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Proteins of Cytochrome Oxidase in Fractions Prepared with Deoxycholate from Bovine Heart Submitochondrial Particles*

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Abstract

The heme *a* content of cytochrome oxidase-containing fractions of varying purity obtained from bovine heart submitochondrial particles with deoxycholate was correlated with the intensity of protein bands obtained on polyacrylamide gel electrophoresis of the preparations dissolved in phenol : acetic acid : water. It is concluded that cytochrome oxidase contains two noncovalently associated proteins. Neither of these proteins is electrophoretically identifiable with the noncatalytic protein of Green *et al.* [12].

Introduction

Several groups of investigators have shown that cytochrome oxidase can exist in different physical states, depending on the surrounding milieu [1-3]. The propensity for aggregation, whether it exists because of hydrophobic amino acid residues on the exterior or because of bound phospholipid, might be expected *a priori* of proteins that are components of membrane systems. This characteristic, however, has led to such great difficulty in isolation and characterization of the proteins that there is still no agreement as to what constitutes the smallest structural or catalytic unit of cytochrome oxidase, and whether the structural and catalytic units are the same entity.

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Criddle and Bock [4] and Ambe and Venkataraman [5] in 1959 were the first to note the polydisperse nature of their cholate-solubilized and salt-fractionated cytochrome oxidase preparation. On ultracentrifugation, they found five to six peaks, each of which was associated with the characteristic spectrum of the oxidase. Conversion of most of the protein to the smallest species was accomplished by treatment with high sodium dodecyl sulfate concentrations, and an approach-to-equilibrium determination on the polydisperse protein showed the smallest protein to have a molecular weight of 72,000. Takemori *et al.* [6] confirmed Criddle and Bock's findings by reporting that in cholate a different preparation of oxidase also behaved as a polydisperse system. When, however, the anionic surfactant was replaced by a nonionic one, the oxidase moved with a single, sharp, green boundary of $S_{20,w} = 21.9$, the molecular weight of which was determined to be 530,000. Since the minimal molecular weight with respect to iron was 100,000, Takemori *et al.* concluded that they were dealing with a pentamer. Tzagoloff *et al.* [7] found that cytochrome oxidase prepared with amyl alcohol, cholate, deoxycholate, and ammonium sulfate and analyzed in the presence of taurocholate was monodisperse, had a molecular weight of 290,000 uncorrected for lipid content, and was a dimer with respect to heme *a* content.

Another way of studying the structure-function relationship of cytochrome oxidase has been by correlation of electron micrographic appearance with physicochemical measurements and with assay of catalytic activity. McConnell *et al.* [2] observed that purified oxidase exhibited subunits of three sizes, a 100 Å unit, a 50 Å unit, and a unit smaller than both of these. The relative amounts of each was influenced by the amount of lipid and surface active agent present. In a similar study, Sun *et al.* [1] concluded that the ability of purified cytochrome oxidase to form aggregated membranous structures depended on the presence of sufficiently high phospholipid and sufficiently low ionic strength. A more informative study in this respect was that of Seki [3]. Seki confirmed the findings of Orii *et al.* [8] that nonionic surfactant-dispersed cytochrome oxidase was a $S_{20,w} = 21$-22 species tetrameric in heme *a* and that graded amounts of sodium dodecyl sulfate depolymerized it, first to a 16-17 $S_{20,w}$ dimeric species and then to a 5.7 $S_{20,w}$ species.

Of direct relevance to the present work is the possible existence of a separate, noncatalytic protein in Complex IV, i.e. cytochrome oxidase. The evidence is sketchy and consists of two preliminary notes [9, 10]. Korman and VandeZande [9] found that treatment of Complex IV with acidified methanol resolved it into two fractions, one devoid of heme and termed by them the nonprosthetic unit, and the other containing 80% of the original iron and copper and termed by them the prosthetic unit. Green *et al.* [11] used this finding to support his hypothesis that all

four of the electron transport complexes contain noncatalytic "core protein".

MacLennan and Tzagoloff [12, 13] reported in 1965 that succinylation of Complex IV resolved it into two fractions: one enriched in copper and depleted in heme *a*, the other enriched in heme *a* and depleted in copper. With characteristic thoroughness, MacLennan and Tzagoloff also considered the possibility that the copper protein may have arisen due to non-specific binding of contaminating ionic copper, and, indeed, when ^{64}Cu was added to the cytochrome oxidase, and the enzyme was subjected to succinylation and gel filtration, specific counts paralleled all of the protein peaks. However, the fractionation procedure in absence of added ionic copper did yield a fraction markedly enriched in copper and depleted in heme *a*, and it seemed unlikely that only one protein fraction preferentially bound contaminating copper.

In 1939 Keilin and Hartree [14] demonstrated that certain inhibitors of the oxidase altered the absorption bands of the oxidase in such a fashion that two components of different reactivity could be invoked. They proposed that cytochrome oxidase consisted of: (1) cytochrome *a* which absorbed strongly at 605 nm relative to 445 nm and did not react with O_2 or the inhibitors CO and HCN, and (2) cytochrome a_3 which absorbed strongly at 445 nm relative to 605 nm and reacted with O_2 and CO in the reduced form and with HCN in both the reduced and oxidized forms. It has not been possible to separate cytochrome oxidase into two heme-proteins, although cytochrome *b*, *c*, c_1, and cytochrome oxidase have been separated from each other.

From the preceding discussion, it should be evident that a method of predicting the number of covalently separate proteins within a complex would be of great value, since the problem would resolve itself into the development of effective separation methods. One would then know how many components to look for and be faced only with the problem of how to separate the several components. What appears to be such a method was developed in 1965 by Takayama *et al.* [15], who adapted to mitochondrial proteins a solubilization method previously found to bring about resolution of water-insoluble viral and ribosomal proteins [16]. Takayama found that exposure of particulate, precipitated mitochondrial electron transport complexes to the solvent system phenol : acetic acid : water = 2 : 1 : 1, w/v/v, resulted in optically clear, homogeneous solutions, and that disc electrophoresis of these solutions on polyacrylamide gel revealed each complex to consist of a surprisingly large number of bands.

Complex IV, the cytochrome oxidase preparation of Fowler *et al.* [17] as modified by MacLennan and Tzagoloff [12] showed two major bands and at least ten minor ones as reported by Takayama *et al.* [15] Sun *et al.* [1] also found two major bands in the preparation of Fowler *et al.* When cytochrome oxidase of wild-type baker's yeast and of a

cytoplasmic "petite" mutant was partially purified by Tuppy and Birkmayer [18] and subjected to disc electrophoresis on polyacrylamide, only two bands were reported, although the original stained gels which might have had small amounts of impurities were not shown. The results were presented only as schematic drawings. Zamudio and Williams [19] separated cytochrome oxidase [20, 21] into five species by preparative electrophoresis on polyacrylamide gel using glycine-Tris*-Emasol 1130 as the buffer-solvent. On analytical polyacrylamide electrophoresis in phenol-acetic acid all five active fractions contained two main bands. Chuang and Crane [22] resolved their lipid-depleted cytochrome oxidase [23] into a phospholipid-, iron-, copper-containing protein of molecular weight 26,500 and another protein with a molecular weight of 55,000 which was free of phospholipid, iron or copper. The latter was tentatively identified as noncatalytic or core protein IV [24]. The separation was accomplished by centrifugation after incubation for 2.5 h at room temperature in the dark with γ-tocopherol, potassium deoxycholate and dithionite added to the Triton X-100-sucrose-Tris · HCl medium.

These observations strongly support the conclusion that the proteins represented by two bands are the fundamental structural units of the oxidase, i.e. a sort of lowest common denominator of all cytochrome oxidase preparations. Because of this possibility, it was felt that these bands deserved reinvestigation: first, to confirm that both bands are indeed parts of cytochrome oxidase, and second, to relate the two bands, if possible, to previously described structural or functional units of the oxidase obtained by milder preparative methods.

Materials and Methods

Submitochondrial particles were prepared from bovine heart by a modification [25] of the original method of Keilin and Hartree [26]. Both the initial centrifugation to sediment large cell fragments and the final centrifugation following adjustment of pH to 5.4, were at 1340 *g*, the former for 30 min, the latter for 20 min. The final sediment of submitochondrial particles was adjusted to 25 mg protein per ml with 0.1 M Na_2HPO_4-KH_2PO_4, pH 7.4.

The *serial extraction* of submitochondrial particles with sodium deoxycholate was accomplished in the following manner: a suspension of submitochondrial particles was stirred at room temperature for 30 min with 10 mg/ml (1%) of sodium deoxycholate added in solid form. The

* Abbreviations: Tris, tris-(hydroxymethyl) aminomethane; Emasol 1130, polyoxyethylene sorbitan monolaurate; Triton X-100, alkyl phenoxy $(ethoxy)_{9-10}$ ethanol.

mixture was then centrifuged at 4°C in the Spinco model L ultracentrifuge at approximately 60,000 *g* for 30 min (30 head, 27,000 rpm). The resulting supernatant fluid was termed Fraction I. The pellet was homogenized at 4°C in an all-glass hand homogenizer with a volume of 0.1 M Na_2HPO_4, pH 8.0, equal to that of the original suspension, and to the resulting homogenate was again added 10 mg/ml (1%) of sodium deoxycholate. Stirring and centrifugation were done as before, and the resulting supernatant fluid was termed Fraction II. This sequence was continued until no further cytochrome could be extracted. Usually five additions were sufficient at the concentrations of sodium deoxycholate used. The final pellet suspended in phosphate became the Residue. The resulting fractions were stored on cracked ice in the cold room until activities were assayed and difference spectra were done. The remainder of each fraction was then stored at −20°C in small aliquots which were thawed as needed.

Cytochrome oxidase was made by the method of Wainio [27].

Protein was determined by the micro-Kjeldahl method, using the factor 6.25 to convert from nitrogen to protein. Protein assays in the serial extracts were done on aliquots extracted with acetone. Since phospholipid was extracted chiefly into the first fractions, it was desired to correct for the unequal contribution of lipid nitrogen to the several fractions.

Lipid was extracted by the method of Brierley and Merola [28], which entailed slow addition of the protein solution to nine volumes of ice-cold acetone, followed by stirring for 15-30 min in the cold, and then centrifuging at low speed to separate the precipitated protein from the aqueous-acetone solution. The pellet was washed once with water and sedimented as before. It was not specifically determined whether this method when applied to the fractions described above, removed most or all of the lipid, as it presumably does when applied to other mitochondrial fractions.

The *preparation of samples* for polyacrylamide gel electrophoresis in organic solvent was as follows: A known aliquot of each fraction was extracted with acetone, washed with water, and then homogenized with a measured volume of water. Aliquots of the resulting suspension were removed for protein analysis, and the remainder of the suspension was centrifuged in the Spinco model L ultracentrifuge at 54,000 *g* for 30 min (40 head, 25,000 rpm). The resulting pellet was set aside until the protein concentration had been determined. When the total protein of the pellet was known, the pellet was taken up in phenol : acetic acid : water = 2 : 1 : 1, w/v/v, of such a volume as to yield the desired protein concentration. Each sample was then made 2 M with respect to urea.

The *concentrations of the cytochromes* c, c_1, b and cytochrome oxidase were measured by the method of Vanneste [29]. The absorption

spectra of the four cytochromes overlap. Therefore, in a mixture of cytochromes, it is possible to measure the concentration of one cytochrome only if appropriate corrections are made for the contributions to the total absorbance at any wavelength of the other three cytochromes.

The activity of cytochrome oxidase was assayed spectrophotometrically by following the rate of oxidation of ferrocytochrome *c* at 550 nm, as described by Davison [30], except that horse heart cytochrome *c* of 97% purity (Type VI, Sigma Chemical Co., St. Louis, Mo.) was used and that initial rates were calculated (mμmoles ferrocytochrome *c* oxidized per second per mg cytochrome oxidase protein). Cytochrome *c* (10.31 mg) was dissolved in 10 ml deionized water plus 10 ml 0.3 M Na_2HPO_4, pH 5.8. This solution was reduced with $Na_2S_2O_4$, aerated, and then kept under N_2 to retard autoxidation. Into a cuvet were pipetted 2.0 ml of the above solution, 0.9 ml deionized water, and the reaction was begun with 0.1 ml of appropriately diluted enzyme fraction. When the effect of Fraction I on the activity of the other fractions was studied, the activity of Fraction I was subtracted.

Each set of the serial extracts was assayed after different times of storage and at slightly different temperatures, while in two instances the assays were done following freezing and thawing of the samples. Therefore, no comparisons of activity as related to heme content were made between series.

Polyacrylamide gel electrophoresis of samples dissolved in phenol : acetic acid : water was done by the method of Takayama *et al.* [15]. The stock solutions were mixed 15 ml A + 5 ml B + 0.1 ml *N,N,N′,N′*-tetramethylethylenediamine.

(A) 3.0 g acrylamide, 80 mg bisacrylamide, 6.0 g urea } diluted with 14 ml glacial acetic acid and made to 30 ml with water

(B) 150 mg ammonium persulfate, 6.0 g urea } diluted to 10 ml with water

1.5 ml of the mixture was pipetted into each tube, layered with 70% acetic acid, and polymerized in a water bath or oven at 45° to 50° C. for 30 min. This procedure yields 7.5% gel. The gels measured 50 mm in length and 6 mm in diameter. Runs were done at room temperature at 2 mA per tube for 20 min, followed by 6 mA per tube for 90 min. In the case of serial extraction, samples contained 100 μg protein per 25 μl volume. Attempts to prepare more concentrated samples so that a smaller volume could be applied and, consequently, sharper resolution obtained, resulted in clouding of the samples and increased inability of the protein to enter the gel pores. The gels were stained by immersion in 0.5% Coomassie Blue in 10% acetic acid at 45° to 50° C. More efficient

destaining was obtained if a small amount of ion exchange resin was added to the bottom of the destaining container.

Electrophoresis of samples in buffer-deoxycholate were done on gels prepared as above, except that urea, acetic acid, and water were not used, and the acrylamide, bisacrylamide, and ammonium persulfate were dissolved in buffer-deoxycholate solution. The reservoirs were also filled with this solution. The sample density was increased sufficiently to permit layering by the addition of surcrose to 2 M. Sample amounts and volumes varied. The gels were stained in aqueous 0.5% Coomassie Blue without prior fixing and destained in water because insoluble deoxycholic acid is produced at low pH. Determinations in SDS-buffer were done in the same way, except that staining and fixing with solutions of low pH was compatible with SDS gels.

"Structural" protein was prepared by method "f" of Lenaz *et al.* [31], except that the original mitochondrial pellet obtained by the method of Crane *et al.* [32] was not divided into heavy beef heart mitochondria and light beef heart mitochondria prior to extraction.

Results and Discussion

The pattern of a representative cytochrome oxidase preparation made by the method of Wainio [27] and separated by electrophoresis on 7.5% polyacrylamide gel after solution in phenol : acetic acid : water is seen in Fig. 1, tubes 2 and 3. The pattern consists of two major bands: a fast moving band designated a and a slower moving, intense band designated b. The estimated amount of protein in these two bands is 70% in b and 30% in a, based on the assumption that the intensity of staining is a fair measure of the protein. This assumption has been shown to be true for Coomassie Blue staining [33]. In addition, there are at least four minor bands which vary in intensity from preparation to preparation, which have been reported by others [1, 13, 15] and which are entirely absent without loss of activity in cytochrome oxidase from yeast [18]. These last results indicate that the minor bands are very likely contaminants and that, at most, two bands need be considered integral parts of the oxidase. It should be noted, however, that one preparation, which on the basis of a minimum molecular weight of 72,000 and a heme content estimated from $\Delta\epsilon$ at 605 nm = 7.6 mM^{-1} cm^{-1} was approximately 100% pure, also showed minor bands. Tuppy and Birkmayer [18] found that yeast cytochrome oxidase could be purified by gel filtration to the point that a preparation showing only the two major bands had a minimal molecular weight of 65,800 on the basis of iron and copper content. No mention was made of the removal of contaminating ionic copper and iron, so that it is possible that this minimal molecular weight is low. However, the good agreement between the minimal molecular

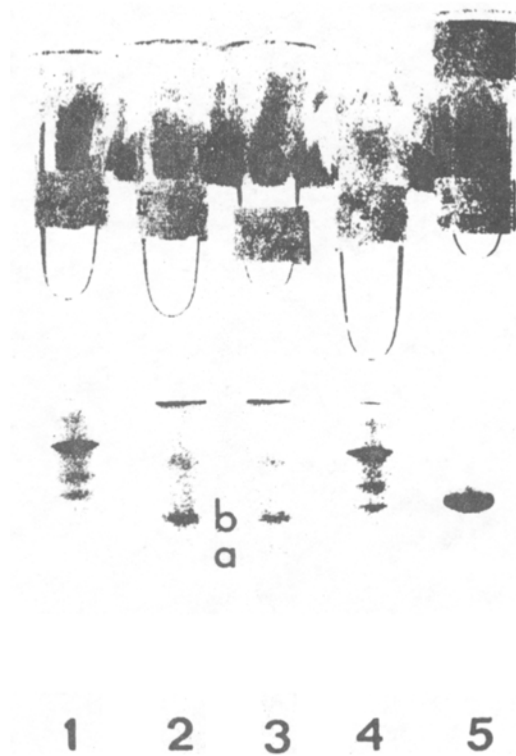

Figure 1. Polyacrylamide gel electrophoresis in phenol : acetic acid of several proteins. Stained with Coomassie Blue. Tubes 1 and 4, "structural" protein; tubes 2 and 3, cytochrome oxidase; for an explanation of a and b see the text; tube 5, cytochrome *c*.

weight calculated in their experiments and the molecular weight of 72,000 of Criddle and Bock [4] should be noted. If 72,000 is the correct value, it may be that the higher minimal molecular weights of some preparations are due to higher content of protein represented by the variable, minor bands, which almost certainly are impurities.

Based on the assumption that the Takayama method of polyacrylamide gel electrophoresis resolves all the noncovalently associated proteins from each other, it was decided to determine whether both or only one of these bands is an integral part of cytochrome

oxidase. Some investigators have tacitly assumed that both bands are proteins of the oxidase merely because both of them are so prominent in the electrophoretic pattern. As explained before, the very solvent system which effects resolution into two components strips the characteristic prosthetic groups, copper and heme, from the protein, and, therefore, direct identification of the bands is not possible. However, only those bands which are integral parts of cytochrome oxidase should parallel properties unique to the oxidase, namely, its characteristic absorption spectrum and its catalytic activity, during fractionation of a mixture of mitochondrial proteins. Previous experiments had shown that serial extraction of particulate mitochondrial proteins with deoxycholate yielded soluble fractions of widely varying cytochrome oxidase concentration [34]. Such fractions, therefore, can be used to relate the specific heme *a* content and the specific oxidase activity to the intensity of the two bands.

Figure 2, A through D, shows the heme *a* contents and specific activities plotted against fraction number of serial deoxycholate extracts, and Fig. 3, A through D, shows the corresponding electrophoretic patterns of samples containing identical amounts of total protein. It should be noted that the two criteria by which the relative concentration of cytochrome oxidase in the fractions is estimated, namely the specific activity and the specific heme *a* content, do not agree well with each other in the later fractions. This is probably due to the fact that deoxycholate, in sufficiently high concentrations, is inhibitory to oxidase activity [35]. The heme *a* content is, therefore, a more reliable index of the amount of oxidase protein present than is the specific activity.

It can be seen that band A parallels heme *a* content. The distribution of band B is not as clearly related to heme *a*. What appears to be a band B is present in large amounts in the first several fractions which contain very little heme *a* or oxidase activity. Band B then decreases in intensity, and increases again parallel with the specific heme *a* content. Repeated attempts to show that band B in the first fractions and band B in the later fractions were two different proteins of nearly the same mobility were not successful. Electrophoresis of a mixture of Fraction I and Fraction IV of Fig. 3A on gels as long as 10 cm repeatedly yielded a single band in the position of band B. The next step should have been to vary the conditions of electrophoresis and to note whether the two bands still moved in the same way. However, this could not be done, because the solvent system used is the only one known to resolve the oxidase into two components. Raising the pH caused precipitation of the protein. Therefore, there were two possibilities: either that band A was the oxidase and band B a contaminant, or that both bands A and B were parts of the oxidase, but that a protein of mobility very similar to that of the protein represented by band B was extracted into the early fractions.

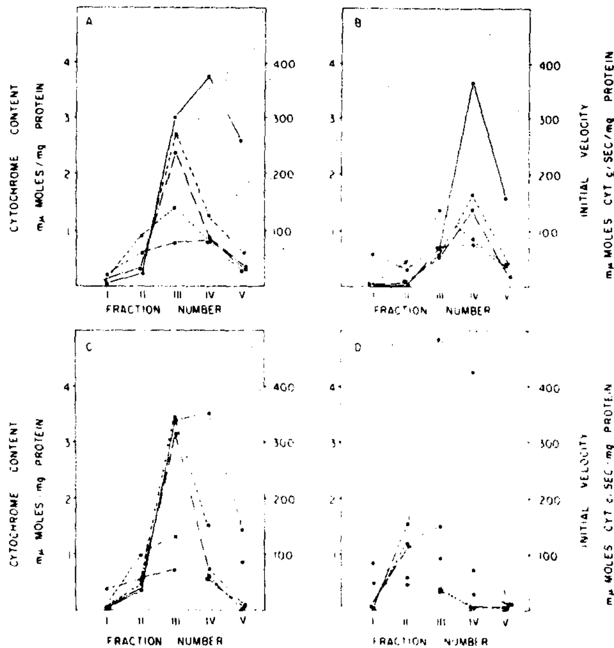

Figure 2. Cytochrome content and cytochrome *c* oxidase activity of successive fractions (I through V) prepared with 1% deoxycholate from beef heart submitochondrial particles. (——) Cytochrome oxidase content; (- - - -) cytochrome *b* content; (– – –) cytochrome c_1 content; (– · –) cytochrome oxidase activity without fraction I added; (· – – ·) cytochrome oxidase activity with fraction I added. The preparations (A through D) are the same as in Fig. 3.

On the basis of the preceding experiments, it was felt that both bands A and B were integral to the oxidase, chiefly because it was not reasonable that deoxycholate should extract one protein (band B) at first in high concentration, then in low concentration, and then in high concentration again.

The next step was to determine whether purified cytochrome oxidase contained a component electrophoretically identifiable as one of the noncatalytic proteins of Green *et al.* [11]. This possibility seemed likely when apparently the same protein was found in oxidase-rich fractions as well as in fractions poor in oxidase and rich in other cytochromes. Tubes 1 and 4 in Fig. 1 show that "structural" protein prepared by the method of Lenaz *et al.* [31] did not yield a single band. Schatz and Saltzgaber [36] also found that they could not purify "structural" protein to the point of homogeneity. If it may be assumed, however, that the most prominent component of the pattern is "structural" protein, then purified cytochrome oxidase does not contain such a component.

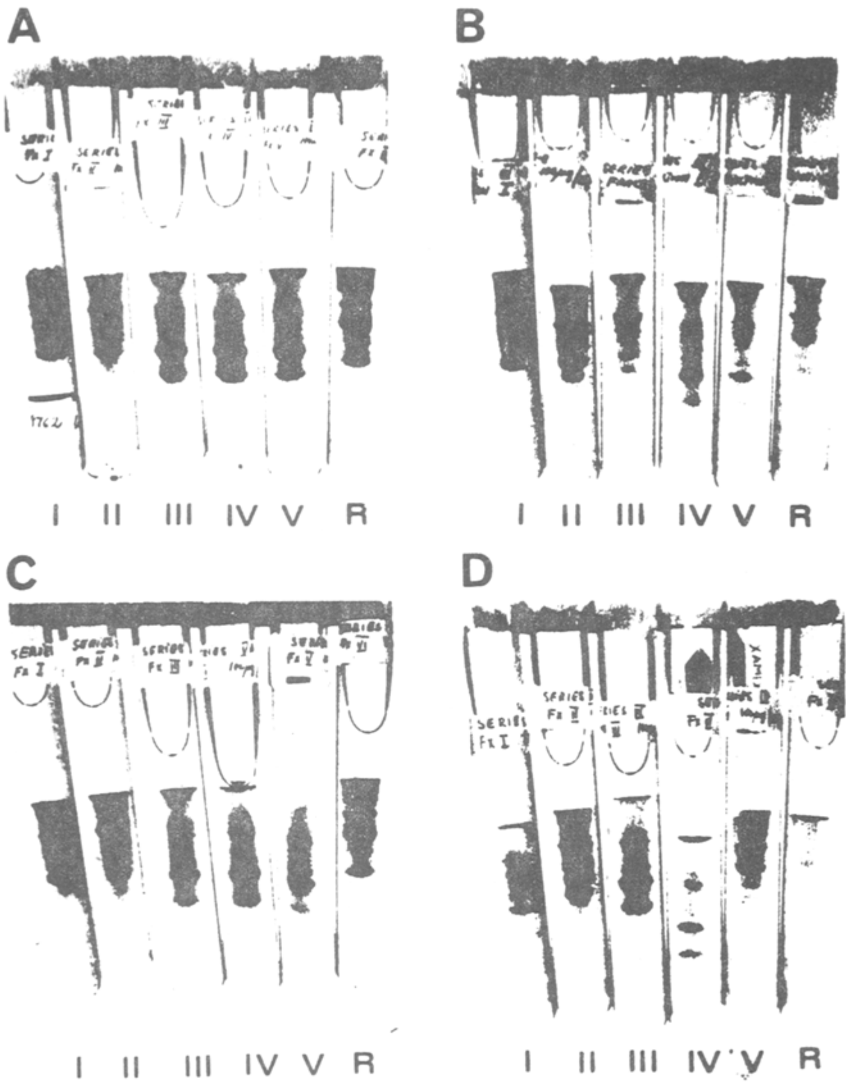

Figure 3. Polyacrylamide gel electrophoresis in phenol : acetic acid of successive fractions (I through V) prepared with 1% deoxycholate from beef heart submitochondrial particles. Stained with Coomassie Blue. R is the residue. The preparations (A through D) are the same as in Fig. 2.

It is concluded that cytochrome oxidase consists of two noncovalently associated proteins, neither of which appears to be the noncatalytic protein of Green *et al.* [11]. These conclusions are, of course, only as correct as the assumptions underlying the method of analysis, namely that the solvent system does not cleave covalent bonds, that the solvent system does achieve resolution of all noncovalently associated proteins within a complex of proteins, and that polymerization does not occur in this solvent system, as it does, for instance, in anionic detergents. There is indirect evidence on all three points. The authors of the method concluded that the solvent system did not cleave covalent bonds based on the observation that each of several well-characterized proteins yielded a single band under identical electrophoretic conditions. Figure 1, tube 5, shows cytochrome *c* subjected to electrophoresis under identical conditions. However, it is more difficult to prove that the solvent system effects complete disaggregation. Probably the best support for that, subject to qualifications, is that the number of components known to constitute Complex III of the respiratory chain equals the number of components demonstrated when Complex III is subjected to electrophoresis after being dissolved in this system [37].

It is not possible to speculate whether the two components are cytochromes a and a_3. As has already been mentioned, the treatment with phenol : acetic acid removes the copper and the heme, with the result that spectral identification of the species can no longer be made.

Attempts to separate cytochrome oxidase into two components under non-denaturing conditions, or at least under conditions such that the prosthetic groups would remain bound to the apoprotein, yielded a single band during initial experiments. Because of this finding and the observation that purified cytochrome oxidase moved as a single boundary on ultracentrifugation, it was concluded that the two components existed as a complex in buffered solutions of a surface active agent, and that the indirect, more laborious method of correlating band intensity on electrophoresis in acidic organic solvent with the specific heme a content might be the only way of tentatively identifying the two components as parts of the oxidase, or as oxidase plus a contaminant. Subsequently, it was found that electrophoresis in the presence of buffer-sodium dodecyl sulfate, also on occasion yielded two major components. In both cases, only the slower band was associated with the characteristic oxidase color. These results were not reproducible, and it is not known what the critical variables were. These results are mentioned only to point out that resolution in buffered surface active agent, under conditions mild enough to preserve catalytic activity, is apparently possible when the correct conditions are obtained. If the two bands demonstrable in organic solvent electrophoresis were the same as demonstrated in buffer-sodium dodecyl sulfate and buffer-deoxycholate, the two cannot be polymers of each other, since only one of the two in buffered surfactant had the oxidase color.

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