Biochimica et Biophysica Acta, 724 (1983) 75–82
Elsevier

BBA 41318

EFFECTS OF *N,N'*-DICYCLOHEXYLCARBODIIMIDE ON ISOLATED AND RECONSTITUTED CYTOCHROME *b*-c_1 COMPLEX FROM BOVINE HEART MITOCHONDRIA

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(Received March 7th, 1983)

Key words: Cytochrome b-c_1 complex; Dicyclohexylcarbodiimide; Membrane reconstitution; Electron flow; H^+/e^- ratio; Proton translocation

N,N'-Dicyclohexylcarbodiimide (DCCD) inhibits the activity of ubiquinol-cytochrome *c* reductase in the isolated and reconstitued mitochondrial cytochrome *b*-c_1 complex. DCCD inhibits equally electron flow and proton translocation (i.e., the H^+/e^- ratio is not affected) catalysed by the enzyme reconstituted into phospholipid vesicles. The inhibitory effects are accompanied by structural alterations in the polypeptide pattern of both isolated and reconstituted enzyme. Cross-linking was observed between subunits V (iron-sulfur protein) and VII, indicating that these polypeptides are in close proximity. A clear correlation was found between the kinetics of inhibition of enzymic activity and the cross-linking, suggesting that the two phenomena may be coupled. Binding of [^{14}C]DCCD was also observed, to all subunits with the isolated enzyme and preferentially to cytochrome *b* with the reconstituted vesicles; in both cases, however, it was not correlated kinetically with the inhibition of the enzymic activity.

Introduction

DCCD inhibits proton translocation by H^+-ATPases in mitochondria [1], chloroplasts [2] and bacteria [3] as well as that by mitochondrial cytochrome *c* oxidase [4] and transhydrogenase [5]. In the case of these ATPases and cytochrome *c* oxidase the inhibition is accompanied by the covalent binding of DCCD to a single glutamic or aspartic acid residue situated in a hydrophobic sequence of amino acids [6]. This residue was postulated to be involved in the mechanism of proton translocation. Lenaz et al. [7] and Price and Brand [8] have reported inhibition by DCCD of H^+ translocation occurring in the cytochrome bc_1 region of the mitochondrial respiratory chain. These groups differ, however, as to the molecular mechanism of the DCCD effect; the former group concludes that there is a parallel inhibition of the electron flow tightly coupled to the proton-transfer reaction whereas the latter postulates a molecular 'decoupling' of the electron transfer from H^+ translocation. More recently, Beattie and coworkers [9,10] have also reported an inhibition of both electron transport and proton translocation by DCCD in yeast cytochrome *b*-c_1 complex and in addition, the covalent binding of DCCD preferentially to cytochrome *b*. We report here further studies on the structural and functional changes caused by DCCD in the isolated and reconstituted cytochrome *b*-c_1 complex.

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Abbreviations: DBH, reduced form of 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone, a synthetic analogue of ubiquinone-2 having a saturated decyl side chain; DCCD, *N-N'*-dicyclohexylcarbodiimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Materials and Methods

Bovine heart mitochondria were prepared according to the method described in Ref. 11. Cytochrome b-c_1 complex was isolated according to the method of Rieske [12] and stored at $-80°C$. The preparation used here contained 6.97 nmol heme b and 3.73 nmol heme c_1 per mg protein. The enzyme was incorporated into asolectin vesicles as described for cytochrome c oxidase [13] except that the concentration of sonicated phospholipid was 105 mg/ml and cytochrome b-c_1 complex was used at a concentration of 7.5 μM heme b. The electron-transfer activity was determined by monitoring the reduction of cytochrome c as the change in absorbance at 550–540 nm using an Aminco DW-2A spectrophotometer fitted with a magnetic stirring device. The cuvette chamber was always thermostatically maintained at 25°C. In the case of the isolated enzyme the experimental sample contained 50 mM potassium phosphate buffer (pH 7.4), 5 μM cytochrome c, 0.4 μM cytochrome b-c_1 complex and 0.25 mg/ml dispersed asolectin as recommended by Rieske [12]. With reconstituted vesicles the composition was: 75 mM choline-chloride, 25 mM KCl, vesicles to give a final concentration of 0.2 μM heme b, 5 μM cytochrome c and 0.13 μM valinomycin. For measurements of uncoupled activities 2.5 μM CCCP was also present. With both systems the reaction was started by addition of DBH, an analogue of ubiquinone-2 [14], at a concentration of 1.7 μM. DBH was reduced according to the method of Trumpower and Edwards [15], dissolved in dimethyl sulfoxide and stored as a 1.13 mM solution at $-20°C$.

Changes in pH of the vesicle suspensions were measured simultaneously with electron-transport activity. A pH electrode (Ingold LoT 405-M3), inserted into the sample cuvette, was connected to a pH-meter (Radiometer PHM-64) and a chart recorder (W + W 600). Measurements of pH were calibrated using freshly prepared 1 mM oxalic acid.

For the isolated enzyme the incubation with DCCD was performed as follows. The sample of the enzyme was diluted to 20 μM heme b with 50 mM potassium phosphate buffer (pH 7.4) and then an ethanolic solution of 0.2 M DCCD was added to give a ratio of 100 mol per mol heme b. Immediately after addition of DCCD, the sample was vigorously mixed and placed in a water bath at 35°C. Small samples of the mixture were taken after the desired time for measurements of enzymic activity. In the case of samples taken for SDS gel electrophoresis, the reaction with DCCD was stopped by diluting the mixture 1 : 1 (v/v) with the sample buffer prepared according to the method of Laemmli [16] and placing the diluted sample in ice. Control samples were incubated with an equal amount of ethanol.

In the case of the reconstituted vesicles, DCCD was added directly to the vesicle suspension (7 μM heme b) from a 0.5 M solution to give a ratio of 400 mol DCCD per mol heme b. The suspension was mixed and placed in a water bath at 35°C. 50-μl samples of this mixture were taken for simultaneous measurements of H^+ translocation and cytochrome c reduction activities. In the case of samples taken for SDS gel electrophoresis, aliquots of 0.5 ml were taken after the desired time of incubation, placed in ice, solubilized with 2.5% (final concentration) sodium cholate, pH 7.8, and layered onto 3.2 ml of 10% sucrose. They were then centrifuged for 20 h at 4°C and 250000 $\times$ g_{max} in the swing-out rotor (6 $\times$ 4.2 ml) of an MSE Prepsin 65 centrifuge. The resulting pellets were dissolved in 100 μl of the sample buffer prepared as described by Laemmli [16] and subjected to SDS gel electrophoresis.

SDS-polyacrylamide gel electrophoresis was carried out using slab or cylindrical gels [16]. Gels were fixed and, when necessary, stained for protein and heme as described previously [17]. Scanning of gels was performed using a locally constructed attachment for an Aminco DW-2A spectrophotometer as described by Broger et al. [18].

Binding of [^{14}C]DCCD (spec. act. 2.0 Ci/mol) to the isolated and reconstituted cytochrome b-c_1 complex was determined following incubation, as described for cytochrome c oxidase [19]. Determination of the [^{14}C]DCCD (spec. act. 1.5 Ci/mol) -binding subunits of both isolated and reconstituted cytochrome b-c_1 complex was performed following incubation, according to the method reported in Ref. 20.

DBH was a generous gift from Dr. B. Trumpower. DCCD was from Fluka A.G., Buchs,

Switzerland, and was further purified by distillation under reduced pressure. Asolectin was prepared from soybean L-α-phosphatidylcholine (Sigma Chemical Co.) as described by Kagawa and Racker [21]. Cytochrome *c* (type VI from horse heart) was from Sigma Chemical Co. [^{14}C]DCCD was from CEA, Gif-sur-Yvette, France. All other chemicals used were of the highest purity commercially available.

Results

The antimycin-sensitive ubiquinol : cytochrome *c* reductase activity of isolated cytochrome *b*-c_1 complex (9.5 electrons/s per heme *b*) was strongly inhibited by DCCD under our experimental conditions. At a concentration of 100 mol per mol heme *b*, DCCD caused an inhibition of 50% after 30 min and almost 100% inhibition after 50 min of incubation. In the absence of DCCD, the enzyme retained full activity throughout the time course of the experiment.

Previous investigations have indicated that the inhibitory effects of DCCD on enzyme function are often accompanied by structural alterations [1,19–23]. The possibility that the above-described inhibition may be related to structural alterations of the enzyme was examined. In the experiment presented in Fig. 1, duplicate samples of cytochrome *b*-c_1 complex were subjected to gel electrophoresis following incubation with either DCCD or, as a control, with the amount of ethanol used to add DCCD. The control (Fig. 1A) gave an electrophoresis pattern similar to those reported by other laboratories [24–28]. It is generally agreed that the two highest molecular weight bands represent core proteins, the third cytochrome *b* and there is some consensus that subunits IV and V correspond to cytochrome c_1 and the Rieske iron-sulfur protein, respectively. Rieske [29] and Trumpower [30] have pointed out, however, that the migration positions of the latter two proteins may be exchanged under certain electrophoresis conditions. To resolve this question for our system, gels were scanned to detect the presence of covalently bound heme. Superposition of the scan of heme absorbance at 400–430 nm and that of Coomassie-blue staining allowed identification of subunit IV as the peptide containing covalently bound heme, i.e., cytochrome c_1. Staining of the gels with tetramethylbenzidine (not shown) confirmed the presence of heme in subunit IV.

As shown in Fig. 1B, following incubation with DCCD the electrophoretic migration pattern of

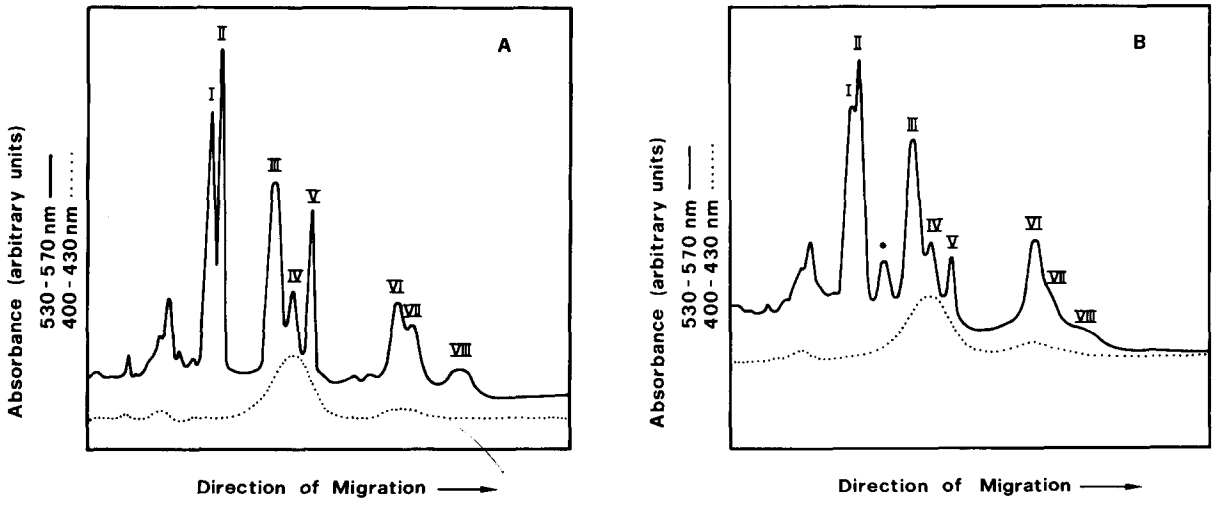

Fig. 1. Effect of DCCD on the SDS-polyacrylamide gel electrophoresis pattern of the isolated cytochrome *b*-c_1 complex. Two samples of the enzyme were incubated with either ethanol (A) or DCCD at a ratio of 100 mol per mol heme *b* (B) as described in Materials and Methods. Directly following the electrophoresis, gels were scanned for heme absorption at 400–430 nm (· · · · · ·) and then stained for protein with Coomassie blue and scanned at 530–570 nm (———). Roman numerals indicate the subunits of the cytochrome *b*-c_1 complex and the asterisk denotes the newly formed band.

TABLE I

MOLECULAR WEIGHTS OF POLYPEPTIDES FOUND IN THE CYTOCHROME b-c_1 COMPLEX INCUBATED WITH DCCD, AS ESTIMATED FROM SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Polypeptide	Molecular weight ($\times 10^{-3}$)
I	52
II	49
* (new band)	42
III	37
IV	33
V	29
VI	14
VII	13
VIII	10

the complex was changed. A new band appeared between bands II and III whereas bands V and VII were either diminished or disappeared completely. The magnitude of this effect depended on the incubation time and amount of DCCD. Table I shows that the position of the newly formed band corresponded to a molecular weight which was the sum of the molecular weights of subunits V and VII. As argued above, subunit V represents the Rieske iron-sulfur protein, whereas subunit VII represents a protein copurifying with cytochrome b [28]. In some experiments there was also a diminution in the intensity of band VIII though this was not found consistently.

Fig. 2 shows that the time courses of appearance of the new band and disappearence of the iron-sulfur protein are concomitant.

In a number of other systems DCCD inhibition was related to the covalent binding of this carbodiimide to the protein. In the system used here, binding of radioactively labelled DCCD was measured following the incubation under conditions identical to those used to obtain inhibition. It was observed that in the case of the isolated cytochrome b-c_1 complex, binding of [^{14}C]DCCD was unspecific, with all subunits labelled approximately to the same extent (data not shown). The onset of binding of DCCD was more rapid than

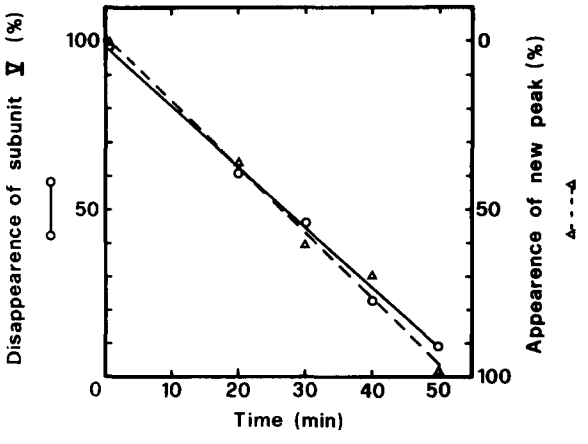

Fig. 2. Time course of appearance of a cross-linked band and dissapearance of subunit V during incubation of the isolated cytochrome b-c_1 complex with DCCD. Incubation conditions were as described in Materials and Methods. Samples of the incubated enzyme were taken at the times indicated and subjected to electrophoresis. The bands corresponding to subunit V (○) and the cross-linked material (△) were integrated and the integrals are shown as a percentage of the zero-time value (for subunit V) or the value after 50 min of incubation (for the cross-linked material).

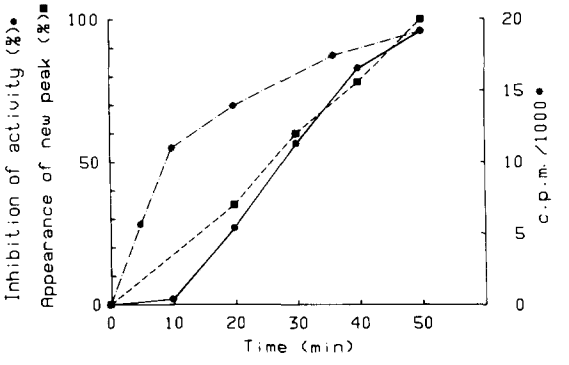

Fig. 3. Comparison of time courses of inhibition by DCCD of cytochrome c reduction by the isolated cytochrome b-c_1 complex, of the appearance of cross-linked material and of binding of [^{14}C]DCCD to the isolated enzyme. DCCD was incubated with the enzyme at the ratio of 100 mol per mol heme b under the conditions described in Materials and Methods. The initial rate of cytochrome c reduction was corrected for antimycin-insensitive activity. The decrease in the reaction rate during incubation with DCCD is shown as a percentage of the rate measured at zero incubation time (●———●). Samples of the incubated enzyme were taken at the times indicated and either subjected to electrophoresis or used for estimation of binding of [^{14}C]DCCD to the enzyme as described under Materials and Methods. The band corresponding to the cross-linked material was integrated and the integrals are shown as a percentage of the value after 50 min of incubation (■— — —■). Radioactivity of covalently bound [^{14}C]DCCD is presented in cpm following corrections for protein content of the samples and the radioactivity of the zero incubation time sample (*–·–·–*).

that of inhibition, this difference being especially noticeable during the first minutes of incubation (see Fig. 3). On the other hand, the cross-linking phenomena correlated kinetically with the inhibition (Fig. 3), suggesting that these two processes may be coupled.

In order to investigate the effects of DCCD on the H^+-pumping activity of the cytochrome b-c_1 complex, the enzyme was reconstituted into phospholipid vesicles. When assayed using the same medium as for the isolated enzyme, the ubiquinol : cytochrome c reductase activity of the reconstituted cytochrome b-c_1 complex was considerably higher (15 electrons/s per heme b). In general, full activity of the cytochrome b-c_1 complex requires the presence of lipid [12], due partly to the lipid solubility of most quinones and partly to restoration of lipid-protein interactions important for the normal functioning of the enzyme. For measurements of H^+ translocation, however, an unbuffered medium had to be used (see Materials and Methods). In this medium the turnover number of the enzyme was lowered significantly (1 electron/s per heme b).

Oxidation of DBH led to the appearance of protons in the external medium. Comparison of the rates of H^+ appearance and cytochrome c reduction gave a ratio of approx. 2 H^+ per electron (1.91 ± 0.16, the mean value and S.D. of nine determinations). In the presence of CCCP this ratio was diminished to 1 (1.03 ± 0.07, the mean and S.D. for nine determinations), thus indicating that one proton was extruded from the vesicle interior for each electron reducing cytochrome c. Appearance of H^+ in the external medium was dependent on the presence of valinomycin and K^+ to prevent the buildup of transmembrane electrical potential. A large internal buffering power was considered important to avoid excessive intravesicular alkalinization.

As shown in Fig. 4, incubation of the vesicles with DCCD led to a time-dependent inhibition of electron-transport activity though at a higher DCCD concentration than with the isolated enzyme. In addition, there was a parallel inhibition by DCCD of the rate of H^+ translocation, though the ratio of approx. 2 H^+ appearing externally per electron (i.e., 1 H^+ translocated vectorially per electron) remained unchanged. The latter observa-

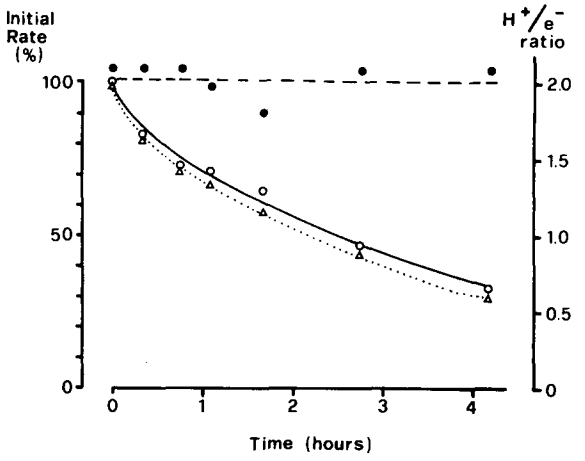

Fig. 4. Time course of the inhibition by DCCD of electron-transfer and proton-translocation activities of purified cytochrome b-c_1 complex reconstituted into asolectin vesicles. Duplicate samples of the vesicles were incubated with either ethanol or DCCD at the ratio of 400 mol per mol heme b under the conditions described in Materials and Methods. The initial rates of electron transfer (○———○) and proton translocation (△·····△) of the DCCD-inhibited samples are shown as percentages of the rates measured with corresponding control samples. The figure shows also the ratios between the rates of proton appearance and electron transfer (H^+/e^- ratio, ●— — —●).

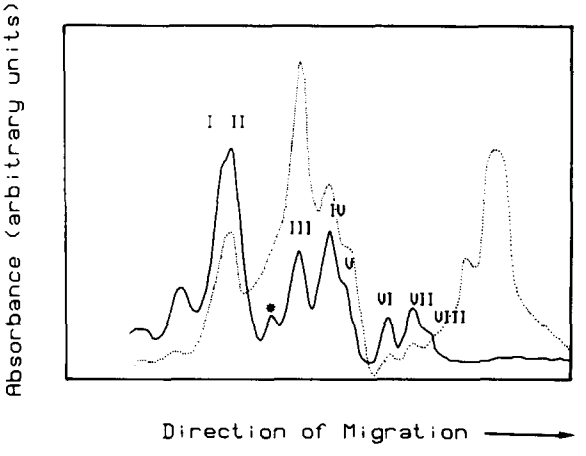

Fig. 5. Identification of the [^{14}C]DCCD-binding subunit(s) in the reconstituted cytochrome b-c_1 complex. Cytochrome bc_1 vesicles were incubated with [^{14}C]DCCD at the ratio of 400 mol per mol heme b for 2 h at 35°C. The samples for SDS-polyacrylamide gel electrophoresis were prepared as described in Ref. 19. The slab gel was fixed, stained with Coomassie brilliant blue, destained as described in Ref. 17 and analysed by autoradiography as detailed in Ref. 20. The dried gel was scanned for Coomassie blue at 570–530 nm (———) and the autoradiogram was scanned at 590–480 nm (·····). Roman numerals represent established subunits of the cytochrome b-c_1 complex and the asterisk denotes the cross-linked material.

tion excludes uncoupling of the vesicle membrane by DCCD.

Solution of DCCD in the membrane lipid would have the effect of lowering the local concentration of DCCD close to the reconstituted enzyme. Instead, the isolated enzyme was incubated with DCCD in the absence of added lipid. This difference in incubation conditions may explain the requirement of the reconstituted system for higher DCCD concentrations to obtain inhibition.

The inhibitory effects of DCCD on the reconstituted cytochrome b-c_1 complex were accompanied by cross-linking of subunits V and VII (as with the isolated enzyme) and by the covalent binding of [^{14}C]DCCD preferentially to cytochrome b (see Fig. 5), although several other subunits were also clearly labelled. To determine whether the binding of DCCD or the DCCD-induced cross-linking were related to the observed inhibition, the time courses of these processes were compared (see Fig. 6). This experiment showed that the covalent binding of DCCD was complete already after 1 h of incubation, whereas the onset of both inhibition of H^+ translocation and the formation of cross-linked material continued for at least 4 h. Thus, only cross-linking and not binding of DCCD correlated kinetically with the inhibition of enzymic activity of the reconstituted cytochrome b-c_1 complex.

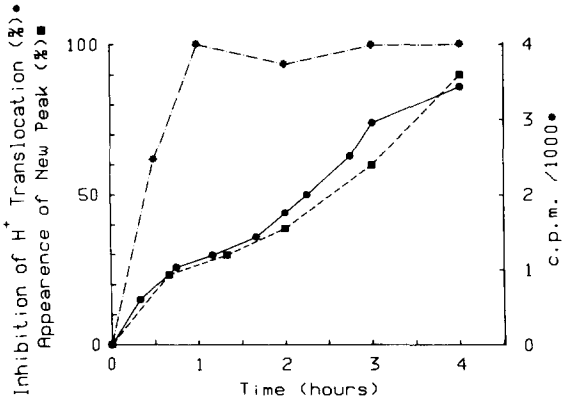

Fig. 6. Comparison of the time courses of inhibition by DCCD of H^+ translocation by the reconstituted cytochrome b-c_1 complex, of the appearance of a cross-linked material and of binding of [^{14}C]DCCD to the reconstituted enzyme. Proteoliposomes were incubated with DCCD at the ratio of 400 mol per mol heme b under conditions described in Materials and Methods. The initial rate of H^+ extrusion measured with the zero incubation time sample was taken as full activity and the percent inhibition relative to this is shown (●———●). Samples of the incubation mixture were also taken and analysed for changes in the subunit pattern of the enzyme and of covalent binding of [^{14}C]DCCD. The new band of cross-linked material was integrated and the integral values were normalized against the corresponding integrals of the two core proteins. The integral of the cross-linked band is presented as a percentage of its value after 4 h of incubation. The latter was assumed to be approx. 90% of the maximal integral of fully cross-linked subunits V and VII (■— — —■). The radioactivity of covalently bound [^{14}C]DCCD is presented in cpm. These values were corrected for protein content and for the radioactivity found in the zero incubation time sample (*–·–·–*).

Discussion

The effects of DCCD on the isolated cytochrome b-c_1 complex

DCCD is a well established reagent for the modification of chemical groups in proteins. Initial activation of groups such as carboxylic, phosphate or thiol may have a number of consequences [31]. The dicyclohexyl-*O*-isourea formed is highly vulnerable to nucleophilic attack from either water or some other nucleophile located in close proximity. Only the latter would result in the formation of a new covalent bond within the protein, but in both cases dicyclohexylurea would be generated. An alternative but much slower process is the shift of the activated group to one of the N atoms (the so-called '*N*-acyl shift') resulting in the stable binding of the dicyclohexylurea to the protein. The requirement of this process for the absence of nucleophiles in the vicinity of the activated group dictates that it can only occur in a highly hydrophobic environment.

The incubation of DCCD with the isolated cytochrome b-c_1 complex results in the cross-linking between subunits V and VII. This would indicate that the polypeptides involved are in close contact. As there is a direct correlation between the kinetics of inhibition of ubiquinol : cytochrome c reductase activity by DCCD and of cross-linking, it seems that these two phenomena may be linked. One should be aware of the fact, however, that a nondetectable internal cross-linking within one of the subunits may also have occurred. Nonspecific covalent binding of [^{14}C]DCCD to the isolated cytochrome b-c_1 complex was also observed. This differs from the findings of Beattie

and Clejan [9] for the isolated enzyme from yeast, namely, a preferential binding of DCCD to cytochrome *b*. Moreover, the kinetics of DCCD binding did not correlate with those of inhibition (Fig. 3), indicating that these two phenomena are unrelated.

The effects of DCCD on the reconstituted cytochrome b-c_1 complex

In the cytochrome *b*-c_1 complex reconstituted into phospholipid vesicles, the electron-transport activity is coupled to vectorial H^+ translocation, though the mechanism of the latter phenomenon has not yet been clarified.

Two models have been proposed for H^+ translocation by the cytochrome bc_1 region of mitochondria. The proton-motive Q-cycle [32] proposes a sequence of chemical reactions vectorially organized in the inner mitochondrial membrane in such a way that protons are taken from the matrix and released into the external phase. On the other hand, the indirectly coupled H^+-pump model [33,34] suggests that the energy released on ubiquinol oxidation is conformationally coupled to H^+ translocation through the enzyme.

In the case of reconstituted vesicles, it is more difficult to envisage how H^+ translocation could occur via a Q-cycle-type mechanism; the vital formation of ubisemiquinone on the inner side of the membrane may not occur here, since the substrate is added externally as the fully reduced quinol. Our observations would thus be more easily reconciled with an H^+-pump-type model. The parallel inhibitions by DCCD of electron transport and H^+ pumping would be consistent with the fact that there is no change in the ratio of the number of protons translocated per electron (2 H^+ appearing per e^- transported, i.e., 1 H^+ extruded from the vesicle interior), despite inhibition by DCCD of the rate of H^+ extrusion.

Price and Brand [8], on the other hand, found that DCCD caused a decrease in the H^+/e^- ratio in intact mitochondria. Such a decrease would also explain the observation of Lenaz et al. [7] that DCCD inhibited H^+ translocation by the cytochrome *b*-c_1 complex in submitochondrial particles, although the rate of succinate oxidation was unaffected by the reagent. The apparent discrepancy between the findings reported by these workers and us may be accounted for as follows. It is widely agreed that the ratio of the number of H^+ translocated vectorially per electron at the level of the cytochrome *b*-c_1 complex in mitochondria is 2. In reconstituted vesicles, however, this number was found to be 1 by us, by Leung and Hinkle [35] and by Beattie and Villalobo [10]. It has been proposed [8] that there may be two distinct pathways of H^+ translocation in the cytochrome bc_1 region of the mitochondrial respiratory chain, one sensitive, the other insensitive to DCCD and each contributing one H^+ to the overall stoichiometry. It may be that in reconstituted vesicles only the DCCD-sensitive system is operating. Thus, DCCD would lead to full inhibition of H^+ pumping in reconstituted cytochrome cytochrome *b*-c_1 complex vesicles, but only a partial inhibition in mitochondria.

As shown above, the inhibitory effects of DCCD on the reconstituted cytochrome *b*-c_1 complex were accompanied by two types of structural alteration. First, subunits V and VII were cross-linked and second, DCCD was bound to the complex, preferentially to cytochrome *b*. Fig. 6 shows, however, that whilst cross-linking occurs with a similar time course to that of inhibition, binding of DCCD is much more rapid. This observation indicates that the inhibition was not related to the binding, but rather to the cross-linking phenomenon. Our conclusions differ from those of Beattie and Clejan [9] who postulated a role of cytochrome *b* in H^+ translocation, based on its binding of DCCD without, however, demonstrating a kinetic correlation between DCCD binding and inhibition of enzymic activities.

DCCD-induced cross-linking between the iron-sulfur protein and subunit VII of the reconstituted cytochrome *b*-c_1 complex indicates that these two polypeptides must be in close contact also in the membrane-bound enzyme.

In conclusion, our data show that reconstituted cytochrome cytochrome *b*-c_1 complex is inhibited in both its electron-transport and H^+-translocation activities following treatment with DCCD and that this inhibition can be correlated with cross-linking of subunits V and VII. We would like to stress, however, that the multiplicity of structural alterations caused by DCCD in this system (intersubunit cross-linking, covalent binding and

possibly intrasubunit cross-linking) suggests that extreme care is required in correlating the structural and functional effects of this substance.

Acknowledgments

The authors wish to thank Dr. B. Trumpower for the generous gift of DBH. This study was supported by grant N. 3.739-080 from the Schweizerischen Nationalfonds and by the Sandoz Stiftung.

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