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STOICHEIOMETRY OF DICYCLOHEXYLCARBODIIMIDE-ATPase INTERACTION IN MITOCHONDRIA

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1. The oligomeric dicyclohexylcarbodiimide (DCCD)-binding protein of mitochondrial ATPase was studied using (a) the relationship between [^{14}C]DCCD binding and inhibition of ATPase activities and (b) the analysis of the kinetics of inhibition. 2. The [^{14}C]DCCD binding to bovine heart mitochondria is linearly proportional to the inhibition of ATP hydrolysis up to a 50% decrease of the original activity resulting in 0.6 mol DCCD bound covalently to the specific inhibitory site (Houštěk, J., Svoboda, P., Kopecký, J., Kužela, Š. and Drahota, Z. (1981) Biochim. Biophys. Acta 634, 331–339) per mol of the fully inhibited enzyme. 3. Kinetics of the inhibition of both the ATPase activity (heart and liver mitochondria) and ADP-stimulated respiration (liver) reveal that 1 mol DCCD per mol ATPase eliminates both the synthetic and the hydrolytic activities. It is inferred that the activity-binding correlation underestimates the number of DCCD-reactive sites. 4. The second-order rate constant of the DCCD-ATPase interaction (*k*) is inversely related to the concentration of membranes, indicating that DCCD reaches the inhibitory site by concentrating in the hydrophobic (phospholipid) environment. 5. At a given concentration of liver mitochondria, comparable *k* values are obtained both for the inhibition of ATP hydrolysis ($k = 5.35 \cdot 10^2$ M$^{-1} \cdot$min^{-1}) and ADP-stimulated respiration ($k = 5.67 \cdot 10^2$ M$^{-1} \cdot$min^{-1}). 6. It is concluded that both the synthetic and the hydrolytic functions of ATPase are inhibited via a common single DCCD-reactive site. This site is represented by one of the several polypeptide chains forming the oligomer of the DCCD-binding protein. The inhibitor-ATPase interaction does not exhibit cooperativity, indicating that the preferential reactivity towards DCCD is an inherent property of the inhibitory site.

Introduction

In mitochondria, chloroplasts and bacteria the proton-translocating ATPase is responsible for the reversible conversion of energy of the transmembrane proton gradient to the chemical energy of ATP (see Refs. 1, 2 for reviews). The DCCD-induced inhibition [3] of the ATPase proved to be an important phenomenon for elucidation of both the structural and functional relationships between the catalytic part of the enzyme (F_1) and its membrane-bound protonophoric moiety (F_0). With the aid of [^{14}C]DCCD, a subunit of F_0 directly involved in the proton translocation was identified as a highly hydrophobic low-molecular-weight (approx. 8000 Da) protein. The amino acid composi-

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; F_1, water-soluble, catalytic part of ATPase; F_0, membrane-integrated moiety of ATPase; NCCD, *N*-(2,2,6,6-tetramethylpiperidyl-1-oxyl)-*N'*-(cyclohexyl)carbodiimide; SDS, sodium dodecyl sulphate; Da, dalton.

tion as well as the secondary structure of this protein, generally referred to as the DCCD-binding protein, was determined and Glu-59 (beef heart) was found to be the only DCCD-modified residue of the molecule (for reviews see Refs. 4 and 5). In the native membrane DCCD-binding protein is present in excess with respect to F_1, being probably arranged in F_0 as a hexamer [6,7].

Under the conditions where [^{14}C]DCCD inhibits the F_1-linked activities solely by blocking the proton translocation through F_0 [8], the maximal inhibitory effect is associated with radioactive labelling of only a fraction of DCCD-binding proteins [6,9–15]. However, the data are controversional and the accurate stoicheiometry of the DCCD-ATPase interaction is not known. In fact, 1 or 2 mol DCCD bound per 6 mol DCCD-binding protein were shown to be required for the maximal inhibition of the ATPase hydrolytic activity [6,9–11,13–15]. Furthermore, even substoicheiometric amounts of DCCD bound to the isolated mitochondrial ATPase (Complex V [14]) were sufficient to eliminate the P_i-ATP exchange activity, indicating that the inhibitory effect might not be due only to the covalent binding of DCCD to the inhibitory site [6,14,16].

The present study shows that the uncertainties concerning stoicheiometry of the DCCD-ATPase interaction as approached by the activity-binding correlations could be overcome by the analysis of the reaction kinetics. The results obtained indicate that the interaction of 1 mol DCCD with 1 mol ATPase of the mitochondrial membrane eliminates the activity.

A part of these results has already been published as a symposium report [17].

Materials and Methods

Preparation of mitochondria

Bovine heart mitochondria were isolated according to the procedure 3 of Smith [18] and rat liver mitochondria according to Schneider and Hogeboom [19].

Incubation with DCCD

Mitochondria were freeze-thawed three times and sedimented by centrifugation for 15 min at 30000 × *g* (at 0°C). Incubation with DCCD (added as an ethanolic solution; 10 μl/ml) was performed in a medium containing 0.25 M sucrose/10 mM Tris-HCl/1 mM EDTA (pH 7.4) in glass test-tubes. Binding of [^{14}C]DCCD to mitochondria washed subsequently with 90% acetone and 2.5% trichloroacetic acid was measured as described earlier [20]. In control experiments the mixing of mitochondria with acetone (0°C) stopped the [^{14}C]DCCD binding immediately. The acetone washing removed about 90% of phospholipids, whereas the protein recovery was better than 95%. When the binding to mitochondrial phospholipids was measured, the binding of [^{14}C]DCCD to membrane proteins was subtracted from the total [^{14}C]DCCD binding (mitochondria washed twice with 2.5% trichloroacetic acid only).

Analytical methods

ATPase activity was measured in the presence of an ATP-regenerating system [21]. A medium containing 1.35 μmol ATP/0.8 μmol $MgCl_2$/2.7 μmol KCl/1.6 μmol phospho*enol*pyruvate/33 μmol sucrose/7 μmol Tris-HCl (pH 7.4) was preincubated with 3.5 units pyruvate kinase for 10 min at 28°C. The reaction was started by the addition of 0.1 mg mitochondrial protein (final volume 0.27 ml). After 1 min the reaction was stopped by 30 μl 50% trichloroacetic acid and tubes were placed on ice. After a 1 min centrifugation at 12000 × *g* (Eppendorf microcentrifuge) 0.25 ml aliquots of the supernatant were taken for determination of inorganic phosphate [22].

Mitochondrial respiration was measured polarographically at 28°C using a Clark oxygen electrode [23]. The incubation medium contained 80 mM KCl, 3 mM $MgCl_2$, 5 mM potassium succinate, 4 mM potassium phosphate, 1 mM EDTA, 0.4% bovine serum albumin (fatty acid free), 20 mM Tris-HCl (pH 7.4) and 1.5 mg mitochondrial protein (the last addition) in a final volume of 0.8 ml. The ADP-stimulated respiration was taken as the difference between the rates of respiration after and before the ADP addition.

Concentration of the strong antimycin-binding sites was determined fluorimetrically in the presence of 1 mg of bovine serum albumin per ml [24].

Protein concentration was determined by the method of Lowry et al. [25] with bovine serum albumin as standard.

Numerical analysis

Nonlinear regression was performed to determine unknown kinetic parameters. The method of direct search [26] was used to minimize the sum of squares of residuals. As a system of differential equations is involved, the estimation of unknown parameters had to be performed by means of numerical integration in a computer. The semiimplicit Runge-Kutta method according to Rosenbrock [27] was applied. The results obtained by means of the modular systems of programmes BIOKIN [28] are summarized in Table I, together with the estimate of individual confidence limits of the parameters at the 95% level of reliability:

$$\bar{k}_i - t_{1-\alpha/2}\cdot S(\bar{k}_i) < k_i < \bar{k}_i + t_{1-\alpha/2}\cdot S(\bar{k}_i)$$

where $S(\bar{k}_i)$ represents the standard deviation of the parameter estimate (k_i) and $t_{1-\alpha/2}$ the appropriate t value from the t-distribution at $1-\alpha$ probability level.

Materials

[^{14}C]DCCD of specific radioactivity 32.5 mCi/mol was prepared according to Ref. 29. Antimycin A (type III) and bovine serum albumin (fatty acid free) were obtained from Sigma; ATP, phospho*enol*pyruvate and pyruvate kinase were from Reanal. Other chemicals were analytical grade.

Results

Correlation between the [^{14}C]DCCD binding and inhibition of ATPase activity in heart mitochondria

The time course of both the [^{14}C]DCCD binding and inhibition of hydrolytic activity of the membrane bound ATPase was measured at 28°C (Fig. 1A). At all [^{14}C]DCCD concentrations tested the binding of the inhibitor was completed within 2 h of the incubation. In agreement with a similar experiment performed at 0°C [30], no more than 50% of the added [^{14}C]DCCD remained bound to mitochondrial membrane proteins, while no significant labelling of phospholipids was detected (not shown). The inactivation of the mitochondrial ATPase was both time- and concentration-dependent [3] and at higher [^{14}C]DCCD concentrations more than 90% of the ATPase activity became inhibited. As the ATPase released by chlo-

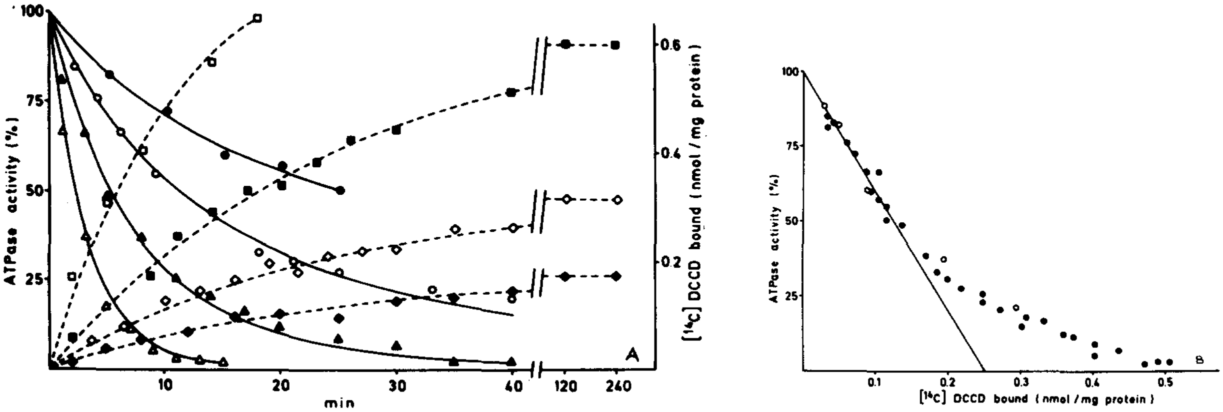

Fig. 1. Binding of [^{14}C]DCCD and inhibition of ATPase activity in heart mitochondria. A. The time course of both the inhibition of ATPase activity (●——●, ○——○, ▲——▲ and △——△) and [^{14}C]DCCD binding (◆------◆, ◇------◇, ■------■ and □------□) to mitochondria (1 mg protein/ml) incubated with 0.37 μM (●, ◆), 0.68 μM (○, ◇), 1.3 μM (▲, ■) and 3.0 μM (△, □) [^{14}C]DCCD at 28°C. The inhibition by DCCD was corrected for the increase of the activity of controls incubated in parallel (1.43–1.65 μmol ATP hydrolysed/min per mg protein), and for the activity insensitive to DCCD (7%). Solid lines refer to the theoretical curves fitted to the experimental points according to the model B (Table I). B. The above data (●), and [^{14}C]DCCD binding and ATPase activity determined after 4 h of incubation of heart mitochondria (1 mg protein/ml with 0.05–1.10 μM [^{14}C]DCCD at 28°C (○), were replotted to find out the relationship between the [^{14}C]DCCD binding and inhibition of ATPase activity. Linear regression was performed to extrapolate all the points within the range 0–0.14 nmol [^{14}C]DCCD bound/mg protein to zero activity (intercept value=0.25±0.007 nmol [^{14}C]DCCD bound/mg membrane protein).

roform [31] from DCCD-treated mitochondria retained its hydrolytic activity (not shown) the inhibition under the conditions used (pH 7.4) was not due to the formation of derivatives of F_1 by DCCD [8].

The relationship between the binding of [^{14}C]DCCD and the inhibition of the ATPase activity is demonstrated in Fig. 1B. The extrapolation of the initial linear part of the curve to zero activity yielded a value of 0.25 ± 0.007 nmol [^{14}C]DCCD bound per mg mitochondrial proteins, whereas the value for the complete inhibitory effect was approx. 0.50 nmol [^{14}C]DCCD bound per mg protein.

In order to compare the two values with the amount of ATPase in the membrane, the content of the enzyme was estimated with the aid of strong antimycin-binding sites which exist in bovine heart mitochondria in an equal quantity with ATPase [24]. The value obtained was 0.29 ± 0.05 nmol antimycin sites per mg membrane protein, giving a ratio of about 1 mol [^{14}C]DCCD bound to membrane proteins per mol inactivated ATPase (as extrapolated from the initial linear part of the curve in Fig. 1B). This value, however, involves the interaction of [^{14}C]DCCD with protein sites which do not belong to the F_0 (Ref. 20; see Discussion), resulting in 0.6 mol [^{14}C]DCCD bound to the specific inhibitory site per mol inactivated ATPase. On the other hand, the activity-binding correlation takes into account only the covalently bound inhibitor. Therefore, the attempts were made to define the DCCD-ATPase interaction independently by analysing the kinetics of the inhibitory effect.

Analysis of the kinetics of the DCCD-ATPase interaction

In kinetics of an irreversible inhibitor-enzyme interaction an approach [32] to the reaction mechanism currently used is to evaluate pseudo-first-order rate constants (k') of the reaction as a function of the inhibitor concentrations. Recently, this approach was successfully used to analyze the kinetics of the DCCD-induced inhibition of hydrolytic activity of the isolated F_1 [33], as well as the proton translocation through bacterial F_0 [34]. Fig. 2 indicates that also in mitochondria the rates of inactivation of ATPase activity by DCCD follow the pseudo-first-order kinetics at the beginning of the reaction. The plot of k' versus DCCD concentrations (Fig. 3) suggests that the reaction order with respect to DCCD is close to one. The second-order-rate constant of DCCD-ATPase interaction is $k = 9.50 \cdot 10^4\ M^{-1} \cdot min^{-1}$ (Fig. 3).

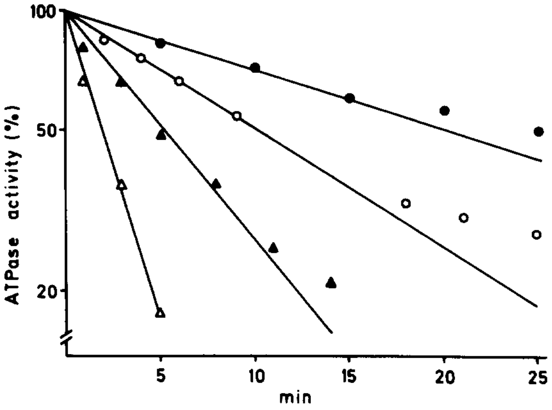

Fig. 2. Semilogarithmic plot of the inhibition of ATPase activity in heart mitochondria by various concentrations of [^{14}C]DCCD. Data from Fig. 1 were used.

However, the deviation of the experimental points in Fig. 2 from linearity indicates that the simple pseudo-first-order kinetic treatment does not fully express the time course of the inhibition. In fact, concentration of DCCD declines significantly during the reaction because: (a) the inhibitor is not present in large excess over the DCCD-binding sites of F_0; and (b) the pH-dependent degradation of DCCD exists in the aqueous medium, the rate of which is enhanced by mitochondrial phospholipids (Kopecký, J., unpublished data). As a consequence of these side reactions, and of a condensing reaction occurring probably at the inhibitory site (see Discussion), no more than 50% of added DCCD remained bound after completion of the reaction (Fig. 1).

To verify the above conclusions derived from the pseudo-first-order treatment of the DCCD-ATPase interaction (Figs. 2 and 3) the set of the analysed inhibitory data was fitted numerically to more realistic second-order-kinetic models of the DCCD-ATPase interaction (Table I; models A–C). Models A and B (as based on the results of the pseudo-first-order treatment) suppose that 1 mol DCCD per 1 mol ATPase eliminates completely its activity. In the model C the possibility that 2 mol

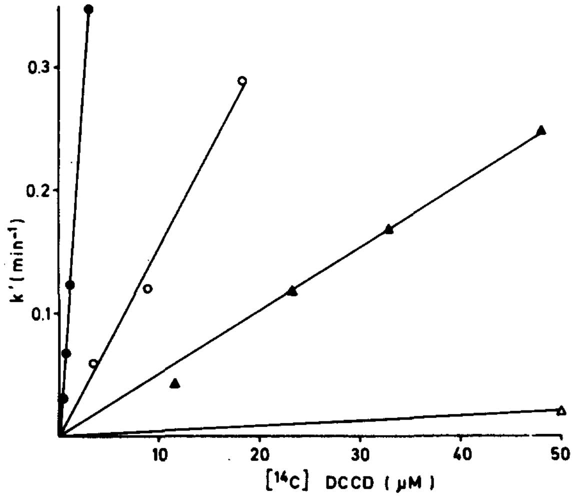

Fig. 3. The plot of the pseudo-first-order rate constants of the DCCD-ATPase interaction (k') in heart mitochondria as a function of DCCD concentration. Values of k' (28°C; 1 mg membrane protein/ml) were derived from the initial linear part of the curve in Fig. 2 (●——●). Mitochondria were also incubated at 0°C with 3.4–18 μM DCCD at 1 mg membrane protein/ml (○——○), with 15–48 μM DCCD at 5 mg protein/ml (▲——▲), and with 50–400 μM DCCD at 50 mg protein/ml (△——△). The k' values were determined from the semilogarithmic plots as in Fig. 2. The second-order rate constants of DCCD-ATPase interaction (k) were: $9.50 \cdot 10^4$ $M^{-1} \cdot min^{-1}$ (●——●); $1.56 \cdot 10^4$ $M^{-1} \cdot min^{-1}$ (○——○); $5.00 \cdot 10^3$ $M^{-1} \cdot min^{-1}$ (▲——▲); and $4.47 \cdot 10^2$ $M^{-1} \cdot min^{-1}$ (△——△), respectively.

DCCD per mol ATPase are required is also tested because up to a 50% inhibition of the original activity the concentration of DCCD added exceeds approximately twice the concentration of the inactivated enzyme molecules (Fig. 1). Model A consists of a single reaction of DCCD with ATPase, and only the second-order rate constant (k) of this reaction is computed. In models B and C the process of DCCD degradation following the pseudo-first-order kinetics is also included and the two parameters, i.e., the value of k and the pseudo-first -order rate constant of DCCD degradation (k_d), are computed.

The second-order rate constants of the DCCD-ATPase interaction as computed from models A–C do not differ too much (Table I) and their values ($k = 6.32 \cdot 10^4$ $M^{-1} \cdot min^{-1}$ to $k = 9.45 \cdot 10^4$ $M^{-1} \cdot min^{-1}$) are very similar to those of the second-order rate constant as derived from the pseudo-first-order treatment of the same data (Fig. 3). However, by comparing the individual confidence limits of k using the sum of the squared deviations ($\Sigma\Delta^2$) as a criterion of the fitting, it is clear (Table I) that the model B (see Fig. 1A for the plot of the calculated inhibitory curves) is better than the model A and much better than model C. As follows from the comparison of the models A and B the involvement of DCCD degradation is essential for a good fitting of the latter (see Discussion).

It is evident that both the pseudo-first-order

TABLE I

NUMERICAL ANALYSIS OF THE KINETICS OF THE INHIBITION OF ATPase ACTIVITY

The inhibitory data from Fig. 1A were subjected to the numerical analysis (see Material and methods) according to the models described (A–C). C_A and C_I represent actual concentrations of the active enzyme and inhibitor, respectively; k, the second-order rate constant of the DCCD-ATPase interaction; k_d, the first-order rate constant of the degradation of DCCD; and $\Sigma\Delta^2$, the sum of squares of residuals. The initial concentration of active enzyme molecule (C_{A_0}) was 0.29 μM.

Model	Mathematical description	Estimated parameters and its individual limits		$\Sigma\Delta^2$
		$k(M^{-1} \cdot min^{-1})(\times 10^4)$	$k_d(min^{-1})(\times 10^{-3})$	
A	$dC_A/dt = -k \cdot C_A \cdot C_I$	6.32±2.90	–	946
B	$dC_A/dt = -k \cdot C_A \cdot C_I$ $dC_I/dt = -k \cdot C_A \cdot C_I - k_d \cdot C_I$	7.20±0.40	4.90± 3.90	218
C	$dC_A/dt = -k \cdot C_A \cdot C_I^2$ $dC_I/dt = -k \cdot C_A \cdot C_I^2 - k_d \cdot C_I$	9.45±3.27	2.98±12.1	6227

treatment and the numerical analysis of the kinetic data lead to the same conclusion about stoicheiometry of the DCCD-ATPase interaction and the k value. Therefore the simple pseudo-first-order treatment was used to analyze the inhibition data in further experiments.

Effect of temperature and concentration of membranes on the kinetics of the DCCD-ATPase interaction

The second-order rate constants of the DCCD-ATPase interaction (1 mg protein/ml) measured at 28°C ($k = 9.50 \cdot 10^4$ $M^{-1} \cdot min^{-1}$) and 0°C ($k = 1.56 \cdot 10^4$ $M^{-1} \cdot min^{-1}$) indicate that the rate of the reaction approximately doubles when increasing temperature by 10°C (Fig. 3). A similar effect was shown for the DCCD-induced inhibition of the ATP-driven NAD^+ reduction by succinate [3].

The second-order rate constant was also strongly dependent on the used concentration of membrane. As compared with the rate of the reaction at 1 mg membrane protein/ml ($k = 1.56 \cdot 10^4$ $M^{-1} \cdot min^{-1}$) a 3-fold and 35-fold decrease of the constant ($k = 5.00 \cdot 10^3$ $M^{-1} \cdot min^{-1}$ and $k = 4.47 \cdot 10^2$ $M^{-1} \cdot min^{-1}$) due to a change in the protein concentration to 5 and 50 mg per ml, respectively, was observed (Fig. 3; 0°C).

Kinetics of the inhibition of the ADP-stimulated respiration and ATP hydrolysis in liver mitochondria

The rate of the ADP-stimulated respiration in rat liver mitochondria was used as a measure of the synthetic activity of ATPase (Fig. 4A, B). The inhibition by DCCD obeyed the pseudo-first-order kinetics (Fig. 4A) and the plot of the pseudo-first-order rate constants (k') as a function of DCCD concentrations suggested (Fig. 4B) that 1 mol DCCD per mol ATPase eliminates the synthetic activity. The second-order-rate constant of the DCCD-ATPase interaction (50 mg mitochondrial protein/ml; 0°C) was $k = 5.67 \cdot 10^2$ $M^{-1} \cdot min^{-1}$ (Fig. 4B). A similar value of the constant was determined on the basis of the DCCD-induced inhibition of ATPase hydrolytic activity when measured in rat liver mitochondria ($k = 5.35 \cdot 10^2$ $M^{-1} \cdot min^{-1}$; Fig. 4B) or in bovine heart mitochondria ($k = 4.47 \cdot 10^2$ $M^{-1} \cdot min^{-1}$; Fig. 3) under identical conditions.

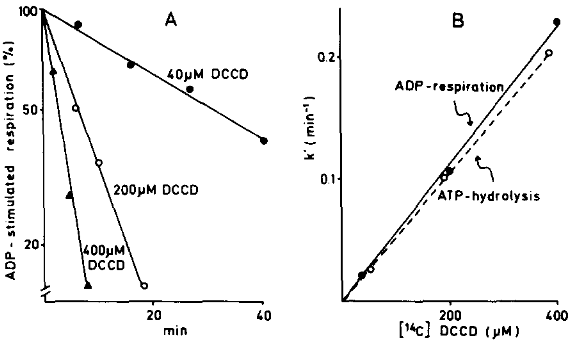

Fig. 4. (A) Semilogarithmic plot of the ADP-stimulated respiration at various DCCD concentrations; (B) plots of k' versus DCCD concentrations for the inhibition of the ADP-stimulated respiration and ATPase activity in liver mitochondria. ADP-stimulated respiration (A,B): freshly isolated rat liver mitochondria (50 mg protein/ml) were incubated with DCCD at 0°C. At indicated time intervals aliquots were taken for the measurement of respiration and the data of inhibition were plotted semilogarithmically (A). The values of k' (derived from A) were plotted as a function of DCCD concentration (B). The rates of respiration in controls in the absence and presence of ADP were 15 and 80 nmol O_2/min per mg protein, respectively. Control values did not change significantly during a 40 min incubation at 0°C. The DCCD-induced inhibition of respiration was abolished by FCCP (1 µM). ATPase activity (B): freeze-thawed rat liver mitochondria were incubated with DCCD as above and ATP hydrolysis was measured. Specific activity of the control was 0.70 µmol ATP hydrolysed/min per mg protein. The second-order rate constants (k) of the DCCD-ATPase interaction were $5.67 \cdot 10^2$ $M^{-1} \cdot min^{-1}$ and $5.35 \cdot 10^2$ $M^{-1} \cdot min^{-1}$ for the ADP-stimulated respiration and ATP hydrolysis, respectively (B).

Discussion

The correlation between the binding of [^{14}C]DCCD to mitochondrial membrane proteins and the inhibition of the ATP hydrolysis indicates that up to a 50% inhibition the binding is linearly related to the inhibition induced. When extrapolated to the maximal inhibition, the binding of 1 mol [^{14}C]DCCD per mol enzyme is obtained. However, it has been shown [20] that as a consequence of the inhibition up to 50% the two membrane proteins are significantly labelled in heart mitochondria: (a) a monomeric form of the DCCD-binding protein of F_0 (about 60% of bound radioactivity; see Fig. 3 of Ref. 20), the labelling of which is proportional to the inhibition of the ATPase activity [12,14,20]; and (b) a 33000 Da protein which apparently does not belong to the components of the mitochondrial ATPase [35]. Hence, the [^{14}C]DCCD binding to the specific inhibitory site of F_0 at the full inhibition results in approx. 0.6 mol [^{14}C]DCCD bound per mol ATPase. It is inferred that a part of [^{14}C]DCCD attacking the inhibitory site remains covalently bound (*N*-acylurea; see Ref. 5) whereas some of the DCCD-activated carboxyl groups (*O*-acylisourea) might undergo a nucleophilic attack by an adjacent reactive group (phospholipids; intra- or intersubunit interactions) to form an inhibitory condensing product and the ^{14}C label is lost. This possibility was already extensively discussed [6,14,16] and appears to be also the case of some other inhibitory effects of DCCD, for example, the inhibition of the hydrolytic activity of isolated F_1 where DCCD-induced intramolecular cross-linking was recently observed [36].

The appearance of the non-linear part of the curve of the activity-binding correlation is in accordance with an abrupt beginning [20] of the labelling of the 16000 Da component of F_0 which represents an aggregate of the DCCD-binding protein. The aggregate is resistant to the dissociation by SDS [37] and is not directly involved in the DCCD-induced inhibition [12,20].

In the light of the expected condensing reaction occurring at the inhibitory site, the conclusion about stoicheiometry of the DCCD-ATPase interaction as derived from the activity-binding correlations remains inaccurate. However, both the pseudo-first-order treatment of kinetics of the inhibition of the ATPase activity and the numerical fitting of the data to the second-order kinetic models strongly suggest that 1 mol DCCD per mol of ATPase eliminates its activity. This holds true especially if the process of DCCD degradation is also included.

The inverse relationship between the amount of the mitochondrial membrane present in an aqueous suspension and the value of the second-order rate constant of the DCCD-ATPase interaction (k) might be explained by assuming that only DCCD concentrated in the hydrophobic membrane environment [38] can reach the inhibitory site. Therefore, at a fixed volume of the mitochondrial suspension an increase in the amount of membranes results in a decrease of the actual DCCD concentration in the hydrophobic (phospholipid) environment surrounding the inhibitory site. Therefore, the value of k (calculated from the DCCD concentration related to the total volume of the mitochondrial suspension) decreases.

The kinetic analysis of the inhibition of the ADP-stimulated respiration revealed that, similarly to the inhibition of ATPase hydrolytic activity, 1 mol DCCD per mol ATPase eliminates the synthetic activity. As the k values obtained from the inhibition of the ADP-stimulated respiration and ATP hydrolysis are similar, both processes have to be inhibited via a common DCCD-reactive site. This conclusion is further supported by the correlations between the DCCD binding and the inhibition of ATP hydrolysis and P_i-ATP exchange in submitochondrial particles [15].

In a view of the present kinetic study, which uses the catalytic activities of membrane bound F_1 as an indirect way to follow the proton translocation through F_0, it is obvious that the preferential reactivity of a single inhibitory site of the DCCD-binding oligomer cannot be explained by cooperativity of DCCD binding. This conclusion is in accordance with a nonrandom labelling by [^{14}C]DCCD of the individual polypeptide chains in the native membrane [12,20], and with the spin-label experiments using NCCD [7]. Taken together, these data provide strong evidence in favour of the intrinsic structural asymmetry of the DCCD-binding oligomer when connected to the F_1 part of the enzyme. However, the detailed arrangement of the oligomer still remains to be elucidated. To understand the involvement of its individual polypeptide chains in proton translocation and the functional and structural relationships between F_0 and F_1 the inhibitory studies at the level of the intact ATPase need to be compared with the direct measurements of passive proton transport through isolated F_0 [34,39] and F_1-depleted membranes [15,40].

References

1 Kagawa, Y. (1978) Biochim. Biophys. Acta 505, 45–93
2 Senior, A.E. (1979) in Membrane Proteins in Energy Trans-

duction (Capaldi, R.A., ed.), vol. 2, pp. 233–278, Dekker, New York
3 Beechey, R.B., Robertson, A.M., Holloway, C.T. and Knight, F.G. (1967) Biochemistry 6, 3867–3879
4 Fillingame, R.H. (1980) Annu. Rev. Biochem. 49, 1079–1113
5 Sebald, W., Machleidt, W. and Wachter, E. (1980) Proc. Natl. Acad. Sci. USA 77, 785–789
6 Sebald, W., Graf, T. and Lukins, H.B. (1979) Eur. J. Biochem. 93, 587–599
7 Sigrist-Nelson, K. and Azzi, A. (1979) J. Biol. Chem. 254, 4470–4474
8 Pougeois, R., Satre, M. and Vignais, P.V. (1980) FEBS Lett. 117, 344–348
9 Fillingame, R.H. (1976) J. Biol. Chem. 251, 6630–6637
10 Altendorf, K. (1977) FEBS Lett. 73, 271–275
11 Graf, T. and Sebald, W. (1978) FEBS Lett. 94, 218–222
12 Norling, B., Glaser, B. and Ernster, L. (1978) in Frontiers of Biological Energetics (Dutton, P.L., Leigh, J.S., Scarpa, A., eds.), vol. 1, pp. 504–515, Academic Press, New York
13 Sigrist-Nelson, K., Sigrist, H. and Azzi, A. (1978) Eur. J. Biochem. 92, 9–14
14 Kiel, R. and Hatefi, Y. (1980) Biochemistry 19, 541–548
15 Kopecký, J., Glaser, A., Norling, B. and Ernster, L.C. (1981) FEBS Lett. 131, 208–212
16 Partis, M.D., Bertoli, E. and Griffiths, D.E. (1980) J. Gen. Microbiol. 116, 233–236
17 Drahota, Z., Kopecký, J., Svoboda, P., Pavelka, S., and Houštěk, J. (1981) in Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria, Bari (Palmieri, F., ed.), pp. 223–226, Elsevier/North-Holland Biomedical Press, Amsterdam
18 Smith, A.L. (1967) Methods Enzymol. 10, 81–86
19 Schneider, W.C. and Hogeboom, G.H. (1950) J. Biol. Chem. 183, 1123–1128
20 Houštěk, J., Svoboda, P., Kopecký, J., Kužela, Š. and Drahota, Z. (1981) Biochim. Biophys. Acta 634, 331–339
21 Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329
22 Lindberg, O. and Ernster, L. (1956) in Methods in Biochemical Analysis (Glick, D., ed.), pp. 1–22, Wiley Interscience, New York
23 Estabrook, R.W. (1967) Methods Enzymol. 10, 41–48
24 Vàdineanu, A., Berden, J.A. and Slater, E.C. (1976) Biochim. Biophys. Acta 449, 468–479
25 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
26 Wang, B.C. and Luus, R. (1978) Am. Inst. Chem. Eng. J. 26, 619–626
27 Seinfeld, J.H., Lapidus, L. and Hwang, M. (1970) Ind. Eng. Chem. Fundam. 9, 266–275
28 Votruba, J. (1981) Acta Biotechnologica 2, 103–116
29 Fillingame, R.H. (1975) J. Bacteriol. 124, 870–883
30 Svoboda, P., Kopecký, J., Houštěk, J. and Drahota, Z. (1979) Biochem. Biophys. Res. Commun. 89, 981–987
31 Beechey, R.B., Hubbard, S.A., Linnett, P.E., Mitchell, A.D. and Munn, E.A. (1975) Biochem. J. 148, 533–537
32 Levy, H.M., Leber, P.D. and Ryan, E.M. (1963) J. Biol. Chem. 238, 3654–3659
33 Pougeois, R., Satre, M. and Vignais, P.V. (1979) Biochemistry 18, 1408–1412
34 Sone, N., Yoshida, M., Hirata, H. and Kagawa, Y. (1979) J. Biochem. 85, 503–509
35 Houštěk, J., Pavelka, S., Kopecký, J., Drahota, Z. and Palmieri, F. (1981) FEBS Lett. 130, 137–140
36 Yoshida, M., Poser, J.W., Allison, W.S. and Esch, F.S. (1981) J. Biol. Chem. 256, 148–153
37 Glaser, E., Norling, B. and Ernster, L. (1981) Eur. J. Biochem. 115, 189–196
38 Beechey, R.B., and Knight, I.G. (1979) J. Bioenerg. Biomembranes 1, 89–100
39 Sone, N., Hamamoto, T. and Kagawa, Y. (1981) J. Biol. Chem. 256, 2873—2877
40 Glaser, E., Norling, B., Kopecký, J. and Ernster, L. (1981) Eur. J. Biochem. 121, 525–531