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# REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PURIFICATION OF SUBUNITS OF OLIGOMERIC MEMBRANE PROTEINS

## THE NUCLEAR CODED SUBUNITS OF YEAST CYTOCHROME *c* OXIDASE

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## SUMMARY

Reversed-phase chromatography of the subunits of an oligomeric membrane protein such as yeast cytochrome *c* oxidase requires additional sample handling techniques which are not necessary for soluble proteins. This paper considers these and discusses (1) methods for the removal of ballast material by preliminary batchwise extraction with solvent mixtures similar to those used for reversed-phase elution; (2) the chromatographic heterogeneity induced by partial cysteine oxidation; (3) the removal of tightly bound proteins from the stationary phase; and (4) the generation of an elution system with continuously variable selectivity based on acetonitrile–1-propanol ratios (0.05% triethylamine, 0.05% trifluoroacetic acid).

These methods are designed to simplify complex mixtures of hydrophobic proteins prior to chromatography and to purify them chromatographically in high yield.

## INTRODUCTION

The purification of proteins and peptides requires the use of multiple high-resolution techniques. Although separations based on charge (ion exchange) and molecular weight (*e.g.*, gel-permeation chromatography) have long been used in biochemistry, it is only recently that means for the high-resolution separation of proteins based on their relative hydrophobicities have been developed. These separations, effected by reversed-phase chromatography, were pioneered by Nice and O'Hare[1]. Their separations with acetonitrile and sodium phosphate (pH 2.1), and those of Molnár and Horváth[2], with acetonitrile and orthophosphoric acid as eluents have been useful for both the purification of low-molecular-weight proteins and the resolution of peptide digests[3]. Modifications of these original methods have included the use of ion pairing reagents[4,5], trifluoroacetic acid buffer systems[6,7], ethanol–formic acid[8], and solvents more powerful than acetonitrile (*e.g.*, propanol) as eluents for the purification of hydrophobic proteins[6].

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Our work centers around the biogenesis and structure of yeast cytochrome *c* oxidase, a seven-subunit hetero-oligomeric enzyme from the inner mitochondrial membrane. The three largest polypeptide subunits (I, 54,000 daltons; II, 31,000 daltons; III, 29,500 daltons[9]) are encoded on mitochondrial DNA[10], are extremely hydrophobic[9], and are usually purified by gel-exclusion chromatography in sodium dodecyl sulphate (SDS)[11]. The lower-molecular-weight polypeptide subunits (IV, 14,500 daltons; V, 12,500 daltons; VI, 9500 daltons; VII, 4500 daltons) are encoded on nuclear DNA[10] and are difficult to isolate in high yield, because they are similar in size, charge and amino acid composition[9]. In studies described here, our goal was to develop a high yield method for the purification of each of the these polypeptide subunits, based on reversed-phase high-performance liquid chromatography (RP-HPLC). Our prefractionation and chromatographic separation methods should be widely applicable to other supramolecular structures, in general, and to oligomeric membrane proteins, in particular.

## EXPERIMENTAL

### *Apparatus*

All analyses were performed on a high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.), including a Model 720 systems controller, data module, U6K injector with a 2-ml loop, Model 450 absorbance detector, and two Model 6000 A pumps. A Waters $\mu$Bondapak $C_{18}$ column (250 × 4 mm) was used.

### *Chemicals*

Guanidine · HCl, trifluoroacetic acid and triethylamine were obtained (Sequanol grade) from Pierce (Rockville, IL, U.S.A.). HPLC-grade acetonitrile, propanol and all other solvents were obtained from Burdick & Jackson (Muskegon, MI, U.S.A.). Cholic acid was obtained from Sigma (St. Louis, MO, U.S.A.) and recrystallized[9]. The product was used as a 20% solution of its sodium salt (pH 8.3) in water. Pancreatic ribonuclease was also obtained from Sigma. Water was purified with a Milli-Q System (Millipore, Bedford, MA, U.S.A.). All other chemicals were obtained at the highest purity available.

### *Methods*

*Pancreatic ribonuclease.* Commercial pancreatic ribonuclease was dissolved in 0.1 *M* Tris acetate buffer (pH 8.5), reduced by the addition of solid dithiothreitol (DTT) to a concentration of 10 m*M*, and incubated at room temperature for 1 h. Alkylation with iodoacetic acid was carried out under nitrogen as follows. The protein was suspended at a concentration of 5 mg/ml in 0.1 *M* Tris acetate, 6 *M* guanidine · HCl and 2 m*M* DTT (pH 8.5) and incubated at 37°C for 30 min. Iodoacetic acid (50 m*M*, neutralized with sodium hydroxide) was added to 5 m*M* and incubation was carried out at 37°C for an additional 30 min. Reduction–alkylation was then repeated as above using 1 m*M* DTT and 2.5 m*M* iodoacetic acid. The remaining iodoacetic acid was quenched by the addition of DTT to 10 m*M*. All samples were adjusted to pH 2.7 with 10% trifluoroacetic acid (TFA) prior to chromatography.

*Cytochrome oxidase fractionation.* Holocytochrome *c* oxidase was prepared from yeast submitochondrial particles following octyl-Sepharose chromatography as described elsewhere[12,13]. Subunits IV, V, VI and VII were isolated from the holoenzyme by extraction with acetonitrile as discussed under Results. Briefly, the enzyme [200 nmol of haem *a* per ml in 50 m*M* sodium 3(N-morpholino)propanesulfonate, 0.5 m*M* EDTA, 0.5% sodium cholate (pH 7.0) (MOPS buffer)] was incubated with acetonitrile (40%) for 1 h at 4°C. The mixture was then centrifuged in 1.5-ml polypropylene centrifuge tubes for 10 min at 15,000 rpm (27,000 $g_{max}$) in an SS34 rotor of a Sorvall RC-2B centrifuge. The supernatant was retained and the pellet reextracted twice, as before, by resuspension in the original volume of MOPS buffer and addition of the appropriate acetonitrile solution. Supernatants from all extractions were pooled and lyophilized prior to HPLC or SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This fraction contained subunits IV and VI and was designated $S_{46}$. The final pellet was resuspended in 0.2 volumes of distilled water and lyophilized. It contained subunits I, II, III, V and VII and was designated $P_1$. Subunits V and VII were extracted from $P_1$ as described under Results. Briefly, the $P_1$ pellet was resuspended in its original (oxidase) volume of 1.25% triethylamine and 1.25% trifluoroacetic acid (1.25% TEA–TFA; see below). One volume of either acetonitrile (0.05% TEA, 0.05% TFA) or the mixed solvent, acetonitrile–1-propanol (1:1, 0.05% TEA, 0.05% TFA) was added to the suspension and the mixture was stirred for 4 h at 4°C. After centrifugation (as above), the supernatant was retained and the pellet is extracted twice more. Supernatants from all extractions were pooled and lyophilized from 0.2 volumes of water, as above. This fraction contained subunits V and VII and was designated $S_{57}$. The remaining pellet ($P_2$) contained subunits I, II and III. It was lyophilized from 0.2 volumes of water and saved for further analysis.

*HPLC of proteins.* The buffer system for all separations was 0.05% TEA–TFA. Aqueous and organic TEA–TFA solutions were prepared by adding equal volumes of neat triethylamine and neat trifluoroacetic acid to the solvent. Programs for the gradients were generated on a Waters systems controller. In all cases, solvent A consisted of 5% acetonitrile in 0.05% TEA–TFA. For pancreatic ribonuclease, the flow-rate was 0.7 ml/min with a linear gradient from 0 to 50% B (0.05% TEA–TFA in acetonitrile) at 1%/min. For $S_{46}$, the same solvent B was used, but the linear gradient was started 5 min after sample application and at a rate of 1%/min from 0 to 70% B; the flow-rate was 0.5 ml/min. For $S_{57}$, the flow-rate was also 0.5 ml/min and the linear gradient started after 5 min, but solvent B was 0.05% TEA–TFA in acetonitrile–1-propanol (1:1); the gradient was from 0 to 70% B in 70 min (0.61%/min).

Samples for HPLC analysis were prepared as follows. Neat pancreatic ribonuclease was dissolved directly in the mobile phase; reduced or alkylated protein solutions were adjusted to 0.1% TFA with 10% TFA prior to their application to the reversed-phase column. Supernatant $S_{46}$ was dissolved in 6 *M* guanidine · HCl–10 m*M* DTT and incubated for 1 h at room temperature; it was acidified to 0.1% TFA prior to injection. Supernatant $S_{57}$ was dissolved directly in 8 *M* guanidine · HCl–10 m*M* DTT–0.05% TFA. All samples were filtered through 0.45-μm Millipore HA filters by centrifugation in a Centrifugal Filter from Rainin Instruments (Woburn, MA, U.S.A.). The oxidase-derived fractions ($S_{46}$, $S_{57}$) were resuspended in twice the volume of the initial enzyme.

Material precipitated in the column was removed by the injection of 1 ml of dimethyl sulfoxide (DMSO) into the system after the last protein peak was eluted (*ca.* 60–70 % B). The gradient program was continued (70 % B) until all peaks were eluted.

*Miscellaneous methods*

The products of the extractions ($S_{46}$, $S_{57}$, $P_1$, $P_2$), as well as proteins recovered after HPLC, were analyzed by SDS-PAGE[14,15] on 12.5% acrylamide gels. HPLC samples were taken to dryness in an evacuated centrifuge (Spin-Vac, Savant Instruments) at 45°C prior to their dissociation in SDS sample buffer[15].

Amino acid compositions were determined after hydrolyzing protein samples under nitrogen for 24 h in 6 *N* hydrochloric acid (0.5 ml) containing 1 drop of redistilled phenol.

## RESULTS

*Prefractionation of cytochrome c oxidase*

One problem which may be encountered in the dissociation of an oligomeric integral membrane protein into its polypeptide subunits is the release of polypeptides, which are extremely hydrophobic and may, in their denatured state, be irreversibly adsorbed on the stationary phase during reversed-phase column chromatography. Yeast cytochrome *c* oxidase contains three polypeptide subunits of this type. These subunits (I, II and III) are encoded by the mitochondrial genome and are known to have a low polarity[9,16]. They are adsorbed on, but cannot be eluted from a $C_{18}$ column after their dissociation from the holoenzyme by guanidine · HCl[16], a chaotropic agent which is used routinely during the preparation of the other subunits of the holoenzyme for reversed-phase chromatography (see below).

In order to purify the nuclear encoded subunits (IV, V, VI and VIII) of yeast cytochrome *c* oxidase by RP-HPLC and to avoid the above-mentioned problems associated with the chromatography of subunits I, II and III, we have developed a prefractionation regimen which selectively releases subunits IV, V, VI and VII from the holoenzyme. This regimen involves the treatment of holocytochrome *c* oxidase with acetonitrile at neutral pH to release subunits IV and VI and acetonitrile at acidic pH, or acetonitrile–propanol, to release subunits V and VII (Fig. 1). The effectiveness of different concentrations of acetonitrile in releasing subunits IV and VI from the holoenzyme is shown in Fig. 2. The release of subunits IV and VI is optimal at 40 % acetonitrile and declines on either side of this optimum. It is clear from Fig. 2 that very little, if any, subunit IV or VI protein remains associated with the $P_1$ pellet.

The removal of subunits V and VII from the $P_1$ pellet requires the use of a stronger solvent than that which was effective for subunits IV and VI (see above). Two different solvents have been found effective. The first is acetonitrile acidified to pH 2.7, while the second is an acetonitrile–propanol mixture. From the results of *one* 4-h extraction of $P_1$ with each of these solvents (Fig. 3), it is clear that the latter solvent is the more effective in extracting subunits V and VII from the $P_1$ pellet. However, it is also clear that neither solvent removes all of the subunit V and VII polypeptides from the holoenzyme since both subunits can be found in the $P_2$ pellet with either solvent. After *three* extractions, the acidic acetonitrile solvent extracts 40–

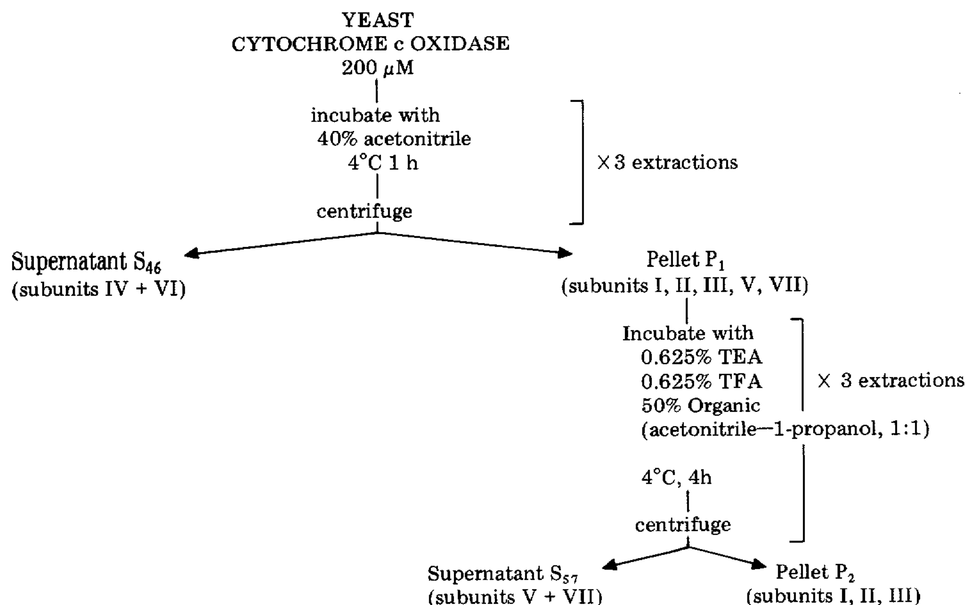


Fig. 1. Prefractionation scheme for removing subunits IV, V, VI and VII from yeast holocytochrome *c* oxidase.

60 % of subunits V and VII present in the $P_1$ pellet while the mixed solvent extracts 80–90 %.

It should be noted that, although the extraction regimen used here is suitable for removing the four nuclear encoded subunits (IV, V, VI and VII) from the three mitochondrially-encoded subunits (I, II and III), the $P_2$ pellet which contains subunits I, II and III and trace amounts of subunits V and VII is nearly intractable to subsequent fractionation. Indeed, subunits I, II and III in this pellet are refractile to solubilization in SDS. It is for this reason that these polypeptides cannot be seen in the electropherogram of $P_2$ pellets, shown in Fig. 3.

*Chromatography of subunit fractions*

Subunit polypeptides in the $S_{46}$ and $S_{57}$ pools are resolved by HPLC on a $C_{18}$ reversed-phase column. Several problems were encountered during the development of conditions for the resolution of pure polypeptide subunits IV, V, VI and VII. These include (1) the presence of multiple peaks due to partial cysteine oxidation; (2) the insolubility of the extracts in the starting buffer; and (3) the incomplete resolution of the polypeptide subunits in TFA–acetonitrile elution systems.

*Cysteine oxidation state.* Heterogeneity in cysteine oxidation was first observed after chromatography of the $S_{46}$ fraction. As shown (Fig. 4), $S_{46}$ which is chromatographed in the absence of DTT pretreatment exhibits multiple peaks. Electrophoresis of each of these peaks on SDS-polyacrylamide gels in the presence and absence of reducing agent has revealed that they correspond to: subunit IV dimer; subunit IV monomer; subunit IV + VI coeluting with subunit VI dimer; and subunit IV monomer. However, after pretreatment with 10 m*M* DTT, $S_{46}$ is revolved into two major

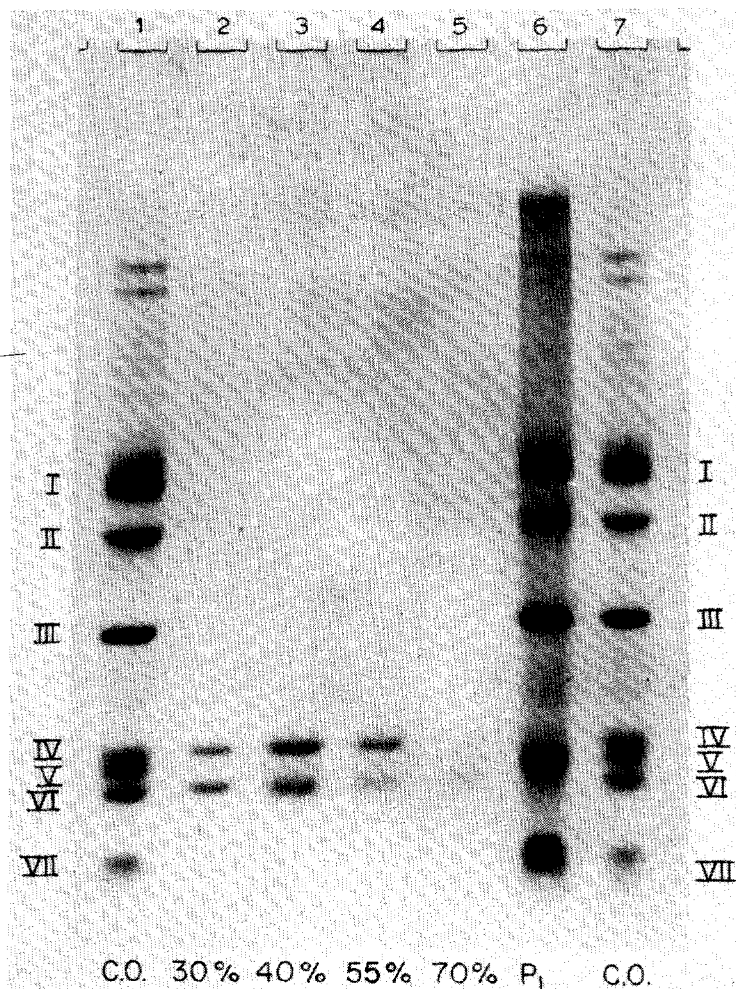


Fig. 2. Effects of acetonitrile concentration on the extraction of subunits IV and VI from yeast cytochrome *c* oxidase. Subunits were extracted from 20 μg of holoenzyme. The extracted subunits were analyzed on 12.5% acrylamide gels by SDS-PAGE. Tracks 1 and 7, 10 μg unfractionated holoenzyme; track 2, 30% acetonitrile extract (one volume 60% acetonitrile, see *Methods*); track 3, 40% acetonitrile extract (one volume 80% acetonitrile); track 4, 55% acetonitrile extract (1.22 volumes acetonitrile); track 5, 70% acetonitrile extract (2.33 volumes acetonitrile); track 6, pellet ($P_1$) from 40% acetonitrile extract.

peaks which correspond to subunit IV and subunit VI monomers. These results emphasize the importance of "fixing" the oxidation state of cysteine residues in polypeptides which are to be purified by RP-HPLC.

A dramatic illustration of the ability of RP-HPLC to discriminate between identical polypeptides with cysteine residues in different oxidation states can be seen in Fig. 5. These results illustrate that this method can differentiate the reduced and carboxymethylated cysteinyl forms of ribonuclease, a protein of similar size to subunits IV and V of yeast cytochrome *c* oxidase. Thus, they demonstrate that even in a

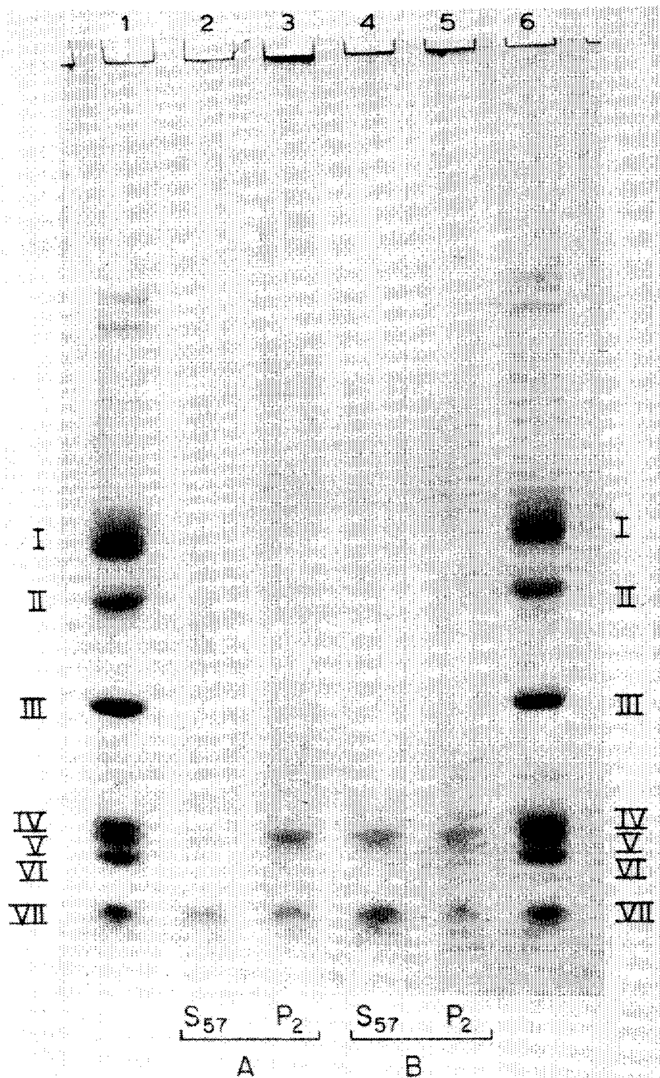


Fig. 3. Effects of propanol on the extraction of subunits V and VII from yeast cytochrome *c* oxidase. Extraction and analysis conditions were as described in Fig. 2. Tracks 1 and 6, 10 $\mu$g holoenzyme; tracks 2 and 3, supernatant (2) and pellet (3) from a single 40 % acetonitrile TEA–TFA extract of $P_1$; tracks 4 and 5, supernatant (4) and pellet (5) from a single 50 % acetonitrile–propanol (1:1) TEA–TFA extract of $P_1$. Note: subunits I, II and III are not dissociable with SDS after exposure to the $S_{57}$ extraction solvents and therefore cannot be seen in either of the $P_2$ pellets shown.

simple system containing one kind of polypeptide heterogeneity can be observed if one is not careful to oxidize or reduce cysteine residues completely.

We, therefore, routinely pretreat both $S_{46}$ and $S_{57}$ supernatants with DTT prior to their separation by RP-HPLC.

*Sample preparation.* The subunit pools ($S_{46}$ and $S_{57}$) prepared during pre-

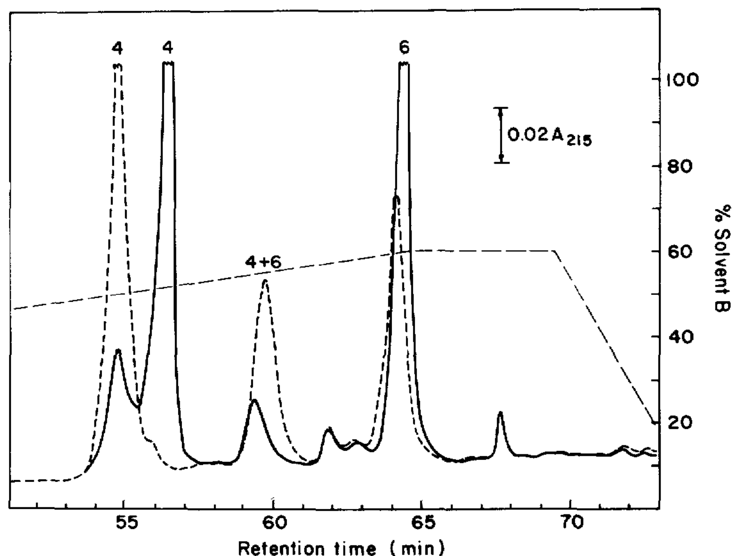


Fig. 4. RP-HPLC of the $S_{46}$ fraction after reduction with dithiothreitol. The conditions are as indicated under *Methods*. Samples were incubated in the presence (——) or absence (– – – – –) of 10 m*M* DTT for 30 min at room temperature prior to chromatography. Numbers 4 and 6 are subunits IV and VI, respectively, identified by SDS-PAGE[15], as described in the text.

fractionation of cytochrome *c* oxidase are insoluble in the initial buffer used for RP-HPLC. However, the subunits in both $S_{46}$ and $S_{57}$ pools become soluble upon addition of 6 *M* and 8 *M* guanidine · HCl, respectively. As observed by Henderson *et al.*[6], denaturation of proteins with guanidine · HCl does not appear to alter their retention times under the chromatographic conditions used here.

However, the use of denatured protein does lead to the accumulation of irreversibly adsorbed material on the column. It has been reported previously that this material could be removed from a $C_{18}$ column by mixtures of acetic acid–guanidine[6]. Because this procedure has been ineffective in our hands, we have adopted an alternative method. Following elution of the last protein peak (*ca.* 60–70%, solvent B), 1.0 ml of DMSO is applied to the column, and the column is washed with 70% solvent B until the DMSO peak is eluted. This procedure leads to the complete removal of insoluble proteinaceous material as determined by the lack of "ghost"[6] peaks when the program is repeated (data not shown)[6], and by the reproducibility of the retention times throughout many (> 100) injections (of up to 2 mg of protein extract per injection).

*Choice of eluents.* The fractionation of the $S_{46}$ pool into Subunits IV and VI is easily accomplished on the $C_{18}$ stationary phase by using acetonitrile (0.05% TEA–TFA) as solvent B (see Fig. 4). However, this eluent does not resolve the mixture of polypeptides present in the $S_{57}$ pool. We have chosen to use the term "mixture" because, as will become apparent below, several proteins migrate together in the region of the SDS gel which contains subunit VII.

Initially, we attempted to use 1-propanol as solvent B to separate the polypeptides in the $S_{57}$ mixture because it has been reported to be an effective eluent for

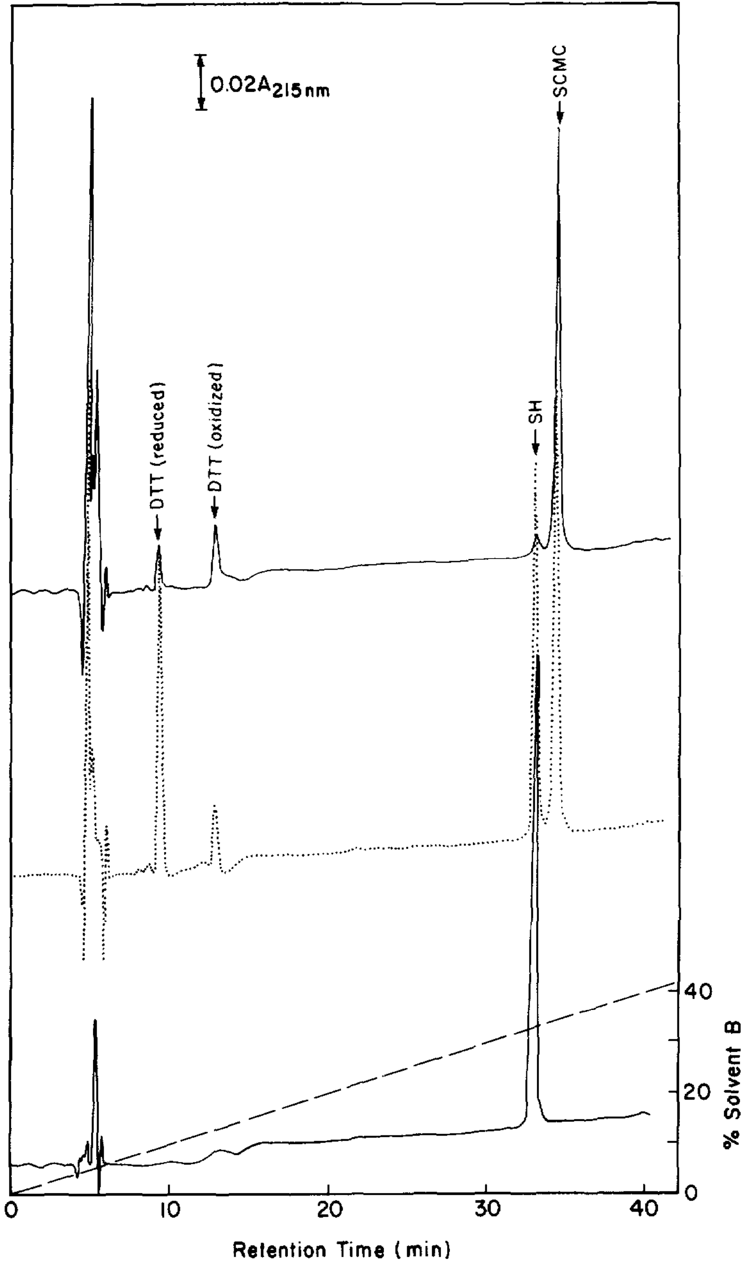


Fig. 5. RP-HPLC of reduced and carboxymethylated pancreatic ribonuclease. Upper tracing: 2.5 μg protein in 0.05% TFA, 10 m*M* DTT; lower tracing; 2.5 μg carboxymethylated protein (acidified reaction mixture); middle tracing: (· · · · ·) 2.5 μg of each. The conditions for the separation are as described under *Methods*.

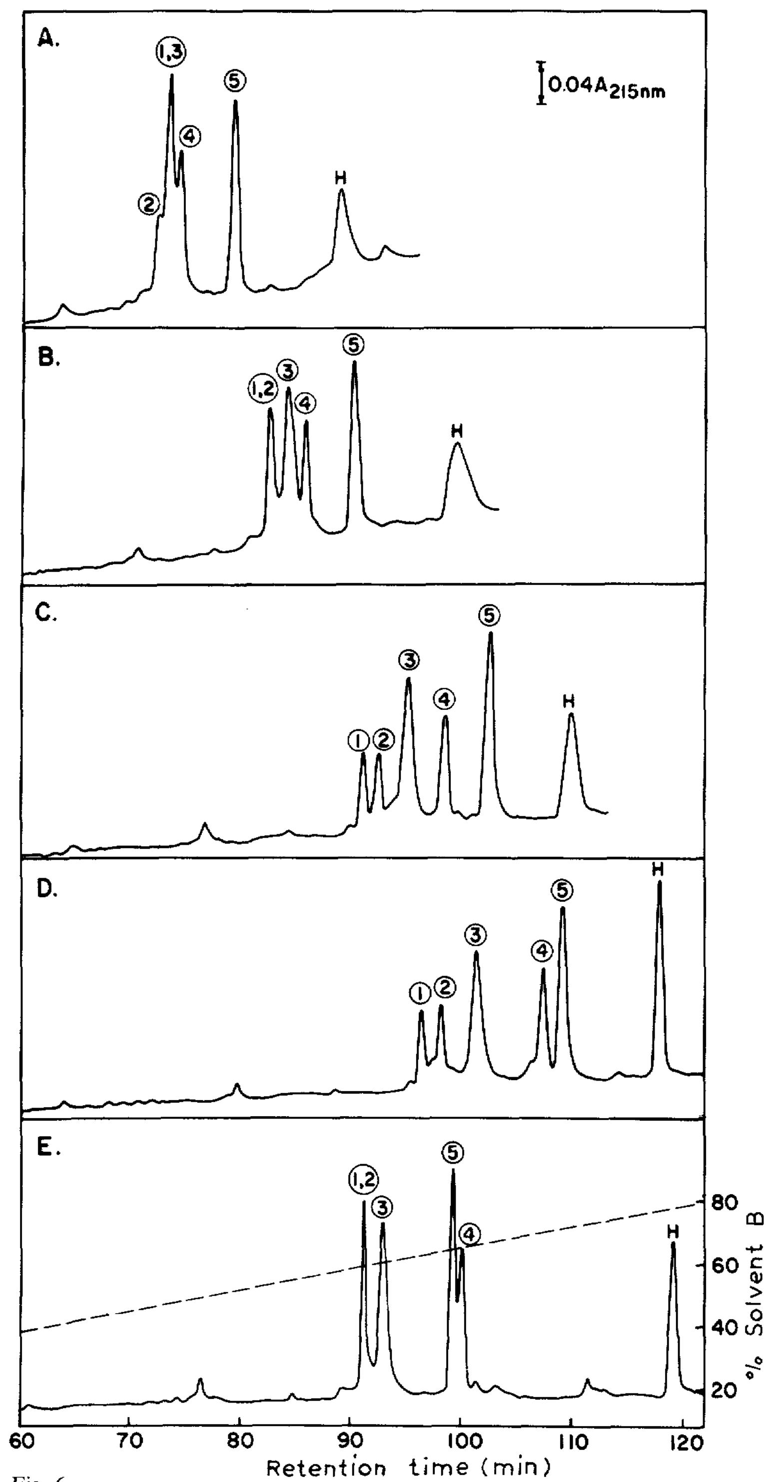


Fig. 6.

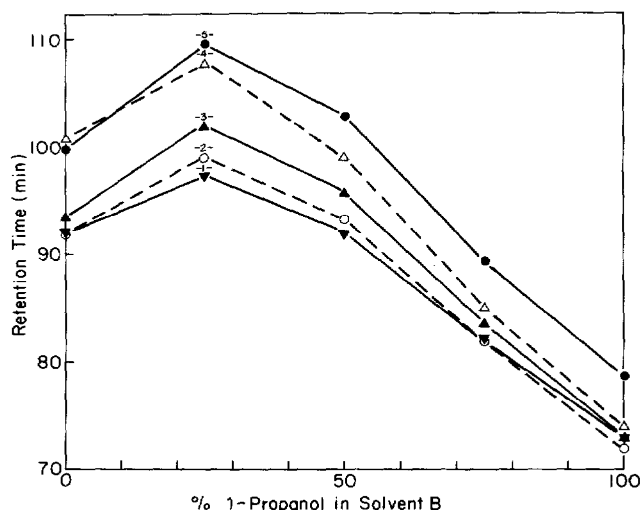


Fig. 6. Effects of acetonitrile–propanol ratios on the fractionation of the $S_{57}$ fraction by RP-HPLC. The $S_{57}$ fraction was prepared from 10 $\mu g$ of cytochrome *c* oxidase and fractionated in the presence of the acetonitrile–1-propanol ratios (0.05% TEA–TFA) in Solvent B which are indicated below. *Left panel*: ratios of acetonitrile to 1-propanol (v/v) are: tracing A, 0:100; tracing B, 25:75; tracing C, 50:50; tracing D, 75:25; tracing E, 100:0; conditions for chromatography are under *Methods*; individual protein peaks are numbered from 1 to 5 (see text); haem *a* is indicated by "H". *Right panel*: the retention times of each peak are plotted as a function of propanol concentration; overlapping peaks obtained at one ratio were resolved by rechromatography of the peak in question in solvent B containing a different ratio of acetonitrile to 1-propanol.

the separation of proteins in RP-HPLC[6]. However, after several unsuccessful attempts to use either 1-propanol or acetonitrile alone as solvent B, we discovered that mixtures of both were very effective in resolving the polypeptides in the $S_{57}$ mixture. Indeed, with acetonitrile–1-propanol mixtures the selectivity of the eluent can be varied over a wide range. As seen in Fig. 6 the chromatographic profiles vary in response to the concentration of 1-propanol in solvent B. When solvent B contains 25–50% 1-propanol, the $S_{57}$ mixture can be resolved into five different protein peaks. All of these represent different polypeptides, as evidenced by the fact that they have unique amino acid compositions (Table I) and different sizes as judged by SDS-PAGE (Fig. 7). From both of these analyses we conclude that peak 3 is subunit V, and peak 2 is residual subunit VI which is left in the $P_1$ pellet and which is extracted with the $S_{57}$ pool. Much to our surprise we observed that the polypeptides present in peaks 1, 4 and 5 have the same apparent molecular weight and migrate with subunit VII in the holoenzyme. Since they have markedly different amino acid compositions and different amino terminal sequences[13] we conclude that they are different molecular species and that what was hitherto thought to be a single polypeptide subunit is composed of three different polypeptides.

The 1-propanol–acetonitrile system used for the resolution of the $S_{57}$ mixture permits good recoveries of all polypeptides, even when chromatographed on a preparative scale (0.6–1 mg total protein per application). We typically recover 90%, or more, of the total $A_{240}$ (absorbance at 240 nm) applied in the integrated areas under

TABLE I

AMINO ACID COMPOSITIONS OF THE POLYPEPTIDES PRESENT IN THE $S_{57}$ FRACTION

Peaks are numbered according to their position in the chromatogram shown in Fig. 6 (left, Panel D). All values were obtained after hydrolyzing each sample for 24 h.

| *Amino acid* | *Mole% of amino acids* | | | | |
|---|---|---|---|---|---|
| | *Peak Nr.* | | | | |
| | *1* | *2* | *3* | *4* | *5* |
| Ala | 10.5 | 10.76 | 10.57 | 9.54 | 14.18 |
| Arg | 3.10 | 6.81 | 5.65 | 6.89 | 5.58 |
| Asx | 4.92 | 12.48 | 8.84 | 8.05 | 4.04 |
| Glx | 5.47 | 16.64 | 13.82 | 10.53 | 6.80 |
| Gly | 10.06 | 1.62 | 8.85 | 9.84 | 2.50 |
| His | 3.29 | 1.18 | 0.87 | 1.57 | 1.80 |
| Ile | 5.04 | 1.81 | 2.33 | 7.46 | 8.62 |
| Leu | 5.18 | 10.89 | 9.21 | 6.65 | 10.99 |
| Lys | 12.00 | 6.39 | 8.54 | 10.94 | 10.42 |
| Met | 0.62 | 0 | 2.47 | 3.69 | 0.15 |
| Phe | 13.53 | 6.65 | 2.72 | 4.91 | 5.32 |
| Pro | 4.93 | 4.01 | 4.37 | 2.31 | 8.18 |
| Ser | 3.49 | 3.98 | 8.76 | 4.52 | 4.79 |
| Thr | 2.25 | 2.63 | 3.01 | 3.49 | 3.37 |
| Tyr | 5.11 | 5.68 | 3.27 | 3.84 | 7.09 |
| Val | 10.95 | 8.47 | 6.70 | 5.76 | 6.20 |

peaks 1, 2, 3, 4, 5 and "H" (haem peak). The residual $A_{240}$ absorbance is represented by protein adsorbed on the stationary phase. It is eluted with DMSO as described above, and does not appear as "ghost" peaks in subsequent chromatograms.

*Purity of isolated subunit polypeptides*

The purity of subunits obtained by the two-step isolation scheme described above has been established by SDS-PAGE (Fig. 7). The polypeptide fractions identified as subunits IV, V, VI or VII in the chromatograms shown in Figs. 4 and 6 migrate as single species on SDS-PAGE. Each of these fractions also has a single amino-terminus and tryptic peptide patterns which are unique[13]. Together, these results clearly establish that each of the subunit fractions obtained by RP-HPLC is pure and consists of only one type of polypeptide species.

DISCUSSION

In this paper we present a new strategy for the isolation of polypeptide subunits from oligomeric integral membrane proteins. This approach makes use of two steps. In the first, polypeptides are removed sequentially from the oligomer by aprotic organic solvents. In the second, the released polypeptides are purified by RP-HPLC. The first step is essentially an elution of polypeptides from the oligomer, based on their hydrophobicities. It is, therefore, similar in principle, to RP-HPLC separations except that the "stationary phase" is composed of the hydrophobic subunits of the

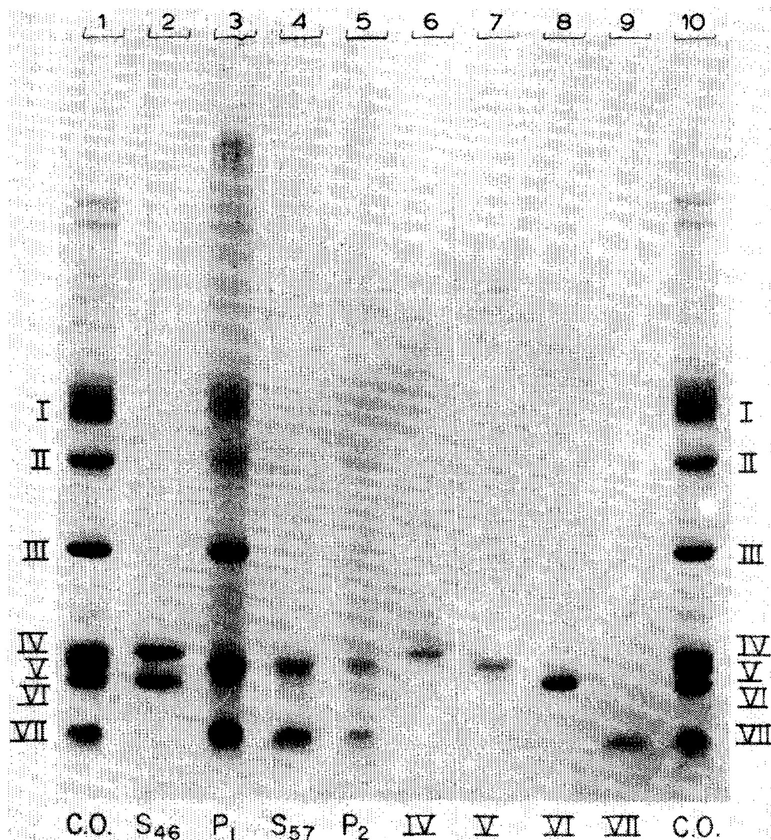


Fig. 7. Fractions obtained during the purification of subunits IV, V, VI and VII of cytochrome *c* oxidase (12.5% PAGE). Tracks 1 and 10, 10 $\mu$g holoenzyme; track 2, $S_{46}$ (40% acetonitrile) from 20 $\mu$g holoenzyme; track 3, pellet ($P_1$) from $S_{46}$ extraction; track 4, $S_{57}$ extract of $P_1$; track 5, pellet ($P_2$) from $S_{57}$ extraction; track 6, 1 $\mu$g subunit IV peak from HPLC of $S_{46}$; track 7, 1 $\mu$g subunit V peak (no. 3 in Fig. 6, trace D) from HPLC of $S_{57}$; track 8, 1 $\mu$g subunit VI peak from HPLC of $S_{46}$; track 9, 1 $\mu$g subunit VII (peak no. 5 in Fig. 6, trace C) from HPLC of $S_{57}$.

oligomer itself, rather than the bonded stationary phase that is used for RP-HPLC in the second step. Since many oligomeric membrane proteins are composed of both hydrophobic polypeptides, which penetrate the lipid bilayer and which may be thought of as a "hydrophobic core"[9], and hydrophobic polypeptides which associate with them by ionic and hydrophobic bonds[17], this step and modifications thereof should be generally useful for the pre-fractionation of a wide variety of membrane proteins.

As applied to cytochrome *c* oxidase, this strategy has permitted us to purify several milligrams of the more hydrophilic subunits (IV–VII) of the oligomer. RP-HPLC of these subunits has also revealed itself to be exquisitely sensitive to the oxidation state of sulfhydryl residues and to the polypeptide heterogeneity in the subunit VII fraction. Indeed, these studies have demonstrated that what was previously thought to be a single polypeptide[15] is, in fact, composed of three different

polypeptides (peaks 1, 4 and 5, Fig. 6)[18]. They also demonstrate that the chromatograms of these polypeptides generated by RP-HPLC provide a very sensitive way of assessing subunit purity. Since polypeptides are resolved on the basis of hydrophobicities by RP-HPLC, this procedure provides a useful complement to SDS-PAGE, in which proteins are separated on the basis of molecular weight. Indeed, when used together, these two techniques allow the unequivocal demonstration of the purity of subunit fractions from yeast cytochrome *c* oxidase.

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