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Triple inhibitor titrations support the functionality of the dimeric character of mitochondrial ubiquinol-cytochrome *c* oxidoreductase

P. Nieboer and J.A Berden

E.C. Slater Institute for Biochemical Research, University of Amsterdam, Amsterdam (Netherlands)

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The ubiquinol-2 or duroquinol oxidoreductase activity of mitochondrial ubiquinol-cytochrome *c* oxidoreductase was titrated with combinations of antimycin, myxothiazol and *N,N'*-dicyclohexylcarbodiimide (DCCD). A statistical model has been developed that can predict the activity of the complex treated with all possible combinations of these inhibitors. On the basis of the measured titration curves the model had to accommodate interaction between the two protomers of the complex. The titrations confirm that treatment with DCCD results in the modification of a certain site in one of the two promoters of the bc_1 dimer, thereby blocking one antimycin A binding site without inhibiting electron transfer. Modification of both antimycin A binding sites of the dimer is apparently required for inhibition of electron transfer through the complex, just as modification of both myxothiazol-binding sites is required for full inhibition. The conclusion can be drawn that mitochondrial ubiquinol-cytochrome *c* oxidoreductase is a functional dimer, consisting of electrically interacting protomers.

Introduction

The bc_1 complex of several organisms has been studied intensively. The mitochondrial enzyme catalyses the electron transfer from ubiquinol to cytochrome *c* and, coupled to this activity, it translocates protons across the inner mitochondrial membrane from the matrix to the cytoplasmic side [1]. From the study of two-dimensional crystals [2] and from sedimentation velocity experiments [3] we know that the isolated complex is a structural dimer. The redox behavior [4,5] and EPR studies of the redox components of the enzyme [5] indicate that the dimeric structure is also present in the intact membrane. In the past, the requirement of a dimeric structure for electron transfer and proton translocation has been extensively discussed [6–10].

The study presented here shows that the inhibitor titration curves of the quinol : cytochrome *c* oxidoreductase activity of sub-mitochondrial particles cannot be understood and simulated without the assumption of a functional dimer. The fact that in sub-mitochondrial particles most bc_1 complexes are oriented inside-out has no effect on the rate of reduction of added cytochrome *c* or the shape of the inhibition curves, under the experimental conditions used.

The finding that DCCD affects the antimycin-binding site of only one of the two protomers of the dimer presented a good tool to formulate the description of the complex titration behaviour, obtained with the use of these inhibitors in various concentrations in combination with myxothiazol.

Correspondence: J.A. Berden, E.C. Slater Institute for Biochemical Research, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DQH_2, duroquinol.

Materials and Methods

Materials and preparations

DCCD and horse-heart cytochrome *c* (Type VI) were obtained from Sigma, USA. Myxothiazol and Antimycin A from Boehringer Mannheim and Duroquinol from ICN. Ubiquinol-2 was prepared in our laboratory by A.F. Hartog.

Sub-mitochondrial particles from bovine heart were prepared using the standard procedures [11–13] and stored in small aliquots submerged in liquid nitrogen. Only the amount of particles needed for an experiment was thawed at the time and superfluous particles were discarded.

Activity measurements

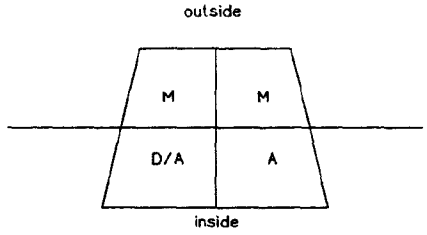
The reaction between ubiquinol-2 or duroquinol and cytochrome *c* was recorded using an Aminco DW2 dual-wavelength spectrophotometer, equipped with a Poptronics ADC412 data-acquisition system. The reduction of cytochrome *c* was followed at the wavelength pair 550 nm minus 540 nm. The reaction was started by the addition of cytochrome *c* to the stirred cuvette. The reaction medium consisted of 100 mM potassium phosphate buffer (pH 7.4), 50 μM duroquinol or 20 μM ubiquinol-2, 2 mM KCN and 15 μM cytochrome *c*. The concentration of the sub-mitochondrial particles was between 0.2 and 2 mg of protein per ml. As background reaction, the reaction between the two substrates was measured in the presence of sub-mitochondrial particles and a two-fold excess of both myxothiazol and antimycin A.

The activity of the SMPs at the concentrations of substrate and acceptor mentioned above was 1.3 μmol cytochrome *c* min^{-1} mg^{-1} with DQH_2 as substrate and 5.1 μmol min^{-1} mg^{-1} when Q_2H_2 was used as substrate. The turnover numbers were 64 and 250 s^{-1}, respectively.

Inhibitor treatment

DCCD was added at a concentration of 100 μM, and incubated for 30 min on ice before addition of the sample to the assay mixture. The protein concentration of the SMP during incubation was between 20 and 50 mg protein ml^{-1} (protein according to Lowry [14]). With this procedure the binding of DCCD to the complex was maximal. Various levels of DCCD occupation were reached by mixing DCCD-treated particles with particles not treated with DCCD, immediately after incubation and just before the preparation of the assay mixture.

Antimycin and myxothiazol were added, in appropriate concentrations, to the assay mixture 5 min prior to the start of the activity measurement, induced by addition of oxidized cytochrome *c*.



A = antimycin A
D = N,N dicyclohexylcarbodiimide
M = myxothiazol

Fig. 1. Model of the dimeric ubiquinol:cytochrome *c* oxidoreductase. This figure indicates the occupation possibilities of the four binding sites of the dimer. A, antimycin A; D, *N*, *N'*-dicyclohexylcarbodiimide; M, myxothiazol.

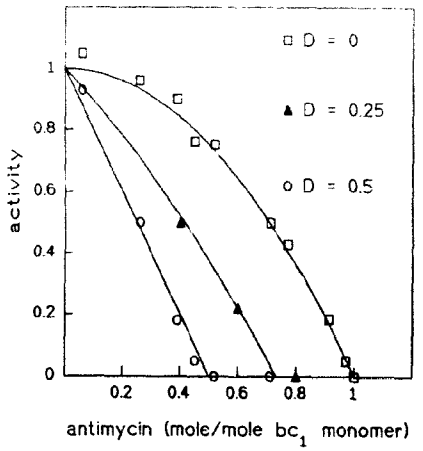


Fig. 2. The titration with antimycin of the rate of cytochrome *c* reduction by DQH_2 in sub-mitochondrial particles in combination with various amounts of bound DCCD. The reduction of cytochrome *c* (15 μM) by duroquinol (40 μM) was measured as described in Materials and Methods. The DCCD concentration (D) is expressed as the relative quantity of DCCD-modified bc_1 monomers. The plotted curves correspond to the predicted values according to the model.

The model

In order to describe the complex titration behaviour of the electron transfer activity of the bc_1 complex we developed a model on the basis of the following assumptions: (1) The model has to take into account all the possible ways of occupation of the bc_1 complex that occur when antimycin A, myxothiazol and/or DCCD are added in various amounts and combinations. Fig. 1 gives a schematic representation of the bc_1 dimer. The possible sites of occupation for the three inhibitors used are indicated. Myxothiazol (M) can occupy two binding sites at center o (outside), antimycin A (A) can bind to both center i (inside) sites. (2) The effect of DCCD is described as the result of modification of one of the two center i sites, thereby blocking the binding of antimycin A to this site [15]. In order to construct the model several additional assumptions were made:

(a) The inhibitors antimycin and myxothiazol bind with very high affinity, such that all added inhibitor (when the amount is (sub)stoichiometric) is bound to the enzyme. DCCD binds covalently, so the binding of DCCD is irreversible.

(b) No cooperativity takes place. Bound inhibitor does not influence the binding of additional inhibitor to unoccupied sites. The cooperativity of the binding of antimycin reported in [16] does not, within experimental error, affect the level of bound ligand due to the very high binding constants of both the T-state and R-state. Also, the shape of the inhibition curve is not significantly influenced by the cooperativity when

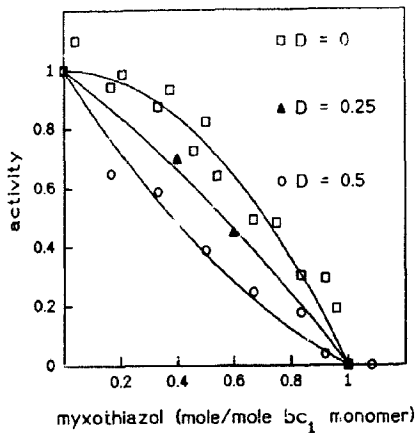


Fig. 3. Titration with myxothiazol of the rate of cytochrome *c* reduction by DQH_2 in sub-mitochondrial particles, in combination with various amounts of bound DCCD. The reduction of cytochrome *c* (15 μM) by duroquinol (40 μM) was measured as described in Materials and Methods. The DCCD concentration (D) indicated is expressed as the relative quantity of DCCD-modified bc_1 monomers. The plotted curves correspond to the theoretical values predicted by the model.

quinol rather than succinate is the substrate (cf. Ref. 17).

(c) There is no preference for one of the two possible binding sites for each inhibitor.

Results

Titrations with antimycin and myxothiazol in the presence of various amounts of DCCD

Titration of electron transfer activity of the mitochondrial bc_1 complex in sub-mitochondrial particles with either myxothiazol or antimycin results in a hyperbolic inhibition curve. This shape can either be due to an overcapacity of the inhibitor-sensitive step or to the requirement that both binding sites of the dimer be occupied to block electron transfer. Using combinations of inhibitors, e.g., myxothiazol and DCCD (Fig. 3), overcapacity as the source of hyperbolicity can be excluded. Duroquinol and ubiquinol-2, substrates with different affinities for bc_1, gave the same hyperbolic titration curves.

DCCD alone has no effect on electron transfer through the bc_1 complex of sub-mitochondrial particles at the concentrations we used (50 μM–1 mM). Only at very high concentrations inhibition of electron transfer occurs [15], probably due to cross-linking [18,19]. At the concentrations we used, however, DCCD completely blocks the reduction of cytochrome *b* via centre i (in the presence of myxothiazol, see Ref. 15) without affecting steady-state electron transfer from quinol to cytochrome *c*. After DCCD treatment only half stoichiometric binding of antimycin A to the complex is possible [18] indicating that DCCD modifies half of the antimycin binding sites. Also the EPR signal of the antimycin-sensitive semiquinone is reduced to half its amplitude [15]. As is clear from Fig. 2, DCCD treatment influences the shape of the antimycin titration curve drastically. Also the curvature of the myxothiazol titration curve is strongly affected by DCCD (Fig. 3).

The model

The model predicts the activity of each possible population of the inhibitor-treated bc_1 complex by using simple statistical rules. In order to simplify the graphical presentation of the model we divided the populations into two groups: the populations treated with DCCD (Fig. 4) and those that were not treated with DCCD (Fig. 5). In the Figs 4 and 5 the activity of each population is indicated with a number between 0 and 1, corresponding with no and full activity, respec-

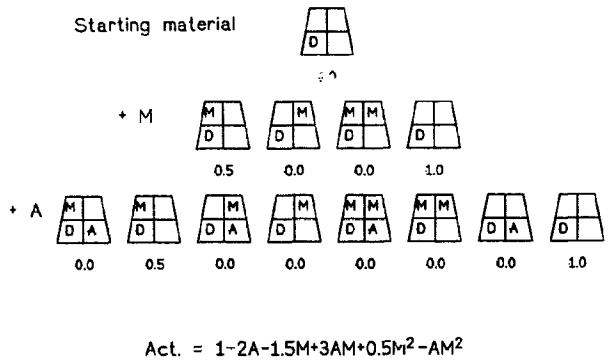


Fig. 4. All possible occupations of the ubiquinol:cytochrome *c* oxidoreductase dimer with antimycin and myxothiazol after treatment with DCCD. Each figure corresponds to one of the possible occupations. The number beneath each figure indicates the activity of the indicated form of enzyme, zero corresponding to no activity and one corresponding to full activity. The expression given for the activity of the enzyme is based on simple statistical rules, as explained in Results.

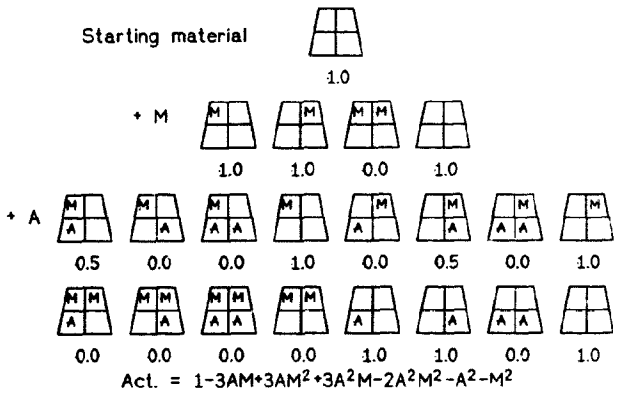


Fig. 5. All possible occupations of a ubiquinol:cytochrome c oxidoreductase dimer with antimycin and myxothiazol. Each figure corresponds with one of the possible occupations. The number beneath each figure indicates the activity of the indicated form of enzyme, zero corresponding with no activity and one corresponding with full activity. The expression given for the activity of the enzyme is based on simple statistical rules, as explained in Results.

tively. For some of these populations we know from previous experiments the activity factor:

Full activity bc_1 complex in the absence of any inhibitor or containing only DCCD bound to the enzyme.

No activity All populations of enzyme to which myxothiazol or antimycin A is bound at stoichiometric amounts (= 2 mol per dimer).

Half activity It is known that the bc_1 complex can function as a monomer [20]. So, if one of the protomers of the dimer is fully occupied (both centers i and o occupied) the activity will be half.

The activity of the other populations was indicated by the shape of the several titration curves presented in this section. With the presented model it is possible to predict the activity of the bc_1 complex treated with

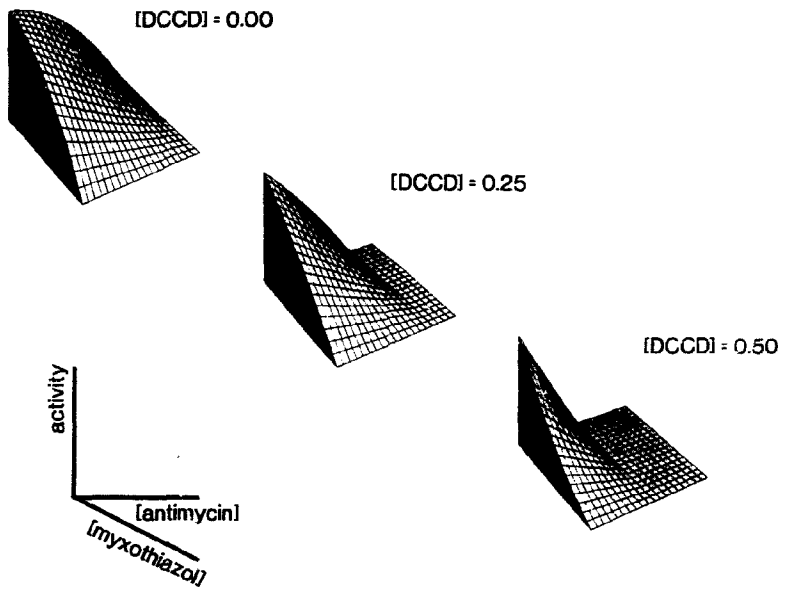


Fig. 6. Three-dimensional representation of the effect of combinations of antimycin, myxothiazol and DCCD on the duroquinol:cytochrome c oxidoreductase activity of the mitochondrial bc_1 complex. The three three-dimensional plots show the effect on the electron transfer activity of the mitochondrial bc_1 complex of all possible combinations of antimycin and myxothiazol, for various amounts of bound DCCD, as predicted by the model.

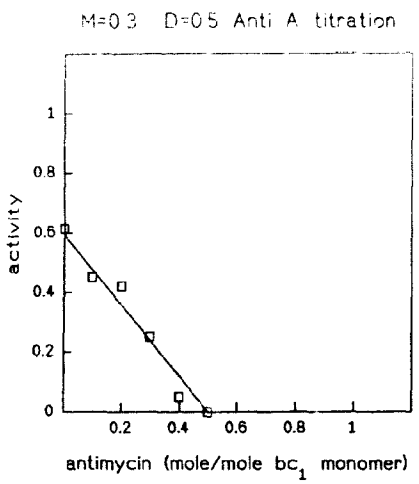


Fig. 7. Titration with antimycin of the electron transfer activity of the bc_1 complex after partial occupation with myxothiazol and full occupation with DCCD. Duroquinol: cytochrome *c* oxidoreductase activity was measured, as explained in Materials and Methods. The plotted curve corresponds to the values predicted by the model. The concentrations of bound myxothiazol (M) and DCCD (D) are indicated at the top of the figure.

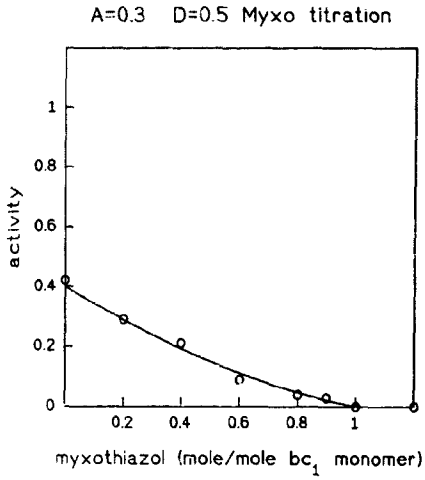


Fig. 9. Titration with myxothiazol of the electron transfer activity of the bc_1 complex after partial occupation with antimycin and full occupation with DCCD. Duroquinol: cytochrome *c* oxidoreductase activity was measured as explained in Materials and Methods. The plotted curve corresponds to the values predicted by the model. The concentration of bound antimycin (A) and DCCD (D) are indicated at the top of the figure.

each possible combination of myxothiazol, antimycin A and DCCD. The formula gives the mathematical behaviour of the model:

$$\text{Activity} = \{2D*(1-2A-1.5M+3AM+0.5M^2-AM^2)\}+\{(1-2D)*(1-3AM+3AM^2+3A^2M-2A^2M^2-A^2-M^2)\}.$$

A is normalized concentration of bound antimycin ($0 \leqslant A \leqslant 1$); M is normalized concentration of bound myxothiazol ($0 \leqslant M \leqslant 1$); D is normalized concentration of bound DCCD ($0 \leqslant D \leqslant 0.5$).

Fig. 6 gives a representation of the predicted activity as a function of the amounts of bound inhibitor.

The curves in Figs. 2, 3 and 7–10 correspond to the theoretical model presented in Materials and Methods. Figs. 2 and 3 correspond to all the possible flanks of the three-dimensional plots (Fig. 6).

In Figs. 7–10 several cross-sections of the model are plotted and compared with the measured activities. From Figs. 2, 3 and 7–10 it can be concluded that the presented model corresponds well with the experimental results.

In order to obtain electron transfer data for the situation where the occupation with DCCD is half, a mixture was made of DCCD-treated sub-mitochondrial particles and normal sub-mitochondrial particles. Since DCCD reacts very slowly with the bc_1 complex it was possible to mix the two types of particles prior to the addition of myxothiazol and/or antimycin A. Also, the dilution of DCCD after addition to the cuvette gives us the certainty that unbound DCCD has no opportunity to modify the bc_1 complex of the sub-mitochondrial particles that are not treated with DCCD. The dilution cannot result in dissociation of DCCD since DCCD binds covalently to the bc_1 complex. Mixing DCCD-treated particles with DCCD-free particles results in an inhomogeneous population of particles. But since

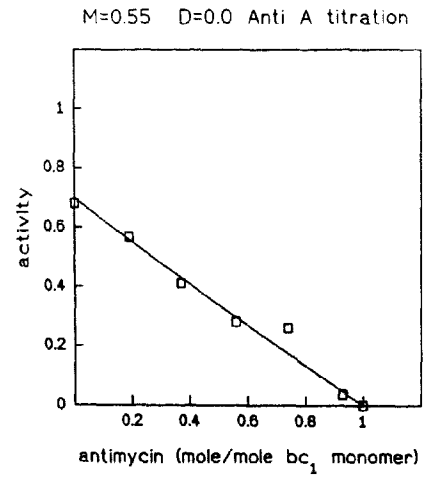


Fig. 8. Titration with antimycin of the electron transfer activity of the bc_1 complex after partial occupation with myxothiazol. Duroquinol:cytochrome *c* oxidoreductase activity was measured as explained in Materials and Methods. The plotted curve corresponds to the values predicted by the model. The concentrations of bound myxothiazol (M) and DCCD (D) are indicated at the top of the figure.

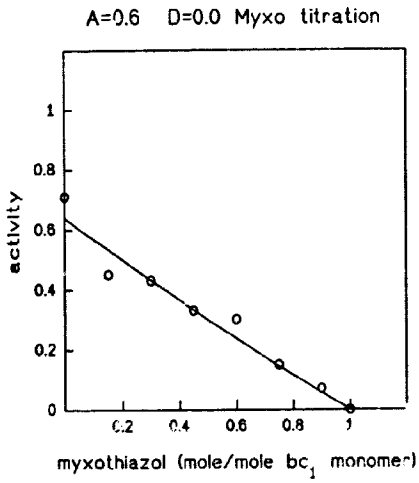


Fig. 10. Titration with myxothiazol of the electron transfer activity of the bc_1 complex after partial occupation with antimycin. Duroquinol:cytochrome *c* oxidoreductase activity was measured as explained in Materials and Methods. The plotted curve corresponds to the values predicted by the model. The concentration of bound antimycin (A) and DCCD (D) are indicated at the top of the figure.

bc_1 complexes within one particle do not influence each other kinetically, this situation is kinetically equal to a situation in which each particle contains both DCCD-containing and DCCD-free bc_1 complexes.

Discussion

The development of the model

In order to explain our experimental results we had to develop a model that corresponds with the measured values for activity. Simple antimycin A or myxothiazol titrations correspond to the curve with the formula $1 - X^2$ were X is A or M (see definitions of A and M in Results). Because of the experimental scatter in the values for electron transfer activity (Figs. 2 and 3) such a formula is not strict. Many other formulae can be used to describe the same experimental data.

DCCD, when used to its full extent, has a marked impact on the shape of the antimycin titration curve (Fig. 3, $D = 0.5$). After use of DCCD the antimycin titration curve is a straight line, ending at half stoichiometric antimycin for full inhibition. This leads to the conclusion that DCCD occupies half of the antimycin binding sites [15,18]. DCCD does not inhibit electron transfer, in contrast to antimycin. The effect of DCCD can be explained with a model, according to which only one of the antimycin A binding sites of the dimer is responsible for inhibition of the bc_1 complex by antimycin A. From the experiment it is not clear whether DCCD specifically binds to the non-inhibitory antimycin A binding site or that, upon binding of DCCD, the site not occupied by DCCD becomes the inhibitory one. The bc_1 dimer contains two antimycin binding sites, to one of which DCCD can bind. DCCD seems to have a preference for one of the sites whereas antimycin A has no preference.

This effect of DCCD on antimycin A binding led to the development of a model that predicts the activity of the complex under all the conditions of occupation of the inhibitory sites. In order to explain all the experimental results it became clear what activity should be ascribed to the several possible populations. Because of the limitations imposed by the triple inhibitor titrations only one model could be found that corresponds to all the experimental data.

In analogy with the effect of DCCD and antimycin A it is assumed also that only one of the myxothiazol binding sites results in full inhibition. This leaves us with the special case, not yet described in this article, were the outside of protomer 1 is occupied in combination with the inside of protomer 2. From the experimental data it became clear that these populations have no enzymatic activity.

An alternative model, in which overcapacity explains the hyperbolic shape of the antimycin- and myxothiazol-titration curves, and no electrical interaction between the two protomers exists, cannot explain the experimental data. In such a model DCCD treatment cannot influence the hyperbolic shape of the myxothiazol titration curve.

From the performed experiments, in combination with the model, we can conclude that the bc_1 complex is a functional dimer. However, this model describes the behaviour of the complex on a statistical basis and does not give any elucidation of the internal reactions. Nevertheless, it is clear that the bc_1 complex is a functional dimer in which the electrical interaction of the two protomers occurs between the two centers i and the two centers o not at a step in between these reaction centers. The model also does not distinguish between a fixed location of either antimycin or myxothiazol and a possible rapid exchange of these inhibitors between the two protomers of a dimeric unit.

The fact that DCCD can only bind to one of the two protomers, and the finding that it does inhibit proton translocation [21–24] without inhibiting electron transfer, leads to the question of whether a functional dimer is required for proton translocation. We may ask the question how the Q-cycle mechanism, which predicts a strict coupling between electron transfer and proton translocation, has to be modified in order to explain the effect of DCCD. In order to find answers to the above questions we are now performing experiments with the bc_1 complex incorporated in liposomes.

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