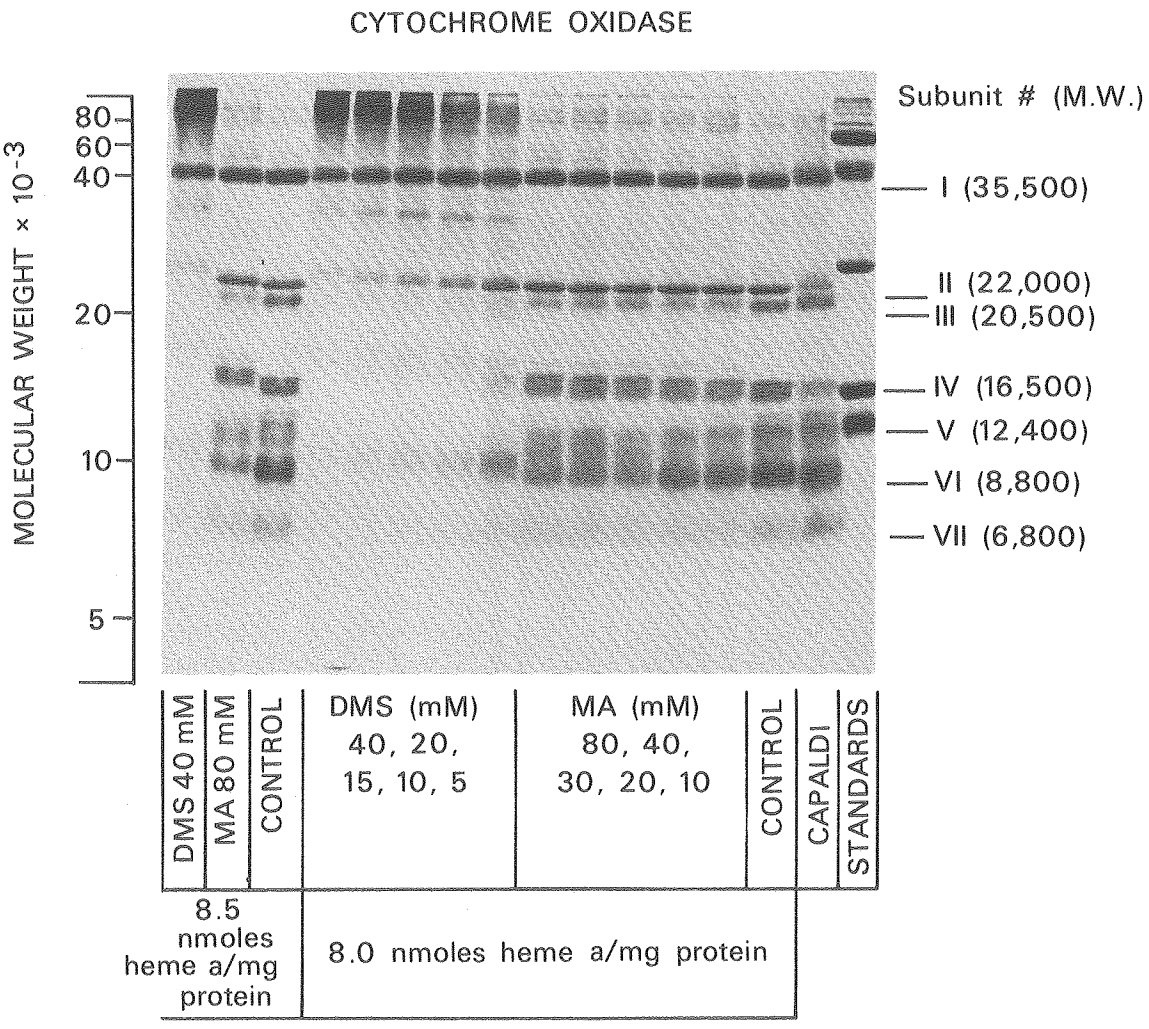
Values are the mean of at least five determinations.

TABLE III

Imidate Treatment of Purified Cytochrome Oxidase and Cytochrome c-depleted Beef Heart Mitochondria

Sample	Imidate	Concentration (mM)	Primary Amino Group Decrease (%)	Separation Gel Excluded Protein (%)	Oxidase Activity Remaining (%)
Purified Oxidase	MA	10	6	1	98
		80	67	1	54
	DMS	5	8	5	71
		40	67	23	18
Beef Heart Mitochondria	MA	10	31	15	96
		80	75	40	78
	DMS	5	17	24	89
		40	60	72	53

Values are the mean of at least five determinations.



XBB770-11453

FIGURE 1

SDS gel electrophoresis of control and imidoester-treated cytochrome c oxidase. Purified oxidase (10 μM) was incubated at various imidate concentrations, dialyzed and then 30 μg protein were loaded per channel. The channel marked 'Capaldi' was the generous gift of Dr. R. Capaldi.

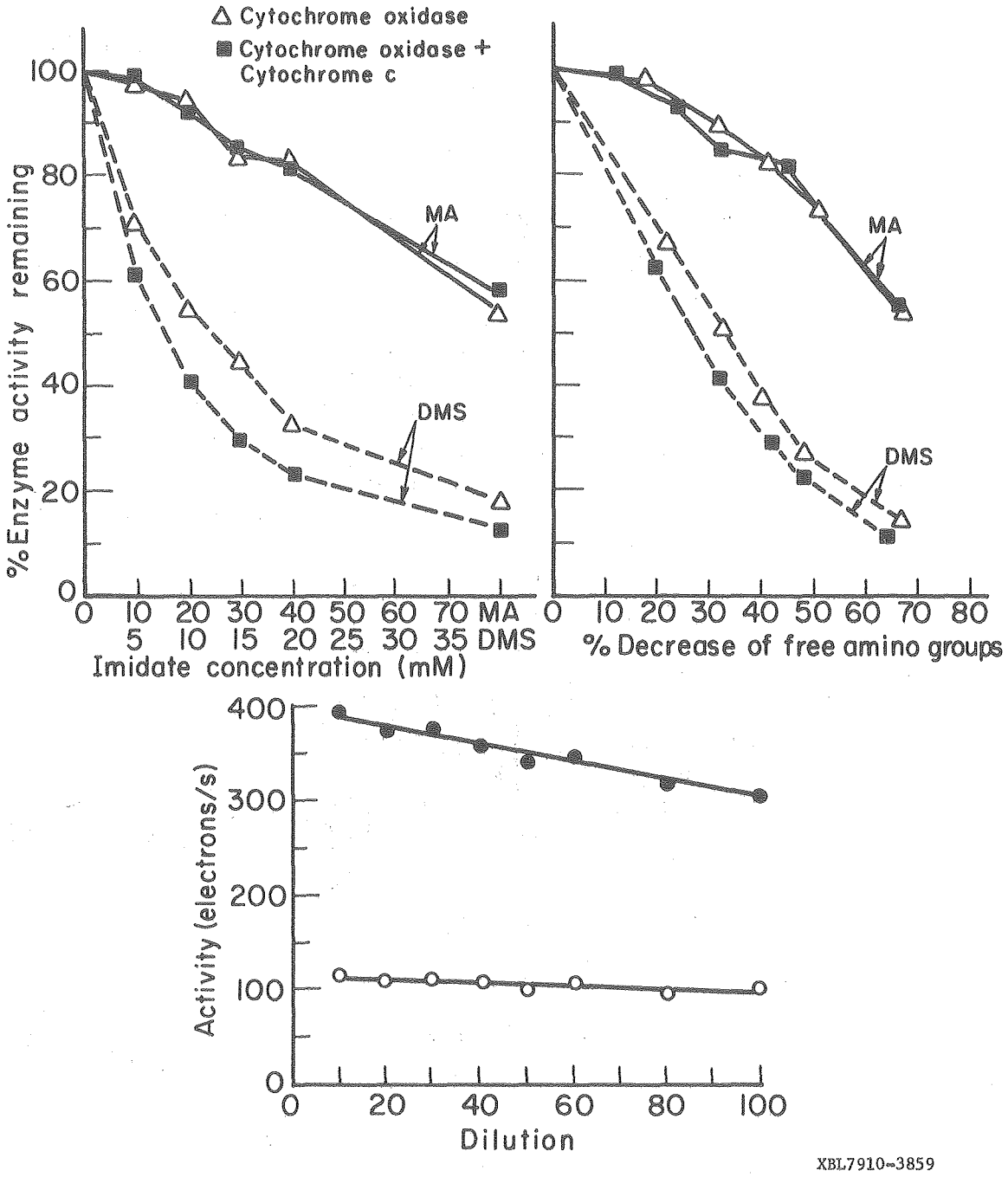


FIGURE 2

Inhibition of ascorbate-TMPD oxidase activity by imidate treatment of cytochrome oxidase. Cytochrome oxidase (10 μM) was incubated either in the absence or presence of cytochrome c (10 μM). When the effect of dilution was studied, the incubation concentration of cytochrome oxidase varied between 0.15 to 15 μM

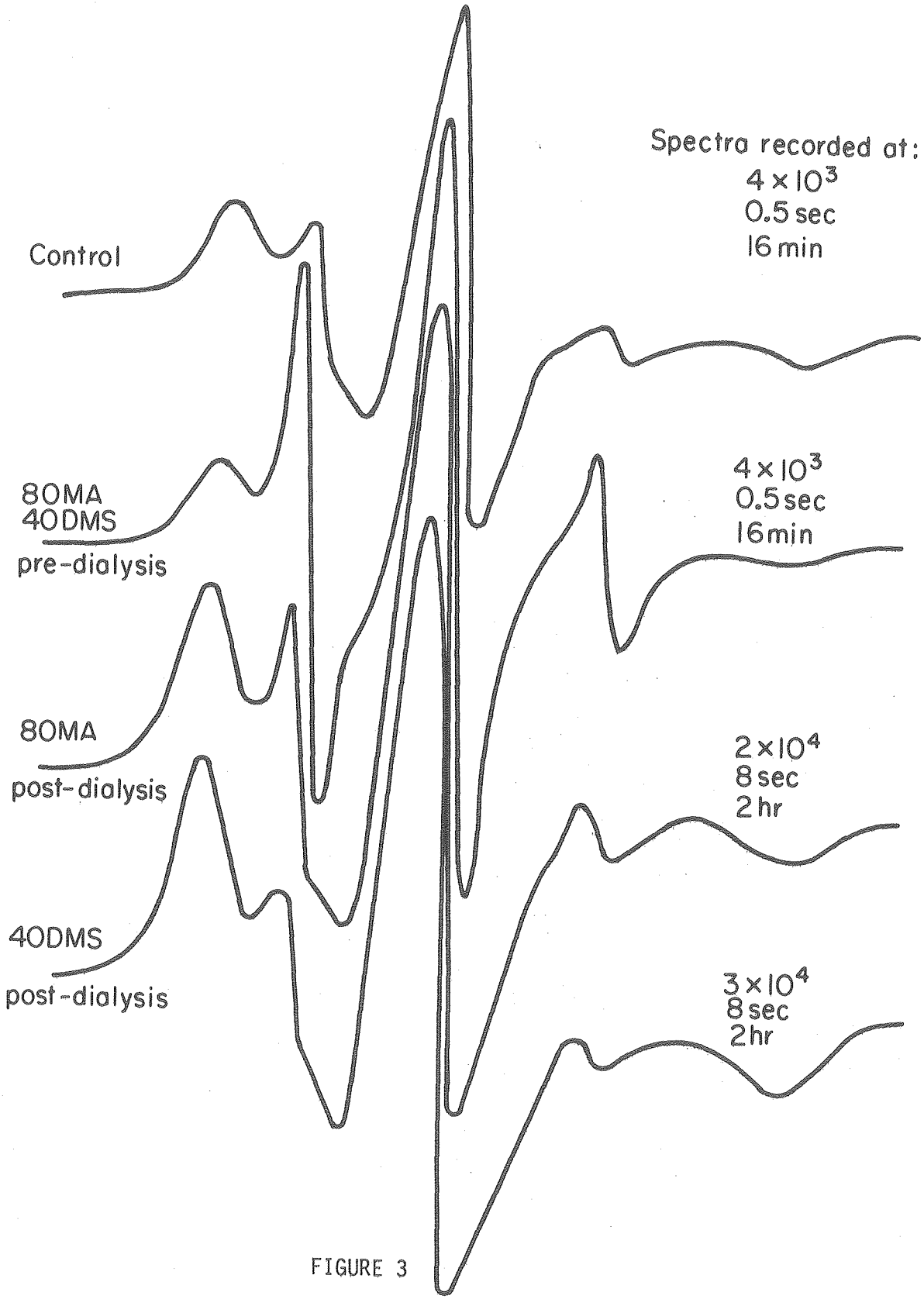
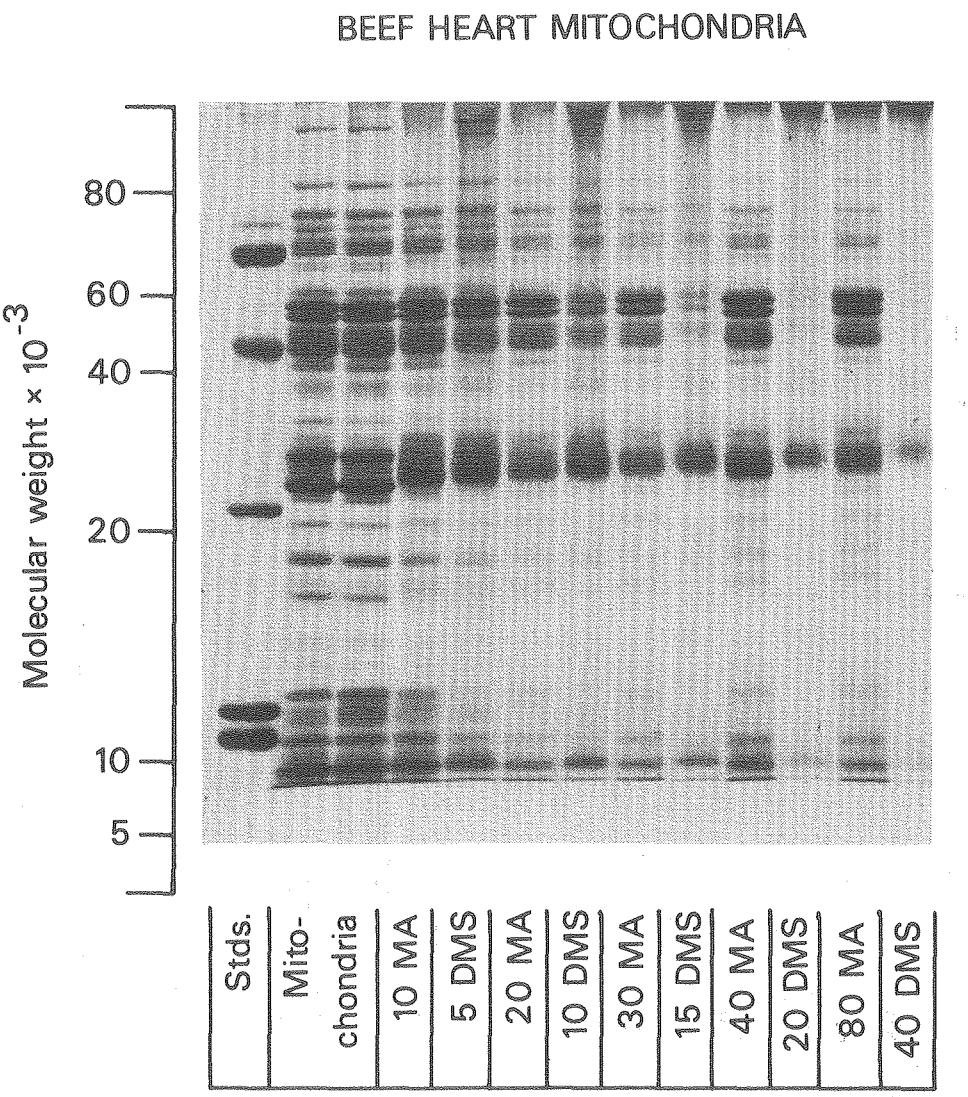


FIGURE 3

XBL799-3796

Electron paramagnetic resonance spectra of control, MA, and DMS-treated cytochrome oxidase labelled with a short-chain maleimide spin label. Spectra were recorded before and after dialysis against 50 mM KH_2PO_4, pH 7.5, 0.25% Tween 20, and 0.05% cholate.



XBB799-12718

FIGURE 4

SDS gel electrophoresis of control and imidate treated beef heart mitochondria. Mitochondria (10 mg/ml) were incubated with various imidate concentrations, dialyzed, and then 30 µg were loaded per channel

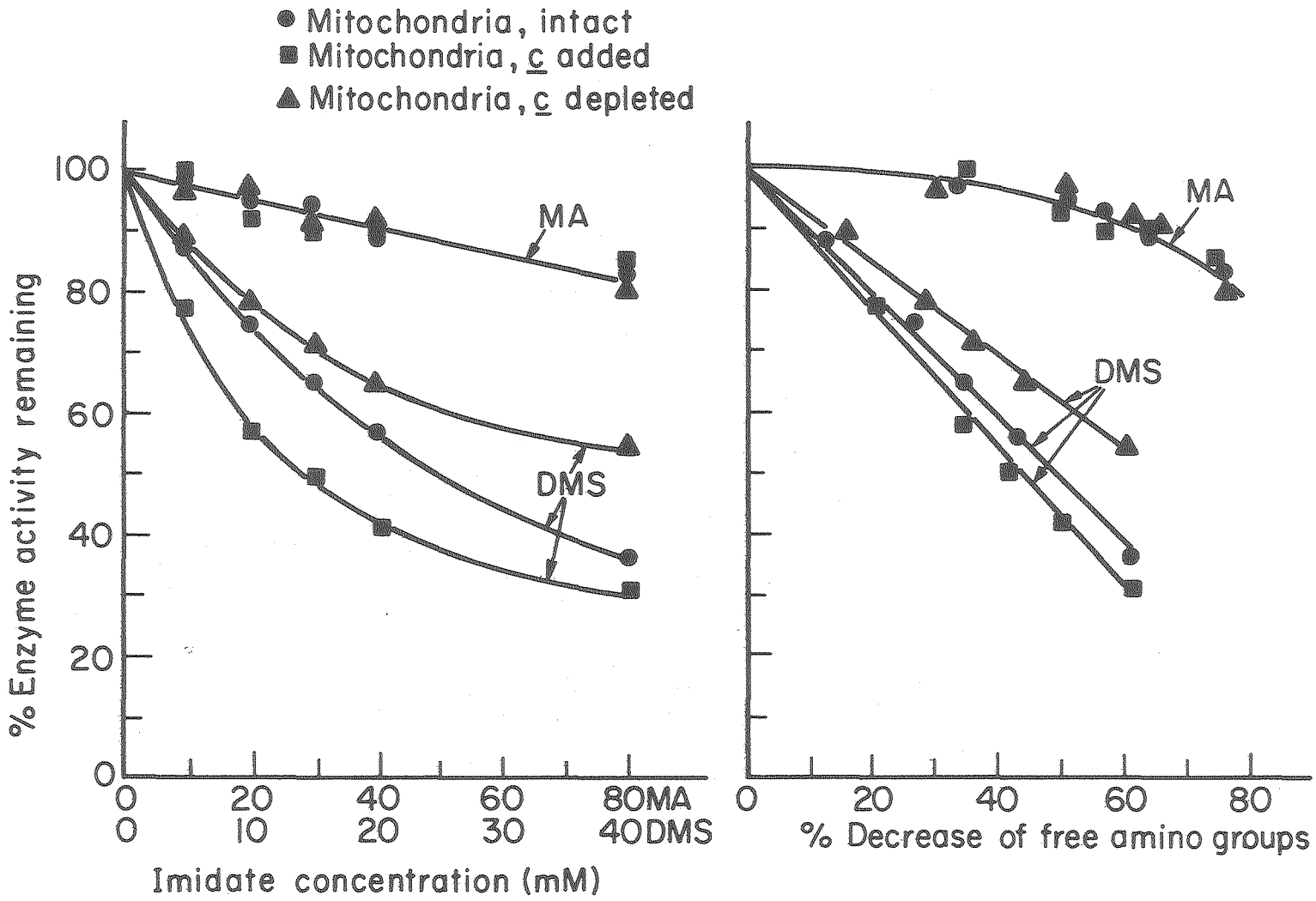


FIGURE 5

XBL799-3795

Inhibition of ascorbate-TMPD oxidase activity in intact, cytochrome c-depleted, and cytochrome c-supplemented beef heart mitochondria. Mitochondria were at 10 mg/ml protein in the incubated mixture.

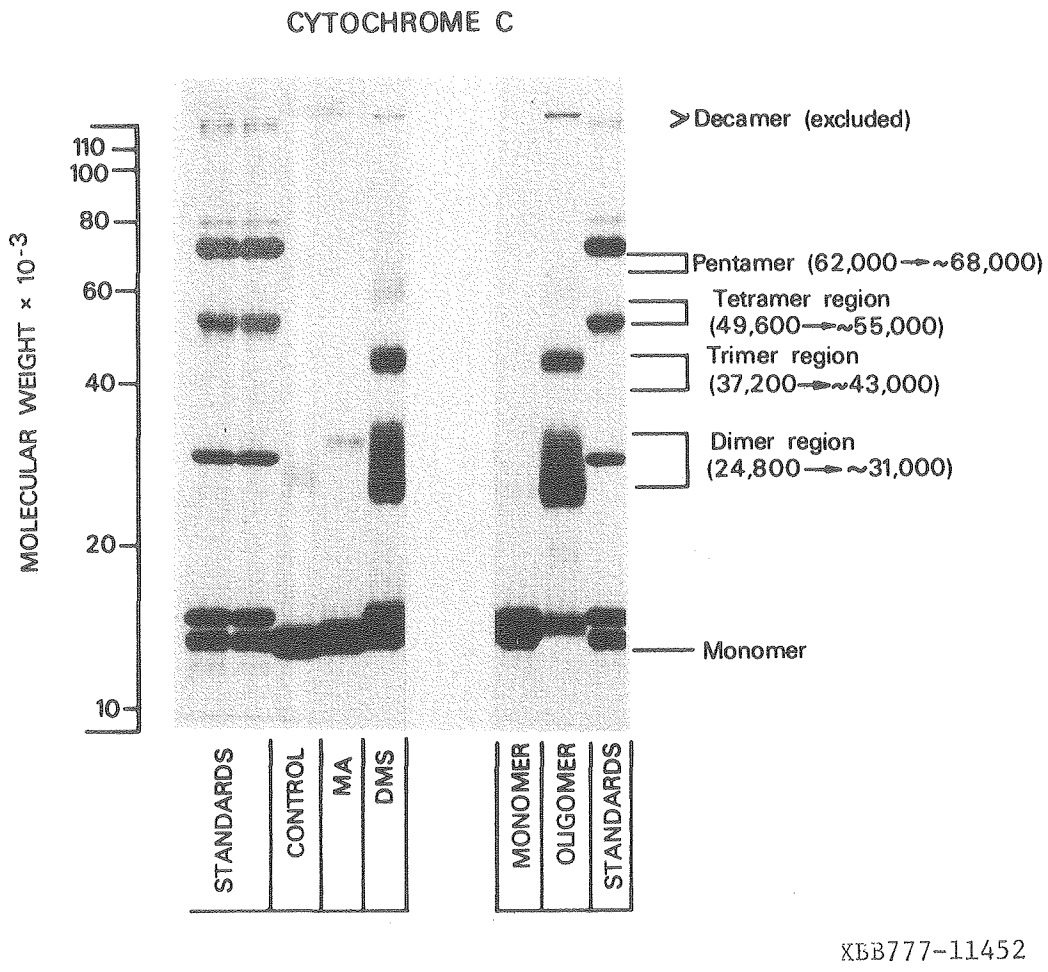


FIGURE 6

SDS gel electrophoresis of imidate-treated cytochrome c. Each channel was loaded with 40 µg protein.

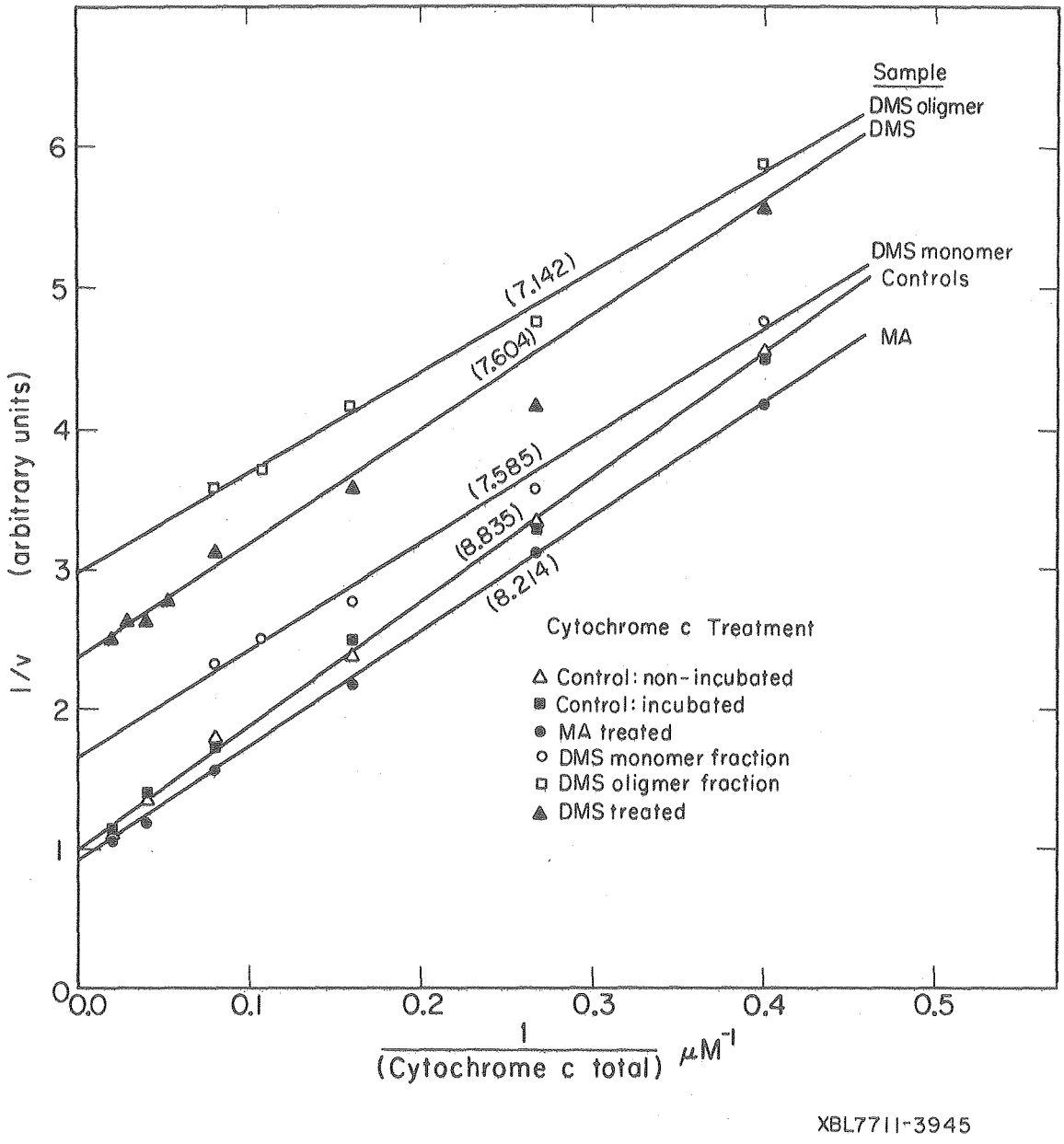


FIGURE 7

Double reciprocal plot of ascorbate-TMPD oxidase activity using MA- or DMS-treated cytochrome c as substrate.

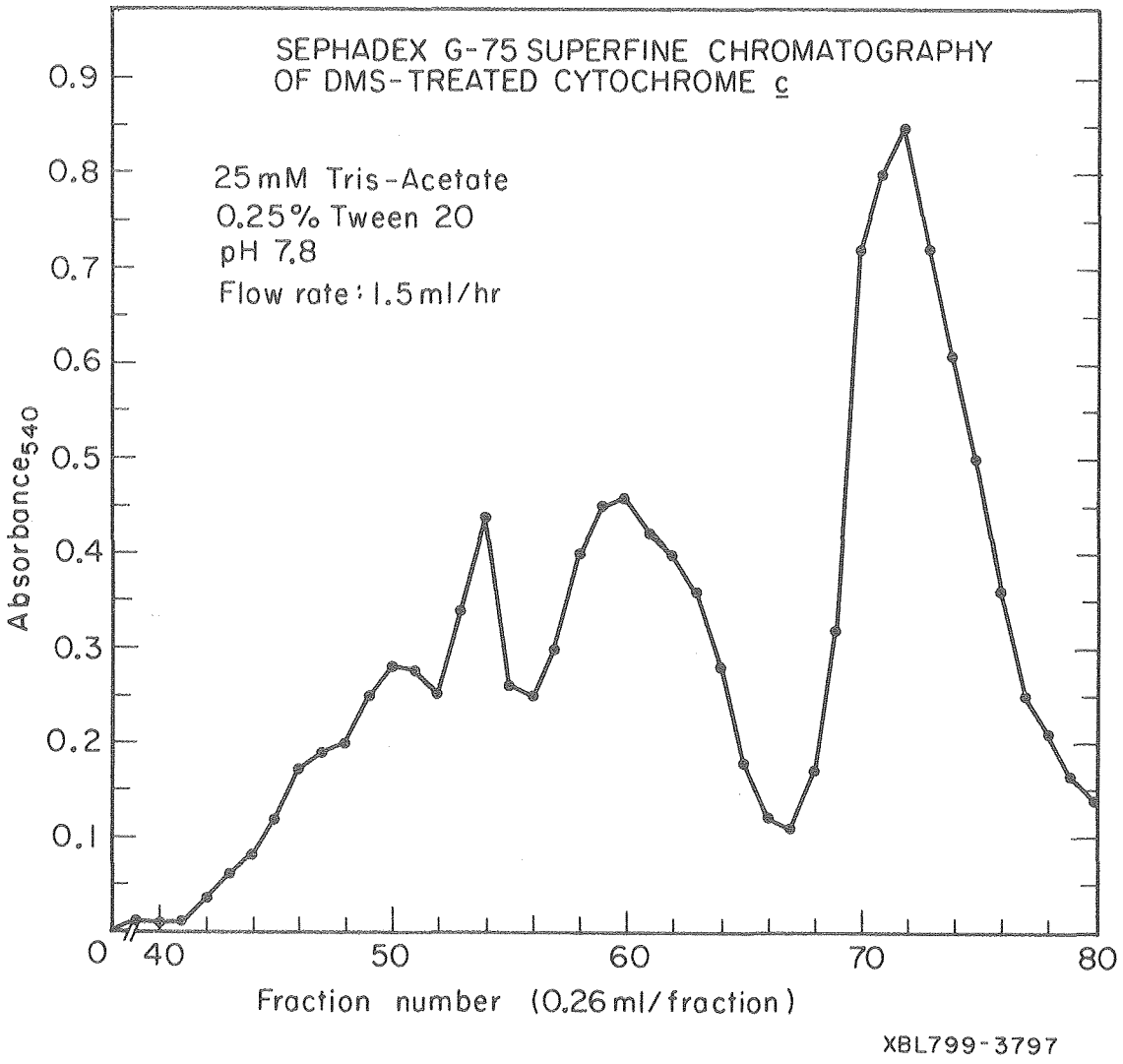


FIGURE 8

Elution profile of DMS-treated cytochrome c
from a Sephadex G-75 Superfine column.