Additional components of bovine heart cytochrome *c* oxidase demonstrated by high-resolution polyacrylamide-gel electrophoresis in the presence of chloral hydrate

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We have shown that aq. 100% (w/v) chloral hydrate (2,2,2-trichloroethane-1,1-diol) dissociates bovine heart cytochrome *c* oxidase. We have developed new procedures of polyacrylamide-gel electrophoresis in the presence of chloral hydrate that permit variation in the pH of the separation, and, by using these procedures, we have observed 15 components in preparations of the enzyme. This number contrasts with the eight bands that were seen on electrophoresis in the presence of SDS (sodium dodecyl sulphate) and urea. We have isolated material from these eight bands and have characterized each by electrophoresis in the presence of chloral hydrate. Twelve of the fifteen components that were seen by electrophoresis in chloral hydrate were identified as constituents of the eight bands seen by electrophoresis in the presence of SDS and urea. Two-dimensional electrophoretic separations confirmed these identifications and showed that the other three components which were resolved as discrete bands by electrophoresis in the presence of chloral hydrate appeared to be diffusely present in the electrophoretic separations performed in the presence of SDS and urea, which suggested anomalous behaviour in that detergent. Trypsin treatment of cytochrome *c* oxidase caused total loss, as observed by electrophoretic separations in the presence of chloral hydrate, of a number of components. The trypsin-sensitive components included all of those that behaved anomalously in the presence of SDS and urea. Chloral hydrate is a potent non-ionic dissociating agent for cytochrome *c* oxidase and its use in polyacrylamide-gel electrophoresis, with variation in the pH of the gel, permits charge-dependent separations that should have general application in the analysis of membrane proteins.

Cytochrome *c* oxidase (EC 1.9.3.1) is the terminal component of the respiratory chain of the mitochondrion. It is a membrane-protein complex and has been isolated from a variety of sources. The subunit structures of these enzymes have been the subjects of intense study, and the cytochrome *c* oxidases from yeast and *Neurospora crassa* have been shown to contain seven subunits (Poyton & Schatz, 1975) and eight subunits (Werner, 1977) respectively.

In contrast, there is substantial disagreement over the subunit composition of bovine heart cytochrome *c* oxidase. Preparations of the enzyme have been shown to yield different numbers of components when analysed by polyacrylamide-gel electro-

phoresis in the presence of SDS, or, as is now more commonly employed, in SDS/urea. Thus six- (Carroll & Racker, 1977; Penttilä *et al.*, 1979; Saraste *et al.*, 1980), seven- (Downer *et al.*, 1976; Yu & Yu, 1977; Chan & Tracy, 1978) and nine- (Poyton *et al.*, 1978) subunit compositions have been proposed.

However, Buse & Steffens (1978) isolated twelve polypeptides from their preparation of the enzyme by gel-filtration procedures, and Verheul *et al.* (1979), who also used preparative-scale gel-filtration procedures, found eleven components in their enzyme.

These analytical procedures have been almost entirely dependent on the action of SDS as a protein-dissociating agent. Clearly, it would be advantageous to have analytical methods that did not depend on

Abbreviation used: SDS, sodium dodecyl sulphate.

this detergent, and we describe here new procedures that employ chloral hydrate (2,2,2-trichloroethane-1,1-diol). Chloral hydrate, at high concentration in aqueous solution, has been found to be an effective membrane-solubilizing agent, and its use in polyacrylamide-gel electrophoresis yields separations that depend on protein charge as well as size (Ballou *et al.*, 1974; Ballou & Smithies, 1977; Booth, 1977). We describe the application of new polyacrylamide-gel-electrophoretic procedures that employ chloral hydrate as a dissociating agent to the analysis of an isolated membrane-protein complex, bovine heart cytochrome *c* oxidase. We show that chloral hydrate is effective in dissociating the enzyme complex and that the dissociated components can be separated by electrophoresis in the presence of chloral hydrate. We further show that the new electrophoretic systems, which permit variation in the pH of the separation, resolve components that co-migrate at the fixed pH of the earlier method described by Ballou & Smithies (1977). In addition, we observe three components that are not separated by polyacrylamide-gel electrophoresis in the presence of SDS/urea. A preliminary report of some of this work has been presented (Landon & Griffin, 1979).

Experimental

Materials

SDS, which was a specially pure SLR grade, and chloral hydrate, which was recrystallized from chloroform before use, were from Fisons Scientific Apparatus Ltd., Loughborough, Leics. LE11 0RG, U.K. Acrylamide and *NN'*-methylenebisacrylamide, which were specially-purified-for-electrophoresis grades, and PAGE Blue G-90 were from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K. Bovine serum albumin, hen's-egg ovalbumin, horse heart cytochrome *c*, horse heart myoglobin and soya-bean trypsin inhibitor were from Sigma (London) Chemical Co. Ltd., Kingston upon Thames, Surrey KT2 7BH, U.K. Bovine trypsin and bovine chymotrypsinogen were from The Boehringer Corporation (London) Ltd., Lewes, East Sussex BN7 1LG, U.K. 1,1,3,3-Tetramethyl-2-thiourea was from Aldrich Chemical Co., Gillingham, Dorset SP8 4JL, U.K.

Methods

Purification of cytochrome *c* oxidase. Cytochrome *c* oxidase was purified from bovine heart tissue exactly as described by Volpe & Caughey (1974).

Electrophoretic methods. All the electrophoretic separations were performed by using a vertical slab-gel apparatus, with gels that were 15 cm wide, 9 cm long and 0.15 cm thick.

(a) Polyacrylamide-gel electrophoresis in the presence of SDS and urea. The method was that

of Swank & Munkres (1971) and used 12.5% (w/v) (total monomer) polyacrylamide gels with an acrylamide/*NN'*-methylenebisacrylamide weight ratio of 9:1. Protein samples were prepared by incubation at 40°C for 1 h in the presence of 2% (w/v) SDS, 1% (v/v) 2-mercaptoethanol and 8M-urea. Gels were stained overnight with 0.1% PAGE Blue G-90 in methanol/acetic acid/water (5:1:4, by vol.) and destained by using several changes of 10% (v/v) acetic acid. Densitometric traces of gels were made at 600nm by using a Helena Quick Scan R & D densitometer (Helena Laboratories, Beaumont, TX, U.S.A.).

For apparent-molecular-weight determinations the following proteins were used as standards: bovine serum albumin (67 000), hen's-egg ovalbumin (45 000), bovine chymotrypsinogen (25 700), horse heart myoglobin (17 000) and horse heart cytochrome *c* (12 300). The CNBr cleavage products of horse heart myoglobin, which were prepared by the method of Gross (1967), with 70% (v/v) formic acid, and which included constituents with mol.wts. 8200, 6200 and 2500 were also used as standards.

(b) New methods of polyacrylamide-gel electrophoresis in the presence of chloral hydrate at pH 4.25, 3.70 and 3.05. The new methods that we have developed are based on a discontinuous aqueous buffer system that was described by Reisfeld *et al.* (1962) and was modified and extended by Jovin (1973). Separating gels were generally 12.5% (w/v) total monomer, with an acrylamide/*NN'*-methylenebisacrylamide weight ratio of 29:1, and contained 100% (w/v) chloral hydrate and 77mM-KOH, adjusted to pH 4.25 with acetic acid (the KOH was neutralized before the addition of chloral hydrate, since the latter compound is unstable in alkali). **Chloral hydrate is toxic, and care should be taken to avoid contact and exposure through breathing.** Gels of different porosity were made by varying the total monomer concentration without changing the ratio of cross-linker. For separating gels at pH 3.70 and pH 3.05, the KOH concentrations were 54mM and 41mM respectively. Photopolymerization of gels was effected by using final concentrations of 10 µg/ml for riboflavin [prepared at 10mg/ml in 100% (w/v) chloral hydrate in water] and 0.1mg/ml for 1,1,3,3-tetramethyl-2-thiourea (prepared at 100mg/ml in chloroform). Brief degassing preceded pouring of the gel solution into an apparatus that used only materials such as polycarbonate, glass, Teflon and silicone rubber, which are compatible with the solvent properties of chloral hydrate (Ballou *et al.*, 1974). An overlay of 70% (w/v) chloral hydrate in separating-gel buffer was applied in order to obtain a flat gel surface and was removed after gelling had occurred. The upper-electrode (anode) buffer was 60mM-β-alanine, adjusted to pH 4.1 with acetic acid,

and lower-electrode (cathode) buffer was 75 mM-KOH, adjusted to pH 4.0 with acetic acid. It is necessary to keep the aqueous electrode buffers separated from the gel in order to prevent swelling. Thus the gel was covered with a 2-cm layer of 70% (w/v) chloral hydrate in upper-electrode buffer and the bottom of the gel was dipped into a trough containing 70% (w/v) chloral hydrate in lower-electrode buffer.

Samples were prepared by the addition of protein solutions to 4 vol. of 125% (w/v) chloral hydrate/1.25% (v/v) 2-mercaptoethanol in 0.12 M-KOH, adjusted to pH 5.45 with acetic acid, and were kept at room temperature for at least 1 h before loading on to a gel. The final protein concentration was usually between 3 and 6 mg/ml. Electrophoresis was performed for 1 h at 1 W and for a further 23 h at 2.5 W (at a maximum of 500 V) by using a constant-wattage power supply.

Staining of the gels was performed with 0.1% PAGE Blue G-90 in 10% (v/v) acetic acid in water. The staining solution was changed after 45 min and staining continued overnight. Destaining and densitometry were performed as described above.

(c) Polyacrylamide-gel electrophoresis in the presence of chloral hydrate at pH 2.3. This method was essentially that described by Ballou & Smithies (1977), which employs 100% (w/v) chloral hydrate in aluminium lactate buffer at pH 2.3. Samples, which were prepared as described by those authors but also included 1% (v/v) 2-mercaptoethanol, were kept at room temperature for at least 1 h before loading on to a gel. The gels were 12.5% (w/v) with respect to total acrylamide monomer, with an acrylamide/*NN'*-methylenebisacrylamide weight ratio of 29:1. Electrophoresis was performed at 215 V for 24 h. The gel was protected from contact with aqueous buffer by an overlay of 70% (w/v) chloral hydrate in electrode buffer and by dipping it into a trough containing the same solution. Staining, destaining and densitometry were performed as described above for the new electrophoresis method employing chloral hydrate.

Isolation of band material after polyacrylamide-gel electrophoresis in the presence of SDS and urea. Band material from separations of components of cytochrome *c* oxidase by SDS/urea/polyacrylamide-gel electrophoresis was isolated and rerun by the new methods of electrophoresis in the presence of chloral hydrate at pH 4.25 and pH 3.05. For separation by electrophoresis in the presence of SDS and urea, a sample solution containing 2 mg of cytochrome *c* oxidase was layered over a 12 cm width of a gel prepared as described above. After electrophoresis, a 6-cm-wide vertical strip was cut from the centre of the gel and stained for 90 min in 150 ml of 20 mM-potassium acetate buffer, pH 3.0, containing 0.2% PAGE Blue G-90. The strip was destained

in two changes of 20 mM-potassium acetate buffer, pH 3.0, followed by two changes of 50 mM-potassium acetate buffer, pH 3.0. The initial lower K^+ concentration was used in order to minimize precipitation of potassium dodecyl sulphate. The bands could then be made visible and those parts of the gel were excised, put into glass tubes and fragmented by using a glass rod. An appropriate volume of 125% (w/v) chloral hydrate/1.25% (v/v) 2-mercaptoethanol in 0.12 M-KOH, adjusted to pH 5.45 with acetic acid, was added together with a small amount of solid chloral hydrate [in order to keep the concentration at 100% (w/v)]. The volume added to each gel piece was sufficient to permit removal of approx. 0.5 ml of solution containing extracted peptide material. Extraction was performed overnight with vigorous shaking. In this way, peptide material was directly extracted into the sample buffer appropriate for the subsequent analysis by electrophoresis in chloral hydrate.

Two-dimensional electrophoretic separations by polyacrylamide-gel electrophoresis. For all types of two-dimensional separation, appropriate samples of cytochrome *c* oxidase were applied over at least half of the width of the first-dimension gel and, after electrophoresis, a central 0.5–0.75-cm-wide vertical section (which contained 45–70 μ g of total protein) was cut out and equilibrated as described below for the particular second-dimension separation.

(a) Chloral hydrate systems in both dimensions. The strip from the first-dimension gel was equilibrated for 30 min at room temperature with gentle shaking in each of four 5 ml portions of 100% (w/v) chloral hydrate/1% (v/v) 2-mercaptoethanol in 96 mM-KOH, adjusted to pH 5.45 with acetic acid.

(b) SDS/urea first dimension, new chloral hydrate system second dimension. The strip was equilibrated at room temperature for 30 min with gentle shaking in 5 ml portions of each of the following solutions of 96 mM-KOH, adjusted to pH 5.45 with acetic acid, and containing: (i) 0.1% SDS, (ii) no addition, (iii) 125% (w/v) chloral hydrate and 1.25% (v/v) 2-mercaptoethanol, (iv) 100% (w/v) chloral hydrate and 1% (v/v) 2-mercaptoethanol. Solutions containing (i), (ii), (iii) and (iv) were used in two, two, one and two portions respectively. Precipitated potassium dodecyl sulphate redissolved on addition of the solution containing (iii).

(c) New chloral hydrate system first dimension, SDS/urea second dimension. The strip was equilibrated, with gentle shaking for 30 min in each solution, four times at room temperature in 5 ml portions of 2% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 0.1 M- H_3PO_4 , adjusted to pH 6.8 with Tris, followed by four times at 40°C in 5 ml portions of the same solution in 8 M-urea. Gels were stained

by the method appropriate for the particular second-dimension separation.

Treatment of cytochrome c oxidase with trypsin

Cytochrome *c* oxidase was treated with trypsin as described by Capaldi (1978), by using a cytochrome *c* oxidase/trypsin weight ratio of 50:1 and incubation at room temperature for 2 h. Cytochrome *c* oxidase was separated from degraded fragments, trypsin and trypsin inhibitor by gel filtration as described by Capaldi (1978), except that Sepharose CL-4B was used.

Results

Polyacrylamide-gel electrophoresis of cytochrome c oxidase in the presence of SDS and urea

A densitometric trace of an electrophoretic separation, in the presence of SDS and urea, of the constituents of cytochrome *c* oxidase is shown in Fig. 1. Eight major bands were found and, in addition, there were small amounts of high-molecular-weight material. The fastest migrating band (VIII) gives an asymmetric peak on the trace, and in other separations was sometimes split into two bands, which suggested that at least two components were present.

Fig. 1. Densitometric trace of the separation of components of cytochrome *c* oxidase by electrophoresis in the presence of SDS and urea

Polyacrylamide-gel electrophoresis in the presence of SDS and urea was performed by the method of Swank & Munkres (1971). Other details are given in the Experimental section. The apparent molecular weights of the components of cytochrome *c* oxidase were as follows: I, 39 000; II, 27 000; III, 21 500; IV, 15 000; V, 12 500; VI, 10 000; VII, 9000; VIII, 6500.

The pattern of resolution of components of cytochrome *c* oxidase that we have obtained was essentially the same as those which have been reported by several other workers. The numbering system that we have used is the same as that employed by Buse & Steffens (1978) and by Verheul *et al.* (1979).

Development of the new methods of polyacrylamide-gel electrophoresis in the presence of chloral hydrate

Earlier methods of electrophoresis in the presence of chloral hydrate have employed either discontinuous (Ballou *et al.*, 1974; Booth, 1977) or continuous buffer systems (Ballou & Smithies, 1977). However, although it was established by Ballou *et al.* (1974) that the protein separations were dependent on charge, the observations that were made were in gels at a fixed pH for the separation. In selecting a discontinuous buffer system, we considered that it would be advantageous to be able to change the pH of the separation gel. Ballou *et al.* (1974) noted that good membrane solubilization was only obtained at high concentrations of chloral hydrate. We have used the same high concentration of chloral hydrate [100% (w/v) in water] throughout the present work.

Chloral hydrate is unstable in alkaline solution and is, therefore, only compatible with acid-pH systems. A system that was initially described by Reisfeld *et al.* (1962) and was subsequently modified and extended by Jovin (1973), which permitted variation of the separation over the range pH 4.15–2.78 appeared to be suitable. In this system the sample can be prepared in a solution that is fixed both in terms of pH and in ion concentration, despite the variation in the pH at which the separations may be effected. Thus a single sample solution can be analysed under the different conditions. From the possible range of pH values for the separation, we have used separations at pH 3.65, pH 3.3 and pH 2.8; these pH values have resulted from separation gels initially at pH 4.25, pH 3.7 and pH 3.05, respectively. When referred to below, the different pH systems are designated by the initial pH of the separation gel. No stacking gel was used, and the sample was prepared at pH 5.45. We have looked at the effects of changing the separation gel by varying the total acrylamide monomer concentration between 6 and 15% (w/v), with a fixed weight ratio of acrylamide to *NN'*-methylenebisacrylamide of 29:1. For most of the separations that are described here, the gels were 12.5% (w/v) with respect to total acrylamide monomer. Protein samples were normally maintained in 100% (w/v) chloral hydrate at room temperature for at least 1 h before being loaded on to the gel. No difference in band pattern was observed when samples were incubated at temperatures up to 60°C for 30 min. Protein samples,

in the solution appropriate for loading on to gels, that were stored at -20°C over a period of several months showed no change in band pattern.

Chloral hydrate/polyacrylamide-gel electrophoresis of cytochrome c oxidase

Densitometric traces of separations of the constituents of cytochrome *c* oxidase that were obtained by the new systems of electrophoresis in the presence of chloral hydrate are shown in Fig. 2. In each case the band pattern that was obtained at a particular pH of the separating gel was more complex than that obtained by separation in the presence of SDS and urea (Fig. 1). Furthermore, the new systems of gel electrophoresis in the presence of chloral hydrate produced band patterns that were more complex than that obtained by using the chloral hydrate/polyacrylamide-gel electrophoretic system of Ballou & Smithies (1977). A densitometric trace of a separation in the latter system, which uses aluminium lactate buffer at pH 2.3, is also shown in Fig. 2.

We considered that the different total numbers of separated bands that were seen in the various systems could have been due to co-migration of certain of the components in a particular system. This possibility was examined by performing two-dimensional separations of components of cytochrome *c* oxidase by electrophoresis in the presence of chloral hydrate, with the separating gel at one pH value in the first dimension and at a different pH value in the second dimension. A photograph of a separation that was performed at pH 4.25 in the first dimension and at pH 3.05 in the second dimension is shown in Fig. 3; 15 components were resolved. Fig. 4 shows a photograph of a two-dimensional separation that was performed in aluminium lactate buffer, pH 2.3, in the first dimension and in the new buffer system at pH 4.25 in the second dimension. In this case, 12 components were resolved. Inspection of these two-dimensional patterns permitted interpretation of the one-dimensional separations (obtained at three different pH values) in terms of the 15 components that could be distinguished in Fig. 3. This information is presented in Fig. 5.

Inasmuch as gel electrophoresis in the presence of chloral hydrate had provided evidence for 15 components in preparations of cytochrome *c* oxidase that had yielded eight or, at most, nine, bands by electrophoresis in the presence of SDS and urea, it was clearly essential that the components seen in the presence of chloral hydrate should be identified in relation to those seen in the presence of SDS and urea. The close proximity of several components after separation by electrophoresis in the presence of chloral hydrate precluded their isolation by excision from gels, whereas isolation by band excision after electrophoretic separations in the

Fig. 2. Densitometric traces of the separations of components of cytochrome *c* oxidase by electrophoresis in the presence of chloral hydrate at different pH values (a) Polyacrylamide-gel electrophoresis in the presence of chloral hydrate was performed by the new method described in the Experimental section, at pH values for the separation gel as indicated. (b) Polyacrylamide-gel electrophoresis in the presence of chloral hydrate at pH 2.3 was performed as described by Ballou & Smithies (1977). Other details are given in the Experimental section.

Fig. 3. Two-dimensional separation of components of cytochrome *c* oxidase by electrophoresis in the presence of chloral hydrate at two different pH values (pH 4.25 and pH 3.05)

Both dimensions of the separation were performed by using the new chloral hydrate/polyacrylamide-gel-electrophoresis method described in the Experimental section. The first-dimension gel was at pH 4.25. In the second dimension, the gel was at pH 3.05. Other details are given in the Experimental section. The fifteen components are numbered as indicated.

Fig. 4. Two-dimensional separation of components of cytochrome *c* oxidase by electrophoresis in the presence of chloral hydrate at two different pH values (pH 2.3 and pH 4.25)

The first dimension of the separation was performed in the presence of chloral hydrate at pH 2.3 as described by Ballou & Smithies (1977). The second dimension was performed by using the new chloral hydrate method at pH 4.25 that is described in the Experimental section. The components are numbered according to the identifications made in Fig. 3. Other details are given in the Experimental section.

presence of SDS and urea was feasible. In relation to this, it was important to know if the presence of SDS in an isolated component would affect its mobility on electrophoresis in the presence of chloral hydrate.

Ballou & Smithies (1977) reported that band patterns which were obtained by electrophoresis in the presence of chloral hydrate were not affected if the material had been previously treated with SDS, and also that the band patterns were unaffected by the presence of up to 1% (w/v) SDS in the sample solution that was loaded on to the gel. We confirmed these observations in relation to the band pattern of components of cytochrome *c* oxidase that were separated by electrophoresis in the presence of chloral hydrate at pH 4.25. Preincubation of cytochrome *c* oxidase in 2% (w/v) SDS at 40°C for 1 h did not affect the band pattern and it was also unchanged when SDS, at concentrations of up to 1% (w/v), was added to the sample solution immediately before loading it on to the gel. The results of these experiments indicated that SDS did not bind to polypeptide components of cytochrome *c* oxidase on electrophoresis in the presence of 100% (w/v) chloral hydrate.

Identification of components present in isolated band material after electrophoresis in the presence of SDS and urea by subsequent electrophoresis in the presence of chloral hydrate

Isolation of components that were present in the bands observable after polyacrylamide-gel-electrophoretic separation of cytochrome *c* oxidase which had been dissociated in SDS and urea was readily accomplished by excision of the particular band, after staining, and subsequent extraction. However, the re-running of extracted material by electrophoresis in the presence of chloral hydrate necessitated care in the choice of the method. The most satisfactory procedure was found to be the one that is described in the Experimental section. Although the purity of the material that was extracted in this way was not directly assessed by electrophoresis in the presence of SDS and urea, the purity of samples that were obtained by analogous procedures, which used solutions containing SDS for the extraction of material, was assessed. The only evidence of cross-contamination of band material after electro-

Fig. 5. Identification of the components of cytochrome *c* oxidase present in the bands obtained after one-dimensional separations by electrophoresis in the presence of chloral hydrate at different pH values

The diagrams represent the protein bands seen after one-dimensional separations performed in the presence of chloral hydrate (a) at pH 4.25, (b) at pH 3.05 and (c) at pH 2.3. Separations (a) and (b) were by the new chloral hydrate method described in the Experimental section, whereas separation (c) was by the chloral hydrate method of Ballou & Smithies (1977). The components present in each band are numbered according to the system presented in Fig. 3. Identifications for (a) and (b) were made from the two-dimensional separation shown in Fig. 3 and those for (c) were made from the two-dimensional separation shown in Fig. 4.

phoresis in the presence of SDS and urea was in relation to band I, where the extracted material contained, in addition to band I, low quantities of band-II and -III material. This cross-contamination, which was probably due to the presence of small amounts of aggregated forms of band-II and band-III components in the region of band I in the original separation, did not hinder the identifications that were subsequently made. Band VIII in the SDS/urea-containing gels was always excised as a single component.

The extracted band material was re-run by gel electrophoresis in the presence of chloral hydrate at pH 4.25 and at pH 3.05, and the identifications that were made are given in Table 1. Although all of the bands from SDS/urea-containing gels were identified in terms of one or more components on the chloral hydrate-containing gels, only very low and variable amounts of staining material (from bands III, V and VI) appeared in the region of components (6 + 7), 8 and 9 on the chloral hydrate-containing gels. The low amounts of staining contrasted markedly with the substantially higher amount of staining of these components that was observed when dissociated cytochrome *c* oxidase was run on the chloral hydrate-containing gels. Band VIII consistently gave a weakly staining band where components 6 and 7 appeared together on 12.5% (w/v) acrylamide gels. Components 6 and 7 could be resolved by electrophoresis at pH 4.25 in 10% (w/v) acrylamide gels and component 6 stained more weakly than component 7. These observations led us to

Table 1. Cross-identification of components of cytochrome *c* oxidase separated by polyacrylamide-gel electrophoresis in the presence of SDS and urea, and in the presence of chloral hydrate

A Band isolated after electrophoresis in the presence of SDS and urea	B Corresponding component* on electrophoresis in the presence of chloral hydrate:		D Chloral hydrate- electrophoresis component* number (as determined from columns B and C)
	at pH 4.25	at pH 3.05	
I	1	1	1
II	2	2	2
III	3	3	3
IV	5	(4 + 5)†	5
V	4	(4 + 5)†	4
VI	(10 + 11)†, 12	(9 + 10)†, (11 + 12)†	10, 12
VII	13	13	13
VIII	(6 + 7)†, (10 + 11)†, (14 + 15)†	(6 + 7)†, (11 + 12)†, 14, 15	6‡, 11, 14, 15

* The component numbering in chloral hydrate is that determined by two-dimensional electrophoresis (at pH 4.25 in the first dimension and at pH 3.05 in the second dimension) as shown in Fig. 3.

† The + sign indicates that these particular components co-migrated in the specified system of electrophoresis in the presence of chloral hydrate.

‡ The evidence for the identification of component 6 (rather than 7) as a constituent of band VIII is presented in the Results section.

identify component 6 as one of the constituents of band VIII.

Two-dimensional polyacrylamide-gel electrophoretic separation in the presence of SDS and urea in the first dimension and in the presence of chloral hydrate at pH 4.25 in the second dimension.

A photograph of the result of this type of two-dimensional separation is shown in Fig. 6. Correlations between components in the two separations were compatible with those listed in Table 1. Of particular significance, however, was the

Fig. 6. Two-dimensional separation of components of cytochrome *c* oxidase by electrophoresis in the presence of SDS and urea followed by electrophoresis in the presence of chloral hydrate at pH 4.25

The first dimension of the separation was performed in the presence of SDS and urea by the method of Swank & Munkres (1971). The second-dimension separation, at pH 4.25, was by the new chloral hydrate method described in the Experimental section where other details are to be found. The first-dimension gel piece is shown in position in the photograph, and it can be seen that bands I and III were not resolubilized and did not enter the second-dimension gel. A cytochrome *c* oxidase standard is on the left-hand side of the second-dimension gel, but retardation of the components that were electrophoresed from the first-dimension gel prevents direct alignment of these components with those in the standard. The regions of staining that are arrowed have mobilities in the second dimension that correspond with components (6 + 7), 8 and 9, and clearly arise from various parts of the first-dimension gel.

appearance of staining material from a number of regions in the first dimension gel that showed up (indicated with arrows in Fig. 6) in the area of the second dimension that corresponded with the observed mobilities of components (6 + 7), 8 and 9. This result suggested that these components, which were resolved as sharp bands in a one-dimensional separation in the presence of chloral hydrate were diffusely present in the separation in the presence of SDS and urea.

Two-dimensional polyacrylamide-gel-electrophoretic separation in the presence of chloral hydrate at pH 4.25 in the first dimension and in the presence of SDS and urea in the second dimension

A photograph of this separation is shown in Fig. 7. The identifications of components that were separated in both of the dimensions were again entirely in agreement with those listed in Table 1. Components (6 + 7), 8 and 9 of the chloral hydrate-containing first-dimension gel generated elongated, diffuse staining regions (indicated with arrows in Fig. 7) in the SDS/urea-containing second-dimension gel. This provided direct evidence for the anomalous behaviour of these components in the presence of SDS and urea, and this evidence complemented that given above for the diffuse presence of these components in one-dimensional separations in that solvent.

*Effect of trypsin treatment of cytochrome *c* oxidase on band patterns after electrophoresis in the presence of SDS and urea and of chloral hydrate*

We have applied the trypsin procedure of Capaldi (1978) to cytochrome *c* oxidase and looked at the band patterns obtained after electrophoresis in the presence of SDS and urea and in the presence of chloral hydrate at pH 4.25. The pattern of the separation in SDS/urea-containing gels was substantially affected by trypsin treatment inasmuch as band VI was eliminated, and bands III and VIII were markedly, and band IV was somewhat, decreased in amounts. The pattern of the separation in the presence of chloral hydrate, which is shown in the form of a densitometric trace in Fig. 8, confirmed these effects and also showed that there was total elimination of components (6 + 7), 8 and 9, none of which appears as a clear band on the one-dimensional separation in the presence of SDS and urea.

Discussion

The present work was initiated in order to establish the feasibility of examining the subunit structure of bovine heart cytochrome *c* oxidase by a procedure that did not depend on dissociation of the complex by SDS. Although chloral hydrate

Fig. 7. Two-dimensional separation of components of cytochrome *c* oxidase by electrophoresis in the presence of chloral hydrate at pH 4.25 followed by electrophoresis in the presence of SDS and urea

The first dimension of the separation was by the new chloral hydrate method at pH 4.25 and the second-dimension separation was by the SDS/urea method of Swank & Munkres (1971). Other details are as given in the Experimental section. The first-dimension gel piece is shown in position in the photograph and it can be seen that components 1 and 3 were not resolubilized and did not enter the second-dimension gel. The numbers above the first-dimension gel show the positions of the components that give streaks of staining material in the second-dimension gel. A cytochrome *c* oxidase standard is on the left-hand side of the second dimension gel and the identities of the bands are given on the far left.

has been shown to be a potent membrane-solubilizing agent (Ballou *et al.*, 1974; Ballou & Smithies, 1977; Booth, 1977), it has not been investigated in relation to a defined membrane protein complex. When we first examined cytochrome *c* oxidase after dissociation in chloral hydrate and electrophoresis at the fixed pH of 2.3 by the procedure of Ballou & Smithies (1977), we obtained a pattern that was similar, in terms of the number of bands, to that found in electrophoresis in the presence of SDS and urea. However, the advantages of variation in the pH at which the separation was performed in the presence of chloral hydrate were clearly established by the more complex patterns that were obtained in our new systems of separation at pH 4.25, 3.70 and 3.25. The results we have presented show that the extra components, which were observed in the separations performed at the higher pH values of our systems, arise by separation of components that co-migrated at pH 2.3. Ballou & Smithies (1977) found that membrane solubilization was better in chloral hydrate at pH values below 4.5. We have seen no indication that dissociation of cytochrome *c* oxidase, which is an isolated membrane-protein complex rather than a membrane preparation, was

affected by the pH (5.45–2.3) at which dissociation was performed.

We have shown that preparations of cytochrome *c* oxidase that gave eight or, at most, nine, bands when examined by electrophoresis in the presence of SDS and urea, contain 15 components that can be separated by the new systems of electrophoresis in the presence of chloral hydrate. These observations depend on the effectiveness of a dissociating agent, chloral hydrate, that has not been widely employed. We have therefore considered the possibility that the additional components that we see arise either by chemical modification by chloral hydrate or as a consequence of partial dissociation of the complex in chloral hydrate.

Ballou *et al.* (1974) showed that amino acids and certain proteins were not modified by exposure to chloral hydrate. In addition, we have seen no evidence from our studies for chemical modification of proteins by chloral hydrate.

In view of the pronounced sieving effect on large protein molecules of a 12.5%-total-monomer-concentration polyacrylamide gel that is further increased by the presence of 100% (w/v) chloral hydrate, the absence of material of very low electro-

Fig. 8. Effect of trypsin treatment on components of cytochrome *c* oxidase

A comparison of components of cytochrome *c* oxidase before and after trypsin treatment is shown. Densitometric traces of electrophoretic separations performed by the new chloral hydrate method at pH 4.25: (a) Standard cytochrome *c* oxidase; (b) cytochrome *c* oxidase after trypsin treatment as described by Capaldi (1978). Cytochrome *c* oxidase was treated with trypsin (1:50, w/w) for 2 h at room temperature. Degraded fragments, trypsin and trypsin inhibitor were removed by gel filtration on Sepharose CL-4B. Other details are given in the Experimental section. Components (6 + 7), 8 and 9, which do not appear after electrophoresis in the presence of SDS and urea, and components 10 and 11, are completely removed by trypsin treatment.

phoretic mobility in our chloral hydrate-containing gels is a sensitive criterion of dissociation. Verheul *et al.* (1979) have noted that components I and III of cytochrome *c* oxidase have a tendency to aggregate in solutions of SDS and that such aggregates do not enter 12.4%-total-monomer-concentration polyacrylamide gels. There was, therefore, no evidence

of such aggregates after the electrophoretic separations in chloral hydrate that we have performed.

We have shown that the separations in chloral hydrate were unaffected both by the presence of SDS in the sample and also by preincubation of the cytochrome *c* oxidase in SDS alone, under conditions that are generally accepted as being appropriate for dissociation of the enzyme. Thus we feel that the identical patterns found after addition of chloral hydrate to components that were previously dissociated by SDS and after the action of chloral hydrate alone, must reflect full dissociation under both sets of conditions.

We therefore believe that the fifteen components seen by electrophoresis in the presence of chloral hydrate are different constituents of the complex. In Table 2 we have compared our observations by electrophoresis both in the presence of chloral hydrate and in the presence of SDS/urea with the results of some other workers.

In agreement with results of other studies, we have found components I, II, III, IV and V to be homogeneous by gel electrophoresis in the presence of chloral hydrate, where we have designated them 1, 2, 3, 5 and 4 respectively. The reversal in order of migration of IV and V in chloral hydrate/polyacrylamide-gel electrophoresis is compatible with the more basic nature of IV, as is apparent from the primary structures of the two components (IV, Sacher *et al.*, 1979; V, Tanaka *et al.*, 1977). In agreement with the results of both Buse & Steffens (1978) and Verheul *et al.* (1979), we saw heterogeneity of band VIII, which was split into four components in chloral hydrate. We also saw heterogeneity of band VI, but not of band VII, which fits better with the results of Buse & Steffens (1978) than with those of Verheul *et al.* (1979).

Our work confirms previous observations that several of the protein bands seen on one-dimensional electrophoresis in the presence of SDS and urea are not homogenous. Because of this, we consider it inappropriate that proposals about subunit structure and subunit stoichiometry of cytochrome *c* oxidase should be based on such one-dimensional separations. As is noted in Table 2, Buse & Steffens (1978) have described different components as 'stoichiometric' or 'sub-stoichiometric', whereas Verheul *et al.* (1979) have used the terms 'major' or 'minor'. At present we do not have data for the relative amounts of the components that we have separated.

In Table 2 we have also noted that some authors believe that certain components of their enzyme preparation are contaminants. With regard to this, we feel that one should reverse judgement about whether a component is a subunit or merely a contaminant until the full functional aspects of a cytochrome *c* oxidase preparation can be established

Table 2. Relationship between components of cytochrome c oxidase separated by electrophoresis in the presence of chloral hydrate with components characterized by other methods

Information sources and comments: A, Buse & Steffens (1978); these authors noted that gel filtration in the presence of SDS permitted quantitative distinction between stoicheiometric (I–V and VII) and substoicheiometric (others) components. B, Verheul *et al.* (1979); these authors noted that gel filtration in the presence of SDS permitted quantitative distinction between major (I–V and VIIa) and minor (others) components. C, Downer *et al.* (1976); these authors also observed additional components designated a and c that migrated between their components IV and V, and V and b, respectively. Components a, b and c varied in amount in different preparations of enzyme and were deemed to be contaminants. D, Capaldi (1978) and Ludwig *et al.* (1979); confirmed results of C above and showed removal of b and c and partial loss of a could be effected by trypsin treatment and gel filtration without loss of electron-transfer activity. E, Tracy & Chan (1979); these authors agree with C about component nomenclature and the nature of a, b and c. F, Penttila *et al.* (1979); these authors also observed components they designated a and c that migrated between their components IV and V, and VI and VII respectively. Removal of components III and b together with most of a and c (components a, b and c were designated as contaminants) was effected by gel electrophoresis in Triton X-100 without loss of electron-transfer activity. Saraste *et al.* (1980) also showed that this preparation method yielded material that exhibited proton-translocation activity and respiratory control when incorporated into liposomes. Merle & Kadenbach (1980a,b) observed twelve bands in cytochrome c oxidase from a number of mammalian sources by using high-resolution gel electrophoresis in the presence of SDS, urea and glycerol. However, they have not presented data specifically for bovine heart enzyme.

Components from electrophoresis in the presence of chloral hydrate		Component designation by:					
		Electrophoresis in the presence of SDS and urea			Gel filtration in the presence of SDS		
		I	I	I	I	I	
1		II	II	II	II	II	
2		III	III	III	III	III	
3		IV	IV	IV	IV	IV	
5		V	V	V	V	V	
4		VI	VI	b	VIa, VIb, VIc	VI	
10, 12		VII	VII	VI	VII	VIIa, VIIb	
13		VIII	VIII	VII	VIIIa, VIIIb, VIIIc	VIIIa, VIIIb, VIIIc	
6, 11, 14, 15		Not resolved					
7, 8, 9							
Information source ...	The present work	The present work	A, B	C, D, E, F	A	B	

and related to each of its polypeptide constituents. This caution is particularly appropriate in view of the fact that there were three components (7, 8 and 9) that we saw by electrophoresis in the presence of chloral hydrate that did not correlate with bands seen after electrophoresis in the presence of SDS and urea. The two-dimensional separation shown in Fig. 6 demonstrates that staining material which corresponded in mobility, in the second-dimension chloral hydrate-containing gel, with components (6 + 7), 8 and 9, was diffusely present in several regions of the first-dimension SDS/urea-containing gel. The reverse-order two-dimensional separation shown in Fig. 7 demonstrated that components (6 + 7), 8 and 9, which appeared as sharp bands in the first-dimension chloral hydrate-containing gel, did not run as discrete components in the second-dimension SDS/urea-containing gel. Despite this evidence for unusual behaviour in detergent, the effect of trypsin, which totally removed components (6 + 7), 8 and 9 (together with some of the

components that behave normally in SDS and urea), suggests that they are proteinaceous in nature. Substantiation of these additional components as true protein constituents of cytochrome c oxidase must await their isolation and full characterization.

In summary, we have shown that chloral hydrate dissociates cytochrome c oxidase. Its use in electrophoretic systems that permit variation in the pH of the separation gel has demonstrated heterogeneity in material that appeared homogeneous on electrophoresis in the presence of SDS and urea. The observations of heterogeneity on electrophoresis in the presence of chloral hydrate are compatible with the results of gel-filtration procedures in the presence of SDS. In addition, we see three components that we believe are proteinaceous in nature, but which do not appear as discrete components on electrophoresis in the presence of SDS and urea. If these three components do prove to be protein constituents of bovine cytochrome c oxidase, then the currently held structural picture of that complex,

which has been almost entirely built up by studies dependent on the effects of SDS as a dissociating agent, will require radical revision.

Our work shows that polyacrylamide-gel electrophoresis in the presence of chloral hydrate is a procedure of very high resolving power. This separation method offers certain advantages over those that use SDS. The latter compound generates protein-detergent complexes and subsequent analyses of these complexes are often impeded by the presence of that detergent. Chloral hydrate, in contrast with SDS, can be readily removed from proteins by dialysis. Clearly, if, as seems likely, the dissociating power of chloral hydrate proves to apply to other membrane protein complexes, then its use will provide a most valuable alternative to the other dissociating agents that are currently employed in the analysis of membrane proteins.

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