Ion-exchange chromatography in the presence of the non-ionic dissociating agent chloral hydrate

Application to a membrane protein, bovine heart cytochrome *c* oxidase

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In previous work we have shown that aq. 100% (w/v) chloral hydrate (2,2,2-trichloroethane-1,1-diol) is a potent non-ionic protein dissociating agent. We have employed it in systems of polyacrylamide-gel electrophoresis and have demonstrated the presence of 15 components in a preparation of bovine heart cytochrome *c* oxidase [Griffin & Landon (1981) *Biochem. J.* 197, 333–344]. Here we describe the use of solutions containing aq. 100% (w/v) chloral hydrate in the ion-exchange column chromatographic separation on CM-cellulose of the α - and β -chains of human haemoglobin, which we have employed as a model protein of known structure. We also describe the use of similar procedures in order to fractionate the polypeptide components of bovine heart cytochrome *c* oxidase. An effective separation has been obtained and we suggest that chloral hydrate-containing solutions could have general application in the ion-exchange-chromatographic analysis of membrane proteins, a procedure that has had restricted use owing to the inadequacy of non-ionic dissociating agents available previously.

Ion-exchange chromatography has been employed widely in the isolation of proteins. Use of solutions containing the non-ionic agent urea has extended applications to dissociated protein complexes. The analysis of the 30S ribosomal subunit from *Escherichia coli* (Hardy *et al.*, 1969) is an impressive example of the potential resolving power of this methodology. However, membrane proteins are generally either poorly soluble or not fully dissociated in urea-containing solutions and ion-exchange-chromatographic analysis of most membrane-protein complexes has been impossible in the absence of a more potent non-ionic dissociating solvent.

Chloral hydrate (2,2,2-trichloroethane-1,1-diol) at high concentration in aqueous solution has been shown to be an effective membrane-solubilizing agent and has had limited application in the electrophoretic analysis of protein constituents of membranes (Ballou *et al.*, 1974; Ballou & Smithies, 1977; Booth, 1977). Recently we have shown that aq. 100% (w/v) chloral hydrate fully dissociates bovine heart cytochrome *c* oxidase and we have described new electrophoretic systems employing this solvent which have been used to demonstrate the

presence of at least 15 polypeptides in a preparation of this membrane-protein complex (Griffin & Landon, 1981). Before this, the analytical methods that had been applied to the complex had almost invariably utilized the anionic detergent SDS, which is incompatible with ion-exchange procedures.

Here we describe the use of aq. 100% (w/v) chloral hydrate as a potent non-ionic protein-dissociating agent in the separation of the polypeptide constituents of protein complexes by ion-exchange column chromatography on CM-cellulose. First we have applied this procedure to the separation of the α - and β -chains of human haemoglobin. We then describe the fractionation of the components of the mitochondrial membrane-protein complex, cytochrome *c* oxidase. A preliminary report of some of this work has been presented (Landon & Griffin, 1979).

Experimental

Materials

Chloral hydrate, which was recrystallized from chloroform before use, was from Fisons Scientific Apparatus Ltd., Loughborough, Leics., LE11 0RG, U.K. CM-cellulose was grade CM-52 from Whatman Ltd., Maidstone, Kent ME14 2LE, U.K.

Abbreviation used: SDS, sodium dodecyl sulphate.

Human haemoglobin was from Sigma (London) Chemical Co. Ltd., Poole, Dorset BH17 7NH, U.K.

Methods

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

Properties of chloral hydrate and choice of conditions for ion-exchange chromatography. Chloral hydrate was used at a concentration of 100% (w/v) in aqueous solution throughout the procedures described below. Its use involves certain precautions and constraints. **Chloral hydrate is toxic and care should be taken to avoid contact and exposure through breathing.** Chloral hydrate-containing solutions dissolve a number of plastic materials and contact was restricted to glass, Teflon and silicone rubber. The solutions also dissolve Sephadex and Sepharose, but cellulose is not affected. As chloral hydrate is unstable in alkaline solution we chose to use acidic buffers and the anion-exchanger CM-cellulose. The viscosity of solutions containing 100% (w/v) chloral hydrate led us to restrict the size of column to 5 cm high $\times$ 2.5 cm diameter, which allowed a flow rate of 20 ml/h without causing problems due to excessive back-pressure. Although the solutions have a significant absorbance at 280 nm, they do permit protein determination by such measurements. Light causes an increase in the A_{280} of chloral hydrate-containing solutions, and exposure to light was minimized during all of the procedures. All operations involving chloral hydrate-containing solutions were at room temperature.

Ion-exchange chromatography in the presence of chloral hydrate. (a) Separation of the α - and β -chains of human haemoglobin. CM-cellulose was equilibrated with 100% (w/v) chloral hydrate/0.2 mM-dithiothreitol in 7.5 mM-KOH, adjusted to pH 5.2 with acetic acid (the KOH was neutralized before the addition of chloral hydrate) and was then packed, under pressure, into a column 5 cm high $\times$ 2.5 cm diameter. The same solution was passed through the column until the effluent had the same conductivity as that of the applied solvent. A sample of 100 mg of human haemoglobin was dissolved in 20 ml of 100% (w/v) chloral hydrate/2.5 mM-dithiothreitol in 7.5 mM-KOH, adjusted to pH 5.2 with acetic acid, and kept at room temperature for 1 h before loading on to the column. After the sample had been applied, 1 column vol. of equilibration buffer was passed through before the initiation of a concave gradient of equilibration buffer against the same buffer, but containing 0.25 M-KCl. The gradient was calculated on the basis of drawing solvent from an open mixing chamber with a reservoir of surface area twice that of the mixing chamber and a total volume in the whole system of 800 ml

(Morris & Morris, 1976). The gradient was formed using an LKB 11300 Ultrograd gradient mixer (LKB Instruments Ltd., South Croydon, Surrey CR2 8YD, U.K.). Fractions (6 ml) were collected at a constant flow rate of 20 ml/h.

Samples were analysed by measurement of A_{280} (for protein) and A_{400} (for haem). The gradient was determined by measurement of conductivity.

Identification of α - or β -chains in samples was effected by polyacrylamide-gel electrophoresis in the presence of chloral hydrate as described by Griffin & Landon (1981) and employed 12.5% (w/v)-total-acrylamide-monomer-concentration gels at pH 4.25.

(b) Separation of components of cytochrome *c* oxidase. The preparation of the CM-cellulose and the column packing was performed as described above and used the same buffer except that it was at pH 4.5. Cytochrome *c* oxidase (105 mg) was dissolved in 13 ml of 100% (w/v) chloral hydrate/2 mM-dithiothreitol in 7.5 mM-KOH, adjusted to pH 4.5 with acetic acid, and was kept at room temperature for 1 h before loading on to the column. The gradient was formed as described above and used the same solutions, but at pH 4.5, and was initiated after 4 column vol. of buffer had been passed through the column.

Fractions (4 ml) were collected at a flow rate of 20 ml/h and were analysed by measurement of A_{280} (for protein) and A_{420} (for haem *a*). The gradient was determined by measurement of conductivity.

Identification of components of cytochrome *c* oxidase that were present in samples was effected by polyacrylamide-gel electrophoresis in the presence of chloral hydrate as described by Griffin & Landon (1981) and employed 12.5% (w/v)-total-acrylamide-monomer-concentration gels at pH 4.25 and 3.05.

Amino acid analysis. Samples of protein were hydrolysed in 6 M-HCl at 110°C for 24 h in evacuated sealed tubes. Amino acid analysis was performed by using an automatic amino acid analyser.

Results

Ion-exchange column-chromatographic separation in the presence of chloral hydrate of the α - and β -chains of haemoglobin

The separation of free haem and the α - and β -chains of haemoglobin that was obtained by ion-exchange column chromatography on CM-cellulose in the presence of chloral hydrate is shown in Fig. 1. The first peak exhibited absorbance at both 280 nm and 400 nm and gave no detectable protein band on electrophoresis. It was therefore identified as free haem. Polyacrylamide-gel electrophoresis in the presence of chloral hydrate at pH 4.25 of

Fig. 1. Separation of the α - and β -chains of haemoglobin by ion-exchange chromatography in the presence of chloral hydrate

Haemoglobin (100 mg) was dissociated in 20 ml of 100% (w/v) chloral hydrate/2.5 mM-dithiothreitol in 7.5 mM-KOH, adjusted to pH 5.2 with acetic acid, and applied to a column (5 cm high $\times$ 2.5 cm diameter) of CM-cellulose equilibrated in 100% (w/v) chloral hydrate/0.2 mM-dithiothreitol in 7.5 mM-KOH, adjusted to pH 5.2 with acetic acid. The dissociated components of haemoglobin were eluted by using a concave gradient (----) of 0–250 mM-KCl in the same buffer at a constant flow rate of 20 ml/h. Fractions (6 ml) were collected and were pooled as indicated by the horizontal bars. Peak identifications were made as described in the text. Other details are given in the Experimental section. —, A_{280} .

samples of material taken from fractions across each of the two major protein peaks showed that the component that was eluted first migrated towards the cathode more slowly than the component that was eluted second. Amino-acid analysis of material from fractions that were pooled as indicated, dialysed exhaustively against water and then freeze-dried confirmed that the less-basic β -chain eluted from the column before the more-basic α -chain.

Electrophoresis in the presence of chloral hydrate of samples of these purified freeze-dried chains showed that a substantial proportion of each consisted of components of mobility lower than that of the monomeric chain. These lower-mobility components were presumably aggregated materials produced during freeze-drying that were not dissociated when re-exposed to chloral hydrate. There was no evidence of this type of aggregate in the samples that were analysed by electrophoresis directly after the column separation.

Ion-exchange column-chromatographic separation in the presence of chloral hydrate of components of cytochrome c oxidase

The separation of components of cytochrome c

Fig. 2. Separation of components of cytochrome c oxidase by ion-exchange chromatography in the presence of chloral hydrate

Cytochrome c oxidase (105 mg) was dissociated in 13 ml of 100% (w/v) chloral hydrate/2 mM-dithiothreitol in 7.5 mM-KOH, adjusted to pH 4.5 with acetic acid, and applied to a column (5 cm high $\times$ 2.5 cm diameter) of CM-cellulose equilibrated in 100% (w/v) chloral hydrate/0.2 mM-dithiothreitol in 7.5 mM-KOH, adjusted to pH 4.5 with acetic acid. The dissociated components were eluted by using a concave gradient of 0–250 mM-KCl in the same buffer at a constant flow rate of 20 ml/h. Fractions (4 ml) were collected and were pooled as indicated by the horizontal bars. Component identifications are given in Table 1. Other details are as given in the Experimental section. —, A_{280} .

oxidase that was obtained by ion-exchange column chromatography on CM-cellulose in the presence of chloral hydrate is shown in Fig. 2. Analysis of the separation was made by polyacrylamide-gel electrophoresis in the presence of chloral hydrate on two types of sample. First samples were taken from specific fractions and analysed directly. The choice of buffer ions used in the ion-exchange separation was made such that direct electrophoretic analysis was possible. Subsequently electrophoretic analysis was performed on the material present in samples from fractions that were pooled as indicated, dialysed exhaustively against water and then freeze-dried. The identifications that were made are given in Table 1. The component numbering (Arabic numerals) is in accordance with that we have used previously for the 15 components of cytochrome c oxidase that we have demonstrated by electrophoresis in the presence of chloral hydrate at pH 4.25 and 3.05 (Griffin & Landon, 1981). For comparison, we have also included the numbering (Roman numerals) applied by some other workers (e.g. Buse & Steffens, 1978; Verheul *et al.*, 1979) to components separated by electrophoresis in the presence of SDS and urea.

water were not unexpected. Irreversible aggregation of certain components of cytochrome *c* oxidase has been noted (Downer *et al.*, 1976; Verheul *et al.*, 1979). This type of problem is best avoided by ensuring that further fractionations are performed in the presence of chloral hydrate or another agent. In preliminary experiments we have found that removal of chloral hydrate by dialysis against 30% (v/v) acetic acid permits fractionation of many of the components by gel filtration in that solvent.

We believe that the potency of aq. 100% (w/v) chloral hydrate will allow membrane-protein analysis by ion-exchange chromatography, and such methodology will be a valuable addition to the limited range of procedures that are compatible with this type of protein.

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