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INHIBITION OF CYTOCHROME *c* OXIDASE FUNCTION BY DICYCLOHEXYLCARBODIIMIDE

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Dicyclohexylcarbodiimide (DCCD) reacted with beef heart cytochrome *c* oxidase to inhibit the proton-pumping function of this enzyme and to a lesser extent to inhibit electron transfer. The modification of cytochrome *c* oxidase in detergent dispersion or in vesicular membranes was in subunits II–IV. Labelling followed by fragmentation studies showed that there is one major site of modification in subunit III. DCCD was also incorporated into several sites in subunit II and at least one site in subunit IV. The major site in subunit III has a specificity for DCCD at least one order of magnitude greater than that of other sites (in subunits II and IV). Its modification could account for all of the observed effects of the reagent, at least for low concentrations of DCCD. Labelling of subunit II by DCCD was blocked by prior covalent attachment of arylazidocytochrome *c*, a cytochrome *c* derivative which binds to the high-affinity binding site for the substrate. The major site of DCCD binding in subunit III was sequenced. The label was found in glutamic acid 90 which is in a sequence of eight amino acids remarkably similar to the DCCD-binding site within the proteolipid protein of the mitochondrial ATP synthetase.

Introduction

Cytochrome *c* oxidase, the terminal member of the mitochondrial respiratory chain, functions to transfer electrons from reduced cytochrome *c* to molecular oxygen while conserving the electrochemical energy released in this reaction for ion transport or ATP synthesis (see Refs. 1 and 2 for recent reviews). Wikstrom and co-workers [3–6] have shown that this enzyme functions as an oxidation-reduction-linked proton pump, setting up a proton gradient and electrical potential across the mitochondrial inner membrane which can be used to drive ATP synthesis.

Considerable progress has been made in determining the structure of bovine heart cytochrome *c* oxidase. Electron microscopy and image reconstruction studies of two-dimensional arrays have provided a low-resolution (25 Å) three-dimensional model of the enzyme [7,8]. Cytochrome *c* oxidase is evidently a Y-shaped molecule arranged in three domains [8]. Two of these domains, the arms of the Y and called the M domains, span the mitochondrial inner membrane. The third domain, the tail of the Y or C domain, extends out from the membrane on the cytoplasmic side of the mitochondrial inner membrane. The enzyme is constituted by two heme moieties and

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

two copper atoms as prosthetic groups, along with at least seven different polypeptides ranging in molecular weight from about 56 000 down to 5 500 [9,10]. The amino acid sequences of most of the polypeptides of bovine heart cytochrome *c* oxidase have been obtained, either by sequencing the protein directly [11,12] or from the corresponding DNA sequence in the case of the three largest subunits which are coded for on mitochondrial DNA (Ref. 13 and Barrell, B.C., personal communication).

The loci of the functionally important prosthetic groups with respect to the different subunits, and within the three-dimensional structure of the protein, are not certain. It remains, therefore, to decide whether all of the subunits of the enzyme are required for cytochrome *c* oxidase activity and what role the important components play. Chemical modification studies provide one approach to identifying functionally important sites in an enzyme molecule. Here we describe the reaction of cytochrome *c* oxidase with $DCCD^+$, a hydrophobic reagent for modifying carboxyl groups of proteins and a reagent shown already to act specifically to inhibit proton translocation in mitochondrial [14], chloroplast [15] and bacterial ATP synthetases [16].

Our results show that DCCD inhibits both electron transfer and proton translocation in bovine heart cytochrome *c* oxidase through reaction with a relatively few sites in the large cytochrome *c* oxidase molecule. These important sites are shown to be in subunits II and III of the enzyme.

Materials and Methods

Enzyme preparations. Bovine heart cytochrome *c* oxidase was prepared according to the methods of Capaldi and Hayashi [17] and Yonetani [18]. Cytochrome *c* oxidase was reconstituted into phospholipid vesicles by the cholate dialysis technique [19, 20]. 40 mg asolectin were dispersed in 100 mM Hepes-NaOH (pH 7.2) and 25 mM potassium cholate by sonication at 4°C. Cytochrome *c* oxidase (1 mg) was added to the dispersion of phospholipids and then the sample was dialysed against the following changes of buffer: (1) for 4 h against 300 vol. of 100 mM Hepes-NaOH, pH 7.2; (2) for 5 h against 300 vol. of 10 mM Hepes-NaOH, pH 7.2, 30 mM KCl and 80 mM sucrose; and (3) for 12–16 h against 300 vol. of 1 mM Hepes-NaOH, pH 7.2, 30 mM KCl, and 80 mM sucrose. Cytochrome *c* oxidase-containing vesicles were resolved by gel filtration through a column of Sepharose 2B as described by Wrigglesworth and Nicholls. [21].

Measurement of electron-transport and protein-pumping activities of cytochrome c oxidase. Electron-transport activity was measured polarographically using the assay conditions described by Vik and Capaldi [22]. Phospholipid vesicles containing cytochrome *c* oxidase were assayed in 50 mM potassium phosphate buffer (pH 7.4) and the respiratory control ratio was determined as described in Ref. 19. All preparations had a respiratory control ratio between 2.8 and 4.0. Samples collected from the Sepharose 2B column have respiratory control ratios as high as 5.0.

Proton pumping of cytochrome *c* oxidase vesicles was monitored in a water-jacketed, stirred cell, using a Beckman SS-2 pH-meter equipped with a combination electrode and a sensitive recorder. Cytochrome *c* oxidase vesicles (0.4 nmol) were incubated in 6 ml of 30 mM KCl (or 100 mM KCl), 80 mM sucrose, 0.5 mM Hepes-NaOH (pH 7.2) and 1 μM valinomycin. Electron transport was initiated by adding 1.5 μM ferrocytochrome *c*. Ferrocytochrome *c* was prepared by adding a few grains of dithionite to a solution of cytochrome *c* (50 mg/ml) dissolved in 80 mM sucrose, 30 mM KCl, and 5 mM Hepes-NaOH (pH 7.2). This solution was passed through a Sephadex G-25 column (26 × 1.2 cm) equilibrated in the same buffer to remove the dithionite.

Reaction of cytochrome c oxidase with DCCD. Reaction of cytochrome *c* oxidase with various concentrations of DCCD, dissolved in methanol, was performed at protein concentrations from 0.1 to 0.25 mg/ml, at 20°C for 1 h and in 80 mM sucrose, 30 mM KCl, 1 mM Hepes-NaOH, pH 7.2, and 0.2% Tween 80. All cytochrome *c* oxidase concentrations were corrected for heme content. The methanol concentration was maximally 0.5% of the total reaction mixture.

Phospholipid vesicles containing cytochrome *c* oxidase (0.1 mg/ml) were reacted with various concentrations of DCCD in methanol for 1 h in 30 mM KCl, 80 mM sucrose and 1 mM Hepes-NaOH, pH 7.2. Aliquots from the same reaction mixture were then assayed for electron-transport and proton-pumping activity. The half-time for inhibition of electron-transport activity was 40 min.

[^{14}C]DCCD labeling of cytochrome c oxidase. Cytochrome *c* oxidase in detergent or incorporated into phospholipid vesicles was labeled with the same ratios of [^{14}C]DCCD to enzyme used in the functional experiments and for 1 h. The reaction mixture was then centrifuged through 10% sucrose, 10 mM potassium phosphate buffer, pH 7.4, at 192 000 × *g* overnight. Triton X-100 (2 mg/mg phospholipid) was added to disrupt the phospholipid vesicles.

The specific activity of the DCCD was 50 mCi/mmol. Its purity was monitored by thin-layer chromatography using heat-activated alumina plates in a solvent of benzene/ethyl acetate (9 : 1, v/v). Unlabeled DCCD was detected by I_2 vapor and all specific activity calculations were corrected for [^{14}C]-dicyclohexylurea content [23].

Gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed by the procedure of Swank and Munkres [24] using both 12.5% acrylamide and *N,N'*-methylenebisacrylamide (10 : 1, v/v) and 15% acrylamide and *N,N'*-methylenebisacrylamide (30 : 1, v/v). Gels were stained and destained as described by Downer et al. [9]. Radioactive gels were sliced into 1 mm thick slices with a Mickle gel slicer. These were dissolved in 1 ml of 15% H_2O_2 at 80°C overnight, then 7 ml Omnifluor (New England Nuclear) (2.66 g/l) in toluene Triton X-100 (2 : 1, v/v) were added to each vial and the radioactivity was measured in a Packard scintillation counter. The amount of [^{14}C]DCCD incorporated into cytochrome *c* oxidase was estimated by summing the amounts of counts in each subunit and dividing this by the measured specific activity. Cytochrome *c* oxidase concentration was determined by loading various known amounts of enzyme (50–250 μg) on companion gels to generate a standard curve of Coomassie brilliant blue staining intensity. The amount of stain in subunits I–IV was then plotted as a function of loaded protein and the pelleted cytochrome *c* oxidase concentration was estimated from this standard curve. This estimation was consistent with values obtained by the method of Lowry et al. [25] on the cytochrome *c* oxidase pellets.

Results

Proton pumping of cytochrome c oxidase in lipid vesicles

Cytochrome *c* oxidase incorporated into lipid vesicles by the dialysis method was found to have a slow rate of electron transfer with cytochrome *c* added to the outside of these vesicles. However, enzyme activity could be increased as much as 5-fold by adding valinomycin and an uncoupling ionophore, FCCP. The slow rate of electron transfer is probably due to protons being consumed by the cytochrome *c* oxidase reaction from the inside of vesicles ($4\ H^+ + 4\ e^- + O_2 = 2\ H_2O$) faster than they can be replaced by their leakage back across the bilayer (see Ref. 5 for details). The uncoupler with valinomycin speeds up to the cytochrome *c* oxidase reaction by increasing the rate at which protons can permeate into the intravesicular space.

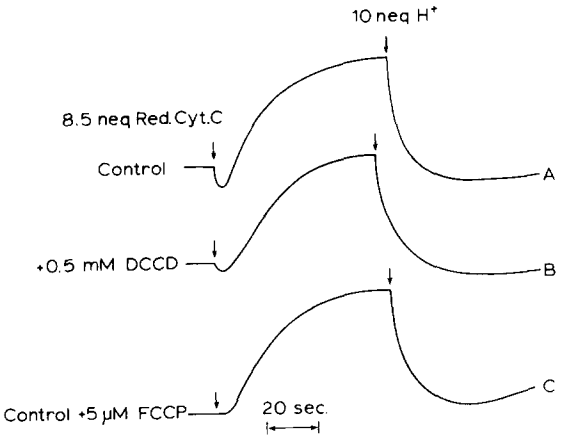

Fig. 1. Changes in extravesicular pH induced by electron transport in cytochrome *c* oxidase-containing vesicles. Phospholipid vesicles containing 0.4 nmol cytochrome *c* oxidase were incubated in the presence of 0.5% methanol (A), 0.5 mM DCCD added in 0.5% methanol (B) and 5 μM FCCP added in 0.5% ethanol (C) and at 20°C. The magnitude of the ejection of protons was measured. The alkalination phase reflects the reaction of protons with electrons and oxygen to form water in the cytochrome *c* oxidase reaction. Red. Cyt. C, reduced cytochrome *c*.

Fig. 1 shows that electron transfer in reconstituted vesicles leads to a rapid acidification of the external medium, followed by a slower alkalination phase as protons leak back across the bilayer and are reacted with electrons to form water. This rapid and transient acidification has been studied in detail by Krab and Wikstrom [26] and others [27,28], who have presented convincing evidence that the initial proton ejection is due to oxidoreduction-mediated proton pumping by cytochrome *c* oxidase. The initial acidification

in Fig. 1 amounted to 0.4–0.5 protons/electron. The alkalination phase that follows was found to depend on the number of reducing equivalents added, as first reported by Krab and Wikstrom [26]. When ferrocytochrome *c* was used in stoichiometric amounts with cytochrome *c* oxidase, to allow only a single turnover of the enzyme, only the acidification phase was observed.

The acidification phase was not observed in vesicles containing FCCP and valinomycin (Fig. 1C). The proton-ejection phase was also inhibited by DCCD (Fig. 1B) as first reported by Casey et al. [20, 29].

Inhibition of proton pumping by DCCD

The effects of DCCD on both electron-transfer activity and the proton-pumping function of cytochrome *c* oxidase are shown in Fig. 2. Both functions were inhibited by DCCD. The reagent inhibited electron transfer in both detergent-dispersed (insert to Fig. 2) and vesicular cytochrome *c* oxidase. The effect was not due to methanol used as a solvent for DCCD or dicyclohexylurea generated by hydrolysis of DCCD in water. These reagents failed to inhibit the enzyme even at high concentrations (i.e., 0.5% for methanol and 1200 mol/mol enzyme for the urea).

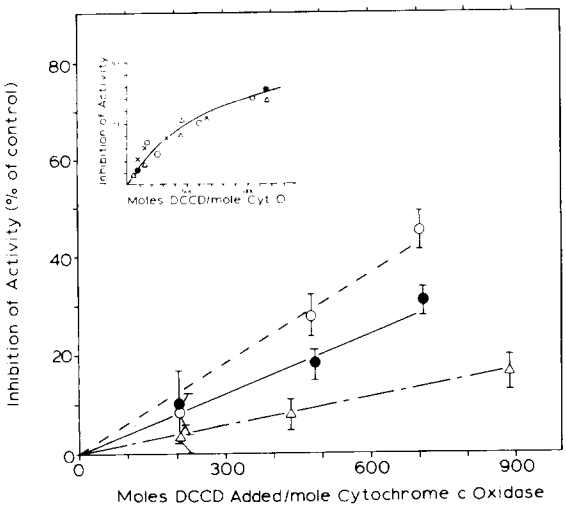

Fig. 2. Concentration dependence of the DCCD inhibition of cytochrome *c* oxidase activity. The main portion of the figure shows the effect of DCCD on proton translocation (○———○) and electron transfer in the absence (△———△) and in the presence (●———●) of FCCP (5 μM) and valinomycin (1 μM). Each point is the average of four experiments on different preparations. The insert shows the effect of DCCD on electron transfer of detergent-solubilized cytochrome *c* oxidase (Cyt. O). The different symbols represent the results of experiments on different preparations of enzyme. Cytochrome *c* oxidase concentrations were determined from heme content. Control rates of electron transfer were 37 μgatoms (O/min per mg protein for detergent dispersed and 28 μgatoms O/min per mg protein for vesicular enzyme.

Fig. 2 shows the inhibition of electron transfer both in the presence and absence of FCCP and valinomycin. It can be seen that the inhibition of electron transfer of the uncoupled enzyme was greater than that of the coupled system. This observation is most likely related to secondary effects of DCCD on the passive permeability of vesicles to protons. The inhibition obtained in the absence of uncouplers could be the average of inhibition of the enzyme by DCCD, and stimulation of active enzyme molecules by an increased leakage of protons back across the bilayer due to the presence of large concentrations of a hydrophobic reagent. The important point is that the leakiness induced by DCCD must be small because vesicles retain 70% of their initial respiratory control (i.e., rate of electron transfer in the uncoupled state divided by the rate in the absence of uncouplers), even at high concentrations of DCCD. Fig. 2 also shows the inhibition of proton pumping in cytochrome *c* oxidase vesicles over a large range of DCCD concentrations. As much as 70% inhibition of the proton-ejection phase was observed in the presence of high concentrations of DCCD. It can be seen that the inhibition of proton pumping was greater than that of electron transfer at all levels of DCCD used.

The effect of DCCD on the absorption spectrum of cytochrome *c* oxidase was monitored over a wide range of reagent concentration. Some nonreducible heme was generated by the reaction of high concentrations of DCCD with the solubilized enzyme indicating some change in heme environment, and by inference, some denaturation of the enzyme. However, this was at most only one-quarter of the observed inhibition of the electron-transport activity.

Incorporation of DCCD into the protein

DCCD reacts predominantly with carboxyl groups in a protein and to a lesser extent with tyrosine and

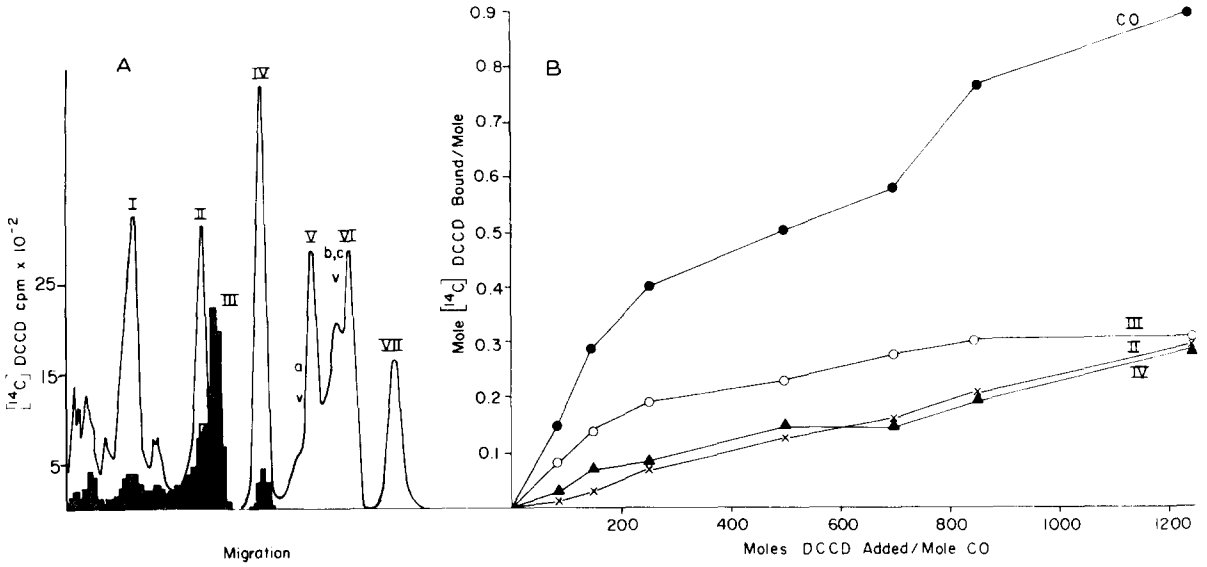

Fig. 3. Labelling of subunits of detergent-solubilized cytochrome *c* oxidase by DCCD. (A) SDS-polyacrylamide gel of DCCD-labelled enzyme. The gel used was 15% acrylamide/bisacrylamide run under the gel conditions of Swank and Munkres [24]. Labelling was done with 80 mol DCCD/mol enzyme. (B) Incorporation of DCCD into different subunits as a function of the concentration of reagent used in the reaction. CO, cytochrome *c* oxidase.

sulfhydryl groups [30]. Carboxyl groups are activated into a highly reactive *O*-acylisourea. When in a hydrophilic environment, this intermediate hydrolyzes to give dicyclohexylurea and regenerate the carboxyl group of the protein [31]. For carboxyl groups in a hydrophobic environment, the *O*-acylisourea can rearrange to form a covalent adduct. Alternatively, it can react with a close nucleophilic center such as an α- or ε-amino group to form an amide bond and thus generate a cross-link within the protein [32]. Either of the last two modifications could cause inhibition of cytochrome *c* oxidase activity.

Radioactive DCCD was used to monitor the covalent modification of cytochrome *c* oxidase (as opposed to cross-linking) and to quantitate this effect. Thus, the enzyme in vesicles or detergent solution was reacted with [^{14}C]DCCD, activities were examined, the protein was dissociated into component polypeptides by SDS and these were resolved by SDS-polyacrylamide gel electrophoresis. Fig. 3A shows the gel profile of a detergent-solubilized cytochrome *c* oxidase which had been reacted with 80 mol of DCCD/mol enzyme. Most of the reagent is clearly incorporated into subunit III. In addition, there is a significant amount of labelling of subunits II and IV.

A quantitation of the incorporation of reagent into subunits II–IV over a range of different DCCD concentrations is given in Fig. 3B. It is evident that the labelling of subunits II and IV relative to III is increased at high concentrations of DCCD. The three subunits are labelled to the same extent by reaction of the enzyme with 1 200 mol of DCCD/mol oxidase. The break evident in the plots of the amount of incorporation of DCCD into subunits II and IV coincides approximately with the concentration of reagent at which a significant amount of nondenatured heme is apparent in samples.

A gel profile showing the labelling of vesicular cytochrome *c* oxidase with 80 mol DCCD/mol enzyme is shown in Fig. 4. A different gel system was used than that employed for experiments in Fig. 3. These gel conditions gave a good separation of subunits II and III but only poor resolution of the smaller components of the complex. Fig. 4B shows the incorporation of reagent into individual subunits over a range of different DCCD concentrations. Subunit III was more labelled than subunit II or IV by factors

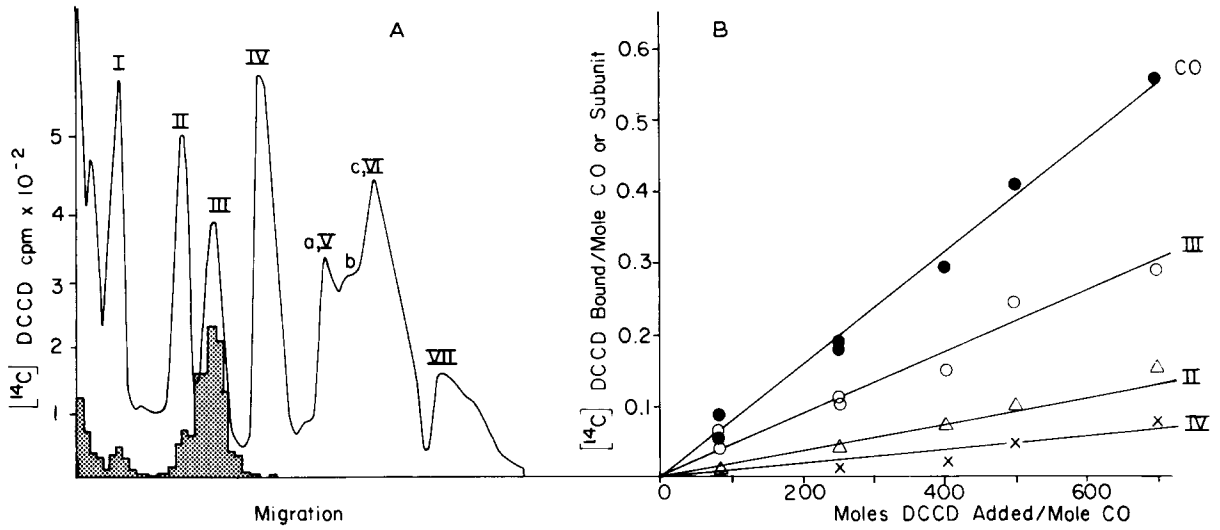

Fig. 4. Labelling of subunits of vesicular cytochrome *c* oxidase by DCCD. (A) SDS-polyacrylamide gels run under the conditions of Swank and Munkres [24]. Labelling was done at 80 mol DCCD/mol enzyme. (B) Incorporation of DCCD into different subunits as a function of the concentration of reagent used in the reaction. CO, cytochrome *c* oxidase.

of 4- and 8-fold, respectively, at low concentrations of DCCD (80 mol/mol) and by 2- and 4-fold, respectively, at high concentrations of reagent (700 mol/mol enzyme).

Gel profiles such as those in Figs. 3 and 4 were always compared with standards of unmodified enzyme subjected to the same manipulations but without DCCD added. No additional bands were seen in the DCCD-treated samples that were not found in the control. Therefore, it is unlikely that there is a significant amount of cross-linking between different subunits due to the reaction of DCCD. There could, or course, be some intrasubunit cross-linking which might modify the enzyme in a way which affects functioning. However, such a modification might be expected to alter the mobility of the polypeptide involved, but no changes in migration of individual polypeptides were observed.

Determination of the total number of sites modified by DCCD

The results shown in Figs. 3 and 4 indicate that there are at least three sites of reaction of DCCD with cytochrome *c* oxidase, on subunits II–IV, and possibly more if any of these subunits have multiple sites of interaction. It is important to know both how many different sites of reaction of DCCD there are and their relative reactivity in order to interpret fully the inhibition studies with this reagent. This requires the purification of labelled subunits and then sequencing to determine the sites of incorporation of the reagent. Obviously, such experiments are long-term projects, particularly as there are difficulties both in cleaving subunits II and III and in separating the many fragments for examination. This work is now in progress and the initial fragmentation experiments with subunits II–IV are presented in the Appendix.

Labelling of subunit II

Incorporation of DCCD into subunit II has been found to occur mainly in the C-terminal portion of the polypeptide within rather hydrophilic stretches of amino acids. There are clearly several sites of labelling with fairly similar reactivity. One of these sites is in fragment CB2-4 containing residues 2–29, a second site is in CB15 or CB16, i.e., in the sequences 185–207 or 208–227.

It has been clearly demonstrated that subunit II provides the binding site for cytochrome *c* on the oxidase complex [33–35]. This interaction has been shown to involve negatively charged residues surrounding the heme cleft on cytochrome *c* [36]. There is also good evidence that the binding of cytochrome *c* to

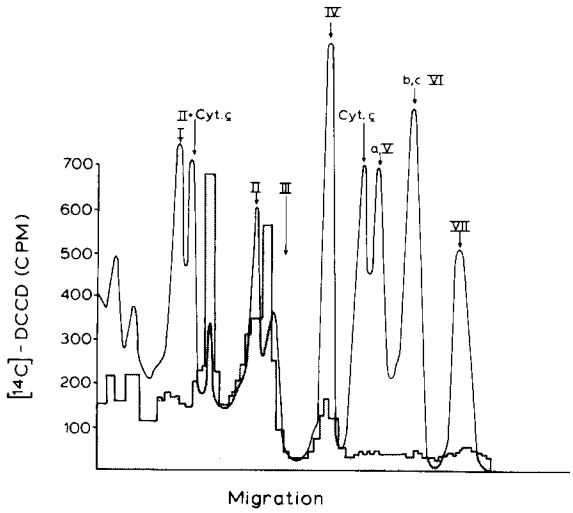

Fig. 5. DCCD labelling of an arylazidocytochrome *c*-cytochrome *c* oxidase complex. A cytochrome *c*-cytochrome *c* oxidase complex was prepared as described by Bisson et al. [34,35]. This complex showed 60% inhibition of electron-transfer activity. A sample of the complex (0.6 mg/ml cytochrome *c* oxidase) was reacted with [^{14}C]DCCD (0.04 mM). for 1 h at 20°C. A control and the labelled cytochrome *c* oxidase were run on SDS-polyacrylamide gels (same conditions as in Fig. 3). There was a 30% decrease in subunit II as judged by staining intensity. The minor component, heavily labelled with DCCD and running just ahead of the II + Cyt. c band, is a 29 000 dalton polypeptide, a contaminant of the preparation. Cyt. c, cytochrome *c*.

cytochrome *c* oxidase has a hydrophobic component [37]. Thus one or more of the negatively charged groups involved in cytochrome *c* binding could be in a sufficiently hydrophobic environment to interact with DCCD.

This possibility was tested by covalently binding arylazidocytochrome to the detergent-solubilized cytochrome *c* oxidase complex prior to labelling with DCCD. This derivative, modified selectively at lysine 13, has been shown to bind to the high-affinity site for the substrate [35]. After SDS-polyacrylamide gel electrophoresis, the covalent cytochrome *c* subunit II cross-linked product can be resolved from other subunits and from unreacted subunit II, which in turn is an internal control in the labelling experiments. As shown in Fig. 5 no labelling of DCCD was found in subunit II that had cytochrome *c* bound to it, although there was labelling of free subunit II. This is good evidence that the DCCD-binding sites on sub-

Ile - Leu - Phe - Ile - Ile - Ser - Glu - Val	CB10 Subunit III bovine enzyme
Leu - Met - Phe - Val - Leu - Ser - Glu - Val	yeast enzyme
Leu - Gly - Phe - Ala - Leu - Ser - Glu - Ala	DCCD binding protein, bovine ATP synthetase
Leu - Gly - Phe - Ala - Leu - Ser - Glu - Ala	yeast enzyme

Fig. 6. A comparison of the sequence of a fragment of subunit III containing the DCCD-binding site with that of the DCCD-binding site in the proteolipid of mitochondrial ATP synthetase.

unit II are in the binding domain for cytochrome *c* on the oxidase complex.

Labelling of subunit III

Fragmentation studies, which are described in the Appendix, strongly suggest that the incorporation of DCCD into subunit III is mainly in a single site. This conclusion rests upon the following observations. There was no incorporation of DCCD into CNBr fragments soluble in 20% acetic acid. This fraction contains nine different fragments making up the N-terminal sequence of residues 1–83. There was essentially no radioactivity associated with the C-ter minal CNBr fragment isolated by gel filtration on Sephadex LH60 in 25% formic acid, 75% ethanol. Almost all of the radioactivity was found to be associated with fragments containing CB10, whether these were separated in fractions soluble in 70% acetic acid or by gel filtration of the 70% acetic-insoluble fragments on Sephadex LH60 in the formic acid, ethanol solution. Sequencing of the fraction rich in CB10 has shown that the DCCD incorporated into this fragment is essentially all bound at glutamic acid 90. Fig. 6 shows the sequence around this glutamic acid residue as determined in the present study. This sequence is compared with the homologous segment of yeast cytochrome *c* oxidase subunit III and the sequences of the DCCD-binding site in the proteolipid protein of both the yeast and beef heart mitochondrial ATP synthetase. The similarity between the DCCD-binding site of subunit III and the site of reaction of this reagent in the proteolipid is obvious.

Labelling of subunit IV

Fragmentation studies have shown that the site of interaction of DCCD is exclusively within CB3, which contains several glutamic acid residues.

Inhibition of electron transfer and proton pumping as a function of the incorporation of DCCD into individual sites

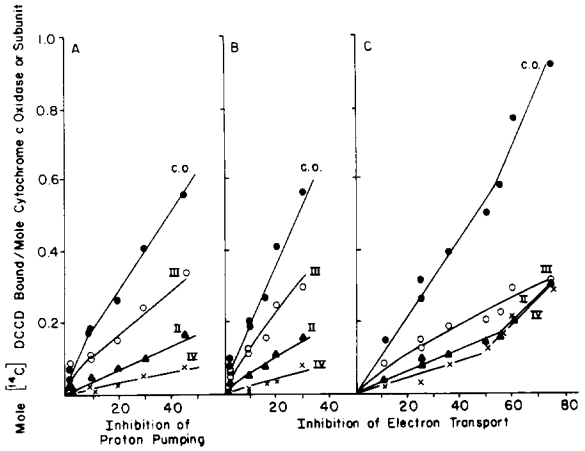

Fig. 7. Inhibition of cytochrome *c* oxidase activity as a function of DCCD bound to each subunit. (A and B) Cytochrome *c* oxidase (c.o.) vesicles. (C) Detergent-solubilized enzyme. Inhibition of proton pumping was calculated as the extent of proton extrusion.

The relationship between inhibition of electron transfer and proton pumping with the incorporation of DCCD into individual subunits and into the cytochrome *c* oxidase complex as a whole is shown in Fig. 7. The results summarized in the previous section show that incorporation of DCCD in subunit III probably involves one major site while labeling of subunit II definitely involves incorporation in several different sites, each with a fairly similar specificity for the reagent. Assuming that there are three sites in subunit II, which is likely to be a minimum, the major site in subunit III would have a specificity for DCCD at least one order of magnitude greater than that of other sites on the complex (in subunits II or IV).

The modification of subunit III is sufficient by itself to account for the observed inhibition effects even assuming that 1 mol of DCCD incorporated into this site per mol of oxidase monomer is required for maximal inhibition of proton pumping. In fact, there is evidence that beef heart cytochrome *c* oxidase shows half of the site reactivity under certain conditions [35], in which case 0.5 mol DCCD/mol subunit III should have a maximal inhibitory effect.

When reacted with higher levels of DCCD, there is a significant incorporation of reagent into sites on subunit II and modification of these sites could influence proton pumping either directly, or indirectly, via inhibition of electron transfer or denaturation of the protein.

Discussion

The recent studies of Wikstrom and co-workers (see Ref. 5 for a review) have shown that electron transfer in beef heart cytochrome *c* oxidase is accompanied by a proton ejection equivalent to one proton being translocated across the membrane for every reducing equivalent added. This electron transfer-coupled proton-pumping function of cytochrome *c* oxidase has now been demonstrated in several laboratories [20,27,28] and is confirmed in this study. Several mechanisms have been proposed to explain the coupling of proton pumping to electron transport in the terminal oxidase. The one we favor considers that reduction of heme *a* causes the uptake of a proton from one side of the membrane (interior of the reconstituted vesicles used here and matrix side of the mitochondrial inner membrane) and that oxidation of this heme then releases the proton along a proton-conducting channel to exit on the opposite side of the membrane (exterior of the reconstituted vesicles or cytoplasmic side of the inner membrane) [5,6].

Several inhibitors of electron transport in cytochrome *c* oxidase are known, including cyanide and azide [38]. These would be expected to decrease proton pumping concomitantly with their effect on the redox properties of the enzyme. Inhibitors of proton translocation, on the other hand, would not necessarily be expected to inhibit electron transfer. In the mechanisms described above, a reagent could block the proton channel responsible for vectorial translocation of protons, but if if the protons are released from the complex by another route, electron transfer would not necessarily be inhibited simultaneously.

The classical inhibitor of the proton-translocating function of enzymes is DCCD, first used by Beechey and co-workers [14] in their study of the ATP synthetase of heart mitochondria. This reagent has been shown to bind to a single glutamic acid residue (aspartic acid in bacterial species) in a very hydrophobic subunit of the membrane sector of the ATP synthetase [39,40]. This specific reaction blocks proton translocation through the ATP synthetase [41].

DCCD was first used in studies of cytochrome *c* oxidase by Casey et al. [20,29]. They showed that

this reagent also inhibits proton translocation in this enzyme. According to Casey et al. [20,29], DCCD inhibited proton translocation without significantly affecting the electron-transport activity. Our experiments confirm that DCCD inhibits the proton-pumping activity of cytochrome *c* oxidase but we find that the reagent also effects a considerable inhibition of electron transfer. In all of our experiments, the inhibition of proton pumping was higher than the inhibition of electron transfer. Thus, not all of the effects of DCCD are due to reaction of sites important for electron transfer. At least one of the sites of interaction of the reagent must be with a group involved in or responsible for maintaining the structural integrity of the proton-translocating pathway.

Casey et al. [29] have recently reported that the reaction of cytochrome *c* oxidase with DCCD occurs almost exclusively in subunit III. This is not the case in our experiments. Subunits II–IV were all labelled by radioactive DCCD in experiments where there was significant inhibition of cytochrome *c* oxidase function. The reason for the discrepancy between the two studies is not clear at present. In both sets of experiments the levels of DCCD covalently bound to protein at any level of inhibition of proton pumping were similar. The difference resides in the observed specificity of the reaction of the reagent. Several factors were tested for their effect on this specificity. DCCD was added to the reaction mixture and the methanol evaporated off before addition of vesicles, in case methanol affected the labelling pattern even at the very low concentrations used. Also, cytochrome *c* oxidase-containing vesicles were resolved on Sepharose 2B and fractions showing only the highest respiratory control ratios were labelled with DCCD. Such preparations would have the highest incorporation of properly oriented enzyme molecules. Neither of these steps significantly altered the relative labelling of subunits.

A preliminary analysis of the number of different sites of modification by DCCD in subunits II–IV is presented. Fragmentation experiments indicate that there is one major site of modification on subunit III, in CB10, and that the residue involved is glutamic acid. This site by far the most reactive single site in cytochrome *c* oxidase (given that there are multiple sites in subunits II) and its modification could account for most of the inhibition observed.

The amino acid sequence of the portion of CB10 containing the DCCD-reactive glutamic acid has been determined. This eight amino acid stretch shows a similarity to the DCCD-binding site in the ATPase proteolipid protein of beef heart (Fig. 6). The fragmentation studies on subunits II–IV are presented in the Appendix. These experiments indicate some of the difficulties encountered in working with hydrophobic membrane proteins, problems which must be overcome before a quantitative analysis of the labelling of cytochrome *c* oxidase with DCCD or other modifying reagents can be completed. The major problem is that these polypeptides are not completely cleaves by chemical methods such as the use of CNBr or by enzymic methods such as trypsin or chymotrypsin treatment. Sites resistant to cleavage are in all cases located in hydrophobic stretches of the sequence. It appears that these stretches of chain do not unfold in solvents such as 70% formic acid (used for CNBr cleavage) or in 1% SDS (used in protease treatment) and thus the labile bond is not available for attack. Approaches are currently being sought to improve the fragmentation procedures.

The importance of subunit III in the functioning of cytochrome *c* oxidase is at present a matter of controversy. Carroll and Racker [42] have claimed that subunit III of beef heart cytochrome *c* oxidase is removed by chymotrypsin cleavage without loss of functioning. However, these cleavage results have not proved to be reproducible in other laboratories (e.g., Ref. 43). It is possible to remove subunit III of the beef heart enzyme by native gel electrophoresis in high concentrations of Triton X-100 [44,45]. Saraste et al. [45] have recently reported that removal of this subunit does not affect electron-transfer activity but that the proton-translocating function of cytochrome *c* oxidase is lost.

Ludwig and Schatz [46] have shown that *Paracoccus denitrificans* contains a cytochrome *c* oxidase with two hemes (an *a* and a_3 heme), two copper atoms but only two subunits. Based on size and on cross-reactivity to antibodies, these two subunits have been shown to be equivalent to subunits I and II of the eukaryote cytochrome *c* oxidases. This two-subunit enzyme shows full electron-transfer activity but translocates protons only with a very low efficiency. This in turn suggests that an additional component (e.g., subunit III) is necessary for the proton-translocating function.

The labelling and sequence data presented here

strongly suggest that subunit III is involved in the proton-translocating function of cytochrome *c* oxidase, as proposed by Casey et al. [29]. However, our labelling studies with DCCD do not at this stage provide unambiguous evidence for this proposal. There are several sites modified by DCCD in subunits II and a site in IV, albeit with a much lower reactivity. Any of these sites could contribute to the observed effects, although none singly can provide the total inhibition seen. The modification of subunit II by DCCD was blocked by a prior covalent attachment of arylazido-cytochrome *c* to cytochrome *c* oxidase in the high-affinity site for substrate cytochrome *c* (previously identified as involving subunit II). It is possible that modification of subunit II by DCCD affects proton translocation but indirectly by affecting electron transfer.

In summary, then, studies reported here confirm the work of Casey et al. [20,29] that the oxidoreduction-linked proton-pumping function of cytochrome *c* oxidase is inhibited by DCCD. Reaction of the enzyme with reagent at the same time inhibited electron transport. The sites of reaction of DCCD are few and include a site on subunit III which is remarkably similar to the DCCD-binding site on the ATP synthetase (the modification of which has been shown to block selectively proton translocation in this complex). At least two sites are modified in subunit II and these are probably in the cytochrome *c* binding domain. The most conservative interpretation of our results is that either subunits II, III or both contribute to the protein-translocating channel of cytochrome *c* oxidase.

Appendix

Introduction

Labelling experiments have shown that DCCD inhibits electron transfer and proton pumping in beef heart cytochrome *c* oxidase through interaction with sites on selected subunits of the enzyme. This appendix describes the results of fragmentation studies started with the long-term objective of defining the sites of interaction of DCCD within the sequences of subunits II–IV. The progress made so far gives us an estimate of the number of sites in each subunit. This information is necessary for interpreting the data in the preceding sections. In the case of subunit III we have sequenced the major site of modification by DCCD. The residue reacted is a glutamic acid in a sequence remarkably like that of the DCCD-binding site in the proteolipid protein of the mitochondrial ATP synthetase.

Experimental section

Separation of [^{14}C]DCCD labelled subunits of cytochrome c oxidase. Cytochrome *c* oxidase (20 mg) was labeled with [^{14}C]DCCD in 30 mM KCl, 80 mM sucrose, 0.2% Tween 80, 2 mM Hepes-NaOH, pH 7.2, at a stoichiometry of 400 mol DCCD/mol cytochrome oxidase for 1 h at room temperature. The enzyme was then centrifuged in a Beckman Model L2-6JV5B ultracentrifuge at 300 000 × *g* overnight. The pellet was resuspended in 5% SDS and 0.2% β-mercaptoethanol. Unlabelled cytochrome *c* oxidase (80 mg) was added to the ^{14}C-labelled enzyme and this mixture was allowed to incubate overnight at room temperature. The dissociated enzyme was centrifuged at 10 000 × *g* for 10 min at room temperature in a SS-34 rotor in a Sorvall R2B centrifuge to remove insoluble material. After 250 mg of sucrose were added to the supernatant, the sample was then loaded onto a Bio-Gel P-60 column (32 × 4.2 cm) with 3% SDS as the eluant [10]. The flow rate was 10 ml/h and 2.3-ml fractions were collected. Fractions were assayed for [^{14}C]DCCD labelling by liquid scintillation counting and for protein by the absorbance at 280 nm. Samples across each peak were examined by SDS-polyacrylamide gel electrophoresis and the peaks of purified subunits II–IV each pooled for further analyses.

CNBr cleavage of ^{14}C-labelled subunits. CNBr cleavage of [^{14}C]DCCD-labelled subunits II and IV (approx. 1–2 μmol) was performed as described by Sacher et al. [48]. Labelled subunit III was cleaved using the same procedure but in 90% formic acid to increase the solubility of this very hydrophobic polypeptide.

Peptides were then separated according to their solubility in acetic acid. Lyophilized CNBr fragments of subunits II and IV were suspended in 10% acetic acid as described by Sacher et al. [48]. The suspension was centrifuged to remove insoluble material and this was dissolved in 70% acetic acid. Fragments of subunit III were initially suspended in 20% acetic acid, insoluble material was then dissolved in 70%

TABLE I

ACETIC ACID SOLUBILITY OF CNBr PEPTIDES OF DCCD-LABELLED SUBUNITS OF CYTOCHROME *c* OXIDASE

Subunit	% cpm soluble in 10–20% acetic acid	% cpm insoluble in 10–20% acetic acid
II	76	24
III	9	91
IV	22	78

acetic acid and protein insoluble in this solvent was dissolved in 90% formic acid. Table I lists the proportion of the counts in the different fractions for each of the subunits.

Gel filtration of subunit II fragments on Bio-Gel P10 in 70% acetic acid. Essentially all of the DCCD incorporated into subunit II was associated with fragments soluble in 10% acetic acid. This material was brought to 70% acetic acid and then subjected to gel filtration on a Bio-gel P10 (200–400 mesh) column (2.5 × 300 cm) in 70%) acetic acid. The eluant was monitored for protein at 280 nm and for radioactivity by scintillation counting.

The elution profile from this column was essentially identical to that reported by Buse et al. [49] using the same separating conditions. This allowed a preliminary identification of fragments by their elution position and based on the sequencing data of Steffens and Buse [50]. Confirmation of the identity of the individual peaks was obtained by amino acid analysis using the procedure described by Spackmann et al. [51] with modifications described by Sacher et al. [48]. For larger molecular weight fragments, the peaks of radioactivity coincided with the peaks of absorbance at 280 nm. For smaller fragments, the size of the DCCD covalently bound was large enough to alter its migration through the column. This led to separation of labelled fragments from unlabelled material. Our results show clearly that there are multiple sites of interaction of DCCD with subunit II. Among the fragments labelled was CB2-4 with a molecular weight in the range 2 800–3 000; either CB15 and/or CB16 with molecular weights in the range 2 250–2 550 and one or more of CB2 + 3, CB3, CB4 and CB9, all with molecular weights in the range 1 300–1 550. A more specific identification of where DCCD is incorporated into subunit II requires the separation of the similarly sized fragments by hydrophobicity using high-pressure liquid chromatography and this work is now in progress.

Labelling of subunit III with DCCD. There was essentially no DCCD incorporated into the 20% acetic acid fraction of subunit III. This fraction has been found to contain CB1-9, the N-terminal one-third of the polypeptide including residues 1–83. The labelled material insoluble in 20% acetic acid was separated into two fractions, one soluble in 70% acetic acid; the second insoluble in the high concentration of acetic acid but soluble in 90% formic acid. The 70% acetic acid-soluble fraction was subjected to gel filtration on Bio-Gel P10 (200–400 mesh) in 70% acetic acid. All of the protein eluted in the void volume, indicating that all of the fragments have a molecular weight greater than 7 000 (the exclusion limit of the column in this solvent). The 70% acetic acid-soluble frac-

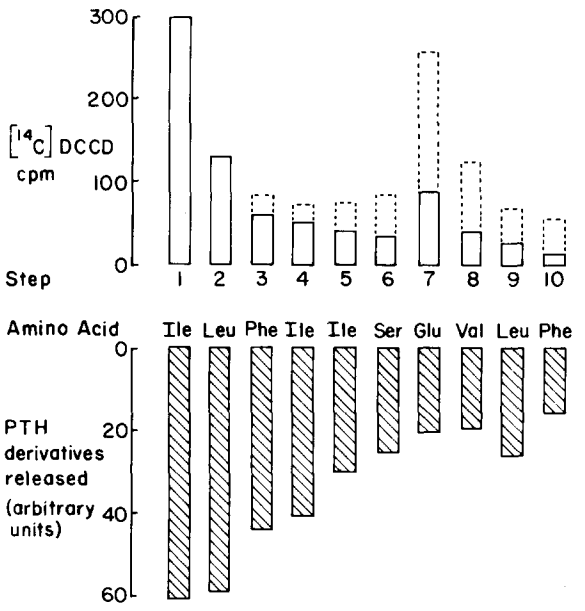

Fig. 8. Edman degradation of [^{14}C]DCCD-labelled CNBr fragment CB10 of subunit III. The solid bars represent ^{14}C radioactivity released at each step. The cross-hatched bars show the relative amount of phenylthiohydantoin (PTH)-reacted amino acids at each step. The dashed bars show radioactivity corrected for decrease in the yield after each step. The ^{14}C in steps 1 and 2 is due to continued washing of protein from the cup. No DCCD-reactive amino acids were released in these steps.

tion was next subjected to sequencing directly. This was performed on a Beckman Model 890C sequencer using a Quadrol protein program [52] with double cleavage [48]. The 2-anilino-5-triazodinone acids were converted at 80°C for 30 min in 1 M HCl. The resulting phenylthiohydantoin derivatives were analyzed by chromatography on thin-layer plates (Merck 60/F-254) using developing solvents described by Sacher et al. [48]. The converted amino acids were assayed for radioactivity using liquid scintillation counting on a Packard liquid scintillation counter. Fig. 8 lists the amino acids identified in each step of sequencing along with the radioactivity released in each step. The major fragment present was CB10, probably joined to CB11 as it was soluble in 70% acetic acid. Also present in much smaller amounts were fragments beginning with CB5, CB6, CB7 and CB9, probably representing CB5-10 or CB5-11, CB6-10 or CB6-11 etc. (CB5, CB6, CB7 and CB9 by themselves would be soluble in 20% acetic acid). Radioactivity was released in a prewashing step and through the first two cycles of sequencing. This is due to washing of noncoupled fragments from the cup rather than to release of labelled amino acids as evidenced by the fact that none of the residues cleaved in the first two cycles are reactive with DCCD. Radioactivity was found in cycle 7 in which glutamic acid 90 was released. The sequencing was continued for a total of 20 steps without further release of radioactivity. The protein left in the cup after this step contained less than 15% of the counts initially present in the sequencer. This experiment clearly identifies a major site of labelling by DCCD within a sequence of subunit III very similar to the DCCD-labelling site in the proteolipid of the mitochondrial ATP synthetase.

The fraction insoluble in 70% acetic acid was dissolved in 88% formic acid, 5% hexafluorobutyric acid and subjected to a second CNBr-cleavage step in order to cleave this material at previously unmodified methionine residues and also at tryptophan residues [53]. Fragments were dissolved in 25% formic acid, 75% ethanol and subjected to gel filtration on a column of Sephadex LH60 (4 X 850 cm) in the same solvent. Fractions of 2.5 ml were collected, read for absorbance at 280 nm and analyzed for radioactivity by scintillation counting. As shown in Fig. 9, the protein eluted in four major peaks. The void volume peak contained 25% of the total absorbance eluting

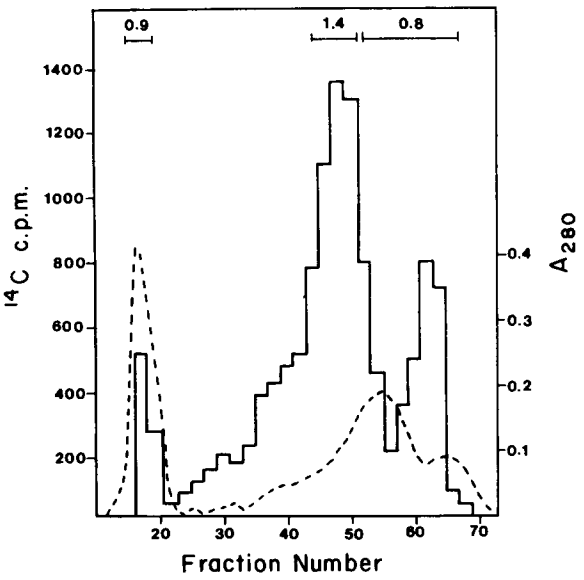

Fig. 9. Separation of CNBr Fragments insoluble in 75% ethanol by gel filtration on a Sephadex LH60 column (4 X 850 cm). The dotted line shows A_{280}, the barograph shows radioactivity in each fraction. The figures above the peaks are protein concentrations in each peak (tubes posted are shown by the lines) obtained by determination according to the method of Lowry et al. [25].

from the column but less than 5% of the radioactivity. This peak was shown to be essentially pure CB11 + 12 by amino acid analysis (Table II). More than 80% of the counts were present in peak B, which from amino acid analysis is concluded to contain CB10 and CB12 as well as slightly smaller fragments derived from these segments by cleavage at tryptophan residues. In summary, the combined studies on subunit III rule out the possibility of significant labelling in CB1-9 or in CB11 + 12, leaving CB10 as the only fragment with high levels of DCCD present. Sequencing in turn locates radioactivity to glutamic acid 90 in this fragment.

Labelling in subunit IV. DCCD incorporated in Subunit IV was distributed in both the 10% acetic acid-soluble and -insoluble fractions and both were subjected to gel filtration on Bio-Gel P10 in 70% acetic acid using the same column used in experiments with subunit II. Fragments were identified by amino acid analysis. Incorporation of DCCD was localized to CB3 which contains several glutamic acid residues.

TABLE II

AMINO ACID COMPOSITION OF THE VOID VOLUME PEAK FROM GEL FILTRATION ON THE 70% ACETIC ACID INSOLUBLE FRACTION OF SUBUNIT III

Amino acid	Fraction [a] 19–23	CB11 + 12 [b]
Asp	3.18	4
Thr	7.92	8
Ser	6.05	7
Glu	6.78	7
Gly	9.28	10
Ala	7.74	6
Cys	0.50	1
Val	7.74	9
Met	1.00	1
Ile	6.62	7
Leu	11.60	10
Phe	16.20	15
His	6.25	6
Lys	1.60	2
Arg	1.86	2
Pro	2.20	1

[a] mol/amino acid per mol methionine. 24 h hydrolyses.
[b] Calculated from the sequence kindly provided by Dr. Bart Barrell.

Further experiments are in progress not only to identify precisely the site of interaction of DCCD in subunit II but in CB3 of subunit IV as well.

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