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Review

Reaction of dicyclohexylcarbodiimide with mitochondrial proteins

Ilmo E. Hassinen and Pertti T. Vuokila

Department of Medical Biochemistry, University of Oulu, SF-90220 Oulu, Finland

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Contents

I. Introduction

Ever since the original observation of Beechey and co-workers [10] on the oligomycin-like effect of dicyclohexylcarbodiimide (DCCD), this topic has been an object of keen interest. DCCD reacts with a specific dicarboxylic amino acid of the DCCD-binding proteolipid on the F_0 part of mitochondrial F_1F_0-ATPase or its bacterial counterpart. Carboxylic groups engaged in hydrophobic membrane-spanning proteins have become a popular model for a proton-conducting channel, and it may be more than a coincidence that all the mitochondrial proton pumping complexes react with DCCD, which results in inhibition of proton pumping. However, as described below, the paradigm behind the use of DCCD in search for proton channels or pump components, not just hydrophobic carboxyls, may not hold good in the light of more recent information.

Since a considerable period of time has elapsed since the last review on DCCD in this journal [2], it

Correspondence to: I.E. Hassinen, Department of Medical Biochemistry, University of Oulu, Kajaanintie 52 A, SF-90220 Oulu, Finland.
Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; complex I, NADH : ubiquinone oxidoreductase; complex III, ubiquinol : cytochrome-*c* oxidoreductase; complex IV, cytochrome-*c* oxidase; complex V, F_1F_0-ATPase; $\Delta E_h'$, redox potential difference; $\Delta\tilde{\mu}_{H^+}$, electrochemical potential of protons; $\Delta\psi$, membrane potential.

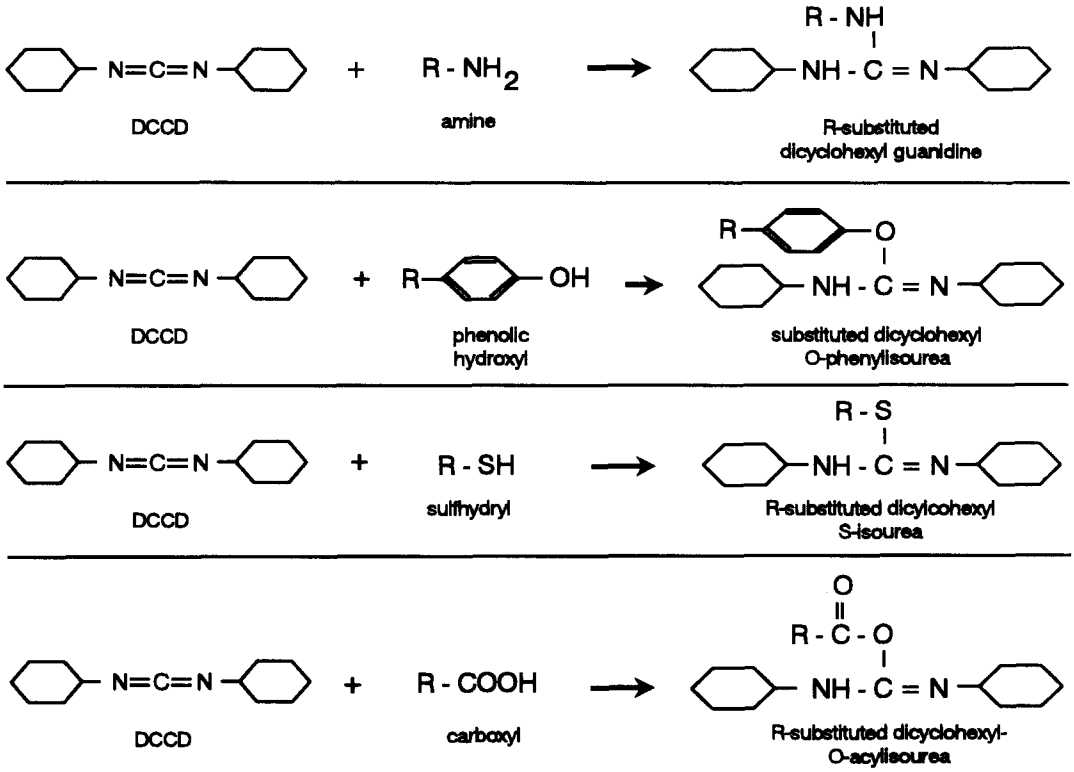

Fig. 1. Reactivity of dicyclohexylcarbodiimide (DCCD) with biomolecules.

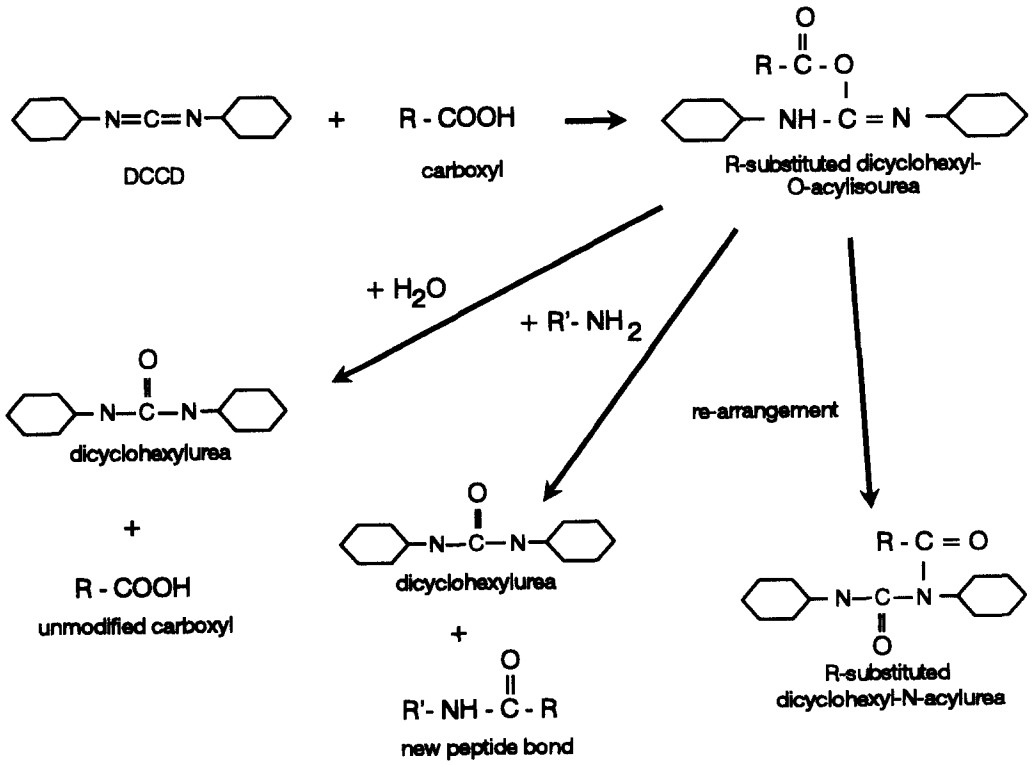

Fig. 2. Reactions of carboxylic groups with dicyclohexylcarbodiimide under different conditions. DCCD becomes permanently bound to the target carboxyl only in the absence of nucleophiles. If the attack is made by an amino-group, a peptide bond is formed. If water reacts before rearrangement of the initial DCCD adduct, dicyclohexyl urea is formed and the target left unmodified.

may be timely to have an update on the information gathered. Although it could have been helpful to give a full review of the structure and function of mitochondria as well, in order to describe the habitat of the DCCD-reactive proteins, this is not practicable without straying from the main issue of the review, which is a commentary on the paradigm of the usage of DCCD for studying mitochondrial proton translocation.

II. Chemistry

For reviews on the chemistry of the carbodiimides the reader is referred to the excellent treatises of Kurzer and Duraghi-Zadeh [70] and Azzi and coworkers [2,87]. We will therefore summarise the reactivity of carbodiimides only briefly in the present context:

DCCD reacts with amino, phenolic, hydroxyl, sulfhydryl and carboxyl groups (Fig. 1). In aqueous solution under neutral or acidic conditions the main reactive groups are the carboxyls and sulfhydryls. Tyrosines also react with DCCD under these conditions, but the rate is very slow [20].

A prominent feature is a reaction with free carboxylic groups which has led to industrial applications of the compounds as stabilisers. Their reactivity with carboxyl and amine groups also resulted in their use in the chemical synthesis of peptides [66] and in the coupling of affinity ligands to chromatographic supports. Water soluble carbodiimides are used for the latter purpose [32].

The reaction with carboxyl groups may have several consequences (Fig. 2). Binding to a carboxylic acid group forms dicyclohexyl-*O*-isourea, which is unstable and may be subjected to nucleophilic attack by water, in which case free dicyclohexylisourea is produced and the protein is not modified.

If the attacking group is an ϵ-amino group from a neighbouring amino acid, a peptide bond is formed. This results in intramolecular or intermolecular cross-link.

In the absence of nucleophiles the activated acyl group is shifted to one of the nitrogen atoms, resulting in stable binding of dicyclohexyl isourea into the protein. This leads to stable labelling of the protein, but can occur only in hydrophobic environments.

In most cases only covalent binding of the label (radioactivity or fluorescence [100]) to the protein under test is sought, because this would be an indication of the existence of a carboxylic group in a non-aqueous environment. Identification of the target polypeptide in a multienzyme complex necessitates polyacrylamide gel electrophoresis or a similar high-resolution separation method, and therefore intermolecular cross-linking would manifest itself. Intermolecular cross-linking by DCCD is not a common finding, however, and intramolecular cross-linking would be difficult to detect. DCCD has not acquired the status of a tool in the cross-linking approach employed in nearest-neighbour analysis of enzyme complexes, for which purpose bifunctional cleavable cross-linkers are more practicable [49,111]. Electrophoretic analysis of the intermolecular cross-linking of proteins is also problematic, because the mobility of the product may be anomalous and not that expected from the molecular weights of the components.

TABLE I

Binding of DCCD in mitochondrial membrane components

n.d., not determined.

Protein/subunit	M_r (kDa)	Binding residue	Reference
Complex I			
ND1 gene product	29	n.d.	140
'ASHI' subunit	19	n.d.	119
9.5 kDa subunit	9.5	n.d.	119
Complex III			
subunit VIII	8	Glu-53	14
cytochrome *b*		Asp-160[a]	126
Complex IV			
subunit III	23	Glu-90[a]	101
Complex V			
subunit *c* of F_0	7.6	Glu-58[a]	113
subunit β of F_1	52	Glu-215	40
NAD(P) transhydrogenase	120	Glu-257	142
		Glu-806[b]	142
Mitochondrial porin (VDAC)	30	n.d.	34
Inner membrane anion channel	n.d.	n.d.	9

[a] In bovine mitochondria.
[b] Not positively identified.

All of the proton-translocating enzyme complexes of the mitochondrial inner membrane react with DCCD, and also one of the outer membrane ion channels binds it (Table I). The individual mitochondrial proton-translocating complexes are discussed in the following in the light of DCCD sensitivity. For narrative reasons they are given in chronological order of the discovery of their DCCD interaction.

III. F_1F_0-ATPase

Mitochondrial F_1F_0-ATPase (ATP synthase) is composed of two major domains, the F_1 part, which contains the catalytic site(s), and the F_0 part, which is embedded in the membrane and houses the transmembrane proton channel.

III-A. Membrane sector

The membrane-embedded F_0 part is an oligomeric structure, the number of subunits is species-specific, and there is some uncertainty about the true number of similar or unlike subunits in the mitochondria. The subunit stoichiometry of F_0 is probably $a_1b_2c_{10-12}$ in *Escherichia coli* [45] and $a_1b_1c_{3-6}$ in the thermophilic bacterium PS3 [64]. Subunit *c* is a DCCD-binding proteolipid which has a molecular mass of 7.6 kDa and is a component of the proton-conducting channel. It is significant that modification of only one subunit in the oligomeric structure is enough to render the whole channel inoperative, indicating that the subunits function in a co-operative manner [66]. The DCCD-binding glutamic acid residue in the mitochondrial peptide (or aspartic acid residue in some bacterial counterparts) has been identified, and the reaction shows high specificity and can be completed at very low concentrations and low temperatures. The proton channel in *E. coli* is probably formed by both the *a* and *c* subunits of F_0. Arg-219, Glu-210 and His-245 in the *a*-subunit and Asp-61 in the *c*-subunit participate in proton transport [61]. The mitochondrial F_1F_0-ATPase contains 14 different subunits, of which seven or eight reside in the transmembranous F_0 part [56,121]. The F_0 subunits are *a* (ATPase-6), *b*, *c* (DCCD-reactive proteolipid), F_6, A6L (product of a similarly named gene in mtDNA), *d*, *e* and an inhibitory subunit [56,121]. The number of *c* subunits in mitochondrial F_0 is uncertain but has been estimated to be six [112]. The mitochondrial subunits F_6, *d*, *e*, A6L are 'supernumerary', and an inhibitory subunit A6L has a hydrophobic region which could constitute a membrane spanning helix [121]. The *a* subunit is a product of the *ATPase-6* gene of mtDNA. The oligomycin sensitivity-conferring protein (OSCP) is homologous with the bacterial δ counterpart of the F_1 domain [121].

Sensitivity to DCCD in the F_0 part is dependent on the Asp-61 in the *c* subunit in *E. coli* or a homologous glutamic acid residue in mitochondria. The chain length of the *c* subunit is variable, the mature form retaining the initiator (formyl)methionine in organisms like *S. cerevisiae* where the subunit is synthetized mitochondrially. In mammalian mitochondria the mature form is trimmed from products of two nuclear genes specifying precursors with different import sequences [49]. Mutation of Asp-61 in bacteria leads to proton tightness in the F_0 channel, while other mutations in this region can lead to steric changes preventing DCCD binding, so that the proton conductivity of the mutant channel is intact or shows reduced activity but has lost its DCCD sensitivity. The Ile-28 residue (in bacteria) is needed for DCCD binding at low concentrations, which means that the peptide chain folds back in the membrane and brings the Asp-61 and Ile-28 into proximity

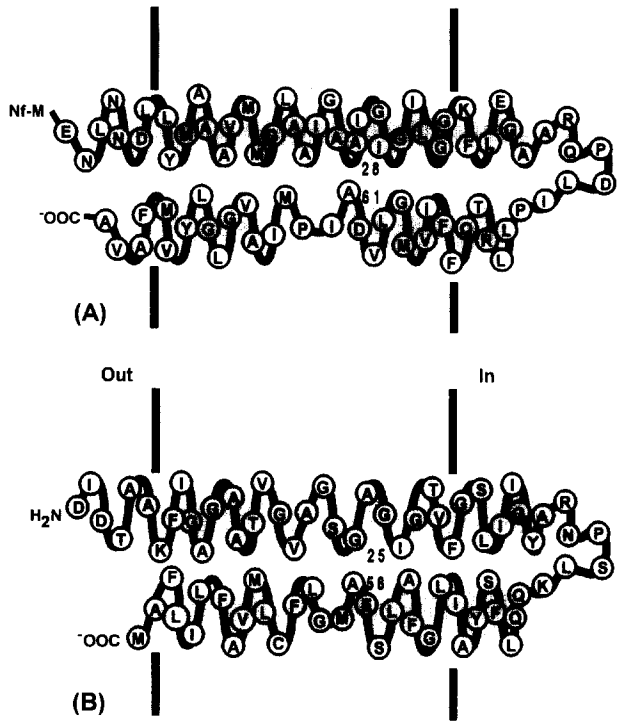

Fig. 3. Folding of the subunit *c* of F_1F_0-ATPase in the coupling membrane. The amino-acid sequences for (A) bovine heart and (B) *E. coli* are shown. The aspartate/glutamate residue homologous to D-61 in *E. coli* is the binding site for DCCD. Mutagenesis experiments on bacteria indicate that the isoleucine homologous to I-28 in *E. coli* is in the proximity of the DCCD reactive group on the other arm of the hairpin structure. The amino-acid four residues from the DCCD-reactive dicarboxylate amino acid in the direction of the carboxy-terminus is usually a helix-breaking proline, glycine or threonine, but the bovine heart mitochondrion has a glycine at a distance of three residues from the DCCD-reactive glutamate. Adapted from Refs. 45 and 49.

(Fig. 3). The determinants for oligomycin sensitivity in bacteria reside in the region of the invariant 'DCCD-binding' aspartate (for references, see Ref. 59). The essentiality of the carboxyl group function in the subunit *c* is emphasised by mutagenesis experiments which show that although elimination of Asp-61 results in proton tightness, a double mutation in which aspartate at position 61 is eliminated but one is generated at position 24, which is normally alanine (on the opposite arm of the peptide hairpin in the membrane) results in a cell growing on succinate at a 60% efficiency as compared with the wild type [79].

The DCCD-binding proteolipid is coded in the nucleus, except in *S. cerevisiae*, where the gene is found in mtDNA [16,78,112,113]. Molecular biology has not succeeded in revealing the ultimate mechanism of proton conduction, which remains unknown. The DCCD-binding proteolipid lacks the β-sheet configuration capable of forming a hydrogen bond lattice across the membrane to act as a proton conveyor [82] by analogy with the ability of ice to conduct protons. It has been suggested that water binding at the bend caused by a

helix-breaking threonine, glycine or proline at four residues from Asp-61 (Fig. 3) is related to proton conduction by analogy to some voltage-gated ion channels [12]. An oligomeric structure is apparently essential to proton conductivity.

III-B. Catalytic domain

ATP synthesis/hydrolysis on F_1F_0-ATPase occurs on the F_1 part of the complex, linked to the F_0 part by a stalk composed of the oligomycin sensitivity-conferring protein (OSCP), coupling factor 6 (F_6), parts of subunits *b*, γ, δ and possibly *d* and ϵ. F_1 has a subunit structure of $\alpha_3\beta_3\gamma\delta\epsilon$. The catalytic site resides in subunit β (with nucleotide binding sites in subunit α) and is also modified by DCCD, which inhibits ATP hydrolysis. The primary structure of the protein is known in several organisms. The DCCD-binding region contains three glutamic residues, of which two become labelled with DCCD in the bovine enzyme, but only one in the thermophilic bacterium PS3 [143]. When a homology search is performed between the DCCD-binding proteolipid and the β subunit of F_1, the surprising finding can be made that a homologue (394-DELSEEDK-401) of the DCCD-binding motif of the *c* subunit of F_0 exists but at a distance of 200 residues from the documented DCCD-binding region. Neither the DCCD-binding nor the DCCD proteolipid-like motifs are in a particularly hydrophobic region [143], which is in accord with observations, that DCCD label incorporation is sluggish and substoichiometric, probably because of the slow rearrangement of the *O*-acylisoureas initially formed or because of rapid nucleophilic attack by neighbouring groups or water molecules, resulting in release of the label. As DCCD binds to the active site of the enzyme, the geometry of the latter places constraints on rearrangement of the molecule. DCCD binding and inhibition is prevented by Mg^{2+} which is considered to be involved in the binding of adenine nucleotides [98]. Thus, the DCCD-reactive carboxyl may be the nucleotide-binding group of the active site.

It may prove significant that the DELSEEDK motif binds a lipophilic cation, the reactive aziridinium form of quinacrine mustard, a phenothiazine analogue with inactivation of the enzyme [18]. Thus, this homologous glutamate-rich sequence in the highly conserved region near the carboxy-terminus is involved in physiological modulation of the enzyme, probably by binding the inhibitor protein IF_1 [18].

IV. Proton-translocating oxidoreductases

IV-A. Cytochrome-c oxidase

Mitochondrial cytochrome-*c* oxidase is a multienzyme complex representing the superfamily of copper and heme-containing oxidases. The enzyme in eukaryotes is comprised of 9 to 13 subunits in one-to-one ratio [63]. The three largest subunits are encoded in mitochondrial DNA [1], the largest one of all (I) containing the ligands for heme *a* and the binuclear centre heme a_3-Cu_B. The low-potential copper (Cu_A) resides in subunit II, which also has a binding site for the substrate, cytochrome *c*.

In 1977 Mårten Wikström made the important observation that in addition to vectorial electron transport from cytochrome *c* on the outer face of the inner mitochondrial membrane to oxygen which is reduced on the matrix side, cytochrome oxidase pumps protons out of the mitochondrion [131]. The proton-consuming oxygen reduction of water has been modelled to fit experimental data and appears to involve an asymmetric cycle in which a two-electron reduced form of the bimetallic cytochrome a_3-Cu_B centre reacts with dioxygen to form a peroxidic adduct P. After being consecutively reduced by one electron to form a three-electron reduced ferryl species F and one more electron and upon consuming four protons, it reduces one O_2 molecule at a time [3]. Only the last two electron transfers are linked to proton pumping, and the model put forward by Babcock and Wikström focuses on the oxygen reduction site with Cu_B as a redox element in the pump. The current models imply that only two of the four electron transfers to dioxygen are productive in proton translocation, and this has been considered by Chan and Li [24] to mean that the pump must be able to distinguish between nonproductive or productive electron transfers or that only half of the electrons pass through the pump site. The former route may therefore be provided by cytochrome *a* and the latter by Cu_A. This, however, implies that the latter route must pump two protons per electron. Chan and Li [23] propose a model in which there is a direct and indirect pump site and the latter is provided by subunit III, to explain the fact that removal of subunit III reduces the proton pumping stoichiometry by one-half.

The chemistry of proton pumping by cytochrome oxidase remains entirely hypothetical at present, but one noteworthy observation is that DCCD interferes with proton pumping more than with electron transfer [21,22]. DCCD is bound to Glu-90 (in mammalian mitochondria) in an evolutionarily conserved motif in subunit III of cytochrome oxidase [101], the sequence being reminiscent of the DCCD-binding site of the *c* subunit of the F_0 part of mitochondrial ATP synthase [103]. This suggests a role for subunit III in proton pumping, but the experimental evidence has been inconsistent ever since the early observations. Subunit III is devoid of redox centres which, as described above, reside in subunits I and II. The model proposed by Chan and Li [23] also implies proximity of subunit III to Cu_A, the putative redox-linked proton pump site.

Cytochrome oxidase deficient in subunit III and incorporated in phospholipid vesicles appeared to have lost its proton pumping properties in certain preparations [108]. Finel and Wikström [44] have shown that ion exchange-purified cytochrome oxidase devoid of subunit III reconstituted in phospholipid vesicles still pumps protons although at low efficiency, and interpreted the result as meaning that subunit III is involved in retaining the oligomeric state of cytochrome oxidase and that only the dimeric enzyme is fully functional. It is not certain, however, whether dimerization is needed for proton translocation [81]. Native *Paracoccus denitrificans* cytochrome oxidase has three subunits [52] corresponding to the mitochondrially coded subunits in eukaryotes, but its two-subunit preparation lacking subunit III is still capable of proton pumping in a DCCD-insensitive process [103]. The *P. denitrificans* subunit III has been documented to bind DCCD both in membranes and in the isolated enzyme, but studies on the DCCD sensitivity of the proton pumping by the three-subunit *P. denitrificans* enzyme have not been reported. The DCCD-binding motif of *P. denitrificans* subunit III has been subjected to site-directed mutagenesis of the conserved DCCD-binding Glu-98, whereupon spheroplasts of the mutant strains were still capable of proton pumping [53]. Deletion of the subunit III gene from *P. denitrificans* results in inefficient assembly and low activity of the enzyme, which suggests a molecular chaperone role for subunit III [51].

Wilson and Bickar [136] consider that although native cytochrome oxidase is dimeric (and the dimerisation may be enhanced by subunit III), and although the removal of the third subunit does not completely abolish proton pumping, the III-less enzyme always has a markedly diminished capacity for proton pumping. They, therefore, suppose that subunit III acts as a part of a pathway or conduit which ensures that protons are taken up by the pump from the matrix side of the membrane. Thus, removal of subunit III would result in a situation in which the pump ejects protons to the cytosolic side but takes them up in a more randomised way, leading to a decrease in efficiency.

The pattern of DCCD labelling of plant mitochondrial cytochrome-*c* oxidase is different from that of mammalian or fungal mitochondria. It was recently reported by Asahi and co-workers [83] that in the seven-subunit enzyme of sweet potato two forms of subunit IV (IVa and IVb) bind DCCD, and it is possible that this plant has two *coxIII* genes.

IV-B. Ubiquinol: cytochrome-c oxidoreductase

The ubiquinol: cytochrome *c* oxidoreductase complex (complex III) of mitochondria is composed of 11 subunits: cytochrome *b*, cytochrome c_1 and the Rieske iron-sulphur protein, which contain the main redox centres, and the 47 kDa, 45 kDa, 13.4 kDa, 9.5 kDa, 9.2 kDa, 8 kDa, 7.2 kDa and 6.4 kDa subunits. Both the 13.4 kDa [145] and 9.5 kDa [144] subunits have been considered to be implicated in ubiquinone binding. The 9.2 kDa and 7.2 kDa proteins are associated with cytochrome c_1, and the former may also be involved in cytochrome-*c* binding.

The effects of DCCD on complex III have been studied by the groups of Lenaz [36,37], Beattie [7,27,28] Azzi [86] and Brand [17,99] and their results are at variance, so that there is no unanimous view regarding the site of binding or mode of action, and the kinetic and thermodynamic approaches give discrepant results.

In 1982 Lenaz and his co-workers [37] reported that DCCD inhibits proton translocation in rat-liver mitochondria and isolated complex III reconstituted in phospholipid vesicles, and similar findings were reported by Beattie and co-workers [27], who showed that DCCD blocks proton ejection in yeast mitochondria and in yeast complex III reconstituted into liposomes [7]. The findings regarding liver mitochondria have been confirmed [28], and similar observations were made with respect to bovine heart mitochondria, in which ^{14}C-labelled DCCD was bound to subunit VIII and cytochrome *b* [28]. Also in complex III from yeast mitochondria DCCD binds to cytochrome *b* [4,5]. The Lenaz group observed that the preferential binding in isolated solubilized bovine heart complex III occurs with subunit VIII [36].

Subunit VIII has an apparent molecular mass of 4–8 kDa and can be extracted with chloroform/methanol. Although Sidhu and Beattie [114] could not find the 8 kDa subunit in yeast complex III, it has been repeatedly found in the bovine heart, and Lorusso et al. [77] considered it an invariant component of complex III. They also confirmed the rapid labelling of the 8 kDa subunit with DCCD. In addition, DCCD can probably cross-link it, and a cross-linked, non-labelled 40 kDa product was also formed from the 12 kDa subunit with the Rieske iron-sulphur protein [77].

Borchart et al. [14] isolated an 8 kDa subunit from complex III and determined its amino-acid sequence. This 78-amino-acid peptide contains one aspartate and two glutamate residues, and the authors suggest that Glu-53, which is located in a β-strand on the membrane surface in a somewhat apolar region, is the DCCD binder, although this has not been verified experimentally. However, if one accepts the paradigm that DCCD binding occurs with a phylogenetically conserved hydrophobic motif in a transmembrane channel which may be involved in a proton pump, this should also be present in the 8 kDa subunit of complex III. A sequence somewhat reminiscent of that motif can indeed be found around Glu-39, but not around Glu-53, although as stated, its DCCD-binding property remains

to be experimentally verified. According to Borchart and co-workers [14], the 8 kDa subunit is in close proximity to cytochrome *b* and is co-isolated with it, but can be purified to homogeneity with their SDS-PAGE modification [109,110].

The binding of DCCD to cytochrome *b* itself has been in dispute for a long time, and has been considered by some workers to be non-specific. Only very recently, however, Wang and Beattie announced the binding of DCCD to Asp-160 of cytochrome *b* (unpublished results, cited in Ref. 126). There is a stretch around Glu-169 in bovine cytochrome *b* (but not in its human counterpart) that shows some homology to the DCCD-binding motif of the *c* subunit (proteolipid) of the F_1F_0 ATPase, while human cytochrome *b* (but not bovine) has a somewhat reminiscent region around Glu-111.

Although this review deals with mitochondrial DCCD-binding proteins, it is appropriate to note some recent information on the cytochrome b_6-*f* complex, which is the chloroplast homologue of mitochondrial bc_1 complex. In chloroplasts the homologue of cytochrome *b* is composed of two proteins: cytochrome b_6 and subunit IV. The former represents the N-terminal part of the corresponding mitochondrial peptide. It may be significant that the proton pumping function of the cytochrome b_6-*f* complex of spinach chloroplasts has been recently found to be inhibited by DCCD, as observed simultaneously in two laboratories [75,125, 126]. The pattern of this phenomenon is in many ways similar to that in the mitochondrial cytochrome bc_1 complex. 75% of maximum inhibition was obtained with 300 mol DCCD per mol of protein (based on cytochrome *f*). Li and co-workers [75] considered the Asp-155 or Glu-166 residues of cytochrome b_6 to be candidates for DCCD binding because they reside in a reasonably hydrophobic region on the lumen side of the thylakoid membrane according to the four-transmembrane helix model of Szczepaniak and Cramer [116]. Wang and Beattie [125] found the DCCD label in a 12.5 kDa peptide fragment also containing the haeme moieties. Sequencing confirmed that the label is indeed incorporated into Asp-155 or Glu-166. The proteolytic digestion which was the prerequisite for this work confirmed the correctness of the four-helix model of Szczepaniak and Cramer [116] for cytochrome b_6. This confirmed DCCD-binding site is not homologous to the corresponding site of the DCCD-binding proteolipid of F_0. Once more, a homology search through the published spinach cytochrome b_6 sequence [130] for the DCCD-binding motif leads to Glu-163 rather than Asp-153, which is not conserved in all cytochromes *b*. This residue corresponds to Glu-162 in bovine cytochrome *b* but not in the human equivalent. The DCCD-binding Asp-155 (Asp-153 according to Ref. 129) of chloroplasts is not conserved in mitochondrial bc_1 complexes.

As mentioned above, it has been recently reported that DCCD binds to Asp-160 in yeast mitochondrial cytochrome *b* [91]. One should note that an aspartate in this region is conserved in yeast and *Aspergillus nidulans* and human cytochrome *b* but not in its murine or bovine counterpart [130]. Bovine cytochrome *b* has a glutamate in position equivalent to Ser-163 of yeast cytochrome *b*. The finding nevertheless strengthens the idea of DCCD being bound to mitochondrial cytochrome *b* and not to some other subunit copurifying with it. The DCCD-reactive carboxyl is in an unusual place (Fig. 4), being in a large extramembranous loop of 50 amino acids on the cytosolic side according to the

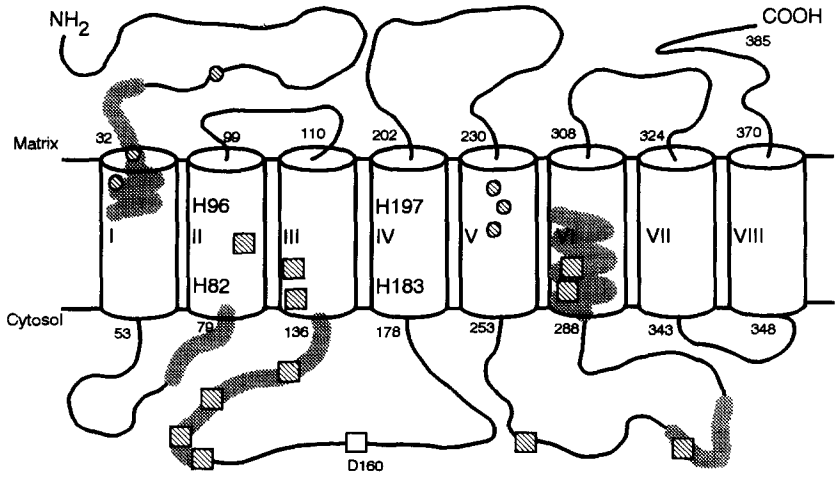

Fig. 4. Schematic presentation of the folding of cytochrome *b* in the inner mitochondrial membrane in relation to the DCCD-binding Asp-160. The thick line shows the highly conserved mytothiazol and stigmatelin-binding region (the Q_o reaction center). The hatched squares depict some of point mutation sites resulting in Q_o inhibitor resistance. The hatched circles show representative sites of point mutations resulting in resistance to Q_i inhibitors. Adapted from Refs. 33 and 60.

8-helix model preferred by Colson et al. [30] and Gennis et al. [31].

As described below in Section IV-C, the DCCD-reactive components of complex I seem to have ubiquinone-binding properties. Cytochrome *b* contains two domains which are involved in ubiquinone binding and have been identified in the primary structure by mutation analysis. Mutagenesis work has shown that determinants of the Q_i site for ubiquinone reduction appear in residues within the amino-terminal peptide and transmembranous helices I and V near the matrix side (Fig. 4). Determinants of the ubiquinol oxidizing Q_o site have been found in several residues in helices III and VI and two large external loops between helices III-VI [33] according to the 8-helix model for cytochrome *b* [60]. The Asp-160 residue that has been reported to bind DCCD is then in one of the large superficial loops near the Q_o domain. The existence of ubiquinone-binding pockets proper and their relation to the primary structure of cytochrome *b* is unclear. The proximity of the DCCD-binding residue to the Q_o domain could result in DCCD interference with ubiquinol oxidation or loss of sensitivity to Q_o inhibitors such as myxothiazol or stigmatellin, but no reports exist on this aspect. As described below in Section IV-C, DCCD binding to complex I competes with inhibitors interfering with ubiquinone binding [11] and some complex III inhibitors affect complex I in a manner suggesting interference with ubiquinone binding [35].

A mechanistic explanation of the effect of DCCD on the proton pumping effected by complex III has been a subject of wide investigation and discussion. The variance in the experimental results regarding subunits labelled in the presence of [^{14}C]DCCD depending on the conditions used has been problematic, and it is also difficult to imagine a 'pump' dependency of complex III, the function of which has been adequately explained by the Q-cycle [80], operating with direct ligand conduction [129] without any need for postulation of a pump. Much of this discussion took place before molecular biology brought us the primary structures of the components involved. It is difficult to explain the repeatedly observed decline in the H^+/e^- ratio under the influence of DCCD without any marked change in the electron transfer rate or proton conductivity of the inner mitochondrial membrane.

The effect on the H^+/e^- ratio came to be seen in a new light when it was found that the DCCD effects verified in kinetic experiments on proton pumping and electron transfer rates in several laboratories could not be confirmed in thermodynamic experiments [17] in which the H^+/e^- ratio was calculated by determining the force ratio of the redox and proton transfer reactions across the cytochrome bc_1 complex. When the electric ($\Delta\psi$) and chemical (ΔpH) components of $\Delta\tilde{\mu}_{H^+}$ are determined from the transmembrane distribution of methyltriphenyl phosphonium and acetate and the ΔE_h across complex III by determining the $\Delta E_h'$ between exogenous ubiquinone$_0$ and cytochrome *c*, $2F \cdot \Delta E_h/\Delta\tilde{\mu}_{H^+} = H^+/e^-$ remains constant (or tends to rise). This indicates that the mechanistic stoichiometry of proton pumping by complex III is not influenced by DCCD. If the stoichiometry were to change due to short circuiting of the hydrogen and electron transfer pathways or the slipping of a 'pump', this should be detected by thermodynamic experiments as well. The latter application has its drawbacks when applied to inhibitor studies, because the reaction under investigation must be in equilibrium. There is ample evidence that this is satisfied under control conditions [90,134] but the reaction would go out of equilibrium at any higher degree of inhibition, and therefore the results must be interpreted with caution. A plausible explanation would be that the DCCD-sensitive component has the role of a channel, similar to that existing in the membranous domain of F_1F_0 ATPase. Inhibition of that channel would force slipping of the redox-driven proton transfer, as evidenced in the kinetic experiments. This interpretation cannot explain the increase in the rate of the redox reaction documented in some reports [17], or its insensitivity [37] but it is in accord with a sensitivity less than that of proton translocation [59]. The existence in *b*-type cytochromes of a conserved sequence homologous to the DCCD binding region of the F_0 domain of mitochondrial ATP synthase makes the channel concept more attractive.

IV-C. NADH: ubiquinone oxidoreductase

Mitochondrial NADH: ubiquinone reductase (complex I) in higher vertebrates is an extremely complex oligomeric enzyme which participates in redox-linked energy conservation by pumping protons out of the matrix space. Typically, more than 30 protein bands can be found in SDS-PAGE analysis of complex I preparations [104]. At present, the primary structures of 34 dissimilar nuclearly coded peptides present in complex I preparations have been determined, mostly by means of sequencing their respective cDNAs [122,123]. In addition, seven subunits are coded by the mitochondrial DNA [24,25]. This means that complex I contains at least 41 dissimilar subunits. It is significant that the corresponding reductase in lower eukaryotes, plants and fungi is simpler, and can be found in both an energy-conserving (proton pumping, mitochondrial complex-I-like) form and a non-conserving form, the latter consisting of a single peptide. Although several models for the functional mechanism of complex I have been proposed [104], the principles are far from resolved (for recent reviews, see Refs. 122,128).

As with other complexes of the respiratory chain, some bacterial NADH dehydrogenases are composed of a smaller number of subunits than those in the mitochondria of higher eukaryotes. Preparations of NADH dehydrogenase from the 'mitochondrion-like' bacterium *P. denitrificans* appear to contain about ten dissimilar subunits and are rotenone-sensitive [137], but recent genetic data suggest that they lack some subunits. A gene cluster from *P. denitrificans* has been cloned and sequenced [121,139,141]. It contains 14 somatic genes coding for complex-I subunits, as deduced from their homology to subunits of bovine complex I, and 6 unidentified reading frames. Plants and fungi possess a rotenone-sensitive, energy-conserving complex I and a rotenone-insensitive, 'non-phosphorylating', single-polypeptide, FAD-containing NADH dehydrogenase. Mitochondria of *S. cerevisiae* have only this NADH dehydrogenase and therefore a non-phosphorylating, rotenone-insensitive NADH : ubiquinone oxidoreductase region in the respiratory chain.

Mitochondrial NADH dehydrogenase can be split by chaotropic agents into hydrophilic flavoprotein and iron-sulphur protein fractions and a hydrophobic fraction [54]. The flavoprotein fraction consists of three polypeptides with molecular masses of 10, 24 and 51 kDa, and the iron-sulphur protein fraction of six polypeptides, 13, 15, 18, 30, 50 and 75 kDa in molecular mass. All the mitochondrially coded and numerous nuclearly coded subunits can be found in the hydrophobic fraction.

Neurospora crassa synthesises in the presence of chloramphenicol a small form of NADH dehydrogenase which is devoid of the mitochondrially-coded hydrophobic subunits [47]. This form still reacts with ubiquinone, although with lower affinity, and the reaction is rotenone-insensitive. Under these conditions the fungus has an operative mitochondrial respiratory chain with a compensationally increased ubiquinone content in the membrane. The analytical and enzyme kinetic data on the 'small' complex I from the chloramphenicol-treated fungus have been interpreted as showing that it lacks several of the membrane-embedded subunits but still has the 'external' subunits needed for reactivity with NADH. The ubiquinone-reactive site of the small form is modified.

The three-dimensional structure of complex I has been approximated by electron microscopy of single particles and membrane crystals. The complete 'large' enzyme is L-shaped, with one arm protruding from the membrane and the other constituting the hydrophobic, membrane-buried part [74].

The organisation of the redox components of complex I is known only partially. There is one mol of FMN per mol of enzyme, probably in the 51 kDa subunit which contains the NADH-binding site [38], but because chaotropic resolution of the enzyme complex alters the EPR properties of the iron-sulphur clusters, it has not been possible to allocate all of them to specific subunits [88]. The primary structure of the 75 kDa subunit suggests that it contains tetranuclear Fe-S centre (N-2 or N-4) and possibly a binuclear centre [107]. The 51 kDa subunit contains a sequence motif conserved in homologous tetranuclear iron sulphur proteins and may therefore contain the N-3 centre [97]. The 36 kDa (ND1) subunit can be labelled with photoaffinity derivatives of rotenone and therefore is probably on the electron transfer pathway and contains the ubiquinone (and piericidin)-binding site [39]. The Fe-S cluster N-1 is present in the small form which is assembled without mitochondrial protein synthesis, which means that both this and the N-3 and N-4 clusters must reside in subunits synthetized in the cytosol. Two new stripped forms of complex I have recently been isolated by Finel et al. [42,43]. One preparation with 22 subunits (subcomplex Iα) has NADH : quinone reductase activity and contains all the EPR-detectable Fe-S clusters including N-2 but not the (mitochondrially coded) ND5 subunit, while the other (subcomplex Iλ), prepared from intact solubilized complex I by sucrose gradient centrifugation, contains the Fe-S clusters but the high-potential N-2 appears in a modified form (Finel et al., data not shown). It was previously thought that cluster N-2 resides in the mitochondrially coded 67 kDa (ND5) subunit [128], but as evidenced by the presence of the same Fe-S cluster in preparations lacking ND5, this can not be the case. Subcomplex Iλ also reduces water-soluble quinones like Q_1. New methods have been developed for the resolution of complex I to subcomplexes, but it remains to be explained, why four of the products of the ND genes of mtDNA have not been found in the Iα and Iλ preparations of complex I.

The small form has a ubiquinone-reactive site which is rotenone-insensitive and thus differs from the physiological electron-donating site for ubiquinone [47], which is probably close to the N-2 cluster, where the rotenone-sensitive $Q^{-\cdot}$ anion has been detected [19,68]. Thus, the complex has two ubiquinone-binding regions bridged by a membranous part consisting of mitochondrially-synthetized hydrophobic subunits. This affords a physical basis for the hypothetical supernumerary ubiquinone cycles in complex I proposed in order to explain the high H^+/e^- translocation ratio [57,69,104].

The initial suggestion of Mitchell [80] of a H^+/e^- ratio of unity for each energy-conserving site has not held up in subsequent research. The net $H^+/2e^-$ ratio between NADH and oxygen must be higher than six, and although the experimentally found H^+/e^- stoichiometry of complex III can be explained by the Q-cycle and its value (two) has been taken as a gold standard for proton pumping stoichiometry in the inner mitochondrial membrane, the observations on

complexes I and IV have not been interpreted mechanistically. The observations of H^+/e^- stoichiometry in complex I are at variance. Wikström [133] found the stoichiometry of $4H^+/2e^-$ by comparing of complexes I and III using matrix alkalinization and membrane potential as criteria, whereas polarographic oxygen electrode measurements in combination with glass electrode measurements as performed by Reynafarje and Lehninger [105] give a $H^+/2e^-$ ratio of 4, and some thermodynamic data even give a ratio of 6 [46,73]. Simultaneous optical read-out of proton extrusion and redox reaction within the spans from NADH to cytochrome *c* and succinate to cytochrome *c* give $H^+/2e^-$ ratios of 8.7 and 3.9, respectively, corresponding to a ratio of 4.8 across complex I [58].

Since these high stoichiometries cannot be explained by a single redox loop, both purely chemiosmotic and pump mechanisms have been considered. The chemiosmotic mechanisms proposed are analogous to the b-cycle [132] or Q-cycle [79] of complex III. Ragan [104] discusses a protonmotive flavin cycle employing high-potential $FMNH^{\cdot}/FMNH_2$ and low-potential $FMN/FMNH^{\cdot}$ couples and high and low-potential Fe-S centres resulting in a $2H^+/2e^-$ ratio of 3. A subsequent internal b-cycle analogue which employs ubiquinone and Fe-S clusters translocates a further $2H^+/2e^-$, so that the total stoichiometry of complex I becomes $5H^+/2e^-$ [104]. This fits with recent experimental data which obviously point to a ratio higher than 4.

The flavin and quinone cycles of complex I have not been fully reconciled with the present view of the three-dimensional arrangement of the subunits in complex I. It is significant that the NADH-reactive, flavin-containing part is external to the membrane and can be removed by NaBr, which would mean that a proton channelling mechanism is needed in the hydrophobic part of the complex.

It has been shown independently in two laboratories [58,138] that complex I is inhibited by DCCD. This can be demonstrated by kinetic experiments on rat liver mitochondria [58] and complex I purified from bovine heart mitochondria [119]. In the former model proton translocation is inhibited more strongly than electron transfer, but this preferential inhibition of H^+ transport cannot be observed in thermodynamic experiments on submitochondrial particles [120]. No satisfactory explanations have been proposed for this discrepancy. Tight mechanistic coupling of H^+ and e^- transport would result in equal inhibition of both modalities, and the effects of DCCD on proton conductance of the membrane are not sufficient to cause the effect on the apparent stoichiometry. One should note that the same dilemma is observed in complex III. The net efficiency of the two protonmotive cycles in complex I could be in principle modulated by short-circuiting or blocking of the pathways with the flavin and quinone cycles, and DCCD could have effects on the fine oligomeric structure to such ends. But if this were so, the kinetics and thermodynamics should give the same results.

DCCD inhibition is accompanied by proportional binding to the enzyme and the inhibition and binding have the same time-course [119]. Vuokila and Hassinen [119] studied the inhibition and labelling of complex I both in submitochondrial particles and in the isolated enzyme, and Yagi and Hatefi [140] inhibition and labelling of the isolated complex I. The results are at variance, perhaps because of differences in the experimental conditions. Yagi and Hatefi [140] observed labelling of two subunits with apparent molecular masses of 29 and 49 kDa. The labelling of the former is faster but does not correlate with the time-course of inhibition, and the subunit concerned has been identified as being coded by the mitochondrial *ND1* gene. Under the conditions used by Vuokila and Hassinen [119] DCCD binds to numerous subunits of the isolated enzyme although the overall binding has the same time-course as the commencement of inhibition. When labelling is performed in submitochondrial particles and the enzyme is studied after subsequent purification, however, only two labelled subunits are found, one with an apparent molecular mass of 21.5 kDa and the other 13.7 kDa [119], as estimated from their mobility in the denaturing polyacrylamide gel electrophoresis system of Laemmli [72].

There are three subunits of complex I that bind DCCD. Partial amino-acid sequencing of the 29 kDa DCCD-binding subunit has identified it as the *ND1* gene product although the exact binding residue has not been identified [140]. Involvement of the ND1 subunit fits with the data of van Belzen and Albracht, who found that the DCCD effect is reminiscent of rotenone and piericidin, and double titration experiments with piericidin and DCCD indicate that they compete for the same site in the enzyme [11].

A partial amino-acid sequence has also been determined for the '21.5 kDa' subunit (Vuokila, P.T. and Hassinen, I.E., data not shown). This fits with the sequence of the 'ASHI' subunit recently determined by Walker et al. [123] by molecular cloning, which gives a molecular mass of 18.7 kDa. Under the conditions used by these authors the mobility in the gel corresponds to a molecular mass of 19 kDa.

The primary structure of the '13.7 kDa' subunit has also been determined recently, a partial sequence (Vuokila, P.T. and Hassinen, I.E., data not shown) has been found to be identical to that of the '9.5 kDa' protein isolated from bovine heart complex III by Borchart et al. [15]. It is remarkable that this subunit has not been implicated in DCCD binding previously. It is an 81-amino-acid peptide which contains four

glutamate residues and one aspartate residue, all of them in putative extramembranous domains according to the model proposed by the above authors [15]. The DCCD-binding residue has not been identified, but both the primary sequence and the hydrophobicity plot make Glu-22 a likely candidate. This subunit may have only one fully penetrating transmembranous helix and an amphiphilic helix probably located on the surface of the membrane. If this is the DCCD-binding region, the 9.5 kDa protein becomes in this respect similar to NAD(P) transhydrogenase, which binds DCCD in a superficial domain. It has been shown recently [118] that the '9.5 kDa' protein is identical to the small molecular mass ubiquinone-binding protein studied in detail by Yu and co-workers [144]. Suzuki and Ozawa [115] have purified from complex I a ubiquinone-binding protein having an apparent molecular mass of 14 kDa. In light of the identity the '13.7 kDa' subunit [119] with the '9.5 kDa' ubiquinone-binding subunit of complex III the possibility of subunit sharing between the complexes should be scrutinised more closely. It is always possible that the existence of the 9.5 kDa subunit in complex I is a contamination, but the recent independent observation by Heinrich et al. [55] that *N. crassa* complex I contains a 9.5 kDa subunit with a sequence highly homologous with that isolated by Borchart et al. [15] from bovine heart complex III lends support to the presence of a similar subunit in the mammalian complex I. One should also remember that the observed stoichiometry between proton translocation and electron transfer in complex I calls for additional redox loops, and an additional Q-cycle or b-cycle-type mechanism has been proposed [104]. If this were the case, complex I would need Q-binding components in addition to the known domain in the *ND1* gene product. Complex I has been recently shown to be involved also in functions other than redox-linked energy conservation, as exemplified by its content of the a prokaryote-type acyl carrier protein [107], which probably has a real function in mitochondrial fatty-acid metabolism not directly linked to oxidative phosphorylation [147]. It remains to be established whether complex I and complex III really share subunits, but subunit sharing in enzymes of different function has been documented in other systems previously. The need for structured Q/QH· binding and transport between complexes I and III fits into this picture. This finding once more raises the question of the exact number of the multitude of subunits in the proton-translocating complexes. It could be that two DCCD-binding subunits of complex I are involved in ubiquinone binding. It might be more than a coincidence that this small, ubiquinone-binding peptide has been observed mitochondrial complex I preparations from two different organisms. The assembly of complex I in the context of the DCCD-binding subunits is illustrated in Fig. 5.

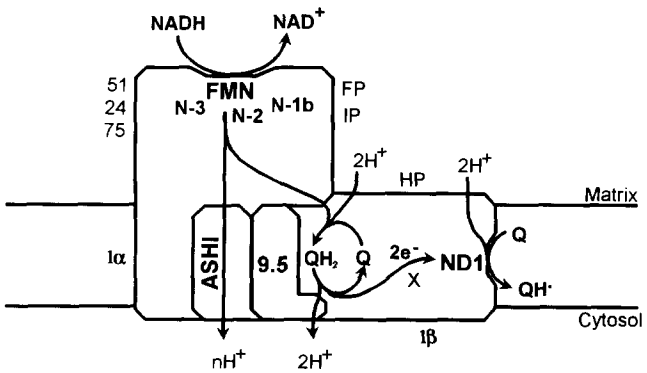

Fig. 5. Proposed scheme for complex I in the inner mitochondrial membrane, showing the DCCD-binding subunits. The 9.5 kDa subunit may be involved in ubiquinone binding as in complex III and may be needed for an internal Q cycle. The ASHI subunit contains a hydrophobic DCCD-binding glutamic residue which may be linked to proton channelling. ND1 is on the rotenone and piericidin-sensitive path to ubiquinone. 1α and 1β refer to the two subcomplexes obtained by the method of Finel et al. [42]. The total number of subunits is at least 41. Modified from Refs. 122 and 128.

The primary structures of the DCCD-binding subunits of complex I can now be evaluated in terms of their common DCCD-binding motifs. The *ND1* gene product of the human mitochondrion contains 11 glutamate and 3 aspartate residues and 7 hydrophobic stretches which could be putative transmembrane helices. There is a cluster of three glutamates, Glu-202, Glu-204 and Glu-206, in a location which could be predicted to be at the membrane surface, and one glutamate, Glu-143 in a hydrophobic region. The three-glutamate cluster 'FDLAEGESE-206' bears some resemblance to the DCCD-binding sequence of the *c* subunit of F_0 but is not in the typical position deep in the membrane. Weiss and co-workers [48] have modelled the ubiquinone-binding region of ND1 in comparison with the bacterial ubiquinone-reducing glucose dehydrogenase (Fig. 6). They consider the invariant Arg-134 and Glu-143 (in human ND1) to be necessary for ubiquinone binding. Walker [122], however, considers the sequence relations between ND1 and the bacterial glucose dehydrogenase too weak and nonsignificant to be used as a proof for ND1 contribution to ubiquinone binding.

The ASHI subunit has Glu-120 at the edge of a hydrophobic 20-amino-acid stretch in a sequence 'FMFWGETTY', which has some resemblance to a family of sequence motifs homologous to the DCCD-binding site of the *c* subunit of the F_1F_0 ATPase.

The data accumulated so far indicate that two of the DCCD-binding subunits of complex I belong to the family of DCCD-binding proteins with typified sequence motifs and properties, but the third does not seem to be structurally related. It is noteworthy that all

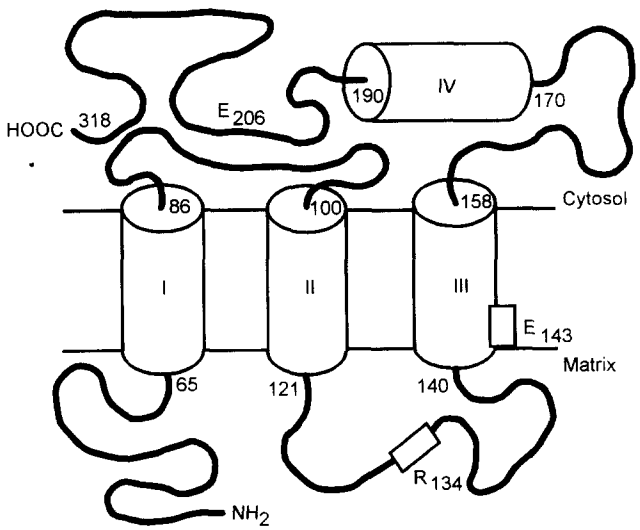

Fig. 6. Diagram of the ubiquinone-binding domain of the ND1 subunit of complex 1 in the mitochondrial inner membrane. The invariants Glu-143 and Arg-134 are thought to be involved in ubiquinone binding. The DCCD-reactive residue has not been identified, but it could be Glu-143 in view of indications of the proximity of DCCD binding to the ubiquinone reactive site or Glu-206 which is in a hydrophobic region. This model has been proposed by Friedrich et al. [48].

these three have some connection to ubiquinone binding [12,144]. Some of the evidence of the DCCD-reactive proteins in complexes I and III cited above point to very similar subunits in these complexes. Further recent evidence point to the same direction, namely, complex III inhibitors myxothiazol and the tridecyl analogue of stigmatellin inhibit both NADH : ubiquinone reductase and ubiquinol : cytochrome-*c* reductase activities [35].

IV-D. Energy-linked transhydrogenase

The mitochondrial energy-linked transhydrogenase resides in the mitochondrial inner membrane and is a reversible redox-linked proton pump, probably having the physiological function of NADP reduction at the expense of NADH. The driving force proper is the electrochemical potential of protons.

$$NADP^+ + NADH + nH^+_{out} \leftrightarrow NADPH + NAD^+ + nH^+_{in}$$

Thus, although the equilibrium constant of the scalar transhydrogenase reaction is near unity, the poise of the reaction in energised mitochondria is on the side of $NADP^+$ reduction, the value of n being one. The bovine enzyme is a homodimer composed of monomers of 109 kDa and containing distinct binding sites for NAD^+ and $NADP^+$. As could be expected from its proton pump character, the enzyme has been subjected to testing with DCCD [89].

Mitochondrial energy-linked transhydrogenase is indeed inhibited by DCCD and is protected from DCCD attack by NAD(H), its analogues and competitive inhibitors of the enzyme (5′-AMP, 5′-ADP), and the DCCD-modified enzyme does not bind to NAD^+-agarose [94,95]. The transhydrogenase is modified by DCCD in submitochondrial particles, resulting in label incorporation. The H^+ translocation moiety of the enzyme becomes inhibited when 1 mol of DCCD is bound to 1 mol of transhydrogenase, hydride ion transfer requiring approx. 2 mol of DCCD for complete inactivation [93]. According to Hatefi and co-workers, DCCD modifies the NAD(H)-binding site [94–96], but there is some disagreement between the research groups of Hatefi and Rydström regarding the relation of DCCD binding to the substrate binding site and the preferential inhibition of proton pumping [90]. The primary structure has been determined and tryptic peptides containing DCCD label have been isolated [142]. The enzyme has an extensive hydrophobic domain in the middle of the molecule and has been modelled as forming 14 transmembranous helices. The 432-residue amino-terminal part and 207-residue carboxy-terminal part constitute large extramembranous

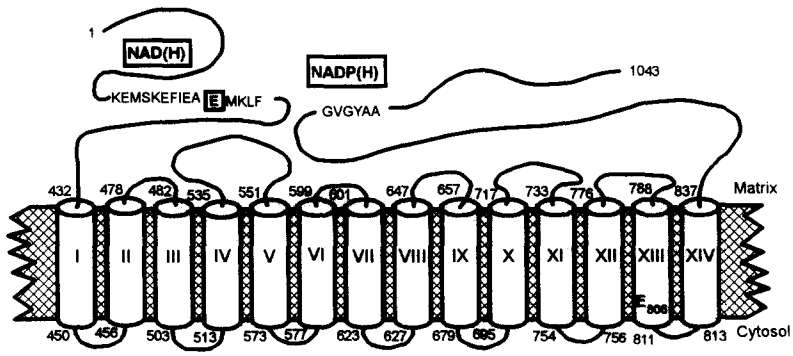

Fig. 7. Schematic diagram of the location of the DCCD-binding domains in the mitochondrial energy-linked transhydrogenase. Folding in the membrane is according to the 14-helix model of Yamaguchi et al. [142]. The DCCD-binding motif near the NAD(H) site is shown. The other putative DCCD binding residue, probably Glu-806, is in helix 13. There are four nucleotide-binding consensus sequences of which one is shown near the NADP(H) site.

domains on the matrix side (Fig. 7). The readily reacting DCCD-binding site (Glu-257) near the NAD(H)-binding region is unusual in that it resides in a rather hydrophilic span on the matrix side of the membrane. The location of the other DCCD-binding site [93] has not been determined. When the primary structure is analysed for homology with respect to the DCCD-binding proteolipid of complex V or subunit III of complex IV, the resemblance is only faint. According to Hatefi and co-workers [142], the residue modified under conditions resulting in preferential inhibition of proton translocation could be Glu-806 in helix XIII.

As in complexes III and IV, DCCD influences proton pumping more than the redox reaction [89]. This is apparent from experiments in which transhydrogenase reconstituted to liposomes was assayed in the absence and presence of an uncoupler [92]. Since proton translocation can be inhibited by 85% without any effect on hydride transfer, catalytic activity and proton pumping cannot be obligatorily linked. The transhydrogenase enzyme still presents some unsolved problems, for as evidenced by DCCD-binding experiments, the NAD(H)-binding site is in the N-terminal extramembranous domain, which also possesses 'nucleotide binding' $\beta\alpha\beta$ folds. Surprisingly, the NADP(H)-binding site may reside in the C-terminal extramembranous domain, which has two consensus nucleotide-binding sequences. The NADP(H) binding probably occurs near the (Gly^{1003}–Gly^{1008}) sequence [62]. Thus the cosubstrate binding sites are formed by opposite ends of the 1043-residue peptide. The preferential inhibition of proton pumping would be unlikely due to blocking of the extramembranous Glu-257 near the substrate site only. That sequence is homologous with the yeast plasma membrane H^+-ATPase. Binding of DCCD to the intramembranous Glu-806 in helix XIII has not been positively documented. In *E. coli* the enzyme is formed of two subunits which are homologues of the amino-terminal and carboxy-terminal halves of the mitochondrial enzyme [26].

In *Escherichia coli*, *Rhodobacter capsulatus* and *Rhodospirillum rubrum* the transhydrogenase is composed of two peptides. In the two former species the break between them is in the domain which is homologous with the membrane-embedded part of the mitochondrial enzyme, whereas in *R. rubrum* the break is between the amino-terminal hydrophilic domain (T_s) and the membrane-embedded domain (T_m). DCCD binding to the enzyme in these bacteria can be enhanced by NADP(H) and partially inhibited by NAD(H) [91]. The $NADP(P)^+$-enhanced modification is located in the membranous T_m peptide and probably leads to a decrease in the rate of release of NADP from the enzyme so that this becomes rate-limiting. NAD(H)-protectable DCCD inhibition is different in mitochondria and *R. capsulatus*, as all the nucleotides in the former which are known to bind to the catalytic site (NAD^+ and its acetylpyridine analogue, NADH and its acetylpyridine analogue and 5′-AMP) protect the enzyme, but the photosynthetic bacteria *R. capsulatus* or *R. rubrum* gain no protection from these nucleotides [91]. Thus, the sequence flanking Glu-257 in mitochondria, although reminiscent of other DCCD-binding motifs [127], is not conserved in the transhydrogenase of photosynthetic bacteria and is probably not involved in proton translocation [91].

V. Mitochondrial anion channels

V-A. Mitochondrial porin

Mitochondrial porin, a channel-forming outer membrane protein [146] is typified by its property of binding some kinases such as glycerol kinase [41] and hexokinase [76] and has a putative role in the formation of contact sites between the inner and outer mitochondrial membranes [135]. It is also identical to the voltage-dependent anion-selective channel (VDAC) present in the outer mitochondrial membranes of all eukaryotes [29]. This protein forms voltage-dependent pores in artificial phospholipid membranes, pores which are reduced in diameter at higher voltages across the membrane [106], although it is not evident how a potential can develop across the outer mitochondrial membrane. It has been proposed that a Donnan potential may be involved in the regulation of the porin channel [29]. The mitochondrial porin (VDAC) bears no resemblance to the bacterial porins.

Palmieri and co-workers [34] showed that the porcine heart mitochondrial '35 kDa' protein which is labelled at low concentrations of DCCD is porin. It is known that also porin from rat liver, rat hepatoma and yeast mitochondria bind DCCD [85].

The significance of the DCCD-binding group in porin is not known, because DCCD-labelling of the protein does not modify its pore-forming, ion selectivity or voltage-gating characteristics in lipid bilayers [85]. However, it affects porins capacity to bind hexokinase [84]. The porin channel probably forms as a 13-strand cylinder composed of 12 antiparallel β-sheet strands and one α-helix. One molecule of porin is sufficient for one channel of a diameter of 3 nm [13]. The carboxy-terminal transmembranous part has a conserved sequence motif with homology to the DCCD-binding proteolipid of F_0 and containing two aspartates (Asp-282 and Asp-264 and one glutamate (Glu-280) which has been regarded as a putative DCCD-binding site [84]. The putative DCCD-binding aspartates would then be arranged rather superficially on either side of the membrane. There is also one glutamate in the middle of the membrane (Glu-152) in

a non-related sequence, but there are no experimental data regarding the position of the DCCD bound to the protein. Site-directed mutagenesis of Asp-282 does not alter the conductance of the channel but has a slight effect on its selectivity [13]. Now, the primary structure of porin is known in several species, and it is evident that the aspartates near the carboxy-terminus which has been considered to bind DCCD are not conserved. Asp-282 is found only in *Saccharomyces cerevisiae*. Asp-264 is conserved in *N. crassa* and human porin, but not in *Dictyostelium discoideum*, an aerobic microorganism with tubular mitochondria but not related to fungi or higher eukaryotes [65,67,117]. The conserved aspartates/glutamates in porin are near the amino terminus, so that the DCCD-reactive region might be there, contrary to the expectations based on sequence similarities

V-B. Inner membrane anion channel

The inner membrane contains an anion channel (IMAC) with wide substrate specificity and a permeability regulated by protons and Mg^{2+} (for references, see Ref. 8). IMAC is an electrophoretic uniport which can be activated by alkaline pH or depletion of the matrix of divalent cations, and it is inhibited by DCCD [9]. Interpretation of this finding remains speculative until more is known of the molecular detail of IMAC which has not yet been purified.

VI. Conclusions

As a tool for identifying proton translocating components in the respiratory chain, DCCD has not been particularly successful in revealing reaction mecha-

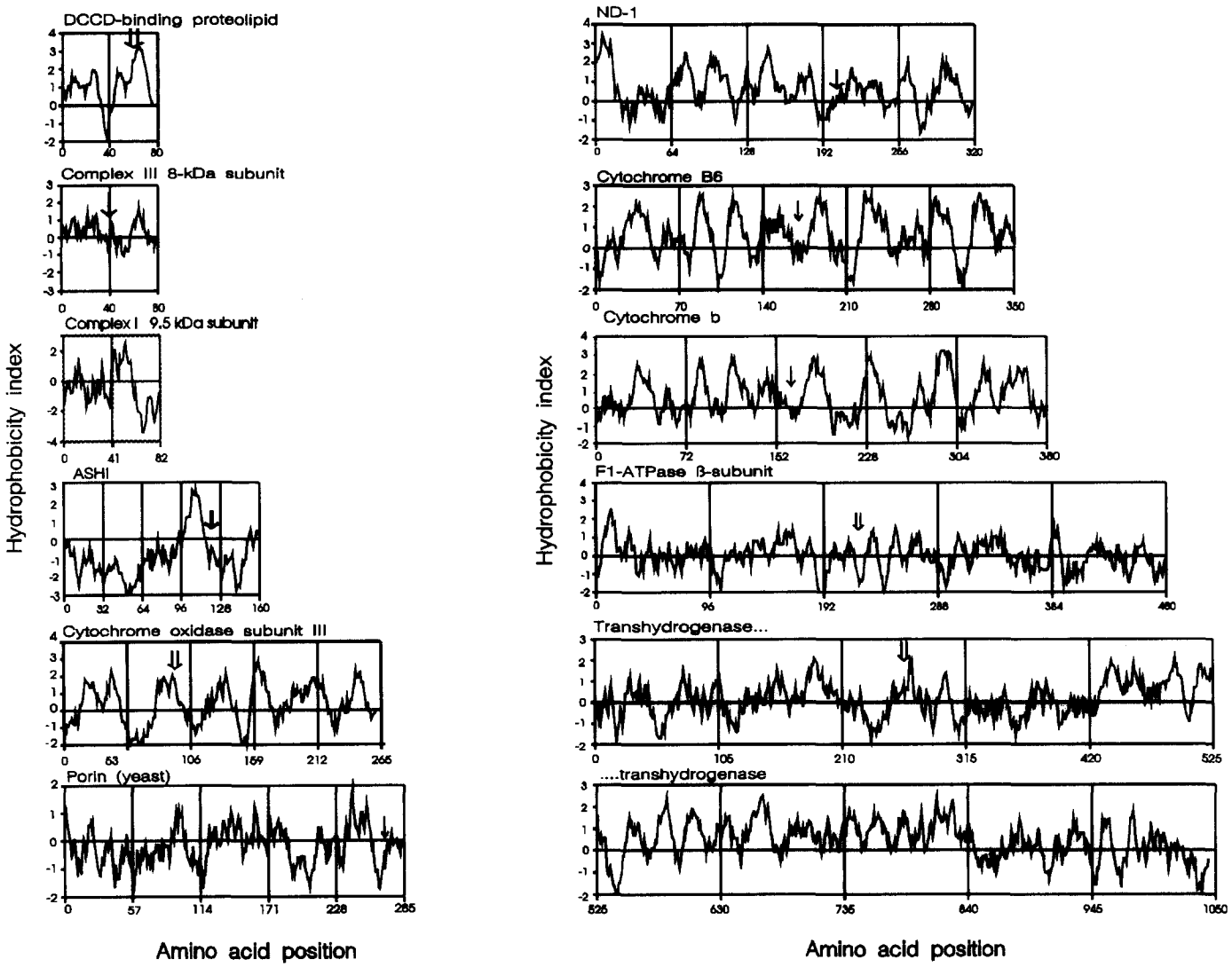

Fig. 8. Hydrophobicity profiles of the DCCD-binding mitochondrial components. The hydrophobicity index table of Kyte and Dolittle [71] was used with a window length of six. The putative and documented DCCD-binding residues are marked with (↓) and (⇓), respectively. Note that the transhydrogenase sequence has been cut after residue 525 and is presented in two parts.

nisms. All this effort may turn out to be useful, however, in that it emphasises the existence of a group of hydrophobic membrane proteins which, although being diverse in size and design, may have common properties or ancestry. The DCCD-binding components of the mitochondrial respiratory chain are not only typified by acidic residues in a hydrophobic milieu but also have often sequence motifs with can be traced to the DCCD-binding proteolipid of F_1F_0-ATPase. As DCCD has mostly been of use in labelling experiments, and cross-linking in a multimeric complex with tens of subunits may pass undetected, only the carboxyls that are in a hydrophobic environment are revealed. All of the mitochondrial DCCD-binding proteins have transmembranous domains, but the documented DCCD-reactive dicarboxylic amino acid is not always located in a transmembanous domain (Fig. 8). The amino-acid sequences of the variety of DCCD-binding proteins do not show any close general homology. Still, in many but not all cases the binding of DCCD occurs in a short-sequence motif which bears some resemblance to the corresponding motif in the DCCD-binding proteolipid of F_0 (Table II). Mutagenesis experiments on the hairpin-shaped DCCD-binding proteolipid of F_0 and DCCD-resistant bacterial strains show that not only the nearest neighbours in the peptide backbone determine the micro environment of an amino-acid residue for covalent binding of DCCD. Because of folding of the protein in the membrane, determinants of DCCD binding can be found at some distance from the DCCD-binding residue. Our knowledge of the primary structures of biologically important proteins is expanding in a rapid pace due to the productivity of the present DNA sequencing methods, but obtaining three-dimensional structures is a much more demanding task. The short-sequence motifs summarised in Table II are not prerequisites for DCCD binding. Only in the *c* subunit of the F_0 domain of ATP synthase and subunit III of cytochrome-*c* oxidase is the documented or putative DCCD-binding dicarboxylic amino-acid residue buried deep in the membrane (Fig. 8), but as most experimental evidence shows in the case of the latter protein, this location is still not proof of a role in proton translocation. Thus, DCCD binding hydrophobic carboxyls may also occur in membrane proteins because the origin of the proteins in proton transloca-

TABLE II

DCCD-binding motifs in subunits of DCCD-sensitive mitochondrial enzymes

Subscripts indicate the amino-acid position in the mature protein and * indicates the carboxy-terminus. The underlined characters represent residues confirmed as being labelled by DCCD. Boldface characters represent potential DCCD binders, since they reside in hydrophobic motifs reminiscent of the DCCD-binding sequence of the *c* subunit of F_0.

Protein	Species	Sequence
F_0 *c* subunit	Bovine	YAI LG FAL S E AMGLFLC$_{65}$
	Yeast	LG FAFV E A
	PS3	IG VALV E A
	E. coli	IV MGLV D A
F_1 β subunit	Bovine	IIAI LGMDEL S EEDKL$_{404}$
	N. crassa	IIAI LGMDEL S EADKTVE$_{443}$
	Bovine	GVGERTRE GND LY HEM$_{216}$
	N. crassa	GVGERTRE GND LY HEM$_{239}$
Cytochrome b_6	Spinach	PDAIPVIGS PLV E LL RGSA$_{169}$
Cytochrome *b*	Bovine	LSAIPYIGTN LV E WIWGGFSV$_{170}$
	Human	LSAIPYIGTD LV Q WIWGGYS$_{169}$
ASHI	Bovine	AFM LFMFW VG ETY PA$_{124}$
ND1	Human	TP FDL A EGE SELVSG$_{210}$
bc_1 8 kDa protein	Bovine	L VQA AVPA TS ESPVL DL$_{45}$
bc_1 9.5 kDa protein	Bovine	VITYS LSPFEQRA$_{25}$
Porin	Yeast	L GVGSSF DALKS E PVH$_{273}$
	N. crassa	AAIA YNVLLREG VTLGV$_{259}$
		ATHKV GT SF TFES$_{283}$*
		GASF DTQKL D QAT H$_{273}$
	Human	GKNVNAGGHKLGLGL EFQA$_{281}$*
		GIKLTLSALLDGKNVN AGGH$_{271}$
CO III	Bovine	IL FII S E V
	Human	IL F ITS E VFFF$_{94}$
	Yeast	L M FVL S E V
	N. crassa	IL F IVS E V
	PS3	F LGG ETVLLFF
Transhydrogenase	Bovine	EMSKEFF IEA EMKL FAL$_{263}$
	Bovine	SYSGWALCA EGFLL$_{810}$

tion can be traced to a proton channel, one of the most ancestral ion channels, since the biological machinery for conserving combustion or light energy relies on proton pumping. Due to the general diversity of sequence and molecular size, the relationships between the DCCD-binding proteins must be regarded as distant. The original central motif may have lost its importance in evolution, as seen in the β-subunit of F_1-ATPase and the energy-linked transhydrogenase, in which the experimentally confirmed DCCD-binding site is far removed from the 'DCCD-binding' motif. Much remains to be revealed by means of site-directed mutagenesis, which so far, except for the c-subunit of the F_0 moiety of the F_1F_0-ATPase, has yielded negative results which have disproved some previous suggestions.

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