Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206–210.
McClare, C. W. F. (1971) *Anal. Biochem. 39*, 527–530.
Melchior, V., Collingshead, C. J., & Cahoon, M. E. (1980) *J. Cell Biol. 86*, 881–884.
Mimms, L. F., Zampighi, G., Nozaki, Y., Tanford, C., & Reynolds, J. A. (1981) *Biochemistry 20*, 883–840.
Morrissett, J. D., David, J. S. K., Pownall, H., & Gotto, A. M. (1973) *Biochemistry 12*, 1290–1299.
Morrissett, J. D., Jackson, R. L., & Gotto, A. M. (1975) *Annu. Rev. Biochem. 44*, 183–207.
Nelson, G. J. (1979) in *Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism*, p 300, Robert E. Krieger Publishing Co., New York.
Noble, R. P. (1968) *J. Lipid Res. 9*, 693–700.
Racker, E. (1979) *Methods Enzymol. 55*, 699–711.
Razin, S. (1972) *Biochim. Biophys. Acta 265*, 241–296.
Redgrave, T. G., Roberts, D. C. K., & West, C. E. (1975) *Anal. Biochem. 65*, 42–49.
Rudel, L. L., & Morris, M. D. (1973) *J. Lipid Res. 14*, 364–366.
Small, D. M. (1967) *J. Lipid Res. 8*, 551–560.
Smith, R., Dawson, J. R., & Tanford, C. (1972) *J. Biol. Chem. 247*, 3376–3381.
Steele, J. C. H., & Reynolds, J. A. (1979a) *J. Biol. Chem. 254*, 1633–1638.
Steele, J. C. H., & Reynolds, J. A. (1979b) *J. Biol. Chem. 254*, 1639–1643.
Watt, R. M., & Reynolds, J. A. (1980) *Biochemistry 19*, 1593–1598.
Watt, R. M., & Reynolds, J. A. (1981) *Biochemistry 20*, 3897–3901.
Waugh, D. A., & Small, D. M. (1982) *J. Lipid Res. 23*, 201–204.
Weber, K., & Osborn, M. J. (1969) *J. Biol. Chem. 244*, 4406–4412.
Yeagle, P. L., Langdon, R. G., & Martin, R. B. (1977) *Biochemistry 16*, 3487–3491.

Lipid and Subunit III Depleted Cytochrome *c* Oxidase Purified by Horse Cytochrome *c* Affinity Chromatography in Lauryl Maltoside†

D. A. Thompson and S. Ferguson-Miller*

ABSTRACT: Cytochrome oxidase is purified from rat liver and beef heart by affinity chromatography on a matrix of horse cytochrome *c*–Sepharose 4B. The success of this procedure, which employs a matrix previously found ineffective with beef or yeast oxidase, is attributed to thorough dispersion of the enzyme with nonionic detergent and a low density of cross-linking between the lysine residues of cytochrome *c* and the cyanogen bromide activated Sepharose. Beef heart oxidase is purified in one step from mitochondrial membranes solubilized with lauryl maltoside, yielding an enzyme of purity comparable to that obtained on a yeast cytochrome *c* matrix [Azzi, A., Bill, K., & Broger, C. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2447–2450]. Rat liver oxidase is prepared by hydroxyapatite and horse cytochrome *c* affinity chromatography in lauryl maltoside, yielding enzyme of high purity (12.5–13.5 nmol of heme *a*/mg of protein), high activity (TN = 270–400 s^{-1}), and very low lipid content (1 mol of DPG and 1 mol of PI per mol of aa_3). The activity of the enzyme is characterized by two kinetic phases, and electron transfer can be stimulated to maximal rates as high as 650 s^{-1} when supplemented with asolectin vesicles. The rat liver oxidase purified by this method does not contain the polypeptide designated as subunit III. Comparisons of the kinetic behavior of the enzyme in intact membranes, solubilized membranes, and the purified delipidated form reveal complex changes in kinetic parameters accompanying the changes in state and assay conditions, but do not support previous suggestions that subunit III is a critical factor in the binding of cytochrome *c* at the high-affinity site on oxidase or that cardiolipin is essential for the low-affinity interaction of cytochrome *c*. The purified rat liver oxidase retains the ability to exhibit respiratory control when reconstituted into phospholipid vesicles, providing definitive evidence that subunit III is not solely responsible for the ability of cytochrome oxidase to produce or respond to a membrane potential or proton gradient.

Cytochrome *c* oxidase (EC 1.9.3.1) is a complex, multisubunited enzyme, which contains four metal centers and spans the inner mitochondrial membrane. Electrons liberated during metabolism are transferred from cytochrome *c* through cytochrome oxidase to molecular oxygen, resulting in formation of water and conservation of energy by mechanisms not fully understood. Elucidation of the structure–function correlations in this elaborate protein has been difficult. Much controversy still exists concerning the number of subunits required for the native functions of the enzyme, and their operational roles, as well as the significance of the biphasic steady-state kinetic pattern observed for the interaction of cytochrome *c* with cytochrome oxidase (Nicholls, 1964, 1965; Ferguson-Miller et al., 1976; Errede & Kamen, 1978; Antalis & Palmer, 1982). Studies on the activity of cytochrome oxidase have been complicated by its functional dependence on the hydrophobic environment. An absolute requirement for specific phospholipids has been postulated (Awasthi et al., 1971; Robinson et al., 1980; Fry & Green, 1980; Vik et al., 1981; Robinson, 1982), and oxidase activity has been proposed to be further influenced by interactions with the membrane, with other membrane proteins, and with itself (by formation of a dimer consisting of two complete sets of subunits) (Robinson & Capaldi, 1977; Bisson et al., 1980; Wikstrom, 1981; Ferguson-Miller et al., 1982).

† From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824. *Received December 30, 1982.* This research was supported by National Institutes of Health Grant GM 26916 to S.F.-M.

0006-2960/83/0422-3178$01.50/0

In order to address existing ambiguities concerning which properties of the enzyme are basic to its function, and which are merely related to species or preparation techniques, we have developed an alternative method of purification that reproducibly yields an enzyme of higher activity and purity and lower lipid content than previously reported. This has been achieved by using a detergent (lauryl maltoside) and a technique (horse heart cytochrome *c* affinity chromatography) not before successfully applied to the preparation of beef cytochrome oxidase. Published reports indicate horse cytochrome *c* affinity resin is not effective in purifying oxidase from beef heart or yeast sources (Weiss & Kolb, 1978; Bill et al., 1980, 1982; Azzi et al., 1982) but is very effective for isolation of the enzyme from *Neurospora crassa* (Weiss & Kolb, 1979). It seemed likely that the major obstacle for other tissues might be difficulty in fully solubilizing and dispersing the proteins of the mitochondrial membranes. Studies in our laboratory on a number of alkyl glycoside detergents have shown that lauryl maltoside is exceptionally well suited for use in chromatographic purification (Rosevear et al., 1980; Van Aken et al., 1980; Ferguson-Miller et al., 1982), because of its ability both to extensively disperse cytochrome oxidase and to suppor high levels of electron transfer activity (Thompson et al., 1982). Using lauryl maltoside and an affinity matrix of horse cytochrome *c* linked via lysine residues to Sepharose 4B, we obtain cytochrome oxidase from beef heart mitochondrial membranes in one chromatographic step with a purity and yield similar to that achieved using an affinity resin prepared from yeast cytochrome *c* linked to a matrix via its sulfhydryl residue (Bill et al., 1980, 1982; Azzi et al., 1982).

Employing the same resin and detergent, we have developed a procedure for purifying rat liver cytochrome oxidase in several steps that gives a uniquely active and delipidated enzyme, depleted of subunit III. We have analyzed the characteristics of the purified rat liver enzyme in terms of subunit composition, lipid content, molecular weight, and its ability to exhibit respiratory control after reconstitution into phospholipid vesicles. The kinetic profile of the purified enzyme has been compared to that of oxidase in the native membrane, the detergent-solubilized membrane, and soybean phospholipid vesicles. The data clarify the roles of lipid, subunit III, and assay conditions in determining the activity of cytochrome oxidase.

Experimental Procedures

Chemicals. Lauryl β-D-maltoside was synthesized according to Rosevear et al. (1980). Cholic acid and deoxycholic acid (Sigma, St. Louis, MO) were recrystallized at least 3 times from hot 95% ethanol and hot 80% acetone, respectively. Cytochrome *c* (horse heart, Sigma type VI) was purified by chromatography on carboxymethylcellulose according to the procedure of Brautigan et al. (1978). Polymerized cytochrome *c* was removed immediately before use by gel filtration, in the reduced form, on Sephadex G-75 superfine in 25 mM Tris–cacodylate, pH 7.9. Dimethyl sulfoxide was treated with Amberlite MB-3 prior to use (both from Mallinckrodt, Paris, KY) (Fleischer, 1979). Other chemicals were the best grades available from the sources indicated: asolectin (Associated Concentrates, Long Island, NY); hydroxyapatite (Bio-Gel HTP), acrylamide, and methylenebis(acrylamide) (Bio-Rad Laboratories, Richmond, CA); protein molecular weight standards (Bethesda Research Laboratories, Inc., Rockville, MD, and Sigma); Sepharose 4B, cyanogen bromide, phenylmethanesulfonyl fluoride, lipid standards, cacodylic acid, carbonyl cyanide *m*-chlorophenylhydrazone, and valinomycin (Sigma); *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (Eastman Chemicals, Rochester, NY); fluorescamine (Roche Diagnostics, Nutley, NJ); reagents for phospholipid extractions and analysis (Mallinckrodt, Paris, KY).

Spectral Measurements. UV and visible spectra were recorded on either a Perkin-Elmer 559 or Aminco-DW2a UV/vis spectrophotometer with Midan T microprocessor. The extinction coefficients for absolute spectra of cytochrome aa_3 (dithionite reduced or air oxidized) were taken from the spectra published by van Buuren et al. (1972): cytochrome aa_3 (reduced), $\Delta\epsilon$(605–650 nm) = 40 mM^{-1} cm^{-1} and $\Delta\epsilon$(440–490 nm) = 204 mM^{-1} cm^{-1}; cytochrome aa_3 (oxidized), $\Delta\epsilon$(420–490 nm) = 140 mM^{-1} cm^{-1}. The extinction coefficients for difference spectra (dithionite reduced minus air oxidized) for cytochromes *b* and aa_3 were those of von Jagow & Klingenberg (1972): cytochrome *b*, $\Delta\epsilon$(560–575 nm) = 23.4 mM^{-1} cm^{-1}; cytochrome aa_3, $\Delta\epsilon$(605–630 nm) = 24.0 mM^{-1} cm^{-1}.

Assay Methods. The electron transfer activity of cytochrome oxidase was measured polarographically as described by Ferguson-Miller et al. (1976, 1978). Steady-state kinetic measurements were performed under the conditions detailed in the table and figure legends. Turnover numbers (TN)[1] were calculated from velocities measured in nanomoles of O_2 per second by multiplying by 4 (to give the nanomoles of cytochrome *c* required to reduce 1 nmol of O_2) and dividing by the total nanomoles of cytochrome aa_3 present in the reaction vessel.

Respiratory control of affinity-purified rat liver cytochrome oxidase was measured after reconstitution into preformed asolectin vesicles by the dialysis procedure of Casey et al. (1979), with the modification that the lipid to protein ratio was increased to 40 mg of asolectin/0.54 mg of aa_3. Phospholipid vesicles containing cytochrome oxidase (0.023–0.035 nmol of aa_3) were added to 1.75 mL of assay buffer (40 mM KCl, 10 mM Hepes, pH 7.2, 0.1 mM EDTA, 5.6 mM ascorbate, and 0.28 mM TMPD). Cytochrome *c* (0.025 μmol), carbonyl cyanide *m*-chlorophenylhydrazone (5.6 μM), and valinomycin (1.1 μM) were added as required.

Affinity Chromatography of Solubilized Beef Heart Mitochondrial Particles. Keilin–Hartree particles were prepared from beef heart essentially according to Ferguson-Miller et al. (1976), with the exception that a Waring blender was used to disrupt the tissue instead of hand grinding in a mortar. The particles were suspended at 60 mg/mL biuret protein (Jacobs et al., 1956) in 100 mM potassium phosphate, pH 7.8, and frozen at −20 °C. They were thawed and extensively dialyzed against cacodylate–Tris or sodium Bicine, pH 7.9, at the concentration to be used in the chromatography. The mitochondrial particles were made 20 mg of protein/mL (biuret), and 2 mg of lauryl maltoside or 5 mg of Triton X-100 was added per mg of protein. The solution was stirred for 30 min and then centrifuged at 40000*g* for 30 min. The supernatant, which contained 95–100% of the total oxidase in lauryl maltoside and 70–90% of the total oxidase in Triton X-100, was diluted to 1.5 mg of protein/mL (biuret) in 0.75% (15 mM) lauryl maltoside or 1% Triton X-100. The protein was chromatographed as described in the legend to Figure 1 on an affinity matrix (1.5 × 10 cm) of horse cytochrome *c*–Sepharose 4B (80 nmol of cytochrome *c*/mL of Sepharose)

[1] Abbreviations: DPG, diphosphatidylglycerol (cardiolipin); EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Me_2SO, dimethyl sulfoxide; PI, phosphatidylinositol; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; TN, turnover number; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TS, 50 mM Tris-HCl, pH 8, and 0.33 M sucrose; TSH, 50 mM Tris-HCl, pH 8, 0.33 M sucrose, and 1 mM histidine.

prepared according to Weiss & Juchs (1978), using cyanogen bromide activated Sepharose 4B prepared according to Cuatrecasas et al. (1968).

Rat Liver Cytochrome Oxidase Purification. Rat liver inner mitochondrial membranes were prepared according to the procedure of Sottocasa et al. (1967), as modified by Felgner et al. (1979). The membranes were suspended at 30 mg of protein/mL (biuret) in 0.33 M sucrose, 50 mM Tris-HCl, pH 8, and 1 mM histidine (1× TSH), made 20% in Me_2SO, and frozen at −65 °C (Fleischer, 1979). The membranes were thawed undisturbed on ice and then diluted ~3-fold by addition of 0.66 M sucrose, 100 mM Tris-HCl, pH 8.0, and 2 mM histidine (2× TSH). The membranes were pelleted by centrifugation at 10000*g* for 15 min, then gently dispersed in 2× TSH, and pelleted at 20000*g* for 20 min. The pellet was resuspended at a final concentration of 20 mg of protein/mL in 0.5 M potassium chloride, 1 mM PMSF, and 1× TSH. The suspension was made 5 mM in lauryl maltoside, and incubated 10 min undisturbed on ice, and then centrifuged at 40000*g* for 1 h. The liquid green pellet obtained was resuspended and diluted to a final concentration of 20 mg of protein/mL in 0.5× TSH plus 1%, or usually 0.5%, sodium cholate. The suspension was dialyzed 1:3 against the same buffer without cholate for 1 h and then centrifuged at 40000*g* for 1 h. The pellet contained 80–100% of the cytochrome aa_3 and was resuspended in and dialyzed overnight against a 100-fold excess of 50 mM Tris-HCl, pH 8.0, and 0.33 M sucrose (TS) containing 10 mM potassium phosphate. The suspension was made 40 mg of protein/mL (biuret) with TS plus 70–100 mM lauryl maltoside and 10 mM potassium phosphate, stirred on ice for at least 30 min, and centrifuged at 40000*g* for 30 min. A small insoluble pellet was discarded. The supernatant was diluted to 4 mg of protein/mL (biuret) in TS plus 10 mM potassium phosphate and 20 mM lauryl maltoside and applied to a hydroxyapatite column (6 nmol of aa_3/mL of hydroxyapatite) equilibrated at 4 °C with the same buffer containing 15 mM lauryl maltoside. After washing with 2.5 column volumes of the same buffer, the cytochrome aa_3 was eluted with a linear gradient of 3.3 column volumes of potassium phosphate, pH 8.0, 10–600 mM, in the above buffer. Fractions containing cytochrome aa_3 with a 280 nm/420 nm absorbance ratio of ≤3.0 were pooled. The cytochrome aa_3 fractions were dialyzed overnight vs. a 100-fold excess of 25 mM cacodylate–Tris, pH 7.9, and 5% sucrose, with two changes of buffer. The enzyme was chromatographed on an affinity matrix of Sepharose 4B–horse heart ferrocytochrome *c* (Weiss & Juchs, 1978), as detailed in the legend of Figure 2. Fractions containing cytochrome aa_3 with a 280 nm/420 nm ratio of ≤2.0 were pooled, and the enzyme was concentrated in an ultrafiltration cell by using an Amicon XM-300 ultrafilter and washed with several volumes of 25 mM Tris-HCl, pH 8.0, and 5% sucrose, to exchange the sample buffer, remove excess lauryl maltoside, and concentrate the oxidase.

$NaDodSO_4$–Polyacrylamide Electrophoresis. The subunit composition of purified rat liver oxidase was examined by the Laemmli discontinuous $NaDodSO_4$–polyacrylamide gel electrophoresis system (1970) on a Bio-Rad vertical slab gel electrophoresis apparatus. Sample preparation and electrophoresis conditions are detailed in the legend of Figure 3. Visualization of the peptides was achieved by scanning 280-nm absorption of the unstained gel, Coomassie Blue staining, fluorescamine labeling (Ragland et al., 1974), and silver staining (Wray et al., 1981).

Phospholipid Analysis. Phospholipids were extracted from purified rat liver cytochrome oxidase (6 mg of protein in 25 mM Tris-HCl, pH 8.0, and 5% sucrose, plus lauryl maltoside) according to the procedure of Awasthi et al. (1971). In some cases, when lauryl maltoside in the extract made thin-layer chromatography difficult, the lyophilized oxidase was preextracted with anhydrous acetone, which removes detergent but no detectable phosphorus from the oxidase (Awasthi et al., 1971). An aliquot of the lipid extract (250 μL; 0.5 μg of P) was chromatographed in two dimensions in the solvent system of Parsons & Patton (1967) on Supelco Redi-Coat 2D precoated TLC plates (0.25 mm). The plates were preextracted with methanol–methylene chloride, 1:1, and activated by heating at 120 °C for 1 h. Lipids were visualized by spraying with potassium dichromate (0.6%) in sulfuric acid (55% by weight) and charring at 180 °C for 20–30 min and were identified by comparison to standards. Total extractable phosphorus was quantitated by using the procedure of Bartlett (1959) or Ames (1966). Total phosphorus present in the oxidase was also determined without extraction by directly ashing the protein and measuring phosphorus by the procedure of Ames (1966).

Molecular Weight Determination by Gel Filtration. Molecular sieve chromatography of purified rat liver oxidase (0.1 mL of 7 μM aa_3) was performed on a Sephacryl 300 column (0.7 × 43 cm) in 100 mM KCl, 10 mM Tris-HCl, pH 7.8, and 1 mM EDTA, at a flow rate of 5 mL/h. Lauryl maltoside was 0.04% during the chromatography of the oxidase and during the calibration procedure. The column was calibrated with the following molecular weight standards: Blue Dextran, 2 000 000; thyroglobulin, 669 000; ferritin, 440 000; catalase, 250 000; aldolase, 158 000; alcohol dehydrogenase, 140 000; hemoglobin, 69 000; bovine serum albumin, 68 000; ovalbumin, 43 000; cytochrome *c*, 12 500.

Results

Affinity Chromatography of Solubilized Beef Heart Mitochondrial Particles. Solubilized beef heart mitochondria were chromatographed on horse ferrocytochrome *c*–Sepharose 4B in lauryl maltoside or Triton X-100 (Figure 1). Cytochromes *b* and c_1 were not significantly retained on the reduced cytochrome *c* matrix, while cytochrome aa_3 was very efficiently bound under the low ionic strength conditions used ($\mu \sim 0.0225$ M). After washing and eluting with a salt gradient, the yield of oxidase was 25% when lauryl maltoside was the detergent used [≥9 nmol of heme *a*/mg of protein (Lowry)], and 20% when Triton X-100 was used (≥7 nmol of heme *a*/mg of protein). The results show that both lauryl maltoside and Triton X-100 are competent in dispersing mitochondrial proteins sufficiently for chromatography, when used at high detergent to protein ratios. However, the loss of oxidase during the binding step in Triton X-100 was consistently at least 2-fold greater than that which occurred in lauryl maltoside under all buffer conditions tested, indicating that Triton X-100 does not disperse the mitochondrial membrane proteins as well as lauryl maltoside.

Cacodylate–Tris at 25 mM, pH 7.9, proved to be the best ionic environment for this chromatography in lauryl maltoside (Figure 1A). Beef heart cytochrome oxidase obtained under these conditions had a 280 nm/420 nm ratio of 2.7 and 9.5 nmol of heme *a*/mg of protein. Because of the absorbance of Triton X-100 at 280 nm, it was necessary to use another method to follow protein elution and to change to a different buffer that would not interfere with protein measurements. Sodium Bicine buffer was chosen since unlike Tris, Bicine allows fluorescamine protein assays to be performed. With Triton X-100 as the detergent, 45 mM Bicine gave maximal oxidase binding and yield (Figure 1B). The oxidase obtained

Table 1: Purification of Cytochrome *c* Oxidase from Rat Liver Mitochondria

purification steps	total oxidase (nmol)	overall yield (%)	turnover number[a] (s^{-1})		heme *a*/protein[c] (nmol/mg)
twice washed inner mitochondrial membranes	517	100	880		0.4
5 mM lauryl maltoside extracted pellet	406	79	500		0.7
1% cholate extracted pellet	287	56	330		1.5
hydroxyapatite chromatography	124	24	470	530[b]	7.0
cytochrome *c* affinity chromatography	79	15	350	630[b]	13.0

[a] Maximal turnover numbers [mol of cytochrome c·(mol of cytochrome aa_3)$^{-1}$·s^{-1}] were measured in 1.75 mL for 50 mM potassium phosphate, pH 6.5, 2.8 mM ascorbate, 0.56 mM TMPD, and 40 μM cytochrome *c*. Lauryl maltoside was added to the assay mixture before the addition of oxidase at concentrations of 1.1 mM for crude preparations and 0.20–0.25 mM for hydroxyapatite and affinity-purified oxidase. [b] As in footnote *a* plus sonicated asolectin vesicles (1 mg). [c] Lowry protein (Lowry et al., 1951).

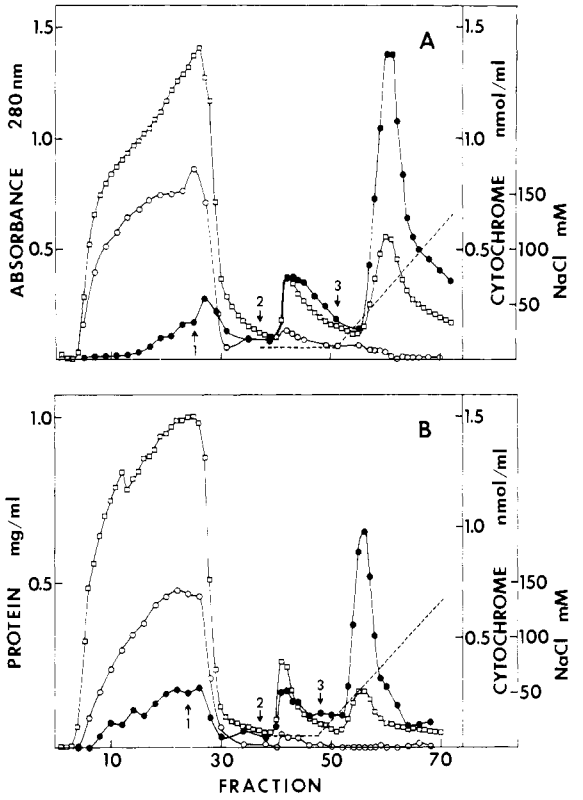

FIGURE 1: Chromatography of solubilized beef heart mitochondria on horse ferrocytochrome *c*–Sepharose 4B. (A) Lauryl maltoside solubilized mitochondria (175 mg of protein) were applied to the affinity column (1.5 × 10 cm) reduced with 5 mM ascorbate and equilibrated on the bench top in cold 25 mM Tris–cacodylate, pH 7.9, 5% sucrose, and 15 mM lauryl maltoside at a flow rate of 1 column volume/h. The column was washed with (1) 50 mL of the same buffer and (2) 50 mL of 11 mM NaCl in the same buffer and eluted with (3) 90 mL of a 11–131 mM NaCl gradient in the same buffer. Fractions of 4 mL were collected, and fractions 59–63 were pooled. Protein was estimated by 280-nm absorbance (□). Concentrations of cytochrome *b* (○) and cytochrome aa_3 (●) were calculated from difference spectra. (B) Triton-solubilized mitochondria (145 mg of protein) were applied to the affinity column equilibrated in 45 mM sodium Bicine, pH 7.9, 5% sucrose, and 1% Triton X-100 and were washed and eluted with the NaCl concentrations used in (A). Fractions 54–59 were pooled. Protein was estimated by fluorescamine determination (□). Cytochrome *b* (○); cytochrome aa_3 (●); NaCl concentration (---).

under these conditions had only 7.0 nmol of heme *a*/mg of protein. Higher ionic strength (77 mM Bicine; $\mu \sim 0.038$ M) caused loss of most of the oxidase during loading. In contrast, with lauryl maltoside, 77 mM Bicine allowed good retention of oxidase and yielded an enzyme with 9 nmol of heme *a*/mg of protein.

The enzyme prepared in lauryl maltoside is significantly more active (TN = 180 s^{-1}) than that prepared in Triton X-100 (TN = 70 s^{-1}), even when the latter is preincubated

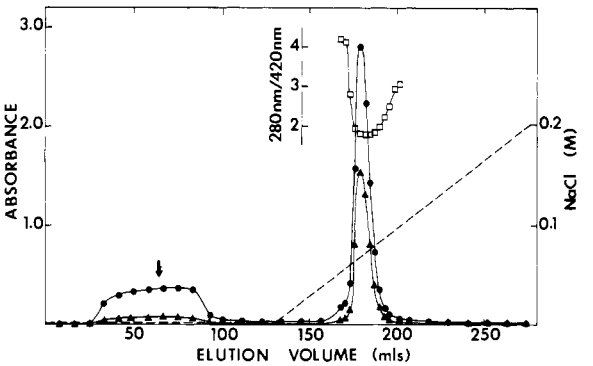

FIGURE 2: Cytochrome *c* affinity chromatography of rat liver cytochrome *c* oxidase. Cytochrome oxidase [164 nmol of aa_3 at 1 mg of protein/mL (Lowry); 8–9 nmol of aa_3/mL of Sepharose] obtained from hydroxyapatite chromatography of solubilized, fractionated inner mitochondrial membranes was applied to a cytochrome *c*–Sepharose 4B column (1.5 × 21 cm) prepared according to Weiss & Juchs (1978). The column was reduced with 5 mM ascorbate and equilibrated on the bench top in cold 25 mM Tris–cacodylate, pH 7.9, 15 mM lauryl maltoside, and 5% sucrose. The column was washed (arrow) with 3 column volumes of the buffer and eluted by the addition of a 4 column volume linear gradient of 0–0.2 M NaCl (---). Protein (●) and cytochrome oxidase (▲) were monitored by their absorbances at 280 and 420 nm, respectively. The 280 nm/420 nm ratio of these absorbances (an indication of the degree of purity) is also shown (□).

and assayed in lauryl maltoside and 50 mM potassium phosphate, pH 6.5 (Experimental Procedures). When assayed in the presence of asolectin vesicles and lauryl maltoside, the lauryl maltoside enzyme had a TN of 400 s^{-1} and the Triton enzyme had a TN of 250 s^{-1}.

Purification of Rat Liver Cytochrome Oxidase. A method for purifying rat liver cytochrome oxidase in lauryl maltoside was developed which makes maximal use of the binding capacity of the horse ferrocytochrome *c* resin, by employing it as the last purification step. Table I presents a summary of the data for a typical cytochrome oxidase purification. Extraction of the mitochondrial membranes with a low concentration of lauryl maltoside (5 mM) in the presence of 0.5 M KCl selectively solubilizes ~90% of cytochromes *b* and c_1, leaving cytochromes aa_3 in the membrane fraction. Residual cytochromes *b* and c_1 could be further removed by extraction with sodium cholate. Total solubilization of the oxidase is achieved by treatment with an increased concentration of lauryl maltoside, yielding a green, optically clear solution that is retained by hydroxyapatite. Elution with a linear gradient of potassium phosphate in lauryl maltoside gives a peak of cytochrome oxidase that is largely free of cytochromes *b* and c_1, but the average heme to protein ratio is only 7–8.4 nmol of heme *a*/mg of protein. The elution profile for the final purification step of cytochrome oxidase on an affinity matrix of Sepharose 4B–horse ferrocytochrome *c* is shown in Figure 2. Under the conditions of low ionic strength and high detergent concentration used, very tight binding between the

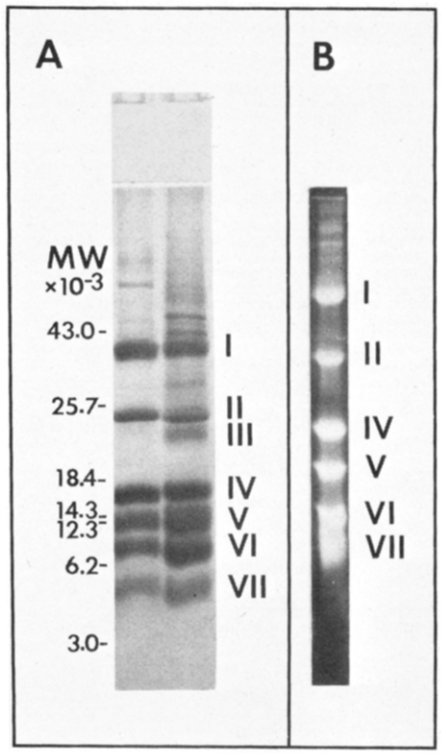

FIGURE 3: Subunit composition of rat liver cytochrome oxidase as revealed by $NaDodSO_4$–polyacrylamide electrophoresis using the discontinuous buffer system of Laemmli (1970). (A) Coomassie blue stained rat liver oxidase (13.5 nmol of heme *a*/mg of protein) (left lane) and beef heart oxidase (9 nmol of heme *a*/mg of protein) (right lane). The separating gel (1.5 × 100 × 140 mm) contained 0.2% $NaDodSO_4$ and gradients of 10–22% acrylamide and 5–15% sucrose. Purified cytochrome oxidase was precipitated in 10 volumes of cold 5% Cl_3CCOOH to remove it from bulk lauryl maltoside which interferes with $NaDodSO_4$ binding. The oxidase was resuspended in 5% $NaDodSO_4$, 5% β-mercaptoethanol, and 5% glycerol in sample buffer, dissociated by sonication at 10–15 °C for 30 min in a bath type sonicator, and electrophoresed for 1 h at 15 mA and then for 3.5 h at 20–25 mA. The sample wells, stacking gel (6% acrylamide), and separating gel are shown. Numbering of subunits (I–VII) is according to Downer et al. (1976). Molecular weight designations are those of standard proteins electrophoresed on the same gel: ovalbumin, 43 000; α-chymotrypsinogen, 25 700; β-lactoglobulin, 18 400; lysozyme, 14 300; cytochrome *c*, 12 300; bovine trypsin inhibitor, 6200; insulin (β), 3000. (B) Fluorescamine-labeled rat liver oxidase. The Cl_3CCOOH-precipitated enzyme was labeled with fluorescamine, dissociated, electrophoresed on a 12.5% acrylamide gel, and then photographed (unfixed) in UV light. The separating gel in shown. Numbering of subunits is as in (A).

highly dispersed cytochrome oxidase and its substrate is promoted. The cytochrome oxidase eluted with the sodium chloride gradient is spectrally pure with an average heme to protein ratio of 12.3–13.5 nmol of heme *a*/mg of protein and a very low 280 nm/420 nm absorbance ratio of 1.9. The turnover number of the purified enzyme is 270–400 s^{-1} and can be further stimulated to 350–650 s^{-1} by the addition of preformed asolectin vesicles.

Physical Properties of Rat Liver Cytochrome c Oxidase. Figure 3A shows the Coomassie Blue stained subunit composition of purified rat liver oxidase compared to that of beef heart enzyme prepared by a modification of the method of Hartzell & Beinert (1974). The beef heart enzyme is resolved into seven major subunits. The rat protein contains only six major subunits, in the presence or absence of up to 6 M urea, and is missing subunit III of apparent molecular weight 22 000 [30 000 as determined by DNA sequence estimates (Thalenfeld & Tzagoloff, 1980)] that is present in the beef heart enzyme. There is little or no aggregated material at the top of the gel or high molecular weight species that might suggest that lack of subunit III is the result of poor entry of this hydrophobic subunit into the gel, or aggregation and subsequent anomalous migration. Figure 3B shows an $NaDodSO_4$ gel run on oxidase that was prelabeled with fluorescamine. The lack of visualization of a M_r 22 000 peptide indicates that its absence is not a function of a particular staining procedure. A similar array of subunits is observed by staining the gel for protein with silver and by scanning the absorbance of the unstained gel at 280 nm (not shown).

Total phosphorus content of the oxidase is 3.0 mol of P/mol of cytochrome aa_3, as determined by analysis of the total lipid extract of the enzyme (Awasthi et al., 1971). Phosphorus analysis performed directly on the unextracted protein (Ames, 1966) is in agreement, revealing 3.1 mol of phospholipid present per mol of cytochrome aa_3. Two-dimensional thin-layer chromatography of the total lipid extract showed cardiolipin (DPG) and phosphatidylinositol (PI) were the only detectable phospholipids spots, indicating that at most 1 mol of DPG and 1 mol of PI are associated per mol of cytochrome oxidase.

Gel filtration of purified rat liver cytochrome oxidase on Sephacryl 300 revealed the presence of a single 420 nm absorbing species having an apparent molecular weight of 310 000 ± 30 000 with no correction made for the contribution of the lauryl maltoside micelle to the Stokes radius of the protein (see Discussion).

Kinetic Properties of Rat Liver Cytochrome Oxidase. The kinetics of reaction of the purified, lipid-depleted oxidase with horse cytochrome *c* are shown in Figure 4A. A clearly biphasic plot is obtained in 50 mM potassium phosphate, pH 6.5. At lower ionic strength (25 mM cacodylate–Tris, pH 7.9), nonlinear kinetics are also observed, but the overall activity is much lower than in the native state, and the two phases are not as well resolved. This can be seen by comparing the behavior of the enzyme in the purified form (Figure 4A) with that of oxidase in intact membranes without any detergent present (Figure 4B). In cacodylate buffer, cytochrome *c* has a much higher turnover and affinity for the membrane-bound cytochrome oxidase than for the purified form (note the scale is reduced by a factor of 10 compared to Figure 4A). In contrast, in phosphate buffer the overall turnover and kinetic behavior of the enzyme appear not to have changed greatly during the purification procedure [membrane-bound oxidase (Figure 4B), $K_{m1} = 3 \times 10^{-7}$ M, $K_{m2} = 1 \times 10^{-5}$ M, and TN = 350 s^{-1}; purified oxidase (Figure 4A), $K_{m1} = 2 \times 10^{-7}$ M, $K_{m2} = 3 \times 10^{-6}$ M, and TN = 400 s^{-1}].

Figure 4C compares the kinetics of the purified oxidase in cacodylate buffer to those of oxidase in the native membrane, in lauryl maltoside solubilized membranes, and in reconstituted phospholipid vesicles. Under these buffer conditions, it appears that the major results of purification and delipidation are an overall decrease in catalytic activity and an increase in the K_m for cytochrome *c* in the second kinetic phase. Lipid repletion partially restores the activity of the purified oxidase to a level closer to that of oxidase in solubilized membranes and also decreases the K_m of the second kinetic phase.

Discussion

Cytochrome c Affinity Chromatography. Horse cytochrome *c* affinity chromatography has been used to obtain beef heart cytochrome oxidase with a purity of 9.5 nmol of heme *a*/mg of protein. The most effective chromatographic procedure utilizes the ability of the nonionic detergent, lauryl maltoside, to disperse and stabilize the enzyme under conditions of relatively low ionic strength (Rosevear et al., 1980; Ferguson-

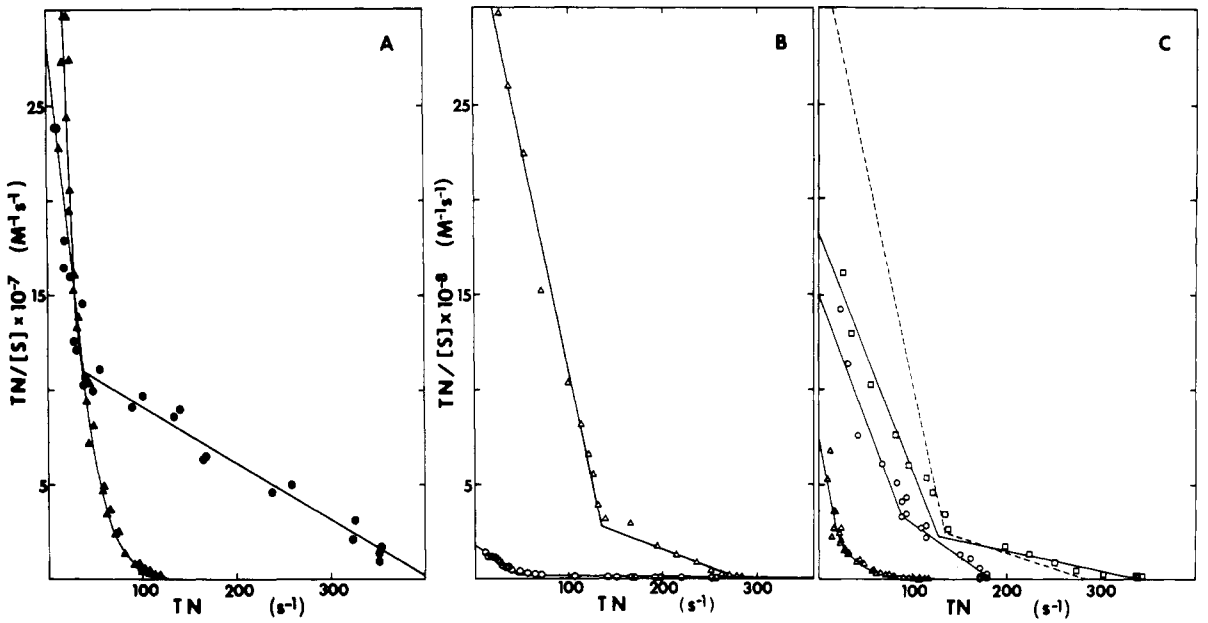

FIGURE 4: Eadie–Hofstee plots of the kinetics of oxidation of cytochrome *c* by rat liver cytochrome oxidase. (A) Cytochrome *c* affinity purified oxidase (0.025 nmol) assayed in 50 mM potassium phosphate, pH 6.5 (●), or 25 mM cacodylate–Tris, pH 7.9 (▲). Oxygen consumption was measured polarographically as indicated under Experimental Procedures in the presence of 0.25 mM lauryl maltoside, 2.8 mM ascorbate, and 0.56 mM TMPD at 25 °C. (B) Rat liver inner mitochondrial membranes (freshly prepared) containing 0.05 nmol of aa_3 assayed in phosphate (○) and containing 0.03 nmol of aa_3 assayed in cacodylate (△) under the same conditions as above but without added detergent. (C) Comparison of purified oxidase (△), purified oxidase incorporated into phospholipid vesicles (○), and oxidase in lauryl maltoside solubilized mitochondrial membranes (□), assayed in 25 mM cacodylate–Tris, pH 7.9. The data for purified oxidase are the same as shown in (A). Phospholipid-reconstituted oxidase (0.015 nmol) was assayed as in (B) with the addition of 5.6 μM CCCP and 1.1 μM valinomycin to the assay mixture. Oxidase in mitochondrial membranes (10 mg of protein/mL) was solubilized with lauryl maltoside (1 mg of detergent/mg of protein) in 25 mM Tris-HCl, pH 8.0, 165 mM sucrose, and 0.5 mM histidine. The assay was performed as in (A) but with 0.026 nmol of oxidase and 0.11 mM lauryl maltoside. The protein to detergent ratio used was that yielding maximal oxidase activity. The kinetic plot for intact mitochondrial membranes from (B) is shown for comparison by the dashed line.

Miller et al., 1982). Weiss & Kolb (1979) have previously demonstrated that Triton X-100 provides a good dispersant for cytochrome oxidase from *Neurospora crassa*, allowing the application of horse cytochrome *c* affinity chromatography to this enzyme. Our results show that the procedure can also be applied to the beef heart enzyme when high detergent to protein ratios are employed. Other workers (Bill et al., 1980, 1982) have suggested that the previous lack of success of cytochrome *c* affinity chromatography in the purification of the yeast or beef oxidase is the result of blockage of the lysine-rich binding site for oxidase on cytochrome *c* by cross-linking of the cytochrome to the cyanogen bromide activated resin via essential lysine residues. They have shown that good purification of oxidase from these sources can be achieved if *Saccharomyces cerevisiae* (yeast) cytochrome *c* is linked to thiol-Sepharose 4B via its single sulfhydryl group, which is some distance from the cytochrome oxidase binding domain. However, our data and those of Weiss and co-workers demonstrate that resin prepared by using limited cyanogen bromide activation and horse heart cytochrome *c* is equally effective. Weiss & Juchs (1978) show that it is essential to hydrolyze some of the reactive cyanate esters on the resin before cytochrome *c* is added in order to avoid multipoint linkages between the protein and the resin that might lead to inaccessibility of cytochrome *c* to the oxidase. Thus, the success of the yeast cytochrome *c* method may be accounted for mainly by the low density of cross-links between the thiol resin and cytochrome *c* and the high ratio of detergent to protein used for solubilization and chromatography. Using the horse cytochrome *c* we achieve a beef heart cytochrome oxidase binding capacity of 0.065 nmol of aa_3/nmol of cytochrome *c*, greater than that reported by Weiss & Kolb (1979) or Bill et al. (1980, 1982). The fact that cytochromes *b* and c_1 do not bind to the ascorbate-reduced resin is convincing evidence that the separation is based on specific interactions (Weiss et al., 1978) and not simple ion exchange or hydrophobic binding (Ozawa et al., 1975; Rascati & Parsons, 1979). We conclude that linkage via lysine residues occurs fairly randomly in this procedure, allowing a large proportion of the cytochrome *c* to bind correctly with the oxidase. Aside from the obvious economic advantage of using horse rather than yeast cytochrome *c*, the imino ester linkage of lysine to the resin is much more stable than the disulfide linkage, permitting the long-term storage and reuse of the resin.

Cytochrome *c* affinity chromatography is applied as a final step in the purification of rat liver cytochrome oxidase, making maximum use of the capacity of the resin and resulting in good yield of a highly purified enzyme (routinely 13 nmol of heme *a*/mg of protein) from a relatively impure state (~7 nmol of heme *a*/mg of protein). The cytochrome oxidase binding capacity (up to 0.12 nmol of aa_3/nmol of cytochrome *c*) achieved under these circumstances is twice that obtained when the column is used as an initial step in purifying the enzyme from whole mitochondria. Furthermore, a single step purification as performed by our procedures in lauryl maltoside or Triton X-100, or as described by Bill et al. (1980, 1982), gives a less pure enzyme at no higher yield, with much more lipid associated (Azzi et al., 1982). When affinity chromatography is coupled with hydroxyapatite chromatography for the purification of rat liver oxidase, the enzyme obtained has several unique properties compared to oxidase prepared by conventional techniques. Important among these is its lipid-depleted, highly active state. Hydroxyapatite chromatography at low ionic strength appears to be particularly important for removing phospholipid from the enzyme. Evidently the strong interaction of phospholipid with the positively charged calcium ions favors its removal from the enzyme. Some denaturation and irreversible binding of oxidase to the column also occur

at this step, resulting in somewhat low yields. However, addition of 100 mM NaCl to the equilibration and elution buffers gives much higher yield without diminishing the capacity of the hydroxyapatite for oxidase binding (Bernardi, 1971). Under these conditions, the same heme *a* to protein ratios can be obtained in the subsequent affinity chromatography, although considerably more lipid remains associated with the enzyme (5 mol of P/mol of aa_3). This is still a low lipid content, and therefore the inclusion of salt in the hydroxyapatite step is recommended for the preparation of oxidase when a highly delipidated enzyme is not required.

Association of Phospholipid with Cytochrome Oxidase. The rat liver cytochrome oxidase prepared by the method described is consistently highly active (TN = 270–400 s^{-1}) and very low in lipid [1 mol of DPG and 1 mol of PI per mol of oxidase (aa_3)]. Other methods of purification of the rat liver enzyme have yielded oxidase with 8–9.5 mol of phospholipid/mol of enzyme (Hochli & Hackenbrock, 1978; Nagasawa et al., 1979). Cytochrome oxidase prepared from beef heart mitochondria by classical procedures usually contains 25–75 mol of phospholipid/mol of enzyme (Brierley & Merola, 1962; Yu et al., 1979; Robinson & Capaldi, 1977). A number of methods have been developed to remove phospholipid from purified beef oxidase (Tzagoloff & MacLennan, 1965; Awasthi et al., 1971; Hartzell & Beinert, 1974; Yu et al., 1975; Robinson & Capaldi, 1977; Fry & Green, 1980; Robinson et al., 1980). A minimum level of 0.5–2 mol of phospholipid/mol of enzyme has been obtained by these techniques, but the resulting enzyme is usually relatively inactive (TN $\leq$ 40–100 s^{-1}). Analysis of the bound phospholipid has shown that cardiolipin remains tenaciously associated with the oxidase. Because its depletion results in a significant loss of activity, and adding it back gives higher activities than obtained with other individual phospholipids, some researchers have proposed that cardiolipin is essential for the function of cytochrome oxidase (Awasthi et al., 1971; Robinson et al., 1980; Fry & Green, 1980; Vik et al., 1981; Robinson, 1982). In contrast, Watt et al. (1978) have reported recovery of activity after complete substitution of cardiolipin with phosphatidylcholine in yeast cytochrome oxidase. However, the low activities obtained in all these lipid repletion experiments make it difficult to draw definite conclusions regarding the precise role of cardiolipin. The rat liver oxidase containing only 1 mol of cardiolipin but retaining high activity and responsiveness to added phospholipid may thus prove to be a useful tool for further investigating the lipid requirement. In fact, we have obtained rat liver oxidase with 0.75 mol of DPG/mol of aa_3 by subjecting the enzyme to a repeat affinity chromatography step, without loss of activity (TN = 400 s^{-1}) (T. Carlson, unpublished data). These findings indicate that a stoichiometric association of DPG with oxidase may not be a specific requirement for electron transfer activity.

Kinetic Changes Related to Purification. Rat liver oxidase containing 1 mol of DPG/mol of aa_3 retains the ability to interact with cytochrome *c* with two kinetically distinct affinities similar to those observed with the membrane-bound enzyme, when assayed under maximal turnover conditions in 50 mM potassium phosphate, pH 6.5. When assayed in 25 mM cacodylate, pH 7.9, conditions designed to favor tight binding of cytochrome *c* to oxidase, the overall activity of the enzyme and the contribution of both kinetic phases are considerably diminished compared to the native enzyme. Vik et al. (1981) have previously reported a differential decrease in the magnitude of the low-affinity phase upon lipid depletion of beef heart oxidase and a concomitant loss of cytochrome *c* binding. The hypothesis formulated by these authors (Bisson et al., 1980; Vik et al., 1981; Fuller et al., 1981), namely, that a second cardiolipin molecule tightly associated with the oxidase is essential for, and the major component of, the low-affinity binding site for cytochrome *c* on the enzyme, is not in accord with our observations of a well-defined second phase in the kinetic analysis performed in phosphate buffer or with the observation that both kinetic phases in cacodylate are greatly diminished in turnover number in response to lipid depletion. However, the increase in apparent K_m of the second kinetic phase could relate to lower affinity binding of a second cytochrome *c*. This interpretation of the kinetics must be qualified, however, since the K_m values cannot be unequivocally determined from these complex plots.

The lower turnover of the purified enzyme in cacodylate may reflect decreased accessibility of the bound cytochrome *c* to the lipophilic reducing agent TMPD in the delipidated enzyme, while in 50 mM phosphate reduction by TMPD occurs mainly in solution (Ferguson-Miller et al., 1978; Brautigan et al., 1978). This postulate is supported by the observation that oxidase reconstituted into phospholipid vesicles has greatly increased turnover numbers compared to the purified enzyme, when assayed in cacodylate but not in phosphate. An effect on TMPD reduction rates alone, however, cannot account for the activity loss during purification observed in these studies or by others, since it has been demonstrated that electron transfer between hemes *a* and a_3 is very sensitive to changes in the lipid content of the enzyme (Yu et al., 1975) and loss of activity measured in the absence of TMPD also is correlated with removal of cardiolipin (Robinson, 1982).

When assayed in phosphate buffer, the membrane-bound oxidase and the purified form show very similar kinetic constants and overall activity, but during the course of solubilization and purification much higher turnover numbers are observed (Thompson et al., 1982; see Table I). Dramatic dependence of turnover rates on the nature of the detergent and detergent to protein ratios has been observed previously (Mason & Ganapathy, 1970), emphasizing the inherent difficulty in using maximal activities as a measure of the degree of "nativeness" of the enzyme. Completeness of dispersion is clearly a factor in determining apparent activity (Vik & Capaldi, 1980) as well as more subtle effects on enzyme conformation or monomer–dimer interconversions (Thompson et al., 1982).

Monomer Form of Cytochrome Oxidase. A number of investigators have considered the idea that the native, active form of cytochrome oxidase may be a dimer (Robinson & Capaldi, 1977; Bisson et al., 1980; Ferguson-Miller et al., 1982; Wikstrom, 1981). The apparent molecular weight of the rat liver oxidase in lauryl maltoside is found to be ~300 000 by gel filtration, in agreement with the value obtained for the beef heart enzyme under the same conditions (Rosevear et al., 1980). It is difficult to assess whether this represents a monomer [M_r ~200 000 (Buse et al., 1982)] or dimer of oxidase without an accurate estimate of bound detergent and given the uncertainty regarding the contribution of the micelle to the Stokes radius of a large asymmetric protein (Nozaki et al., 1976; le Maire et al., 1980). Sedimentation equilibrium studies were therefore performed (Suárez et al., 1983) at different solvent densities (Reynolds & Tanford, 1976) to rigorously determine the molecular weight of both the protein and the associated micelle. The lauryl maltoside micelle is found to have a molecular weight of 76 000, slightly larger than previous estimates from gel filtration (Rosevear et al., 1980), and less than a single micelle of lauryl maltoside is found

associated with the purified rat liver oxidase. Using the value for bound detergent determined by sedimentation equilibrium, we obtain a molecular weight for oxidase from the gel filtration data of ~250000, higher than that predicted for a monomer, but considerably lower than expected for a dimer. The sedimentation equilibrium results (Suárez et al., 1983) indicate that the size of cytochrome oxidase is overestimated by the gel filtration procedure. We conclude that the predominant form of cytochrome oxidase obtained in lauryl maltoside under these buffer and pH conditions is a highly active monomer. An active monomeric species is consistent with the results of target size analysis of cytochrome oxidase by radiation inactivation procedures, which show that high turnover and normal binding of cytochrome *c* are associated with a molecular weight unit even smaller than a monomer (Thompson et al., 1982).

Subunit III. There is a long-standing controversy in the study of cytochrome oxidase concerning the existence and role of subunit III, a peptide coded for by the mitochondrial genome with a molecular weight of 30000 (Thalenfeld & Tzagoloff, 1980) and anomalous behavior on $NaDodSO_4$–acrylamide gels [apparent molecular weight 18000–24500 (Azzi, 1980)]. Capaldi and co-workers (Downer et al., 1976; Capaldi et al., 1977) have demonstrated that the behavior of this peptide in $NaDodSO_4$ gel electrophoresis systems is highly variable, depending on how the sample is treated before it is applied to the gel and the exact conditions under which it is run. Considering the difficulties caused by subunit polymerization, protein insolubility, and inadequate Coomassie Blue staining, we examined the peptide composition of rat liver oxidase using a number of different sample treatments, gel systems, and staining techniques. We have shown that under conditions where subunit III from beef heart oxidase is clearly visualized, no such peptide is seen in the purified rat liver enzyme. At early stages in our purification procedure we observe the presence of this peptide. Merle & Kadenbach (1980) have demonstrated unequivocally that subunit III of rat liver oxidase runs in a similar position to that of oxidases from other sources and can be detected under the buffer and sample preparation conditions used in our electrophoretic analysis. In addition, this peptide is present in rat liver oxidase prepared by other methods (Hochli & Hackenbrock, 1978; Nagasawa et al., 1979). We therefore conclude that chromatography in lauryl maltoside effectively removes not only lipid but also subunit III.

There is some debate regarding the role of subunit III as an essential or auxiliary element in the process by which cytochrome oxidase establishes a potential gradient across the mitochondrial membrane. There is evidence that cytochrome oxidase may be able not only to abstract protons from the matrix to form water from O_2 (Mitchell, 1961; Moyle & Mitchell, 1978) but also to extrude protons from the matrix by means of a proton pump mechanism (Wikstrom, 1977; Wikstrom & Saari, 1977; Sigel & Carafoli, 1978; Azzone et al., 1979). Involvement of subunit III in this latter process has been suggested by labeling studies using DCCD (Azzi et al., 1979; Casey et al., 1981; Prochaska et al., 1981). Our results show that rat liver oxidase depleted of subunit III can be reconstituted into phospholipid vesicles and exhibit respiratory control ratios as high as 9.0. These data are in agreement with those of Carroll & Racker (1977) and Wikstrom and co-workers (Penttila & Wikstrom, 1981; Wikstrom, 1981; Wikstrom et al., 1981; Saraste et al., 1981), who obtained preparations of beef oxidase lacking subunit III by either chymotrypsin digestion or high pH plus high detergent treatment and demonstrated that uncoupling agents stimulated the electron transfer rates of these enzymes. Because the conditions used to remove subunit III in both cases caused loss of other subunits, and possibly other undefined effects, we feel that our data obtained with the rat liver oxidase, from which the subunit was removed without drastic treatment, are particularly significant in establishing the existence of a mechanism other than a subunit III dependent proton pump for producing a proton gradient.

Subunit III has also been implicated in substrate binding by cytochrome oxidase (Wikstrom & Penttila, 1982). Our observations are not in agreement with the suggestion that subunit III has a critical role in the high-affinity binding of cytochrome *c* to cytochrome oxidase, since we find that loss of activity in response to lipid and subunit III removal occurs equally in both kinetic phases, is recoverable by repletion with phospholipid, and is accompanied by relatively small changes in the K_m values of the initial kinetic phase.

Conclusions. The minimum structural requirements necessary for the full native activity of cytochrome oxidase remain to be defined. However, analysis of cytochrome oxidase purified in lauryl maltoside by chromatographic techniques has been useful in demonstrating that neither a dimer form, subunit III, nor tightly bound cardiolipin in excess of 1 mol/mol of oxidase is necessary for most aspects of normal oxidase function. Rat liver oxidase in which these structural features are absent retains the characteristic spectrum of cytochrome oxidase, the capacity to participate in high- and low-affinity interactions with cytochrome *c*, and the ability to develop and respond to a membrane potential gradient.

Acknowledgments

We thank Dr. John Wilson, J. Messer, and M. Stadt for rat liver inner mitochondrial membrane preparations, Steve Castleman for synthesis of lauryl maltoside, and Dr. William Wells for the fluorescamine-labeled oxidase polyacrylamide electrophoresis.

Registry No. Cytochrome *c* oxidase, 9001-16-5; lauryl β-D-maltoside, 69227-93-6; cytochrome *c*, 9007-43-6.

References

Ames, B. (1966) *Methods Enzymol. 8*, 115–118.
Antalis, T. M., & Palmer, G. (1982) *J. Biol. Chem. 257*, 6194–6206.
Awasthi, Y. C., Chuang, T. F., Keenan, T. W., & Crane, F. L. (1971) *Biochim. Biophys. Acta 226*, 42–52.
Azzi, A. (1980) *Biochim. Biophys. Acta 594*, 231–252.
Azzi, A., Bisson, R., & Casey, R. P. (1979) in *Membrane Bioenergetics* (Lee, C. P., Schatz, G., & Ernster, L., Eds.) pp 13–20, Addison-Wesley, Reading, MA.
Azzi, A., Bill, K., & Broger, C. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2447–2450.
Azzone, G. F., Pozzan, T., & Di Virgilio, F. (1979) *J. Biol. Chem. 254*, 10206–10212.
Bartlett, G. (1959) *J. Biol. Chem. 234*, 466–468.
Bernardi, G. (1971) *Methods Enzymol. 22*, 325–339.
Bill, K., Casey, R., Broger, C., & Azzi, A. (1980) *FEBS Lett. 120*, 248–250.
Bill, K., Broger, C., & Azzi, A. (1982) *Biochim. Biophys. Acta 679*, 28–34.
Birchmeier W., Kohler, C. E., & Schatz, G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4334–4338.
Bisson, R., Jacobs, B., & Capaldi, R. (1980) *Biochemistry 19*, 4173–4178.
Brautigan, D. L., Ferguson-Miller, S., & Margoliash, E. (1978) *Methods Enzymol. 53*, 128–164.

Brierley, G. P., & Merola, A. J. (1962) *Biochim. Biophys. Acta 64*, 205–217.

Buse, G., Steffens, G. J., Steffens, G. C. M., Sacher, R., & Erdweg, M. (1982) in *Electron Transport and Oxygen Utilization* (Ho, C., Ed.) pp 157–163, Elsevier/North-Holland Biomedical Press, New York.

Capaldi, R. A., Bell, R. L., & Branchek, T. (1977) *Biochem. Biophys. Res. Commun. 74*, 425–433.

Carroll, R., & Racker, E. (1977) *J. Biol. Chem. 252*, 6981–6990.

Casey, R., Chappell, B., & Azzi, A. (1979) *Biochem. J. 182*, 149–156.

Casey, R., Broger, C., & Azzi, A. (1981) *Biochim. Biophys. Acta 638*, 86–93.

Cuatrecasas, P., Wilchek, M., & Anfinsen, C. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 636–643.

Downer, N. W., Robinson, N. C., & Capaldi, R. A. (1976) *Biochemistry 15*, 2930–2936.

Errede, B., & Kamen, M. D. (1978) *Biochemistry 17*, 1015–1027.

Felgner, P. L., Messer, J. L., & Wilson, J. E. (1979) *J. Biol. Chem. 254*, 4946–4949.

Ferguson-Miller, S., Brautigan, D. L., & Margoliash, E. (1976) *J. Biol. Chem. 251*, 1104–1115.

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

Ferguson-Miller, S., Van Aken, T., & Rosevear, P. (1982) in *Electron Transport and Oxygen Utilization* (Ho, C., Ed.) pp 297–303, Elsevier/North-Holland Biomedical Press, New York.

Fleischer, S. (1979) *Methods Enzymol. 55*, 28–32.

Fry, M., & Green, D. (1980) *Biochem. Biophys. Res. Commun. 93*, 1238–1246.

Fuller, S. D., Darley-Usmar, V. M., & Capaldi, R. A. (1981) *Biochemistry 20*, 7046–7053.

Hartzell, C. H., & Beinert, H. (1974) *Biochim. Biophys. Acta 368*, 318–338.

Hochli, L., & Hackenbrock, C. (1978) *Biochemistry 17*, 3712–3719.

Jacobs, E. E., Jacob, M., Sanadi, D. R., & Bradley, L. B. (1956) *J. Biol. Chem. 223*, 147–156.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

le Maire, M., Rivas, E., & Møller, J. V. (1980) *Anal. Biochem. 106*, 12–21.

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

Mason, H. S., & Ganapathy, K. (1970) *J. Biol. Chem. 245*, 230–237.

Merle, P., & Kadenbach, B. (1980) *Eur. J. Biochem. 105*, 499–507.

Mitchell, P. (1961) *Nature (London) 191*, 144–148.

Moyle, J., & Mitchell, P. (1978) *FEBS Lett. 88*, 268–272.

Nagasawa, T., Nagasawa-Fujimori, H., & Heinrich, P. C. (1979) *Eur. J. Biochem. 94*, 31–39.

Nicholls, P. (1964) *Arch. Biochem. Biophys. 106*, 25–48.

Nicholls, P. (1965) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S., & Morrison, M., Eds.) Vol. 2, pp 764–783, Wiley, New York.

Nozaki, Y., Schechter, N. M., Reynolds, J. A., & Tanford, C. (1976) *Biochemistry 15*, 3884–3890.

Ozawa, T., Okumura, M., & Yagi, K. (1975) *Biochem. Biophys. Res. Commun. 65*, 1102–1107.

Parsons, J., & Patton, S. (1967) *J. Lipid Res. 8*, 696–698.

Penttila, T., & Wikstrom, M. (1981) in *Vetorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria* (Palmieri, F., et al., Eds.) pp 71–80, Elsevier/North-Holland Biomedical Press, Amsterdam.

Prochaska, L. J., Bisson, R., Capaldi, R. A., Steffens, G. C. M., & Buse, G. (1981) *Biochim. Biophys. Acta 637*, 360–373.

Ragland, W. L., Pace, J. L., & Kempner, D. L. (1974) *Anal. Biochem. 59*, 24–33.

Rascati, R. J., & Parsons, P. (1979) *J. Biol. Chem. 254*, 1586–1593.

Reynolds, J. A., & Tanford, C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4467–4470.

Robinson, N. C. (1982) *Biochemistry 21*, 184–188.

Robinson, N. C., & Capaldi, R. A. (1977) *Biochemistry 16*, 375–381.

Robinson, N. C., Strey, F., & Talbert, L. (1980) *Biochemistry 19*, 3656–3661.

Rosevear, P., Van Aken, T., Baxter, J., & Ferguson-Miller, S. (1980) *Biochemistry 19*, 4108–4115.

Saraste, M., Pentilla, T., & Wikstrom, M. (1981) *Eur. J. Biochem. 115*, 261–268.

Sigel, E., & Carafoli, E. (1978) *Eur. J. Biochem. 89*, 119–123.

Sottocasa, G. L., Kuylenstierna, B., Ernster, L., & Bergstrand, A. (1967) *Methods Enzymol. 10*, 448–463.

Suârez, M., Revzin, A. R., Swaisgood, M., Thompson, D. A., & Ferguson-Miller, S. (1983) *Am. Soc. Biol. Chem., Biophys. Soc.*, Abstr. No. 1782.

Thalenfeld, B. E., & Tzagoloff, A. (1980) *J. Biol. Chem. 255*, 6173–6180.

Thompson, D., Suârez-Villafañe, M., & Ferguson-Miller, S. (1982) *Biophys. J. 37*, 285–292.

Tzagoloff, A., & MacLennan, D. (1965) *Biochim. Biophys. Acta 99*, 476–485.

Van Aken, T., Thompson, D. A., Rosevear, P., & Ferguson-Miller, S. (1980) *Am. Soc. Biol. Chem., Biophys. Soc.*, Abstr. No. 2397.

van Buuren, K. J. H., Nicholls, P., & Van Gelder, B. F. (1972) *Biochim. Biophys. Acta 256*, 258–276.

Vik, S. B., & Capaldi, R. (1980) *Biochem. Biophys. Res. Commun. 94*, 348–354.

Vik, S. B., Georgevich, G., & Capaldi, R. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1456–1460.

von Jagow, G., & Klingenberg, M. (1972) *FEBS Lett. 24*, 278–282.

Watts, A., Marsh, D., & Knowles, P. F. (1978) *Biochem. Biophys. Res. Commun. 81*, 403–409.

Weiss, H., & Juchs, B. (1978) *Eur. J. Biochem. 88*, 17–28.

Weiss, H., & Kolb, H. J. (1979) *Eur. J. Biochem. 99*, 139–149.

Weiss, H., Juchs, B., & Ziganke, B. (1978) *Methods Enzymol. 53*, 98–112.

Wikstrom, M. (1977) *Nature (London) 266*, 271–273.

Wikstrom, M. (1981) in *Mitochondria and Microsomes* (Lee, C. P., Schatz, G., & Dallner, G., Eds.) pp 249–269, Addison-Wesley, Reading, MA.

Wikstrom, M., & Saari, H. T. (1977) *Biochim. Biophys. Acta 462*, 347–361.

Wikstrom, M., & Penttila, T. (1982) *FEBS Lett. 144*, 183–189.

Wikstrom, M., Krab, K., & Saraste, M. (1981) *Cytochrome Oxidase: A Synthesis*, pp 152–154, Academic Press, New York.

Wray, W., Boulikas, T., Wray, V., & Hancock, R. (1981) *Anal. Biochem. 118*, 197–203.
Yu, C., Yu, L., & King, T. (1975) *J. Biol. Chem. 250*, 1383–1392.
Yu, C., Yu, L., & King, T. (1979) in *Cytochrome Oxidase* (King, T. E., Orii, Y., Chance, B., & Okunuki, K., Eds.) pp 219–227, Elsevier/North-Holland Biomedical Press, Amsterdam.

Distribution of Proteins among Chromatin Components of Nucleoli†

Mark O. J. Olson* and Barbara A. Thompson

ABSTRACT: The nucleolus contains proteins which are believed to be involved in the organization of its subcomponents. To examine the possible locations of these proteins we have studied the release of proteins and chromatin subunits by digestion of Novikoff hepatoma nucleoli by micrococcal nuclease, deoxyribonuclease I, and ribonuclease A. Micrococcal nuclease digests of nucleoli produced typical sucrose density gradient profiles of nucleosomes showing separation of monomers, dimers, and trimers. The DNA lengths in the monomers, dimers, and trimers were generally the same as previously reported for whole nuclear chromatin of various rat tissues. Protein C23 (M_r 110 000), a putative nucleolus organizer protein which contains highly acidic phosphorylated regions, was rapidly released by micrococcal nuclease. The bulk (approximately 90%) of protein C23 released by micrococcal nuclease sedimented more slowly than mononucleosomes, at approximately 7 S. The remaining portion of protein C23 sedimented with mono-, di-, and trinucleosomes at various extents of digestion. Deoxyribonuclease I also liberated protein C23 from nucleoli. In addition, treatment of nucleoli with ribonuclease A released significant quantities of protein C23 without release of histones. These results suggest that protein C23 is associated with DNA-containing as well as RNA-containing components but that the major portion of the protein is loosely bound or associated with RNP components. However, it also appears to be present in nucleosomes to a small but significant extent. Protein B23 (M_r 37 000), which also contains highly acidic phosphorylated regions, was released by all nuclease treatments but was found to a lesser extent in nucleosome fractions. Another protein of M_r 160 000 (160K) sedimented with di- and trinucleosomes and was present in higher concentrations in nucleolar compared to whole nuclear chromatin. The 160K protein was found predominantly in the pellet after DNase I digestion of nucleoli followed by extraction with 2 M NaCl, suggesting that it is a nucleolar component of the nuclear matrix.

The nucleolus is the subnuclear organelle where preribosomal RNA is synthesized and ribosomes are assembled (Busch & Smetana, 1970). Because of these functions, the nucleolus contains both chromatin and ribonucleoprotein (RNP)[1] subcomponents. The chromatin component contains multiple copies of the genes for ribosomal RNA, although these account for less than 1% of the total DNA in rat nucleoli (Attardi & Amaldi, 1970; Attardi et al., 1965). The remainder of the DNA in nucleolar chromatin is of unknown function but includes sequences which are highly repetitive in nature (Fuke et al., 1979). The RNP components consist of preribosomal RNA at various stages of processing associated with a number of ribosomal and nonribosomal proteins (Warner, 1979; Kumar & Subramanian, 1975; Prestayko et al., 1974).

The proteins of nucleolar chromatin (Olson et al., 1975; Olson & Busch, 1978) and RNP components (Kumar & Subramanian, 1975; Prestayko et al., 1974; Olson et al., 1974) have been examined and categorized to some extent, although it is not precisely known which proteins are involved in organizing subcomponents within the nucleolus. For example, it is likely that certain proteins are responsible for producing the condensed regions of nucleolar chromatin, while other proteins may interact with chromatin and hold preribosomal RNP particles in place during RNA processing and ribosome assembly. Proteins which play these roles might be expected to have special features which allow them to engage in multiple interactions with other macromolecules.

We have isolated and partially characterized two major nucleolar proteins which have characteristics suggesting that they may serve as organizer molecules. These proteins, designated B23 and C23 by Orrick et al. (1973), have regions of high net negative charge (Mamrack et al., 1977, 1979) which may interact with histones in chromatin or ribosomal proteins in nucleolar RNP particles. The proteins are localized predominantly to nucleoli of interphase cells (Olson et al., 1981; Michalik et al., 1981), and protein C23 also resides at the nucleolus organizer regions of chromosomes (Lischwe et al., 1981). A certain portion of protein C23 which is readily extractable with low ionic strength buffers (Rothblum et al., 1977) is largely associated with preribosomal RNP particles (Prestayko et al., 1974; Olson et al., 1974), while another fraction appears to be rather tightly bound to chromatin (Olson et al., 1975; Rao et al., 1982). More recently, it was found that protein C23 binds DNA and appears to have a preference for DNA sequences upstream from the genes for ribosomal RNA.[2] These characteristics, taken together, suggest that protein C23 plays an organizational role in the nucleolus,

† From the Department of Biochemistry, The University of Mississippi Medical Center, Jackson, Mississippi 39216. *Received January 4, 1983.* This research was supported by Grant GM 28349 from the National Institutes of Health.

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; PCA, perchloric acid; RNP, ribonucleoprotein; $NaDodSO_4$, sodium dodecyl sulfate; SSC, standard saline citrate containing 0.15 M NaCl and 0.017 M sodium citrate (pH 7.0); rDNA, genes coding for ribosomal RNA; DNase I, deoxyribonuclease I; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

[2] M. O. J. Olson, B. A. Thompson, and S. T. Case, unpublished results.

0006-2960/83/0422-3187$01.50/0