THE ACTIVE FORM OF CYTOCHROME *C* OXIDASE

EFFECTS OF DETERGENT, THE INTACT MEMBRANE, AND RADIATION INACTIVATION

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ABSTRACT Cytochrome oxidase is a multisubunit, intrinsic membrane protein with a complex function that includes oxidation of cytochrome *c*, reduction of oxygen and generation of a membrane potential. To clarify the relationship of its normal function to protein and membrane structure, we have examined the kinetic behavior of rat liver cytochrome oxidase in the intact inner mitochondrial membrane and in detergent solubilized states. Dissolution of rat liver mitochondrial membranes alters the kinetic parameters of the oxidase in a manner dependent in part on the dispersing agent, and characterized by a large increase in maximal activity which is not attributable to exposure of more oxidase or diminished affinity for cytochrome *c*. The most profound effect of solubilization of the membrane is seen on the low affinity reaction of cytochrome *c*, suggesting that the electron transfer pathway from this site to oxygen is sensitive to alterations in hydrophobic interactions within the oxidase. Purified rat liver and beef heart oxidase exists predominantly in a monodisperse, 300 kilodalton form in laurylmaltoside[1] (Rosevear et al., 1980). However, a smaller, 130 kd species that exhibits high turnover rates equal to the 300 kd form is detected in some beef heart preparations, implying that the dimer may not be essential for high activity. Radiation inactivation studies on purified oxidase reveal a molecular weight for the functional unit of ~70 kd. It is concluded that less than a complete set of subunits may be sufficient for both normal binding of cytochrome *c* and rapid electron transfer to oxygen.

INTRODUCTION

Cytochrome *c* oxidase is a protein of complex structure and function, intrinsic to the mitochondrial inner membrane and capable of transporting electrons from cytochrome *c* to oxygen and contributing to the development of a membrane potential. Even though the enzyme can be purified to a fairly reproducible form, the number of subunits that actually belong to the oxidase and their respective roles remains unclear. Measurement of various kinetic parameters would normally be a means of determining the relationship between function and structure, but oxidase displays very complex kinetics that are highly sensitive to the nature of the amphipathic molecules used to keep it in solution. The substrate, cytochrome *c*, interacts with the oxidase in two different ways, with high and low affinities (Nicholls, 1964; Ferguson-Miller, 1976; Errede and Kamen, 1978) but the physiological significance of the two electron transfer pathways is not obvious, nor is there agreement on what kinetic parameters provide a good measure of the "native state" of cytochrome oxidase. Actual turnover rates (moles of cytochrome *c* oxidized/mol cytochrome aa_3/s) in the intact mitochondrial membrane under physiological conditions can range from 10 to 500 s^{-1} (Nicholls, 1974; Erecinska et al, 1979), yet the solubilized and purified enzymes can give activities as high as 1,100 s^{-1} (see below).

To define the kinetic behavior of cytochrome oxidase further and to relate activity to subunit composition, molecular form, and membrane effects, we compared the kinetic behavior of rat liver oxidase in the membrane-bound and solubilized states in the presence of various detergents, and determined the relationship of activity to molecular size by gel filtration and radiation inactivation. The results show that the second, high turnover phase of the kinetics is most strongly affected by the presence or absence of the intact membrane and the nature of the hydrophobic environment. Gel filtration reveals that the predominant form of the purified enzyme from both rat liver and beef heart oxidase in laurylmaltoside is a ~ 300 kilodalton (kd) species, but smaller forms (< 130 kd) can be detected that have equally high turnover rates.

Inactivation by ionizing radiation has proven to be an effective tool for determining the size of the functional unit of an enzyme (Kempner and Schlegel, 1979). Application of this technique to cytochrome oxidase (Kagawa, 1967) had given very low values of 40–70 kd that were smaller than even the minimum mol wt required to contain two hemes (125 kd), let alone the molecular mass of a dimer (300 kd) proposed to be the active species (Vik and Capaldi, 1977; Bisson et al., 1980; Ferguson-Miller et al., 1981). However, using highly active beef heart cytochrome oxidase in a monodisperse dimer form, we have confirmed the original low estimate for the size of the functional unit of cytochrome oxidase. The results imply that neither a dimer, nor even a complete monomer is

necessary for normal binding of cytochrome *c* and rapid transfer of electrons to oxygen.

MATERIALS AND METHODS

Laurylmaltoside (CMC = 0.16 mM, Rosevear et al., 1980) was synthesized according to Rosevear et al. (1980). Cytochrome *c* (Sigma Type VI) was purified according to Brautigan et al. (1979). Cholate and deoxycholate (Sigma Chemical Co., St. Louis, MO) were recrystallized four times from 95% ethanol and 80% acetone, respectively. Other chemicals were the highest grades available from the sources indicated: cacodylic acid (J.T. Baker Chemical Co., Phillipsburg, NJ), Tris (Calbiochem Corp., San Diego, CA; Ultrol), ascorbic acid (Mallinckrodt Inc., St. Louis, MO), *N,N,N',N'*-tetramethyl-P-phenylenediamine dihydrochloride (Eastman Organic Chemicals Div., Rochester, NY), protein mol wt standards for SDS-polyacrylamide electrophoresis (Bethesda Research Labs, Inc. Bethesda, MD), phospholipids (Sigma), asolectin (Associated Concentrates, Woodside, N.Y.).

Enzyme Preparations

Rat liver mitoplasts were obtained by the procedure of Felgner et al. (1979) and frozen in 20% DMSO at −65°C. Protein was measured by the Lowry procedure (Lowry et al., 1951), corrected for absorbance contributions of the buffer. Beef heart cytochrome oxidase was prepared by a modification of the procedure of Kuboyama et al. (1972) using cholate as the detergent. The enzyme contained 10 nmol heme *a*/mg protein and was stored in 1% laurylmaltoside at −170°C.

Assay Methods

Steady-state kinetic measurements were performed in 25 mM Tris cacodylate, pH 7.9, or 50 mM potassium phosphate, pH 6.5, in the presence of specified concentrations of detergents, 0.5 mM TMPD, and 2.5 mM ascorbate, over a cytochrome *c* concentration range of 0.01–60 μM. Rates of oxygen consumption were measured polarographically as described by Ferguson-Miller et al. (1976, 1978).

Molecular Weight Determination by Gel Filtration

Molecular sieve chromatography of purified rat liver and beef heart oxidase was performed on Sephacryl 300 (Pharmacia Inc., Piscataway, NJ) in 100 mM KCl, 10 mM Tris-chloride, pH 7.8, 1 mM EDTA, 0.1% (2 mM) laurylmaltoside, at a flow rate of 5 ml/h.

Radiation Inactivation

Beef heart cytochrome oxidase was diluted to 36.6 μM in 10mM Tris Cl, 100 mM KCl, 1 mM EDTA, pH 7.8, and 0.5% (10 mM) laurylmaltoside. Samples of 100 μl were placed in plastic vials to give a layer of < 2 mm thick, and were stored in liquid N_2. Irradiation was performed at −170°C with 2 MeV electrons produced by a van der Graaff accelerator (Dow Chemical Co., Midland, MI.) Prior to kinetic analysis, butylated hydroxytoluene (BHT) was added to the frozen sample (to prevent secondary reactions from radicals produced during radiation) to give a final sample composition when thawed of 11 μM cytochrome oxidase, 0.016% BHT, 3.3% ethanol, 1.7% (33 mM) laurylmaltoside, 10 μM Tris Cl, 100 mM KCl, 1 mM EDTA, pH 7.9.

Spectral Measurements

Spectrophotometric determinations were performed on an Aminco DW 2a dual-wavelength/dual-beam spectrophotometer (American Instrument Co., Silver Spring, MD) or a Perkin-Elmer Model 559 Double-beam UV-Visible Spectrophotometer (Perkin-Elmer Corp. Instruments Div., Norwalk, CT). The following extinction coefficients were used to calculate the concentration of purified cytochrome oxidase: $\Delta\epsilon_{mM(422-480)}$ oxidized = 140; $\Delta\epsilon_{mM(445-480)}$ reduced = 200; $\Delta\epsilon_{mM(605-630)}$ reduced = 37. These represent the three most prominent absorption maxima for the enzyme. The extinction coefficients were obtained from the spectra of van Buuren et al. (1972). The concentration of cytochrome *c* was calculated from $\Delta\epsilon_{mM(550-630)}$ reduced = 28.

RESULTS

Kinetic Characteristics of Rat Liver Cytochrome Oxidase in the Intact and Solubilized Membrane

The steady-state kinetics of reaction of cytochrome *c* with rat liver oxidase were examined under conditions that maximize rates of turnover, 50 mM potassium phosphate, pH 6.5 (Davies et al., 1964). Fig. 1 shows an Eadie-Hofstee plot of the data obtained with rat liver inner mitochondrial membranes (cytochrome *c*-depleted mitoplasts) and with the same membranes solubilized with laurylmaltoside. It is apparent that the kinetic parameters of the enzyme are markedly altered by dissolution of the membrane, in a manner that cannot be explained by a simple increase in the amount of available oxidase. The intact and laurylmaltoside-solubilized membranes differ in their apparent K_m values as well as their maximal turnover numbers. The very high activity of the dissolved membranes appears to result mainly from increased turn-

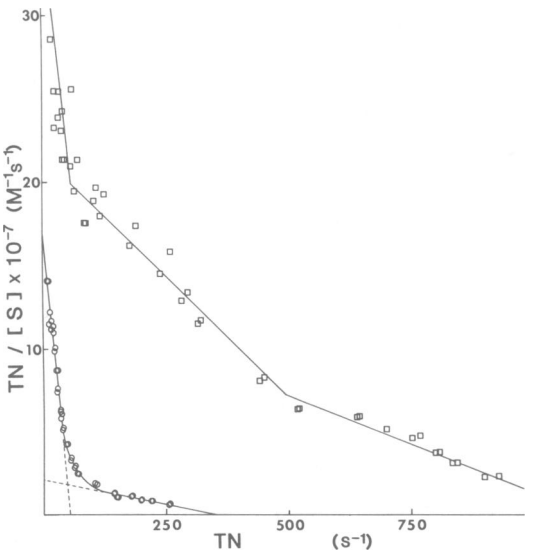

FIGURE 1 An Eadie-Hofstee plot of the kinetics of oxidation of cytochrome *c* by rat liver cytochrome oxidase in 50 mm potassium phosphate, pH 6.5. Rat liver inner mitochondrial membranes (0.05 nmol cytochrome oxidase)(O—O); membranes treated with detergent (□---□). Membranes (40 mg/ml protein) were solubilized in 150 mM laurylmaltoside (~ 2 mg detergent/mg protein) and diluted in the assay to 0.34 mM laurylmaltoside and 0.018 nmol cytochrome oxidase. Rates of oxygen consumption were measured polarographically as described in Methods. Turnover numbers were calculated by multiplying the velocity in nmol O_2/s by four (to convert to nmol cytochrome *c*/s) and dividing by the total nmol cytochrome aa_3 in the assay mixture. The range of horse heart cytochrome *c* concentrations used was 0.07–50 μM.

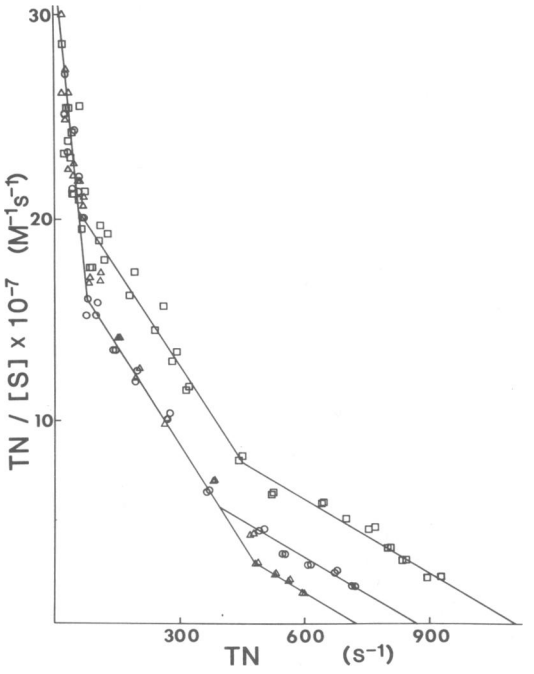

FIGURE 2 An Eadie-Hofstee plot of the kinetics of oxidation of cytochrome *c* by rat liver cytochrome oxidase in inner mitochondrial membranes solubilized with detergent. Assays were performed in 50 mM potassium phosphate, pH 6.5, with: laurylmaltoside at 0.34 mM and oxidase at 0.018 nmol (□—□); deoxycholate at 0.20 mM and oxidase at 0.031 nmol (Δ—Δ); lysophosphatidylcholine-1-oleoyl at 0.11 mM and oxidase at 0.02 nmol (O—O). Prior to assay the membranes were treated with laurylmaltoside as described in Fig. 1, or with deoxycholate (Smith and Camerino, 1963), or with lysophosphatidyl choline, at a detergent:protein ratio of 1 mg:1mg. The protein:detergent ratio was chosen in each case by determining conditions for maximal oxidase activity in a series of samples containing various ratios from 0.2 to 10 mg detergent to mg protein, in 25 mM Tris Cl, 165 mM sucrose, 0.5 mM histidine, pH 8.0. Rates of oxygen consumption were measured as described in Methods. Calculations were as in Fig. 1. Cytochrome *c* concentrations were 0.07–40μM.

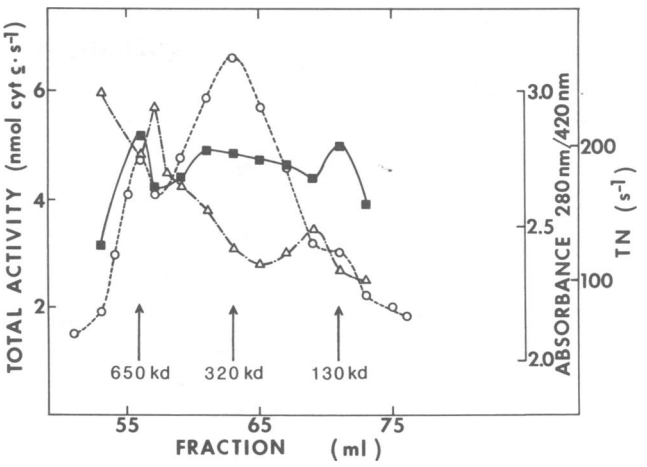

FIGURE 3 Elution profile of beef heart cytochrome oxidase on Sephacryl 300. Oxidase prepared by the method of Hartzell and Beinert (1974) was the gift of Dr. G. Babcock. The enzyme (0.5 ml, 100 μM, 11 nmol heme *a*/mg protein) was chromatographed as described in Methods on a 1.5 × 80 cm column. The molecular mass values indicated with arrows were derived from a plot of log molecular mass vs. elution vol using the following standards: blue dextran (2,000 kd); thyroglobulin (669 kd); ferritin (440 kd); catalase (230 kd); aldolase (158 kd); BSA (68 kd); chymotrypsinogen (25 kd); cytochrome *c* (12.5 kd). Total activity (O—O) was measured polarographically in phosphate buffer as described in Methods, and refers to the activity present in each fraction. Turnover numbers (■—■) were calculated as described in Fig. 1. The ratio of 280 nm/420 nm absorbance (Δ—Δ) was obtained from complete UV-visible spectra of each fraction.

over in the second kinetic phase and the development of a third phase in the kinetic plot. In spite of the complexity of the kinetic picture, a striking feature of the data is an increase in the overall turnover number from 350 s^{-1} to 1,100 s^{-1}, with no large change in the contribution to the activity by the initial high-affinity phase, and no concomitant decrease in apparent affinity for cytochrome *c* in the second (or third) phase that would account for an increased turnover in terms of an increased rate of dissociation of cytochrome *c*. Other detergents of different chemical character, lysolecithin and deoxycholate, give very similar kinetic effects (Fig. 2) but differ in their ability to increase the rate of turnover of the enzyme. For each detergent, a series of detergent-to-protein ratios were tested to determine the conditions required for maximal activation. The maximal turnover rates obtained from the data shown in Fig. 2 are 700 s^{-1} in deoxycholate, 850 s^{-1} in lysophosphatidylcholine, and 1,100 s^{-1} in laurylmaltoside. These results indicate that although the chemical nature of the detergent is a factor in determining the maximal activity in the lower affinity phase, the most profound kinetic effects are produced by simple disruption of the membrane structure and are not dependent on the specific detergent.

Gel Filtration Analysis of Molecular Size

Gel filtration of the purified rat liver oxidase on Sephacryl 300 shows a single species with apparent molecular mass of 300 ± 20 kd.[1] Purified beef oxidase had previously shown a similar state of dispersion (Rosevear et al., 1980; Ferguson-Miller et al., 1981) but some enzyme preparations show evidence of small amounts of two other molecular species (Fig. 3), roughly corresponding to a tetramer (650 kd) and a monomer (130 kd), assuming that the 300 kd form is a dimer containing four hemes. The molecular weights were assigned by calibration of the column with standard proteins, as indicated in the legend to Fig. 3. All the forms were found to have the same maximal activities (TN). In the molecular mass estimates no corrections have been made for the detergent micelle, which has an apparent mol wt by gel filtration of 50 kd (Rosevear et al., 1980). The extent to which the micelle will contribute to

[1]Thompson, D. A., and S. Ferguson-Miller. Purification, structure, and function of rat liver cytochrome oxidase in laurylmaltoside. Manuscript in preparation.

the Stokes radius of this large asymmetric protein is not known (Nozaki et al., 1976). Nevertheless, it is clear that there is an active form of beef oxidase of a relatively small molecular mass < 130 kd.

Radiation Inactivation Analysis

Irradiation of purified beef cytochrome *c* oxidase at −170°C with high-energy electrons resulted in a decrease in activity of the enzyme as measured polarographically under optimal conditions for the determination of maximal turnover (50 mM potassium phosphate, pH 6.5). As shown in Fig. 4, cytochrome *c* oxidase activity exhibits an exponential decay with increasing radiation dose up to 30 Mrads. At the highest dose, 50% of the initial activity (715 s^{-1}) was destroyed. The solid line represents a least squares fit to the data constrained to the value A_D/A_O = 1.0 at $D = 0$. Target theory predicts that the dose required for 37% inactivation is inversely proportional to the volume of the functional unit responsible for the biological activity. The volume can be converted into a molecular mass in daltons by taking into account the density of protein and the temperature at which radiation was performed (Kempner and Schlegel, 1979; Fluke, 1972). The molecular mass estimate for the cytochrome *c* oxidase functional unit obtained from this data is 70 ± 15 kd. Because it has been suggested that the active form of cytochrome oxidase may be a dimer of mol wt ~ 300 kd containing four hemes (Robinson and Capaldi, 1977; Bisson et al., 1980; Ferguson-Miller et al., 1981), the theoretical line for such a molecular mass is shown in Fig. 4. Also shown is the other obvious alternative, that the active species of cytochrome oxidase contains only two hemes and a single copy of each subunit; this predicts a molecular mass of ~ 125 kd.

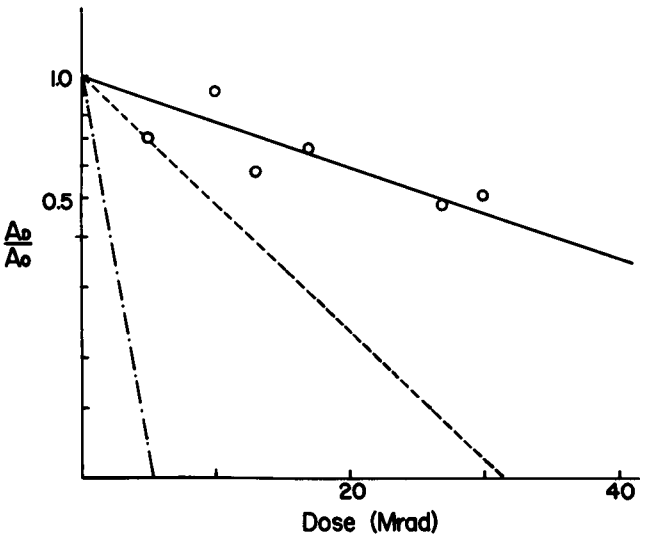

FIGURE 4 Inactivation of cytochrome *c* oxidase by a irradiation with high energy electrons. Samples were frozen at −170°C. Fitted line is from constrained least square analysis (O—O). Rates of oxygen consumption were measured polarographically in 50 mM potassium phosphate, pH 6.5, as described in Methods, with 0.05 nmol cytochrome aa_3, 3.7–30 μM horse heart cytochrome *c* and 1 mM laurylmaltoside. Theoretical inactivation curves are drawn for a target of 125 kd (- - -) and 300 kd (·—·—).

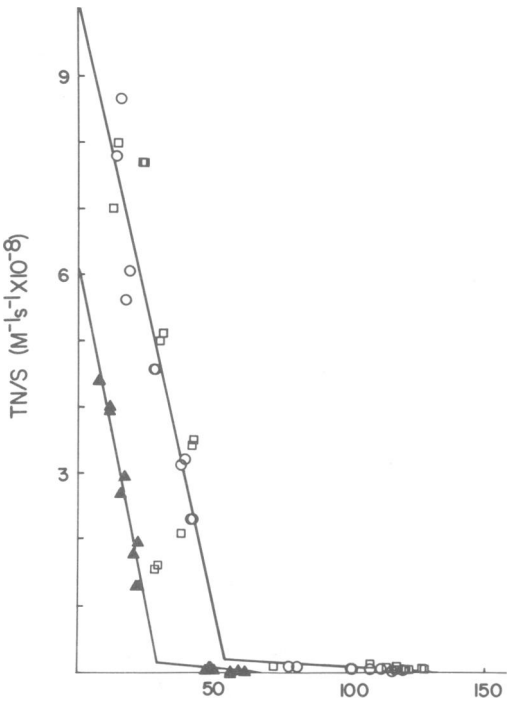

FIGURE 5 Comparison of the steady-state kinetics of oxidation of cytochrome *c* by purified beef heart oxidase before and after irradiation with 30 M rad. Rates of oxygen consumption were measured polarographically in 25 nM Tris cacodylate pH 7.9, 0.05 nmol cytochrome aa_3, 0.01–30 μM horse heart cytochrome *c*, and 1 mM laurylmaltoside. Turnover numbers were calculated as in Fig. 1. Native oxidase (O—O); native oxidase with 0.016% BHT (□—□); irradiated oxidase with 0.016% BHT (▲—▲).

A more complete kinetic analysis of the enzyme was performed at lower ionic strength and high pH (25 mM Tris cacodylate pH 7.9), to detect possible changes in the high affinity binding of cytochrome *c* to the oxidase and to determine whether the two interactions of cytochrome *c* were differentially affected by irradiation. The biphasic dependence of velocity on substrate concentration seen with the native enzyme in an Eadie-Hofstee plot (Fig. 5) reflects two interactions of cytochrome *c* with the oxidase, with differing affinities and possibly at different sites (Nicholls, 1964; Ferguson-Miller et al., 1976, 1978; Errede and Kamen, 1978; Nicholls et al., 1980). Fig. 5 shows that addition of BHT to the enzyme causes no change in the kinetic parameters of the oxidase. In the kinetic plot for the sample irradiated with the highest dose, 30 Mrads, the remaining activity displays the same apparent K_m values as the native enzyme, consistent with the basic premise of the method that the activity remaining after radiation exposure is due to units that have escaped ionization and are fully active (Kempner and Schlegel, 1979). Contrary to expectations, the high affinity binding

of cytochrome *c* and turnover in that phase appears to be less sensitive to radiation damage than the low affinity, high turnover reaction.

The sample that displayed only 50% of the normal activity appeared essentially unaltered in concentration and spectral properties. It was totally reducible by dithionite, but a possibly significant shift was observed in the position of the oxidized Soret peak, from 422 nm in the native enzyme to 420 nm in the highly irradiated sample.

DISCUSSION

To clarify the aspects of protein and membrane structure that are important in the normal functioning of cytochrome oxidase, we have studied the relationship between activity and molecular size of the purified form by gel-filtration, and investigated the nature of the functional unit by radiation inactivation. We find that the enzyme in the intact membrane is not identical in its kinetic properties to the enzyme in membranes dissolved with detergent. It is usually assumed that the increased activity seen when mitochondrial membrane particles are solubilized is the result of increased availability of the enzyme, because of the existence of a considerable proportion of inverted membrane vesicles in many particle preparations. However, the membranes used in these studies are intact mitoplasts in which the cytochrome oxidase should already be totally accessible to cytochrome *c*. Moreover, the kinetic changes seen upon addition of detergent are not those expected for a simple increase in enzyme concentration, which would result in little change in the apparent K_m values and increased V_{max} values of both kinetic phases (Ferguson-Miller et al., 1976). In contrast, we observe that the kinetic characteristics and turnover numbers of the second kinetic phase are much more dramatically affected than those of the initial phase, regardless of the buffer system, pH, or type of detergent used. The large increase in activity caused by detergent addition, with concomitant alterations in the character of the second and appearance of a third phase, suggest that the low affinity interactions of cytochrome *c* with the oxidase involve an electron transfer event that is highly sensitive to the lipid environment as well as to other influences in the membrane, such as surface pressure (Conrad and Singer, 1979) or protein-protein interactions. With regard to the latter, the increase in oxidase activity could be explained as resulting from a change in association with other membrane proteins, or from conformational changes caused by more subtle forces such as loss of surface pressure. However, it is interesting to consider the possibility that a dimer of cytochrome oxidase may exist in the mitochondrial membrane with lower activity than a monomer, rather than higher activity as previously suggested (Robinson and Capaldi, 1977; Bisson et al, 1980; Ferguson-Miller et al., 1981.) The very high turnover rates observed when the membrane is dissolved could then result from conversion of some of the oxidase into a highly active monomer form with different kinetic parameters from the dimer, contributing to the complex kinetic picture. Purification of the enzyme could increase the probability of self-association, resulting in a return to the lower activity dimer form most commonly observed.

The greater sensitivity of the lower affinity kinetic phase to changes in the state of the membrane and the nature of the solubilizing agent, suggests that this interaction of cytochrome *c* involves a lipid-sensitive electron transfer step within the oxidase, as altered binding alone does not appear to account for the large increase in turnover rates. This conclusion is based on a comparison of the apparent K_m values obtained for the membrane-bound and solubilized oxidase (Fig. 1) which show increased rather than decreased apparent affinity for cytochrome *c* in the presence of detergents. Because it has been shown that electron transfer from heme *a* to heme a_3 is inhibited by lipid depletion and certain inactivating detergents (Yu et al., 1975), this electron transfer step seems a likely candidate to be part of the pathway from the low affinity binding site of cytochrome *c* to oxygen. Consistent with this interpretation is the finding of Vik et al. (1981) that depletion of cardiolipin from the oxidase results in preferential loss of the low affinity electron transfer site. The fact that the high affinity reaction of cytochrome *c* is relatively insensitive to detergent treatment of the membranes (this paper) and removal of cardiolipin (Vik et al., 1981) suggests that neither the binding nor the electron transfer pathway from this site involves an easily disrupted hydrophobic interaction.

Evidence that pertains to the question of the active form of cytochrome oxidase is the observation that certain beef heart preparations contain a low molecular mass species (~ 130 kd) detectable by gel filtration and with an activity very similar to the ~ 300 kd form. This species is present in variable and small amounts, so further studies on its composition and kinetics have not yet been possible, but the apparent molecular mass suggests that it has less than a complete complement of subunits.

The possibility that normal cytochrome oxidase activity could reside in a unit smaller than a monomer is supported by independent evidence from analysis of the dose-dependence of inactivation of the enzyme by ionizing radiation. A review by Kempner and Schlegel (1979) documents that this method has given values for the molecular mass of the functional unit of over thirty different enzymes that closely correspond to the size of either the whole enzyme or a subunit known to be responsible for the activity. The results we have obtained with cytochrome oxidase are noteworthy for several reasons. (*a*) They confirm the molecular mass estimate measured previously under different conditions (Kagawa, 1967), emphasizing the reproducibility of the technique. (*b*) By examining the complete kinetic picture in addition to maximal activity we can conclude that the small functional unit revealed by this method is capable not only of high rates of electron

transfer, but also of normal binding of cytochrome *c*. (*c*) Because the studies were performed using an enzyme with very high activity (700 s^{-1}) and nevertheless revealed a molecular mass ~70, the necessity of a dimer form of cytochrome oxidase to achieve these rates appears unlikely. (*d*) We observe that the low affinity reaction of cytochrome *c* is more sensitive to inactivation by irradiation than is the high affinity reaction, implying that the electron transfer pathway from the former site to oxygen involves a larger molecular mass species than from the latter.

The existence of an active species of cytochrome oxidase of ~ 70 kd, capable of binding cytochrome *c* with normal affinities, transferring electrons rapidly, and reducing oxygen to water, is not inconsistent with available information concerning the structure of the enzyme. Winter et al., (1980) have shown by low temperature electrophoresis and specific staining that subunit I (35 kd) and subunit II (24 kd) contain the two copper and two heme prosthetic groups, with the copper atoms residing entirely on subunit II. Sequence analysis of cytochrome oxidase further implicates subunit II as the copper carrying peptide (Steffens and Buse 1979; Tanaka et al., 1975) and several prokaryotic cytochrome oxidases have been isolated in an active form containing only two subunits that appear similar in size to the two largest polypeptides of the beef oxidase (Yamanaka et al., 1979; Sone and Kagawa, 1980; Fee et al., 1980; Ludwig and Schatz, 1980). From these results one can conclude that if a unit smaller than a complete assembly of subunits is capable of full oxidase activity, it is likely to contain at least the two largest polypeptides.

Can any conclusions be drawn concerning the relative positions of the two cytochrome *c* binding sites and the redox centers of the oxidase? A model can be formulated that is consistent with the present data as well as previous cross-linking, steady-state kinetic and low temperature kinetic studies. The low affinity reaction of cytochrome *c*, which is sensitive to cardiolipin removal (Vik et al., 1981), detergent, and radiation (this paper), is proposed to occur on the larger, more hydrophobic subunit I, and to involve transfer of electrons directly to heme *a*. The high affinity reaction of cytochrome *c*, which is less sensitive to changes in the membrane and detergent environment, is proposed to occur on the more hydrophilic subunit II and to involve transfer of electrons directly to the visible copper located on this polypeptide (Winter et al., 1980) and shown to be rapidly reduced by cytochrome *c* (Wilson et al., 1975). This model is consistent with the results of Briggs and Capaldi (1977) demonstrating that cytochrome *c* can be covalently cross-linked to subunit II under conditions that favor formation of the high affinity complex, and with the low temperature studies of Chance et al. (1978), indicating that transfer of electrons from cytochrome *c* to heme a_3 can occur without intervention of heme *a*. This scheme for electron transfer is in agreement with that proposed by Nicholls et al., (1980) but differs from the model of Winter et al., (1980) in the locations of the heme groups, and differs from the interpretation discussed by Vik et al., (1981) in that we are suggesting that subunit I rather than just cardiolipin is involved in the low affinity reaction. The model does not address the important questions of whether the cytochrome *c* interactions are independent or negatively cooperative, what the roles are of the other subunits, and how association of oxidase with itself or other proteins in the membrane might control the rates of electron (or proton) transfer.

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DISCUSSION

Session Chairman: Thomas E. Thompson *Scribe*: Rhoderick E. Brown

GEORGEVICH: I would like you to update some of the information you have on the characteristics of the oxidase isolated in lauryl maltoside, noting especially the lipid and the protein subunit content.

FERGUSON-MILLER: The enzyme we have isolated in lauryl maltoside is the rat-liver oxidase. We are able, without any special effort, to get rid of almost all of the phospholipid, to the point where we have one cardiolipin remaining. The enzyme appears to be missing subunit 3, which has been invoked as being involved in proton translocation. However, in our reconstituted system, we do get respiratory control. We have not looked at the actual proton translocation.

GEORGEVICH: Are there any differences in the lipid or subunit composition in the various aggregation states which you believe to be monomers, dimers, or tetramers?

FERGUSON-MILLER: We have not yet characterized the various forms of the beef-heart oxidase equilibrated in lauryl maltoside with respect to lipid and detergent content or the subunit composition.

GEORGEVICH: The proportion of the total oxidase activity contributed by the low and high affinity phases is about the same before and after irradiation. Doesn't this suggest equivalent target sizes?

FERGUSON-MILLER: We see a slightly greater inactivation of the second phase. In fact, at higher levels of irradiation we get to the point where we have only the first phase left. We're not sure what this means or whether there are some artifactual situations that arise here, but our data at the moment still support the possibility that the second phase of the interaction of cytochrome *c* may require a larger piece of oxidase than the first phase. We proposed in our paper that this might represent subunit I, which is the larger, more hydrophobic subunit, as distinct from the interaction of the high affinity cytochrome *c* with subunit II. We are not willing to make any strong statements on this, and we are still trying to determine whether this more rapid loss of the second phase is significant.

GEORGEVICH: With that in mind, it is interesting that the sizes for subunit I and III as determined by SDS-PAGE are quite different from oxidase subunit sizes deduced from mitochondrial genome sequences. Subunit I would be closer to 57,000 and subunit III to 30,000 daltons. The aggregate size for subunit I plus either subunit II or III is actually quite close to what your inactivation curves predict.

FERGUSON-MILLER: Yes, the molecular mass values from the DNA sequence of the first two subunits, which appear to contain the heme groups and the copper (Winter et al. 1980. *J. Biol. Chem.* 255:11408 – 11414), are larger than we see on gels. A molecular mass of 25.5 kdaltons for subunit II and 57 kdaltons for subunit I adds up to 82 kdaltons, not very far from the number of 70 ± 15 kdaltons. At the moment, our working hypothesis is that the active unit could be the two largest subunits.

GEORGEVICH: It looks as though the oxidase is quite relieved from regulation once it is removed from the membrane by lauryl maltoside. Would you postulate any role for some subunits as regulatory components?

FERGUSON-MILLER: That's a very interesting possibility. The subunits that we are discarding as unnecessary are mostly made in the cytoplasm on the nuclear genome, whereas the subunits we are suggesting are necessary are the ones made in the mitochondria. One could postulate regulation of insertion of the enzyme into the membrane, respiratory control, or interaction with other proteins in the respiratory chain, not to mention proton translocation, as functions of the other subunits.

McINTYRE: In the size measurement by radiation inactivation shown in Fig. 4, it appears that the largest unit for any specific step in the transfer is approximately a 70,000-dalton unit. However, do these data preclude that a larger overall complex is necessary for the complete function?

FERGUSON-MILLER: As to what the measured target size really represents, I can't say. The basis for giving credence to this technique is that empirically it has been found, with at least 30 different enzymes, that a size is obtained that is similar to the known size of an active subunit or, if a multisubunit enzyme is required, a multisubunit association. This applies not only to soluble enzymes, but to membrane-bound enzymes as well. The simplest assumption is that, if more of the enzyme is necessary, then you would see a larger target area.

McINTYRE: Would that not depend somewhat on the assumption that the tightness of the interaction between subunits involved individual steps and whether the high energy of an impact "hit" would transfer readily to an adjacent subunit? Other subunits of the complex may be functionally related, but not physically apposed in a manner that would allow the necessary energy transfer to occur.

FERGUSON-MILLER: I don't think so. If the subunit that you hit is actually an important one, it is gone and its activity effect with it. It doesn't have to transfer any energy to the rest of the enzyme, if the enzyme is dependent on that subunit.

McINTYRE: Therefore, since the 70,000-dalton subunit is required for one of the critical steps in the multi-step chain reaction, a "hit" on this target would block the chain. The data do not preclude that other subunits are essential for other steps in the chain because your assay measures only the complete chain from cytochrome *c* to oxygen.

FERGUSON-MILLER: The data *do* preclude that possibility. The target size will be the *sum total* of all the subunits that could in any way affect activity or cytochrome *c* binding, since the K_m values give a measure of the latter.

McINTYRE: Did you do any studies with the reconstituted system using radiation inactivation?

FERGUSON-MILLER: No. Kagawa, who performed similar experiments in 1967 (Y. Kagawa. 1967. *Biochim. Biophys. Acta.* 131:586 – 588), got this same value of 70,000 daltons using intact mitochondrial membrane. We repeated these experiments because we thought that this value was unbelievably low and might represent minimal activity. However, using our highly active enzyme, we still got 70,000 daltons.

FLEMING: I agree that, when you add the lauryl maltoside, the kinetics look like a three-component system, but I also know from personal experience that drawing lines on Eadie-Hofstee plots can be an exercise in optical illusion. Have you done nonlinear least-squares analysis on the original data to help you decide whether it remains two-component or whether it really does become three-component?

FERGUSON-MILLER: No, we haven't done that. The only point we can really make is that, in the presence of detergent, the enzyme behaves differently. There is a dramatic change in the apparent affinity of the second phase, going from native to purified and to reconstituted enzyme, regardless of what the complex situation is when we simply dissolve the membrane. You are right that we can't interpret this unambiguously. It could be 10 phases for all we know.

FLEMING: How do you interpret the two phases? Do you believe there are two sites or two states?

FERGUSON-MILLER: We know something about what these kinetics mean. You can look at the binding behavior of cytochrome *c* with this enzyme and show that it binds to oxidase with two different affinities, similar to those you predict from the kinetic phases. Therefore, it is reasonable to say that these phases represent two interactions of cytochrome *c* with oxidase. However, we cannot say, from these data or any other that I know of, whether these are distinct, different sites or whether they are interacting, very similar sites. Nor can we say whether electron transfer occurs directly from each of these sites or whether there is only one electron transfer site and each additional cytochrome *c* is actually displacing the first, allowing turnover at higher rates. At present these are equally reasonable interpretations of the data. In addition, it is quite possible that two different forms of the oxidase are present in the detergent-solubilized membranes.

ROBINSON: With respect to Fig. 3, you have concluded that you have a dimer of cytochrome oxidase based upon the fact that it has an elution position corresponding to a 300,000-dalton water-soluble protein. We have done similar experiments in Triton with similar results. Yet, when we do sedimentation equilibrium and correct for bound detergent, we estimate a protein molecular weight of 200,000, which I interpret as being monomeric oxidase. I would say that you probably have a monomer rather than a dimer of oxidase. Would you comment on that?

FERGUSON-MILLER: We have not made the correction for detergent nor done sedimentation analysis. The molecular mass that we can calculate, assuming a single lauryl maltoside micelle is associated with the 300-kdalton form, is ~250 –270 kdaltons. This could be a monomer or a dimer depending on what you assume the molecular weights of the subunits are and what other things are happening as far as the hydrodynamic properties are concerned. At this point, it is valid to say that we could have dimer, monomer, and less-than-monomer in Fig. 3.

ROBINSON: You have a small shoulder which elutes at a position equivalent to 130,000 mol wt for a soluble protein. Have you pooled that fraction and tried to rechromatograph to see if elutes again at the same position to show that it's not just the trailing of the main peak?

FERGUSON-MILLER: No, but it is something we intend to do

because there could be a dynamic equilibrium between the different forms.

ROBINSON: Have you run gels on that to see if you are missing any subunits?

FERGUSON-MILLER: No.

LAZAROW: In view of your finding that the reaction kinetics are different when cytochrome oxidase is soluble instead of membrane-bound, and in view of the suggestion that other subunits might be involved in regulation, it would be interesting to examine the effects of radiation inactivation on the kinetics of the enzyme in intact membranes. The apparent target size might be larger. Do you have any data on this point?

FERGUSON-MILLER: No. Kagawa (1967) only determined a turnover number in his earlier studies with intact membranes, so it might be fruitful to reexamine this system in terms of the complete kinetics.

TROY: Can you comment on how many of the 30 enzyme complexes which have been analyzed by radiation inactivation analyses are membrane-bound? Secondly, what is the molecular weight range? Surely, they are not all 70,000 daltons.

FERGUSON-MILLER: Molecular weights are reported up to at least 250,000. ~5–7 of those 30 proteins are membrane-bound. Recently, J. T. Harmon et al. (1981. *J. Biol. Chem.* 256:7719–7222) reported using this technique to analyze the insulin-binding protein in the membrane. A transition from monomer to dimer representing a change of 100,000 to 250,000 mol wt was demonstrated. In general, the irradiation technqiue shows membrane enzymes behaving as well as soluble enzymes.

McINTYRE: How does the elution profile of the rat-liver enzyme compare with that of the beef-heart enzyme shown in Fig. 4?

FERGUSON-MILLER: With the rat-liver enzyme that is purified in lauryl maltoside we do not see any of these other forms that we get with the beef-heart. We see a single peak in the 300,000 mol wt range.

McINTYRE: So if the beef-heart enzyme is exhibiting some self-associating properties, these properties are not the same as those observed with the rat-liver enzyme?

FERGUSON-MILLER: That is correct. However, our rat-liver enzyme is purer and more lipid-free than any of our beef-heart preparations.

KLAUSNER: Concerning the radiation inactivation of true integral proteins, recently Ellis Kempner and I at NIH have examined reconstituted bacteriorhodopsin. We have examined the radiation inactivation in a crystal to determine the minimum molecular weight necessary for function. When reconstituted as either a multimeric crystalline form or as a monomer, we get 27,000 as the minimum molecular weight.

REFEREE'S QUESTION: How is the point of solubilization of the membrane determined? Under these conditions, how many lipids remain in contact with the enzyme? What is the critical micelle concentration (cmc) of lauryl maltoside?

FERGUSON-MILLER: The cmc of lauryl maltoside is 0.2 mM. We solubilized the membranes by performing a series of experiments to determine where the highest activity was attained. We varied the ratio of detergent:protein with the three different detergents and checked for activity optima. We have not determined how much lipid remains bound to the oxidase using lauryl maltoside as the membrane dissolving agent. We suspect that a resonable amount of lipid remains associated with the oxidase. Possibly the two phases represent enzyme that is mostly associated with detergent and enzyme which is totally associated with lipid.

FREY: How do you explain the proposed regulation of enzyme activity by the extra subunits when many bioenergeticists claim that the enzyme is regulated only by the membrane potential?

FERGUSON-MILLER: These extra protein subunits could be involved in the regulation by the membrane potential. The more water-soluble subunits located near the surface of the enzyme may be particularly good candidates. An alternative function might be regulation of the insertion of the protein into the membrane or may be even a means of communication between the nuclear and mitochondrial genomes.

MANNELLA: In the radiation-inactivation experiments, have you considered looking at the molecular weight associated with loss of respiratory control?

FERGUSON-MILLER: Yes, we have. There are some technical problems to be resolved. We are not sure how to get a reconstituted system with respiratory control that will survive freezing to liquid-nitrogen temperatures and then thawing.