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KINETICS OF INTERACTION BETWEEN THE H^+-TRANSLOCATING COMPONENT OF THE MITOCHONDRIAL ATPase COMPLEX AND OLIGOMYCIN OR DICYCLOHEXYLCARBODIIMIDE

Elzbieta Glaser and Birgitta Norling

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

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Kinetics of interaction between the H^+-translocating component of the mitochondrial ATPase complex and oligomycin or dicyclohexylcarbodiimide were studied in beef heart submitochondrial particles, and the results suggest that the two inhibitors have different binding sites with respect to the membrane and to F_1. Oligomycin seems to be bound to a subunit or a part of a subunit in F_0, which is localized superficially, and which is influenced by F_1, since the presence of F_1 considerably lowers the rate of inhibition. The oligomycin binding site further seems to be influenced by the different conformational states of F_1 occurring during the catalytic cycle of the enzyme. The binding site of DCCD on F_0, on the other hand, seems to be deeply embedded in the membrane and not influenced by F_1.

Synthesis and hydrolysis of ATP by the ATPase complex (F_0F_1) of mitochondria are coupled to H^+-translocation through F_0 (1, 2). Oligomycin (3) and dicyclohexylcarbodiimide (DCCD) (4), inhibitors of the hydrolytic and synthetic activity of the complex, are supposed to exert their inhibitory effect by binding to the F_0 part of the complex (5, 6) and blocking H^+-translocation through F_0 (7-9). It has been demonstrated that DCCD binds covalently to a specific protein component of F_0 (10, 11), which exists in an oligomeric form (12, 13), whereas oligomycin binds reversible (14) to a not yet identified subunit of F_0.

In a recent investigation in our laboratory the stoichiometries of inhibition by oligomycin and DCCD on different F_0-related reactions in beef-heart submitochondrial particles were studied (15, 16). The data indicate that passive H^+-translocation through F_0 is blocked when 2 moles of DCCD are bound per mole of F_0. These results suggest that, in the absence of F_1, F_0 contains two H^+-channels, both of which are equally active in H^+-translocation. Furthermore,

Abbreviation: DCCD, dicyclohexylcarbodiimide

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H^+-translocation coupled to ATP hydrolysis seems to involve a cooperative interaction of the two H^+-channels, since only one DCCD per F_o is needed for complete inhibition of ATPase activity. In the case of oligomycin, both the H^+-translocation coupled to ATP hydrolysis and passive H^+-translocation are inhibited by one mole of oligomycin per mole of F_o. These results suggest that the oligomycin-binding protein may be present only as a monomer in F_o, and thus may be a different subunit than the DCCD-binding oligomer. Alternatively, oligomycin may bind to the same protein as DCCD, but in contrast to the latter, it may induce a cooperative inhibition of the two H^+-channels even in the absence of F_1.

In order to better understand the mechanism of H^+-translocation of H^+-translocation through F_o, the kinetics of inhibition by oligomycin and DCCD of active H^+-translocation coupled to ATP hydrolysis and of passive H^+-translocation were investigated.

Materials and methods

Submitochondrial particles were prepared as described earlier (17). The particles were passed through a Sephadex G-50 column in order to remove the ATPase inhibitor protein (18).

ATPase (19) and NADH oxidase (9) activities were measured according to earlier described methods.

The effect of DCCD on the ATPase and NADH oxidase activities were studied after incubation of DCCD with submitochondrial particles suspended in 0.25 M sucrose and 10 mM Tris-Acetate, pH 7.5. Protein and DCCD concentrations in the various experiments are described in the figure legends. Samples were withdrawn after different times of incubation, for measurements of ATPase and NADH oxidase activities.

Inhibition of ATPase and NADH oxidase activities by oligomycin was estimated continuously from the spectrophotometric traces at protein and inhibitor concentrations as described in the figure legends.

Protein was estimated according to Lowry et al. (20).

Results

Fig. 1 shows the $t_{1/2}$, i.e. the time requried for 50% of maximal inhibition of ATPase and NADH oxidase activities in beef heart submitochondrial particles, for different concentrations of oligomycin and DCCD. NADH oxidase activity is dependent on passive H^+-leak through F_o devoid of F_1 and inhibition of NADH

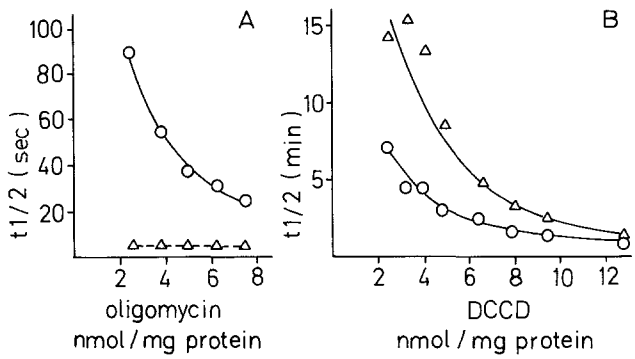

Fig. 1. The half-time ($t_{1/2}$) of inhibition of the ATPase (o-o) and NADH oxidase (Δ-Δ) activities by oligomycin (A) and DCCD (B) in submitochondrial particles.
A. The decrease in ATPase and NADH oxidase activities was measured continuously at a protein concentration of 0.008 mg/ml.
B. Submitochondrial particles were incubated with DCCD at a protein concentration of 1 mg/ml and samples were withdrawn at different times for measurements of ATPase and NADH oxidase activities. In the ATPase assays 1 μM FCCP was present. ATPase activity and NADH oxidase activity were measured as described in Materials and methods.

oxidation by oligomycin or DCCD therefore indirectly reflects inhibition of passive H^+-translocation.

As seen in Fig. 1A, oligomycin, in a concentration range of 2-8 nmol inhibitor/mg protein, gives $t_{1/2}$ for the ATPase activity ranging between 100-20 seconds. The experiments were performed at 0.008 mg particle protein/ml. DCCD affected ATPase activity with $t_{1/2}$ values between 7-1 minutes for the same range of inhibitor concentration. However, these experiments were performed at 1 mg particle protein/ml. Fig. 1B furthermore shows that inhibition of NADH oxidase by DCCD is slower than inhibition of ATPase activity; however the difference is diminished with increasing concentrations of DCCD. In the case of oligomycin, NADH oxidase is inhibited very rapidly; almost maximal inhibition was obtained during mixing time (5 sec.).

Fig. 2 shows the inhibition of ATPase and NADH oxidase activities by DCCD plotted as log activity versus time. At an inhibitor concentration of 3.3 nmol DCCD per mg protein (Fig. 2A) the inhibition of both ATPase and NADH oxidase activities seems to follow pseudo-first-order kinetics as revealed by linear plots. At a lower DCCD concentration, 2.4 nmoles/mg protein (Fig. 2B), only inhibition of the ATPase activity seems to follow pseudo-first-order kinetics,

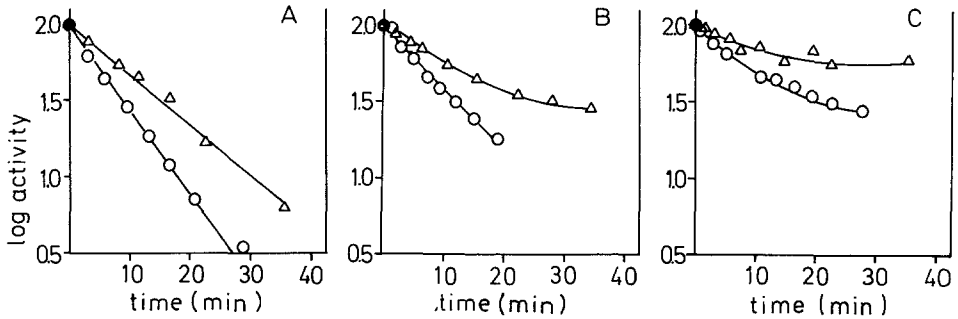

Fig. 2. Inhibition of ATPase (o-o) and NADH oxidase (Δ-Δ) activities by DCCD in submitochondrial particles as a function of time. Submitochondrial particles were incubated with DCCD at the following concentrations: 3.3 nmol (A), 2.4 nmol (B), and 1.6 nmol (C), DCCD per mg protein. The protein concentrations were 0.6 mg/ml (A) and 1 mg/ml (B and C). Other conditions as in Fig. 1.

whereas at the lowest concentration used, 1.6 nmoles DCCD/mg protein (Fig. 2C); inhibition of both activities did not follow pseudo-first-order kinetics. Under the conditions employed no binding of DCCD to the β subunit of F_1 occurred (13), neither was the ATPase activity of F_1 inhibited.

The inhibition of ATPase activity by oligomycin does not follow pseudo-first-order kinetics for any of the oligomycin concentrations used, namely 2.9, 5.0 and 9.5 nmoles oligomycin/mg protein (Fig. 3). In all three cases, there is an initial faster rate of inhibition which leads to about 50% inhibition of the ATPase activity.

Discussion

As reported earlier (15, 16) the stoichiometry of inhibition of passive H^+-translocation through F_0 devoid of F_1 is different for oligomycin and DCCD, indicating different modes of action of the two inhibitors on F_0. Kinetic experiments reported in this paper further demonstrate the different behaviours of the two inhibitors. This is shown by comparing the effect of oligomycin and DCCD on the ATPase and the NADH oxidase activities. Inhibition of the ATPase activity reflects inhibition of active H^+-translocation through complete F_0F_1 complexes, whereas inhibition of the NADH oxidase activity reflects inhibition of passive H^+-translocation through F_0 devoid of F_1.

In the case of oligomycin, inhibition of ATPase activity is much slower than inhibition of NADH oxidase activity. This result indicates that the pre-

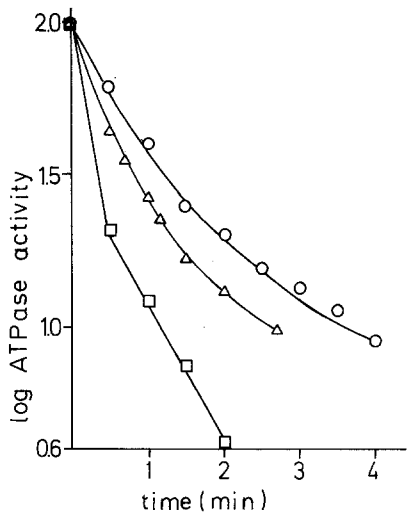

Fig. 3. Inhibition of ATPase activity by oligomycin in submitochondrial particles as a function of time.
ATPase activity was measured, in the presence of 1 μM FCCP, as described in Material and methods. Oligomycin and protein concentration were; 2.9 nmol oligomycin/mg protein and 0.03 mg particle protein/ml (□-□); 5.0 nmol oligomycin/mg protein and 0.0083 mg protein/ml (Δ-Δ); 9.5 nmol oligomycin/ml and 0.0035 mg protein/ml (o-o).

sence of F_1 considerably influences the rate of binding of oligomycin to F_o, the rate being much higher in the absence of F_1. However, the absence of F_1 does not seem to enhance the rate of binding of DCCD to F_o, while on the contrary, DCCD inhibitis NADH oxidase activity more slowly than ATPase activity. This may be due to the fact that two molecules of DCCD need to be bound per F_o in order to completely block passive H^+-translocation through F_o, and that the second molecule of DCCD binds with a lower affinity than the first one.

Another difference, which is very obvious, is that DCCD binds considerably slower to F_oF_1 or F_o than does oligomycin. The reason for this difference may be either that the formation of a covalent bond between the imido group of DCCD and a carboxylic group on the DCCD-binding protein is relatively slow, or that the DCCD-binding site is deeply embedded in the membrane. The rate of inhibition by DCCD can be enhanced by increasing the concentration of DCCD, which is consistent with both alternatives. Oligomycin on the other hand is very rapidly bound, especially in the absence of F_1, which may indicate a superficial binding site for oligomycin close to F_1 which is peripheral in relation to the membrane.

The interaction of DCCD with both F_oF_1 (21, 22) and F_o follows pseudo-first-order kinetics (cf. Fig. 2). However, at an intermediate concentration of DCCD used, the inhibition of NADH oxidase activity did not follow pseudo-first-order kinetics, whereas the inhibition of ATPase activity did. This difference again is probably due to the fact that two molecules of DCCD need to be bound per F_o in order to maximally inhibit NADH oxidase activity, and in this case the excess of DCCD is not sufficient to fulfill the conditions for pseudo-first-order kinetics.

Inhibition of the ATPase activity by oligomycin did not show pesudo-first-order for any of the oligomycin concentrations used, namely 2.9, 5.0 and 9.5 nmoles oligomycin/mg protein which corresponds to a 7, 12 and 24-fold excess of inhibitor over F_oF_1 sites. The plots of log activity versus time were non-linear, showing a fast phase of inhibition of about 50% of the activity. Since the ATPase complex is supposed to exist in different conformational states during catalysis (23), these results may indicate that oligomycin binds with different affinities to the different conformational states.

In conclusion, the results presented in this paper would suggest that oligomycin and DCCD have different binding sites with respect to the membrane and to F_1. Oligomycin seems to be bound to a subunit or a part of a subunit in F_o, which is localized superficially, and which is influenced by F_1, since the presence of F_1 considerably lowers the rate of inhibition. The oligomycin binding site further seems to be influenced by the different conformational states of F_1 occurring during the catalytic cycle of the enzyme. The binding site of DCCD on F_o, on the other hand, seems to be deeply embedded in the membrane and not influenced by F_1.

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