Inhibition of (Na^+,K^+)-ATPase by Dicyclohexylcarbodiimide. Evidence for Two Carboxyl Groups That Are Essential for Enzymatic Activity†

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ABSTRACT: *N,N'*-Dicyclohexylcarbodiimide (DCCD), a reagent that reacts with carboxyl groups under mild conditions, irreversibly inhibits (Na^+,K^+)-ATPase activity (measured by using 1 mM ATP) with a pseudo-first-order rate constant of 0.084 min^{-1} (0.25 mM DCCD and 37 °C). The partial activities of the enzyme, including (Na^+,K^+)-ATPase at 1 μM ATP, Na^+-ATPase, and the formation of enzyme–acyl phosphate (E–P), decayed at about one-third the rate at which (Na^+,K^+)-ATPase at 1 mM ATP was lost. The formation of E–P from inorganic phosphate was unaffected by DCCD while K^+-phosphatase activity decayed at the same rate as (Na^+,K^+)-ATPase measured at 1 mM ATP. The enzyme's substrates (i.e., sodium, potassium, magnesium, and ATP) all decreased the rate of DCCD inactivation of (Na^+,K^+)-ATPase activity measured at either 1 mM or 1 μM ATP. The concentration dependence of the protection afforded by each substrate is consistent with its binding at a catalytically relevant site. DCCD also causes cross-linking of the enzyme into species of very high molecular weight. This process occurs at about one-tenth the rate at which (Na^+,K^+)-ATPase activity measured at 1 mM ATP is lost, too slowly to be related to the loss of enzymatic activity. Labeling of the enzyme with [^{14}C]DCCD shows the incorporation of approximately 1 mol of DCCD per mole of large subunit; however, the incorporation is independent of the loss of enzymatic activity. The results presented here suggest that (Na^+,K^+)-ATPase contains two carboxyl groups that are essential for catalytic activity, in addition to the previously known aspartate residue which is involved in formation of E–P. DCCD most probably modifies these groups by inducing the formation of intramolecular isopeptide bonds involving these groups and nearby amino groups. Modification of the more reactive group interferes with ATP stimulation of the E_2 to E_1 transition at the end of the catalytic cycle; modification at the second site interferes with the initial phosphorylation of the enzyme.

Carbodiimides, in particular *N,N'*-dicyclohexylcarbodiimide (DCCD),[1] have been shown to inhibit a number of ion-transporting ATPases including the bacterial and mitochondrial coupling factors (Roberton et al., 1968; Evans, 1970), various eukaryotic proton pumps (Fanestil & Park, 1981; Schneider, 1983; Sussman & Slayman, 1983), Ca^{2+}-ATPase from sarcoplasmic reticulum (Pick & Racker, 1979; Murphy, 1981), and (Na^+,K^+)-ATPase (Schoner & Schmidt, 1969; Schoner et al., 1972; Contessa & Bruni, 1971).

In many cases, the characteristics of the inhibition caused by DCCD are consistent with its reacting at a site that is important in the binding of the cation substrate. In the cases of the bacterial and mitochondrial proton pumps, the site of modification by DCCD is the membrane-bound sector of the enzyme (F_0) (Cattell et al., 1971). Since DCCD abolishes the proton conductivity of F_0 (Patel & Kaback, 1976), a carboxyl group is implicated in the transport of protons by this enzyme. More recently, DCCD has been shown to react at an additional site in the F_1 portion of this enzyme (Pougeois et al., 1979). Modification of this site inhibits the ATPase activity of soluble F_1 and is thought to occur at or near an ATP binding site (Pougeois, 1983). The DCCD inhibition of the Ca^{2+}-ATPase also suggests that a carboxyl group is important in the transport of calcium since the cation protects the enzyme from inhibition (Pick & Racker, 1979; Murphy, 1981).

In the case of (Na^+,K^+)-ATPase, carboxyl groups are implicated in the binding of both sodium and potassium since the inclusion of either cation in the reaction mixture decreases the rate of inactivation by DCCD (Robinson, 1974; Yamaguchi et al., 1983). The experiments reported here extend the earlier work on DCCD inhibition of the (Na^+,K^+-ATPase and, in particular, focus on the information which can be obtained from the protection afforded by the enzyme's substrates against inhibition by DCCD. The effects of DCCD on the partial reactions of the enzyme are also reported.

Experimental Procedures

Materials. DCCD (99%) was obtained from Aldrich. [^{14}C]DCCD (45 mCi/mmol) was obtained from Research Products International. The specific activity was reduced to either 7.5 or 15 mCi/mmol with unlabeled DCCD, and the resulting solution (in pentane) was stored at −20 °C. [^{32}P]P_i (carrier free) was from New England Nuclear, and [γ-^{32}P]-ATP (carrier free) was from Amersham. The [^{32}P]P_i was neutralized with Et_3N, diluted with unlabeled triethylammonium phosphate to a specific activity of about 0.25 Ci/mmol, and filtered through a 0.22-μm filter to remove polyphosphates. The specific activity of the [γ-^{32}P]ATP was reduced with unlabeled Na_2ATP to 0.4 Ci/mmol for the assay of E–P formation or to 10 mCi/mmol for the Na^+-ATPase assay. The triethylammonium salt of ATP was prepared from the sodium salt (Sigma, vanadate free) by chromatography on Dowex 50W and neutralization with Et_3N. (Na^+,K^+)-

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[1] Abbreviations: CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; DCCD, *N,N'*-dicyclohexylcarbodiimide; EDTA, ethylenediaminetetraacetic acid; E-P, enzyme–acyl phosphate; Et_3N, triethylamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; P_i, inorganic phosphate; SDS, sodium dodecyl sulfate; Cl_3CCOOH, trichloroacetic acid; PAGE, polyacrylamide gel electrophoresis.

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ATPase was purified from dog kidney by the rapid SDS extraction method of Jorgensen (1974). The preparations used in these experiments had specific activities of about 900 μmol h^{-1} mg^{-1}; this activity was >95% inhibitable by ouabain. Analysis of the preparations by polyacrylamide gel electrophoresis in the presence of SDS reveals the presence of only the two subunits of the (Na^+,K^+)-ATPase.

Assays. (Na^+,K^+)-ATPase activity was measured by using a mixture which couples the hydrolysis of ATP to the oxidation of NADH (Barnett, 1970). The assay mixture contained 100 mM NaCl, 25 mM KCl, 2 mM $MgCl_2$, 1.5 mM phospho(enol)pyruvate, 1 mM ATP, 1 mM dithiothreitol, 0.35 mM NADH, 0.1 mM EDTA, 6.4 units/mL pyruvate kinase, and 10.4 units/mL lactic acid dehydrogenase in 20 mM HEPES, adjusted to pH 7.5 with Et_3N. In some instances, the concentration of ATP was reduced to 1 μM in an otherwise identical mixture. K^+ phosphatase was measured by using 1.5 mM *p*-nitrophenyl phosphate in a solution which also contained 25 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM K_2EDTA in 20 mM HEPES/Et_3N, pH 7.5. Na^+-ATPase activity was assayed by using [γ-^{32}P]ATP in 50 μL of a medium containing 100 mM NaCl, 2 mM $MgCl_2$, and 1 mM [γ-^{32}P]ATP in 25 mM HEPES/Et_3N, pH 7.0. The reaction was terminated by adding ammonium molybdate and H_2SO_4, and the resulting phosphomolybdate complex was extracted into benzene/isobutyl alcohol (1:1, by volume) as described by Goldin (1977). A 100-μL aliquot of the organic phase was added to 3 mL of Aquasol (New England Nuclear) and counted for ^{32}P. The temperature for all assays of enzymatic activity was 37 °C.

The formation of enzyme–acyl phosphate (E–P) from [^{32}P]P_i was performed by a modification of the method described by Resh (1982). Enzyme (1.25 μg) was incubated for 30 min at 0 °C in 50 μL of a solution containing 100 μM [^{32}P]P_i, 180 μM ouabain, and 9 mM $MgCl_2$ in 25 mM HEPES/Et_3N, pH 7.0. The reaction was terminated by adding 30 μL of 5 mg/mL bovine serum albumin and 0.9 mL of 10% (w/v) Cl_3CCOOH containing 10 mM H_3PO_4. The Cl_3CCOOH precipitates were collected by centrifugation, washed twice with Cl_3CCOOH/H_3PO_4, dissolved in 50 μL of 1 N NaOH, and counted for ^{32}P in 3 mL of Aquasol.

The formation of E–P from ATP was performed by incubating enzyme (3 μg) for 30 s at 0 °C in 60 μL of a solution containing 100 mM NaCl, 3 mM $MgCl_2$, and 100 μM [γ-^{32}P]ATP in 25 mM HEPES/Et_3N, pH 7.0. The reaction was stopped, and the Cl_3CCOOH precipitates were treated as described for E–P formation from P_i.

Protein was determined by the method of Lowry et al. (1951) in the presence of 1% sodium dodecyl sulfate using bovine serum albumin as a standard.

Reaction of Enzyme with DCCD. Reactions were carried out at a protein concentration of 250 μg/mL in 25 mM HEPES/Et_3N, pH 7.0, at 37 °C. The reaction was initiated by adding an aliquot of DCCD in absolute ethanol to the protein solution. The final concentration of DCCD was 0.25 mM unless otherwise indicated; ethanol was present at 2% (v/v). After incubation for the desired interval, the reaction was stopped by one of two methods. If a single enzymatic assay was to be performed, an aliquot of the reaction mixture was diluted directly into the assay mixture. The minimum dilution was 100-fold. If more than one activity was to be determined in a single experiment, the reaction was stopped by placing an aliquot of the reaction mixture into a tube which was precooled to 0 °C. Aliquots of this sample were subsequently removed for various assays.

Incorporation of [^{14}C]*DCCD into the Enzyme.* Reaction of the enzyme with [^{14}C]DCCD was carried out as with unlabeled reagent except that the concentration of DCCD was 125 μM. The incorporation of DCCD was determined as follows. The reaction mixture (100 μL) was added to 1 mL of ice-cold 10% (w/v) Cl_3CCOOH followed by 30 μL of 5 mg/mL bovine serum albumin. After incubation at 4 °C for 10 min, the precipitate was collected by centrifugation and washed twice by resuspension in 1 mL of 10% Cl_3CCOOH. Finally, the pellet was washed with 1 mL of acetone by vortexing, incubating for 1 h at −20 °C, and centrifuging. The resulting pellet was resuspended in 50 μL of 2% (w/v) sodium dodecyl sulfate and counted in 3 mL of Aquasol to determine the amount of ^{14}C.

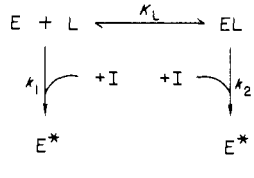
Electrophoretic Analysis of the Products of the DCCD Reaction. After reaction with DCCD, the enzyme was denatured with sample buffer, electrophoresed on a 7% acrylamide gel using the buffer system described by Laemmli (1970), stained with Coomassie blue, and destained. The incorporation of radioactivity into the α subunit of samples labeled with [^{14}C]DCCD was determined as follows. A 1 × 1 cm section of gel which contained the α subunit and the new band which appears slightly below it (see Figure 4) were removed, and the protein contained therein was removed from the gel by electroelution. The amount of protein in each sample was estimated by determining the absorbance at 550 nm of the resulting solution (the color being due to the protein-bound Coomassie blue). The radioactivity contained in each sample was determined by scintillation counting an aliquot of the solution in Aquasol. These measurements allow the determination of the rate at which cross-linking occurs and of the specific radioactivity of the un-cross-linked α subunit.

Analysis of the Data. The pseudo-first-order rate constants for DCCD inactivation of enzymatic activity (k_{obsd}) were determined by fitting the data to a single-exponential decay using nonlinear regression. The standard error of the estimate is reported for k_{obsd}.

Secondary plots of the dependence of k_{obsd} on the concentration of a protecting ligand (L) were also fitted by nonlinear regression to the model

$$k_{obsd} = k_1\frac{K_L}{K_L + [L]} + k_2\frac{[L]}{K_L + [L]}$$

This model was derived from the scheme:



where E is enzyme, E* is inactive enzyme, I is the inhibitor, and K_L is the dissociation constant for L, the protecting ligand. The rate constants for inactivation of E and EL are k_1 and k_2, respectively.

Results

Inhibition of Enzymatic Activity. DCCD was found to cause inactivation of (Na^+,K^+)-ATPase activity (measured by using 1 mM ATP) with a pseudo-first-order rate constant of 0.084 ± 0.007 min^{-1} (mean ± standard deviation of 14 determinations) at 37 °C and 0.25 mM DCCD. Most of the partial reactions of the enzyme were less sensitive to inactivation by DCCD than was (Na^+,K^+)-ATPase activity at 1 mM ATP. (Na^+,K^+)-ATPase activity measured with 1 μM ATP decayed at about one-third the rate observed when 1 mM

Table I: Rate Constants for DCCD Inactivation of Partial Reactions of (Na^+,K^+)-ATPase[a]

reaction	k_{obsd}
(Na^+,K^+)-ATPase/1 mM ATP[b]	1.00
(Na^+,K^+)-ATPase/1 μM ATP[b]	0.32
Na^+-ATPase	0.41
K^+ phosphatase	0.94
E–P from ATP	0.26
E–P from P_i	0.0

[a] The rate of DCCD inactivation of each activity is reported as a fraction of the rate of inactivation of (Na^+,K^+)-ATPase measured by using 1 mM ATP, determined in the same experiment. [b] The concentration of ATP used in the assay.

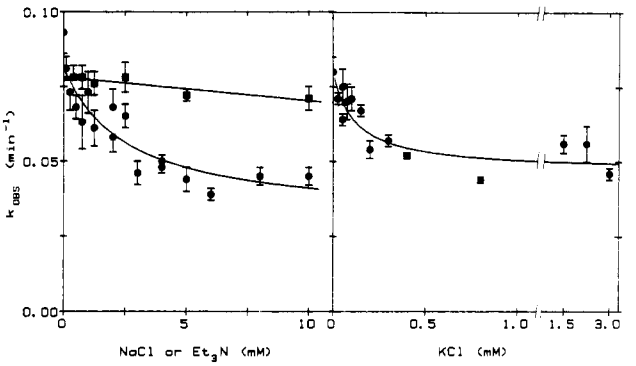


FIGURE 1: Cation protection against DCCD inactivation of (Na^+,K^+)-ATPase activity measured by using 1 mM ATP. The left panel shows the effect of including sodium chloride (●) or triethylammonium chloride (■) in the reaction mixture. The effect of potassium chloride is shown in the right panel.

ATP was used; the same is true for Na^+-ATPase activity (Table I). K^+ phosphatase activity, however, was inactivated at the same rate as (Na^+,K^+)-ATPase measured at 1 mM ATP.

The formation of enzyme–acyl phosphate (E–P) from ATP was also inactivated at about one-fourth the rate at which (Na^+,K^+)-ATPase activity decayed (Table I). Formation of E–P from inorganic phosphate was unaffected by DCCD (Table I). In fact, the level of E–P formed from P_i was still >90% of the initial value after a 2-h incubation with DCCD. (Na^+,K^+)-ATPase activity was inhibited by >90% at 1 mM ATP and by 70% at 1 μM ATP, under these conditions.

Protection against DCCD Inactivation by Substrates. The rate of DCCD inactivation of (Na^+,K^+)-ATPase activity (1 mM ATP) was decreased by including any of the enzyme's substrates in the reaction mixture. Protection in all cases occurred with a concentration dependence, which is consistent with binding of the substrate at catalytically relevant sites. Figures 1 and 2 show the concentration dependence of monovalent cation and ATP protection, respectively. The various parameters for protection by all of the substrates are summarized in Table II. These effects are not due to changes in ionic strength since triethylammonium chloride has no effect on the rate of DCCD inactivation (Figure 1, left panel).

The effects of substrates on the partial reactions of (Na^+,K^+)-ATPase were not studied as extensively. Sodium, potassium, and magnesium were found to protect against DCCD inactivation of (Na^+,K^+)-ATPase activity measured at 1 μM ATP (Table III). The protection by potassium and magnesium is comparable whether activity was measured with 1 μM or 1 mM ATP. Sodium, however, was more effective at protecting activity at the lower concentration of ATP. Protection by ATP against DCCD inactivation of (Na^+,K^+)-ATPase at 1 μM ATP could not be determined since the ATP used for protection would contribute significantly to the concentration of ATP in the assay.

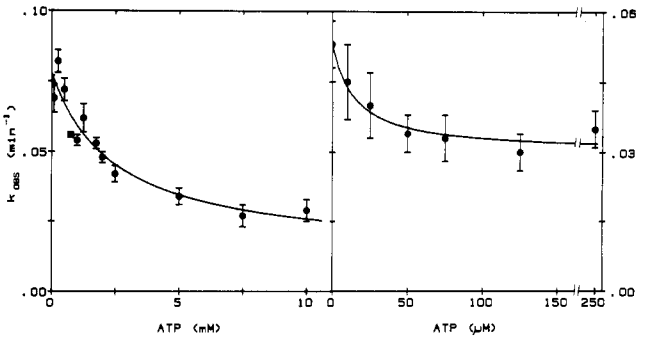


FIGURE 2: Protection against DCCD inactivation of (Na^+,K^+)-ATPase activity measured by using 1 mM ATP by ATP included in the DCCD reaction mixture. The triethylammonium salt of ATP was used. The experiment shown in the left panel was carried out by using the standard reaction conditions. The right panel shows the ATP effect seen in the presence of 5 mM triethylammonium CDTA.

Table II: Protection against DCCD Inactivation of (Na^+,K^+)-ATPase Activity by Substrates[a]

substrate	k_1 (min^{-1}) obsd[b]	k_1 (min^{-1}) fitted[c]	k_2 (min^{-1})[c]	K_L (mM)[c]
sodium	0.093	0.086 ± 0.004	0.030 ± 0.002	1.5 ± 0.3
potassium	0.080	0.078 ± 0.004	0.032 ± 0.002	0.20 ± 0.05
magnesium	0.069	0.072 ± 0.005	0.041 ± 0.003	0.16 ± 0.03
ATP	0.076	0.077 ± 0.003	0.013 ± 0.009	2.2 ± 0.3
ATP (+CDTA)[d]	0.053[e]	0.053 ± 0.002	0.030 ± 0.002	0.015 ± 0.007

[a] The rate constant for DCCD inactivation of (Na^+,K^+)-ATPase at 1 mM ATP was determined at a number of substrate concentrations (as in Figures 2 and 3), and the data were fit to the model described under Experimental Procedures. The standard error of the estimate for each parameter is indicated. [b] The value of k_{obsd} determined experimentally in the absence of substrates. [c] Determined by fitting the data using nonlinear regression. [d] 5 mM triethylammonium CDTA was present during the DCCD reaction. [e] Presumably, this value is much lower than the others because the CDTA adds 20 mM carboxyl groups to the reaction mixture.

Table III: Substrate Protection against DCCD Inactivation of (Na^+,K^+)-ATPase Activity Measured at both 1 mM and 1 μM ATP[a]

substrate	k_{obsd}[b] 1 mM[c]	k_{obsd}[b] 1 μM[c]
none	1.00	1.00
10 mM NaCl	0.24	0.07
10 mM KCl	0.56	0.57
10 mM ATP[d]	0.33	ND[e]
2.5 mM $MgCl_2$	0.67	0.67

[a] Inactivation of the enzyme was performed as described under Experimental Procedures except that the concentration of DCCD was 125 μM. [b] The rate constants are expressed as a fraction of the rate observed in the absence of substrate. These values were 0.040 and 0.018 min^{-1} for activity measured at 1 mM and 1 μM, respectively. [c] The concentration of ATP used in the assay of (Na^+,K^+)-ATPase activity. [d] The triethylammonium salt was used. [e] Not determined (see text).

DCCD-Induced Cross-Linking of the Enzyme. Incubation of the (Na^+,K^+)-ATPase with DCCD leads to the progressive loss of both subunits (Figure 3). Concomitant with this loss is the appearence of Coomassie blue stainable material which does not enter the gel. Traces of intermediates having a molecular weight greater than 121 000 are also apparent. The rate constant for the loss of the α subunit is 0.0052 min^{-1} (125 μM DCCD, 37 °C, average of two determinations), about 10% the rate of inactivation of (Na^+,K^+)-ATPase activity measured by using 1 mM ATP (Figure 4, bottom panel).

Labeling of (Na^+,K^+)-ATPase by [^{14}C]DCCD. [^{14}C]DCCD is incorporated into the enzyme (irrespective of aggregation

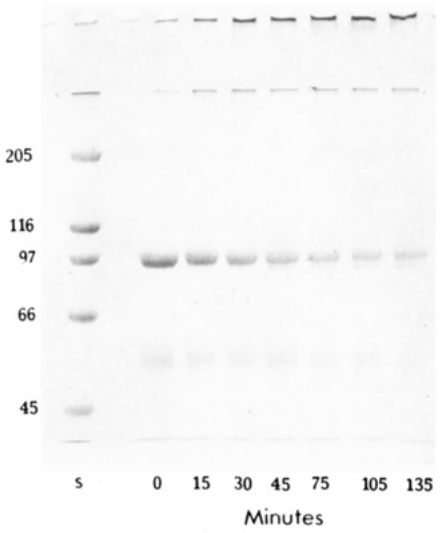


FIGURE 3: Cross-linking of (Na^+,K^+)-ATPase by DCCD. Enzyme was incubated with 125 μM [^{14}C]DCCD (15 mCi/mmol) for the indicated time before being denatured and electrophoresed. Twenty micrograms of enzyme was loaded in each lane. Molecular weight markers are shown on the left.

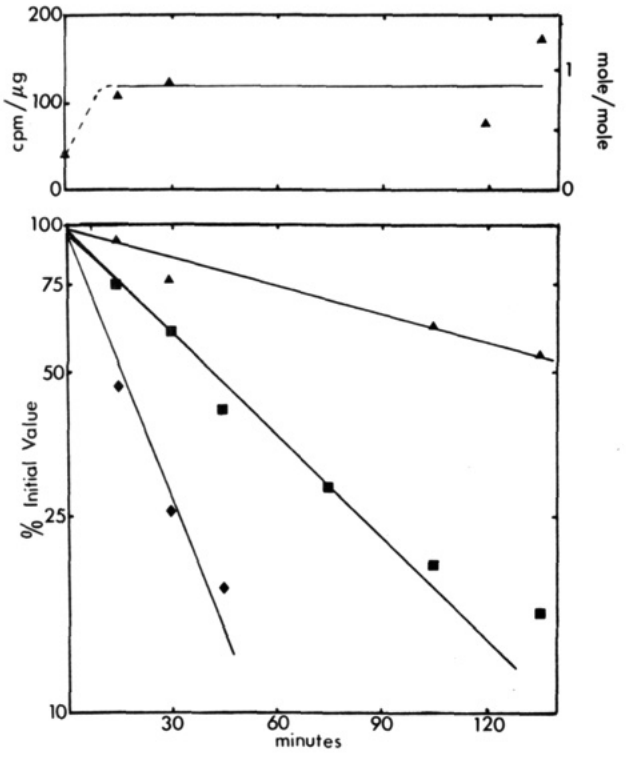


FIGURE 4: (Upper panel) Specific radioactivity of the α subunit of [^{14}C]DCCD-treated enzyme. The values are calculated from determinations of α subunit which remains un-cross-linked (see Experimental Procedures and the lower panel) on the assumption that the α subunit comprises 68% of the enzyme on a weight basis. The caveat regarding calculation of incorportion on a molar basis (legend to Figure 5) also applies here. The lower panel shows the decay of (Na^+,K^+)-ATPase activity measured at 1 mM ATP (♦) and at 1 μM ATP (■) and the loss of α subunit (▲) as a function of incubation time with DCCD (125 μM, 37 °C); the values of k_{obsd} for these processes were 0.033 ± 0.004, 0.012 ± 0.002, and 0.0059 ± 0.0009 min^{-1}, respectively. The data shown in both panels are from analysis of the gel shown in Figure 3.

state) with a pseudo-first-order rate constant of 0.014 ± 0.007 min^{-1} (125 μM DCCD, 37 °C) (Figure 5). The maximum extent of incorporation is 3–6 mol of DCCD per mole of enzyme. If [^{14}C]DCCD-labeled enzyme is analyzed by SDS–PAGE, one observes radioactivity in the cross-linked

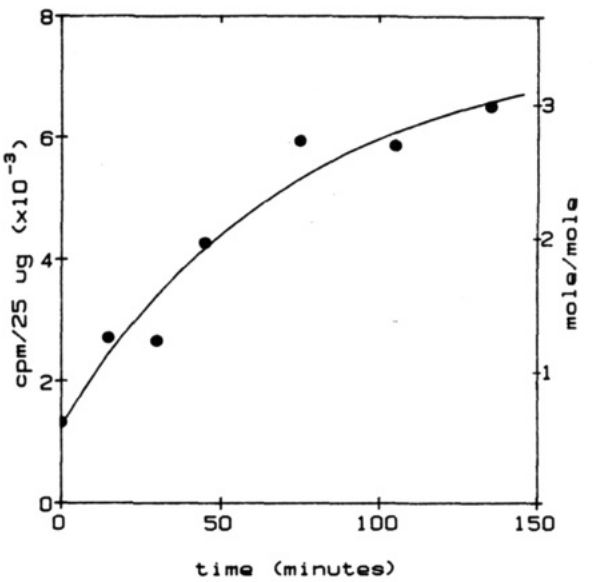


FIGURE 5: Incorporation of [^{14}C]DCCD into (Na^+,K^+)-ATPase, irrespective of aggregation state. Enzyme was incubated with [^{14}C]DCCD (7.5 mCi/mmol) and the radioactivity incorporated determined by the Cl_3CCOOH precipatation method described under Experimental Procedures. The average of two seperate experiments is reported. The solid line represents a nonlinear least-squares fit to the equation $V_t = (1 - e^{-kt})V_f + e^{-kt}V_0$ where V_0, V_f, and V_t are the incorporation of DCCD at time zero, at infinity, and at any time t, respectively; k is the rate constant. The calculated parameters and their standard deviations are V_0 = 1283 ± 465 cpm/25 μg, V_f = 7557 ± 1329 cpm/25 μg, and k = 0.014 ± 0.007 min^{-1}. The stoichometry, in terms of moles per mole, is calculated by assuming a molecular weight of 177 000 (Craig & Kyte, 1980) and that the Lowry determination of protein gives an accurate determination of (Na^+,K^+)-ATPase protein. If the Lowry determination overestimates the true protein concentration (Moczydlowski & Fortes, 1981), then the true values could be as much as twice the values reported here.

material and in the α subunit; labeling of the β subunit is not observed. Labeling of the α subunit amounts to approximately 0.8 mol/mol and is independent of enzymatic activity (Figure 4, top panel).

Discussion

The observation that DCCD inhibits the (Na^+,K^+)-ATPase suggests that carboxyl groups are important in catalysis by the enzyme. In fact, the existence of an active-site aspartate residue, which forms an acyl phosphate during the catalytic cycle, has been known for some time (Post & Kume, 1973). Several lines of evidence indicate that DCCD is reacting at sites other than the active-site aspartyl group. The level of E–P formed from P_i (a measure of intact active-site carboxyls) is unaffected by DCCD under conditions which substantially inhibit (Na^+,K^+)-ATPase activity. Also, if reaction were occurring at the active-site aspartate, one would expect that all partial activities of the enzyme would decay at the same rate as (Na^+,K^+)-ATPase activity measured at 1 mM ATP. This is clearly not the case. Thus, it is likely that DCCD is modifying a group (or groups) other than the active-site aspartate.

All of the results described here can be explained by postulating that modification of the enzyme by DCCD occurs at two sites. These sites differ in their reactivity toward DCCD and the manner in which modification affects enzymatic activity. Of course, arguments based upon kinetics of inactivation cannot formally determine the number of reacting groups; they can only indicate classes of groups with similar reactivity. One group, the more reactive, causes inhibition of (Na^+,K^+)-ATPase measured at 1 mM ATP. The rate of reaction at this

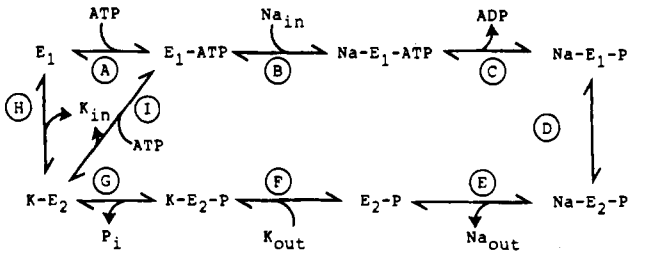


FIGURE 6: Scheme for the reaction mechanism of the (Na^+,K^+)-ATPase proposed by Cantley (1981), among others. The letters enclosed in circles indicate reaction steps for reference in the text. Step I is required to be faster than step H.

site is diminished by the presence of substrates in the reaction mixture. Modification of the enzyme by DCCD at this site interferes with the rate-limiting step (step I, Figure 6) of (Na^+,K^+)-ATPase activity measured at 1 mM ATP without affecting step H which is rate limiting in the case of (Na^+,K^+)-ATPase activity measured at 1 μM ATP (Kanazawa et al., 1970; Robinson, 1976). This would explain why (Na^+,K^+)-ATPase activity measured at 1 μM ATP decays more slowly than activity measured at 1 mM ATP.

The second group, which reacts with DCCD at about one-third the rate of the first group, is responsible for inhibition of the partial activities. The defect caused by modification of this group is in the first half of the reaction cycle (steps A–D). This defect is, most probably, in the rate of the initial phosphorylation of the enzyme (step C). An effect on the formation of the ternary complex Na–E_1–ATP (steps A and B) is unlikely since the concentrations of sodium and ATP used in the experiments were in great excess over their respective half-saturation constants. Finally, a defect in the rate of the E_1 to E_2 conformational transition (step D) or the spontaneous decay of E–P to E + P_i is not likely. A decrease in the rate of either of these steps would lead to inhibition of Na^+-ATPase activity but an increase in the level of E–P formed from ATP. Conversely, stimulation of these steps would result in a decrease in the amount of E–P formed from ATP and an increase (or no change) in Na^+-ATPase activity.

Experiments in which protection by substrates is examined indicate that both of these groups are located near the site(s) which bind substrates. The concentration dependence of sodium and potassium protection against inactivation suggests that DCCD is modifying the site(s) which bind the cation substrates during transport. The values obtained for the half-saturation constants in the protection experiments (1.5 and 0.2 mM, for sodium and potassium, respectively) are very similar to the K_m's for cation stimulation of ATPase activity [at low concentration of the constant cation; for example, see Post et al. (1960)]. These results confirm the observations of Robinson (1974) and Yamaguchi et al. (1983) dealing with sodium and potassium, respectively.

The observation that both sodium and potassium have similar effects on the rate of DCCD inactivation rules out the possibility of conformation-related changes in the reaction of DCCD with the enzyme. The cations are known to place the enzyme in different conformations as detected by a number of different methods [for a review, see Cantley (1981)].

Protection against DCCD inactivation by magnesium (K_L = 0.16 mM) is probably due to interaction at a site which, when occupied, results in the stimulation of ATPase activity. This site has a K_d of 0.15 mM in the absence of other ligands (i.e., the conditions of the DCCD inactivation experiment), although its affinity is decreased (K_d = 1 mM) to a value similar to the K_m for magnesium stimulation of (Na^+,K^+)-ATPase activity if sodium and potassium are added (Grisham & Mildvan, 1974). This is in contrast to the site at which magnesium causes inhibition of ATPase activity. this site has a K_d of 1 mM in the absence of other substrates, and its affinity is decreased (K_d > 5 mM) by their presence (Forgac, 1980).

ATP protection against inactivation by DCCD is consistent with binding at the active site. In the absence of a chelator and in the presence of ATP, the enzyme is most probably in the E_2–P form due to the presence of tightly bound magnesium (Skou, 1974; Karlish et al., 1978). Thus, in the absence of CDTA, the concentration of ATP needed for half-maximal protection against DCCD is about 2 mM, similar to the K_d for ATP binding to E_2–P. However, when CDTA is added to the reaction mixture, the enzyme should be in the E_1 conformation and should express an ATP site having a much higher affinity (Hegivary & Post, 1971; Jensen & Norby, 1971). This is exactly what is observed. The K_L for ATP protection in the presence of CDTA is about 15 μM, a factor of 130 lower than in the absence of CDTA.

The conclusion that two groups can be protected by all of the enzyme's substrates is not suprising in light of the work of Grisham and colleagues [for a review, see Grisham (1979)] which suggests that the binding sites for sodium, potassium, and ATP are all within 5–7 Å of one another, certainly close enough together that two groups could be protected by all of the substrates.

Direct evidence for the number and nature of the groups whose modification is responsible for the loss of enzymatic activity has been unobtainable. Reaction of DCCD with the (Na^+,K^+)-ATPase has two consequences in addition to causing the inactivation of enzymatic activity. The first of these is the cross-linking of both of the enzyme's subunits into large aggregates. Cross-linking is presumably due to the DCCD-induced formation of intermolecular isopeptide bonds between aspartyl and/or glutamyl resudies and the ϵ-amino group of lysyl residues (see below). Cross-linking cannot be related to the inactivation of enzymatic activity. The rate of loss of the α subunit is only one-tenth the rate of inactivation of (Na^+,K^+)-ATPase activity measured at 1 mM ATP and about one-fourth the rate of loss of ATPase activity at 1 μM ATP. The stable, covalent incorporation of DCCD into protein, presumabably as the *N*-acylurea (see below), is also observed. This labeling of the enzyme by [^{14}C]DCCD is not related to the inactivation of the enzyme. One mole of DCCD is incorporated per mole of alpha subunit, and the incorporation is independent of the degree of inactivation of any of the enzyme's activities. DCCD also labels the aggregated enzyme to a greater extent (3–6 mol/mol). Labeling of the β subunit has not been observed although some of the labeling of the aggregated enzyme could be due to incorporation into the β chain.

The nature of the modification responsible for inactivation of enzymatic activity by DCCD has not been directly established, although the formation of intramolecular isopeptide bonds is a likely candidate. Carbodiimides are reactive toward many nucleophiles [for a review, see; Williams & Ibrahim (1981)]; thus, there are many possible sites of reaction in proteins. Residues at which reaction of DCCD has been observed to occur include serine, tyrosine, cysteine, and, most commonly, aspartate and glutamate. Modification of seryl, tyrosyl, or cysteinyl residues cannot be responsible for inactivation of enzymatic activity. Modification of these residues would lead to the incorporation of radioactivity from [^{14}C]DCCD into the protein which should be related to the loss of enzymatic activity. This is not the case (see above). Furthermore, modification of seryl and tyrosyl groups by carbo-

diimides can be reversed by incubation with hydroxylamine (Carraway & Koshland, 1968; Banks et al., 1969). Recovery of enzymatic activity is not observed upon incubation of DCCD-treated (Na^+,K^+)-ATPase with 0.5 M hydroxylamine for up to 4 h (data not shown). The remaining and most commonly reactive (toward carbodiimides) protein nucleophile is the carboxyl group. Reaction of a carbodiimide with a carboxylic acid initially yields an *O*-acylisourea. The *O*-acylisourea can rearrange to form an *N*-acylurea (DeTar & Silverstein, 1966), or it can react further with a nucleophile [see Williams & Ibrahim (1981) and references cited therein]; both reactions have been observed with proteins. The formation of the *N*-acylurea between DCCD and the (Na^+,K^+)-ATPase would lead to the incorporation of radiolabel from [^{14}C]DCCD into the protein. Although this occurs (see above), it is unrelated to the loss of enzymatic activity.

Thus, the only possible reaction which can be responsible for the loss of enzymatic activity is the reaction of the *O*-acylisourea with a nucleophile. The most likely product is an intramolecular peptide bound formed by reaction of the *O*-acylisourea with a nearby amino group. Reaction of the *O*-acylisourea with other protein nucleophiles (i.e., hydroxyls, sulfhydryls, and carboxyls) would lead to the formation of species which are relatively unstable in aqueous solution; however, these products cannot be ruled out on the basis of the current data.

Conclusions

The (Na^+,K^+)-ATPase has been shown to contain at least two essential carboxyl groups in addition to the previously known aspartyl residue which forms an acyl phosphate intermediate during ATP hydrolysis. Modification of one of these groups interferes with the acceleration of the E_2 to E_1 transition by ATP and thus inhibits the complete catalytic cycle without affecting most of the enzyme's partial reactions. Reaction of DCCD with a second group interferes with the initial formation of the phosphorylated intermediate from ATP. Both groups are located near the enzyme's active site, but neither is the aspartate residue which forms the acyl phosphate intermediate. The DCCD-induced modification responsible for inactivation of enzymatic activity is most probably the formation of an intramolecular isopeptide bond.

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Registry No. ATPase, 9000-83-3; DCCD, 538-75-0; ATP, 56-65-5; Na, 7440-23-5; K, 7440-09-7; Mg, 7439-95-4.

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