RELATIONSHIP BETWEEN THE BINDING OF DICYCLOHEXYLCARBODIIMIDE AND THE INHIBITION OF H^+-TRANSLOCATION IN SUBMITOCHONDRIAL PARTICLES

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1. Introduction

Dicyclohexylcarbodiimide is a specific inhibitor of electron transport-linked phosphorylation in mitochondria, chloroplasts and bacteria [1,2]. It forms a covalent adduct with a hydrophobic protein that is a component of the H^+-translocating moiety (F_0) of the ATP synthetase (F_0F_1) complex.

Maximal inhibition of ATPase activity by [^{14}C]-DCCD is associated with radioactive labelling of only a fraction of the total amount of DCCD-binding protein [3–8], and 1 or 2 mol DCCD/6 mol DCCD-binding protein are required for maximal inhibition of the ATPase activity [3–5,7] or the protonophoric function [9] of F_0. Similarly, substoichiometric amounts of DCCD bound to the isolated mitochondrial ATPase complex have been reported to give a complete inhibition of the P_i–ATP exchange activity [10].

The purpose of this study was to compare the DCCD titers of various F_1-linked activities of submitochondrial particles including ATPase, P_i–ATP exchange and H^+-translocation. It will be shown that 1 mol DCCD/mol F_0F_1 gives a nearly maximal inhibition of the F_1-linked reactions. On the other hand, the inhibition of passive H^+-translocation through F_0 in the absence of F_1 requires 2 mol DCCD/mol F_0. The data suggest that H^+-translocation through F_0F_1 is more complex than through F_0 alone.

Abbreviations: F_0 and F_1, components of the mitochondrial ATPase system; DCCD, dicyclohexylcarbodiimide; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone

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2. Materials and methods

'Heavy' beef-heart mitochondria [11], Mg-ATP particles [12] and EDTA particles [12] were prepared as described earlier. The EDTA particles were passed through a Sephadex column in order to remove the ATPase inhibitor protein, and subsequently treated with urea to remove F_1 [13]. For the fluorometric assay of the H^+-translocation, EDTA particles were prepared by sonication in the presence of 1 mg fluorescein isothiocyanate-labelled dextran/mg protein according to [14,15].

For the estimation of [^{14}C]DCCD-binding [16], 10 μl aliquots of particle suspensions (10 mg protein/ml) were diluted with 1 ml of 10% water in acetone (v/v) at 0°C, and sedimented for 5 min at 8000 × *g*. After washing with 2.5% trichloroacetic acid, the pellets were solubilized in an NCS tissue solubilizer and the radioactivity was determined in a liquid scintillation counter.

ATPase and NADH oxidase activities were measured spectrophotometrically at 30°C as in [17,18]. P_i–ATP exchange was measured in a reaction mixture containing 50 mM Tris–acetate (pH 7.5), 50 mM sucrose, 13 mM $MgSO_4$, 16 mM ATP, 4 mM ADP, 5 mM KP_i (pH 7.5), 10^6 cpm $^{32}P_i$ and 0.3 mg protein in a final volume of 0.25 ml. (The exchange reaction was also measured without added ADP as in [10].) Incubation was at 30°C for 7 min. The reaction was stopped by adding 50 μl 2.5% $HClO_4$ and chilling to 0°C. After centrifugation, the incorporation of $^{32}P_i$ into organically-bound phosphate was measured using the isobutanol–benzene extraction method [19].

H^+-translocation in particles containing trapped dextran labelled with fluorescein isothiocyanate was measured according to [15]. An Aminco-Bowman spectrofluorometer (480–530 nm, ratio mode) con-

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nected with a fast-response recorder was used. The response time of the apparatus was below 50 ms. The reaction proceeded in a cuvette with mechanical stirring under a constant stream of nitrogen at 25°C. The reaction medium containing 50 mM KCl, 3 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.4) was deaerated in vacuum before adding it to the cuvette together with 0.6–1.0 mg protein, 0.25 μg valinomycin and 3 mM NADH (final vol. 1.5 ml) [21]. The respiration-driven H^+-translocation was activated by repetitive pulses of 30 μl aliquots of air-saturated reaction medium, and H^+-efflux following anaerobiosis was recorded. The half-time ($t_{1/2}$) of the H^+-efflux was calculated and the $1/t_{1/2}$ was taken as a measure of the rate of H^+ diffusion. Protein was estimated according to [22].

[^{14}C]DCCD (50 mCi/mmol) was purchased from CEA (Gif-sur-Yvette), fluorescein isothiocyanate-labelled dextran (M_r 3000) from Pharmacia (Uppsala), and $^{32}P_i$ from the Radiochemical Centre (Amersham).

3. Results and discussion

Fig.1 shows the relationship between added and bound DCCD in EDTA particles. The particles were incubated with DCCD for 16 h, after which time maximal binding was obtained. It may be seen that the extent of DCCD binding increased nearly linearly with the concentration of DCCD up to a level of ~2 nmol DCCD bound/mg particle protein. Above this level, the extent of binding increased only slightly with increasing DCCD concentration [23]. Under the conditions employed DCCD was bound, as shown in [6,8,16], only to 3 components. Two of these were concluded to be a monomeric and an oligomeric form

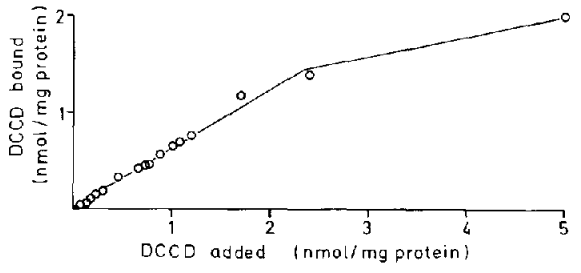

Fig.1. Binding of [^{14}C]DCCD to submitochondrial particles. Submitochondrial particles at 10 mg protein/ml were suspended in a medium containing 0.25 M sucrose, 5 mM $MgSO_4$ and 10 mM Tris–acetate (pH 7.5) and incubated for 16 h at 0°C with [^{14}C]DCCD. The amount of [^{14}C]DCCD bound was estimated as in section 2.

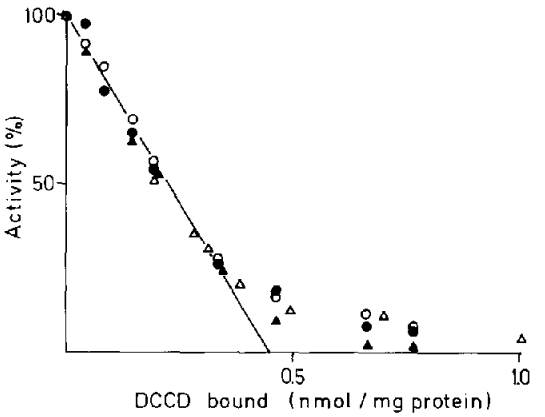

Fig.2. The effect of DCCD on the ATPase and the P_i–ATP exchange activities. ATPase activity in the presence (●——●) and absence (○——○) of FCCP; P_i–ATP exchange activity in the presence (▲– –▲) and absence (△——△) of ADP. The ATPase activity (± FCCP) was 1240 and 590, and the P_i–ATP exchange (± ADP) 126 and 69 nmol . mg^{-1} . min^{-1}, respectively. The ATPase activity is given after subtraction of a DCCD-insensitive portion (= 110 nmol . mg^{-1} . min^{-1}) both in the presence and absence of FCCP. For other experimental conditions, see section 2.

of the DCCD-binding subunit of F_0 [6,8,16]. The third component was not part of the isolated ATPase complex [6,8,16] and was identified as the P_i translocator [24]. This latter protein accounted maximally for ~20–30% of the total binding. It appears, thus, that ~1.5 nmol DCCD are maximally bound to the DCCD-binding protein component of F_0. Since the F_1 content of the submitochondrial particles is ~0.5 nmol/mg protein [25], these data indicate that the particles contain ~3 mol DCCD-binding protein/mol F_1, i.e., that each molecule of F_0F_1 complex contains 3 molecules of DCCD-binding proteins.

The relationship between DCCD binding and inhibition of the ATPase and P_i–ATP exchange reactions is shown in fig.2. The titration curves for both reactions were virtually linear up to ~80% inhibition. Extrapolation of the linear portions of the titration curves to zero activity gave a value of ~0.45 nmol DCCD bound/mg particle protein. The effect of DCCD on the ATPase activity was not influenced by the presence of FCCP, which stimulated the ATPase activity ~2-fold. The P_i–ATP exchange showed the same response to DCCD in the presence and absence of ADP. The inhibition of the P_i–ATP exchange coincided with that of the ATPase. This is in contrast to the findings in [10,26], according to which the P_i–ATP exchange was more sensitive to DCCD than

the ATPase activity. The reason for this discrepancy is not clear.

Assuming an F_1 content of 0.5 nmol/mg protein [25], these data are consistent with the conclusion [5,7,27] that the binding of 1 mol DCCD to each F_0F_1 complex is sufficient to inhibit ATPase-linked H^+-translocation, and that DCCD binds preferentially to one DCCD-binding protomer in each F_0F_1 complex. This preferential binding may be due either to an intrinsic difference between the protomers, or to a negative cooperativity with respect to DCCD-binding induced by DCCD. Data to be reported elsewhere [28] favour the former alternative.

It was of interest to compare the effect of DCCD on the ATPase-linked H^+-translocation with that on the passive H^+-translocation through F_0 as measured in the absence of F_1. In the experiments shown in fig.3 and 4, the H^+-uptake and release induced by oxygen pulses in F_1-depleted submitochondrial particles were followed with the aid of fluorescein isothiocyanate-labelled dextran [15]. The fluorescein probe was trapped inside the particles during sonication, and F_1 was removed by means of urea treatment (see section 2). The particles were suspended in an anaerobic medium containing K^+ and valinomycin. The O_2-induced acidification of the medium inside was reflected by a decrease of fluorescence, the extent of which was proportional to the H^+-concentration inside the particles between pH 6–7.5 (see inset of fig.3); outside this pH range, the response of the probe was non-linear. In accordance with earlier experiments using a glass electrode [21], the $t_{1/2}$ of the fluorescence decrease following anaerobiosis can be taken as an

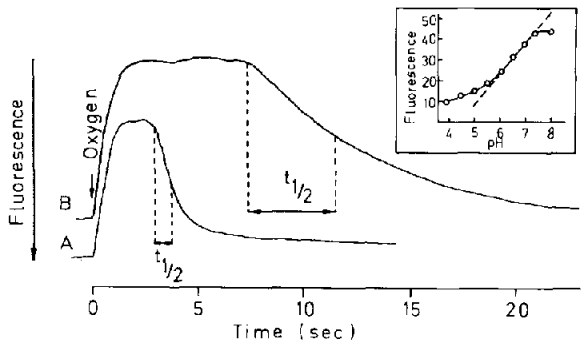

Fig.3. Fluorometric measurements of H^+-translocation induced by oxygen pulses in submitochondrial particles, in the absence (A) and presence (B) of DCCD. For the experimental conditions, see section 2. Inset shows dependence of the fluorescence on the pH of the reaction mixture.

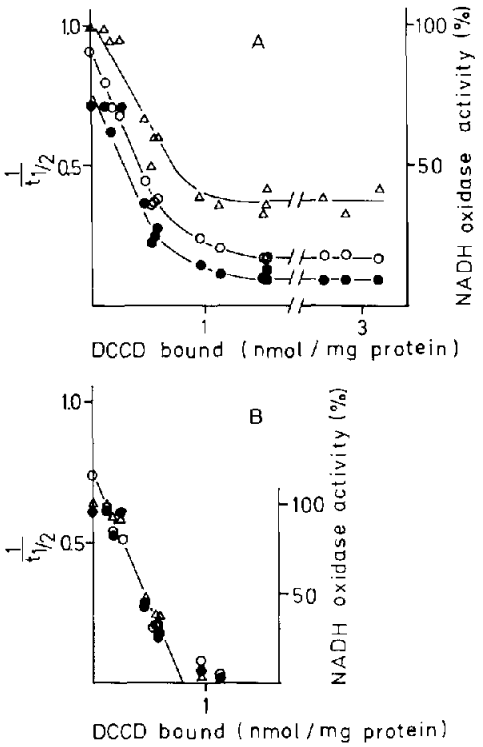

Fig.4. Effect of DCCD on the passive H^+-translocation and NADH oxidase activity in submitochondrial particles depleted of F_1. (A) Effects of DCCD on passive H^+-translocation (expressed as the reciprocal value of $t_{1/2}$) in the presence (●——●) and absence (○ ——○) of bovine serum albumin and on NADH oxidase activity (△——△). (B) The same parameters as in (A) corrected for the DCCD-insensitive portions of the various activities.

inverse measure of the rate of passive H^+-release. The $t_{1/2}$ was increased by DCCD (fig.3); this increase is therefore an expression of the protonophoric activity of F_0.

Fig.4 shows the relationship between DCCD binding and the rate of passive H^+-diffusion (expressed as $1/t_{1/2}$ of fluorescence decrease following anaerobiosis; cf. fig.3). The latter was measured both in the absence and the presence of bovine serum albumin, which lowered the rate of H^+-diffusion without changing the DCCD titer. This effect is probably due to the removal of endogenous free fatty acids which may increase the H^+-permeability of the membrane [29]. Shown in fig.4A is also the effect of DCCD on the respiration of the particles with NADH as substrate. The inhibition caused by DCCD was reversed by FCCP, but not by valinomycin + K^+ (not shown), and can thus be taken as a measure of respiratory

control induced by DCCD due to inhibition of H^+-flow through F_0. It may be seen (fig.4B) that, after subtraction of the DCCD-insensitive portions of H^+-translocation and respiration, the DCCD-titration curves of the two parameters coincide. Extrapolation of the linear portion of the titration curves gives ~0.8 nmol DCCD/mg protein. This value is significantly higher than 0.45 nmol DCCD/mg protein found for the ATPase-linked H^+-translocation (cf. fig.2), and suggests that inhibition of H^+-translocation through F_0 in the absence of F_1 requires 2, rather than 1, molecules of DCCD/molecule of F_0. Further data supporting this conclusion will be presented in [30].

4. Conclusion and comments

Our data indicate that passive H^+-translocation through F_0 is blocked when 2 molecules of DCCD are bound/molecule of F_0. In contrast, active H^+-translocation coupled to F_1-catalyzed ATP hydrolysis (or ATP synthesis) is blocked by 1 molecule DCCD/molecule F_0. These results suggest that, in the absence of F_1, F_0 contains 2 H^+-channels, both of which are equally active in H^+-translocation. F_1-catalyzed ATP hydrolysis (or ATP synthesis) appears either to restrict the H^+-translocating activity of F_0 so that only one channel at a time is operating, or to require a simultaneous H^+-translocation through both channels. The latter alternative would be consistent with the need for >1 H^+ to be translocated for each molecule of ATP hydrolyzed or synthesized [31].

It has been concluded [6,7,27] that the DCCD-binding subunit of F_0 in mitochondria and chloroplasts occurs as a hexamer, i.e., that each F_0F_1 complex contains 6 molecules of the DCCD-binding protein. Our data (fig.1) suggest a lower value, probably as low as 3, which is consistent with data reported for bacteria [3,4,9]. The final answer to this question will require further estimates, taking into account, on one hand, the possible occurrence of low-affinity DCCD-binding protomers in F_0 and, on the other hand, the existence of DCCD-binding proteins that are not part of F_0, e.g., subunit III of cytochrome *c* oxidase [32], the β-subunit of F_1 [33], and the P_i translocator [23] as well as possibly other proteins (cf. [8,16]). In any case, these data show that only a limited number of the DCCD-binding subunits in each F_0 need to be blocked in order to inhibit H^+-translocation, and that this number is further restricted when H^+-translocation is coupled to F_1-catalyzed ATP hydrolysis or ATP synthesis.

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