Kinetics of Inactivation of the F_1F_o ATPase of *Propionigenium modestum* by Dicyclohexylcarbodiimide in Relationship to H^+ and Na^+ Concentration: Probing the Binding Site for the Coupling Ions

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ABSTRACT: Purified F_1F_o ATPase of *Propionigenium modestum* was rapidly inactivated by dicyclohexylcarbodiimide (DCCD) with $k_2 = 1.2 \times 10^5$ M^{-1} min^{-1} at pH 5.6 and 0 °C. Na^+ ions provided specific protection from the modification by DCCD while protons stimulated the reaction. Plots of pseudo-first-order rate constants of inactivtion (k_{obs}) against pH yielded titration curves with $pK(H^+) = 7.0$ in the absence of Na^+ and $pK(H^+) = 6.2$ in the presence of 0.5 mM Na^+. From the dependencies of k_{obs} on Na^+, $pK(Na^+)$ of about 2.5 and 3.3 were obtained at pH 6.5 and 8.0, respectively. These results indicate that DCCD reacts with a protonated group of the enzyme that dissociates with $pK(H^+) = 7.0$ in the absence of Na^+, and that Na^+ ions promote the dissociation of this group. Additionally, higher Na^+ concentrations were required at more acidic pH values to yield half-maximal protection from inactivation. These effects fit a competitive binding model for Na^+ or H^+ at the DCCD-reactive conserved acidic amino acid of subunit c (Glu-65). The active-site carboxylate could either be protonated and modified by DCCD or bind Na^+ which then provides protection. Complementary results were obtained from the effects of Na^+ and H^+ on ATPase activity. The pH–rate profile of v_{max} (with saturating Na^+) indicated an increase of activity with apparent $pK = 6.8$, an optimum around pH 7.5, and decreasing activity with apparent $pK = 8.7$. The remarkable activation of the enzyme by Na^+ ions (20-fold at pH 7.5 and above) indicates that a step involving Na^+ binding to F_o is rate limiting and therefore that the pH effect on v_{max} reflects an effect of pH on the Na^+ binding site. Maximal ATPase activities were about the same at pH 6.5 and 9.0, but about 10 times higher Na^+ concentrations were required at the acidic pH to saturate the enzyme. These results can be explained by an interference of protons (H_3O^+) with Na^+ binding at the active site. The ATPase at pH 9.0 showed positive cooperativity ($n_H = 2.6$) with respect to Na^+ transport and Na^+-activated ATPase activity, indicating an interaction of at least three Na^+ binding sites. The multiple c subunits present in the enzyme could readily provide binding sites for three or more Na^+ ions at a time. A new model for ion translocation involving formation and breakdown of salt bridges at the membrane-embedded Glu^- residue is discussed that can account for Na^+ and H_3O^+ transport.

The ATP synthase or F_1F_o ATPase catalyzes the $\Delta\tilde{\mu}(H^+)$ [or $\Delta\tilde{\mu}(Na^+)$]-driven formation of ATP from ADP and P_i in mitochondria, chloroplasts, or bacteria [for recent reviews, see Fillingame (1990), Penefsky and Cross (1991), and Dimroth et al. (1992)]. The structure and mechanism of these enzymes have been remarkably conserved during evolution. The ATPases consist of the water-soluble F_1 part with the typical subunit composition $\alpha_3\beta_3\gamma\delta\epsilon$, and the integral membrane-bound F_o moiety which for the least complicated bacterial enzymes has the subunit composition ab_2c_{9-12}. The isolated F_1 part catalyzes the uncoupled hydrolysis of ATP, and the F_o part catalyzes the translocation of protons across the membrane. These two partial reactions are tightly coupled in the F_1F_o ATPase complexes.

The ATPase of *Propionigenium modestum* is a typical member of the F_1F_o ATPase family, but uses Na^+ instead of H^+ as the physiological coupling ion (Laubinger & Dimroth, 1987, 1988). At low Na^+ concentrations, however, the enzyme switches from Na^+ to H^+ transport, suggesting a common mechanism for the translocation of both ions (Laubinger & Dimroth, 1989). This conclusion was further corroborated by the formation of hybrids composed of F_1 from *Escherichia coli* and F_o from *P. modestum* using *in vitro* or *in vivo* complementation studies (Laubinger et al., 1990; Kaim et al., 1992). The hybrids exhibited the same specificity with respect to Na^+ or H^+ pumping as the homologous *P. modestum* ATPase. The F_o part is therefore exclusively responsible for this cation specificity. These studies have clearly disfavored the direct coupling mechanism proposed by Mitchell (1976), i.e., a direct participation of the coupling ions in the chemical events of ATP formation, but are in favor of the conformational coupling mechanism proposed by Boyer (1975).

Insight into the mechanism of ion translocation through the F_o moiety has recently been obtained by measuring Na^+ or H^+ translocation with *P. modestum* F_o reconstituted into liposomes. It was shown that F_o from *P. modestum* behaves like a typical transporter, not like a channel, and that a membrane potential is obligatory for unidirectional Na^+ or H^+ fluxes while ΔpNa^+ or ΔpH are unappropriate driving forces (Kluge & Dimroth, 1992).

In spite of considerable efforts, notably with sequencing, chemical labeling, and mutagenesis studies, the amino acids involved in ion translocation through the F_o moiety could not be unequivocally defined until now [see Filligame (1990) and Penefsky and Cross (1991) for reviews]. A comparison of the primary structures of the F_o subunits from proton-translocating ATPases with that of *P. modestum* has not solved this problem, because most of the conserved residues are also conserved in

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the F_o subunits from *P. modestum* [for a review see Dimroth et al. (1992)]. All F_1F_o ATPases contain a conserved acidic amino acid residue in the middle of the C-terminal membrane-spanning α-helix of subunit c that is absolutely essential for function and exhibits unique reactivity toward modification by DCCD [for a review see Fillingame (1990)]. Recently, we observed that subunit c of the *P. modestum* ATPase becomes specifically protected from this modification in the presence of Na^+ or Li^+ ions (Kluge & Dimroth, 1993). As this observation has bearings on the location of the binding site for the coupling ions, we performed detailed studies on the kinetics of the modification of the ATPase by DCCD in relationship to H^+ and Na^+ concentration. The results of these studies reported here strongly indicate that the Na^+ binding site is at the DCCD-reactive amino acid residue (Glu-65) of subunit c.

EXPERIMENTAL PROCEDURES

Materials. Enzymes were from Boehringer (Mannheim, FRG). $^{22}NaCl$ was from Amersham International (Buckinghamshire, U.K.). The cyclohexylammonium salt of NADH (Na^+ content < 0.1%) was from Sigma (Buchs, Switzerland); $MgCl_2$ (Na^+ content < 0.001%), K_2SO_4 (Na^+ content < 0.001%), and KOH (Na^+ content < 0.002%) were from Merck (Zürich, Switzerland). Potassium ATP was prepared from barium ATP (Fluka, Buchs, Switzerland) according to Laubinger and Dimroth (1987). EIPA was a gift from Professor E. Bäuerlein (Munich). The F_1F_o ATPase from *P. modestum* was purified as previously described (Laubinger & Dimroth, 1988).

Determination of ATPase Activity. ATPase activity was determined by a coupled spectrophotometric assay usually containing the following in 1 mL (25 °C): 50 mM potassium phosphate buffer, pH 8, 5 mM $MgCl_2$, 0.25 mM NADH, 15 U lactate dehydrogenase, 10 U pyruvate kinase, 3 mM phosphoenolpyruvate, F_1F_o ATPase (0.3–15 μg), and 5 mM NaCl. Reaction was started by addition of 2 mM ATP.

The dependencies of ATPase activities on Na^+ concentrations were determined between pH 5.5 and pH 6.5 in buffer containing 20 mM each of MES, MOPS, and TRICINE, and between pH 6.7 and pH 9.8 in buffer containing 20 mM each of MOPS, TRICINE, and glycine, both adjusted to the desired pH with KOH and both containing 100 mM K_2SO_4. The amount of endogenous Na^+ was minimized by using chemicals with low Na^+ content (see above) and taking precautions as described elsewhere (Dimroth & Thomer, 1986).

ATPase activities, plotted as $v/(v_{max} - v)$ *versus* $\log[Na^+]$ yielded straight lines with slopes of n_H. (n_H = Hill coefficient, $[Na^+]_{0.5}$ = Na^+ concentration required for half-maximal activity). $[Na^+]_{0.5}$ values were determined according to the logarithmic form of the Hill equation (Segel, 1975) (eq 1).

$$\log \frac{v}{v_{max} - v} = n_H \log[Na^+] - \log K' \qquad (1)$$

When $\log v/(v_{max} - v) = 0$, $v/(v_{max} - v) = 1$, and the corresponding position on the $\log[Na^+]$ axis gives $\log[Na^+]_{0.5}$. K' is a constant comprising the interaction factors and the intrinsic dissociation constant, K.

Inactivation of ATPase with DCCD. A 20-μL sample of purified ATPase (70–100 μg, in 5 mM potassium phosphate buffer, pH 7, 1 mM dithioerythritol, 1 mM diisopropylfluorophosphate, 0.05% (v/v) Triton X-100) or a 94-μL sample of proteoliposomes (3.25 mg of phospholipids and 26 μg of protein, in 20 mM each of K-MOPS, K-TRICINE, K-glycine, pH 6.5, 1 mM dithioerythritol, 5 mM $MgCl_2$) was brought to a final volume of 0.1 mL with the appropriate buffer (described in the figure legends), containing 1–67 μM DCCD, and incubated at 0 or at 25 °C. Samples (0.02 mL) were taken after different times and added to a 0.98-mL assay mixture. Immediately afterward, ATPase activity was determined. Stock solutions (0.1–6.7 mM) of DCCD were prepared in ethanol 1 day before the experiments were performed. Control assays contained the same amount of ethanol (1%).

Evaluation of Rate Constants. Pseudo-first-order rate constants for ATPase inactivation by DCCD (k_{obs}) were calculated by fits of the linear part of the data plotted as log activity *versus* time. The slope of the line is the negative of the rate constant for inactivation. Second-order rate constants (k_2) were calculated as follows: $k_2 = k_{obs}/[DCCD]$.

The data for the pH dependence of the rate constant of inactivation of the *P. modestum* ATPase were fitted to eq 2

$$\log y = \log \frac{YL + YH(K/H)}{1 + K/H} \qquad (2)$$

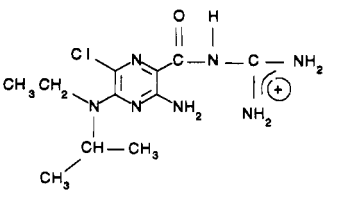
according to Cleland (1979), where y corresponds to the second-order rate constant k_2, YL is the value of y at low pH, YH the value of y at high pH, H is the proton concentration, and K is the dissociation constant of the group whose ionization or protonation decreases activity. K was found by trial-and-error, giving the best fit of the calculated line to the measured data.

Other Determinations. Reconstitution of proteoliposomes and determination of ATP-dependent Na^+ transport were performed as described before (Kluge & Dimroth, 1992) in the following buffer: 20 mM MOPS, 20 mM TRICINE, 20 mM glycine, adjusted with KOH to the appropriate pH. Protein and Na^+ concentrations were determined according to Kluge and Dimroth, 1992.

RESULTS

Inactivation of ATPase by DCCD. Incubation of purified F_1F_o ATPase of *P. modestum* with DCCD resulted in a rapid progressive loss of ATPase activity. About 6–7% of the initial activity was resistant to inactivation by DCCD and can therefore be attributed to the ATPase activity of the DCCD-modified enzyme. A representative experiment performed with 1 μM F_1F_o ATPase and 10 μM DCCD at pH 6.5 and 0 °C is shown in Figure 1. During the first 30 s, where the activity dropped to about 25% of its initial value, the inactivation followed pseudo-first-order kinetics. From the linear part of log activity *versus* time plots (see inset of Figure 1), the pseudo-first-order rate constants (k_{obs}) were derived and used for all of the following calculations. The pseudo-first-order rate constants depended on DCCD concentrations, as anticipated for a second-order reaction between enzyme and the modifying agent. The data for pH 5.6 and 0 °C are shown in Figure 2. Also shown is a marked reduction of the

[1] Abbreviations: DCCD, dicylclohexylcarbodiimide; EIPA, *N*-ethyl-*N*-isopropylamiloride, 3-amino-5-(ethylisopropylamino)-6-chloro-*N*-(diaminomethylene)pyrazinecarboxamide (see formula below).



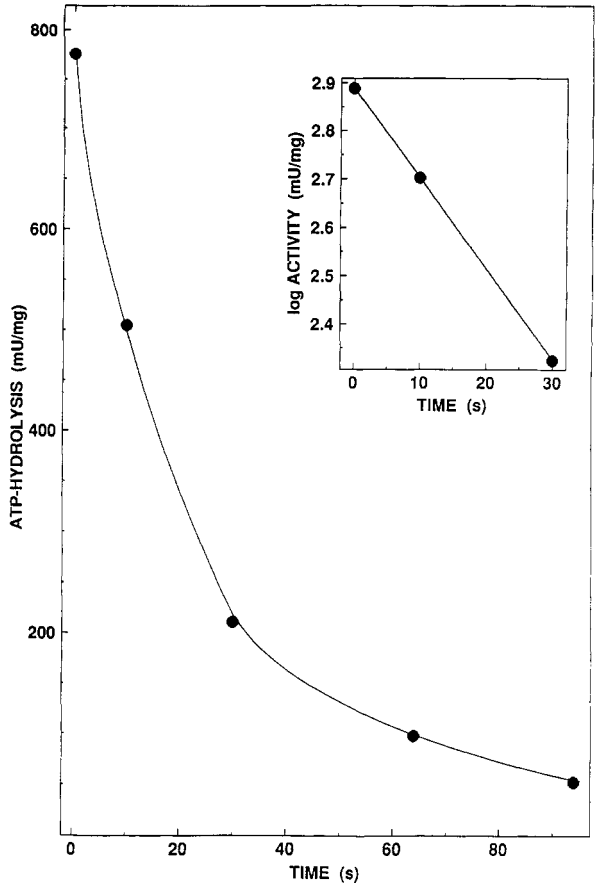


FIGURE 1: Kinetics of ATPase inactivation by DCCD. F_1F_o ATPase of *P. modestum* (1 μM) was incubated with 10 μM DCCD at 0 °C in 25 mM each of MES, MOPS, and TRICINE, adjusted with KOH to pH 6.5. After the indicated incubation times, samples (0.02 mL) were taken and added to 0.98 mL of the assay mixture. ATP hydrolysis activity was determined immediately afterward. Inset: Semilogarithmic plot of activity *versus* incubation time.

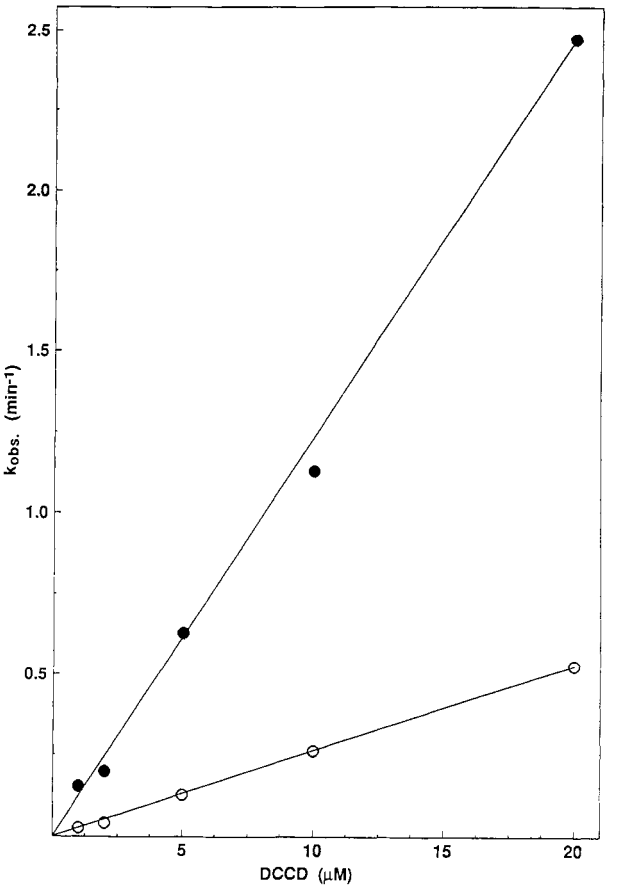


FIGURE 2: Rate constants for inactivation of the ATPase by DCCD. ATPase activities were measured after incubation of the enzyme in 20 mM each of K-MOPS, K-MES, and K-TRICINE, pH 5.6, at 0 °C with 1–20 μM DCCD for the appropriate time intervals to follow pseudo-first-order kinetics (0.2–3 min). The endogenous Na^+ concentration was 25 μM. The inactivation rate constants, k_{obs}, were determined from residual ATPase activities of samples taken after various incubation times as described under Experimental Procedures. Key: (●) no further addition; (○) addition of 2 mM NaCl to the incubation mixture.

rate of inactivation in the presence of 2 mM Na^+ ions. The second-order rate constants calculated from these data are 1.2×10^5 M^{-1} min^{-1} in the absence of Na^+ and 2.6×10^4 M^{-1} min^{-1} in the presence of 2 mM Na^+. Na^+ ions thus protected the ATPase from inactivation by DCCD, which suggests that the reagent reacts at the Na^+ binding site. Please note the enormous rate of the inactivation reaction which is about 7 orders of magnitude faster than the reaction of DCCD with acetic acid at the same pH and temperature (deTer & Silverstein, 1966).

It is also remarkable that the reaction of DCCD with the soluble enzyme was more than 10 times faster than with ATPase incorporated into proteoliposomes (containing 26 μg of protein and 3.25 mg of phospholipids). The reactivity of the soluble ATPase (26 μg of protein) toward DCCD decreased about 10-fold by adding liposomes (3.25 mg of phospholipids) without incorporation of the enzyme into the lipid by the freeze/thaw/sonication procedure (data not shown). These results indicate that DCCD reacts with the target amino acids of the ATPase from the water phase, not from the lipid phase. The reactivity of the ATPase with DCCD was therefore reduced when the aqueous DCCD concentration decreased by dissolving part of the hydrophobic compound in phospholipid. It was, however, independent of whether the ATPase was in close contact with the added phospholipids or not. The high rate of inactivation indicates very specific binding of DCCD to the enzyme, in accord with the specific labeling of subunit c after incubation with the radioactive compound (Laubinger & Dimroth, 1988; Kluge & Dimroth, 1993). The target amino acid on the c subunit of F_1F_o ATPases has been shown to be a conserved aspartate or glutamate residue in the middle of the C-terminal membrane-spanning α-helix (see Fillingname (1990) for review). Glu-65 at the corresponding position of the *P. modestum* c subunit (Ludwig et al., 1990) is therefore the putative DCCD-reactive amino acid residue.

Dependence of k_{obs} on Na^+. If, as suggested by the protective effect of Na^+, the inactivation of the ATPase is in fact due to the reaction of DCCD with the Na^+ binding sites, the inactivation rate constant should change in the Na^+ concentration range in which these sites bind the metal ion. As shown in Figure 3, k_{obs} measured at pH 6.5 and 25 °C in the presence of 10 μM DCCD decreased with increasing Na^+ in the range of 10^{-4} to 3×10^{-3} M to about 5% the rate in the absence of Na^+. The profile followed a titration curve with an inflection point at 300 μM Na^+, indicating that this is the apparent dissociation constant for Na^+ from its binding site on the enzyme. At pH 8.0, the rate constant for ATPase inactivation by DCCD in the absence of Na^+ was about 10 times lower than at pH 6.0 (see below, Figure 4). Higher DCCD concentrations than at pH 6.0 were therefore required to follow the kinetics of inactivation at pH 8.0. At this pH, the Na^+ concentration yielding half-maximal protection from inactivation of the ATPase by DCCD was about 50 μM (not shown). Interestingly, the Na^+ concentration required for

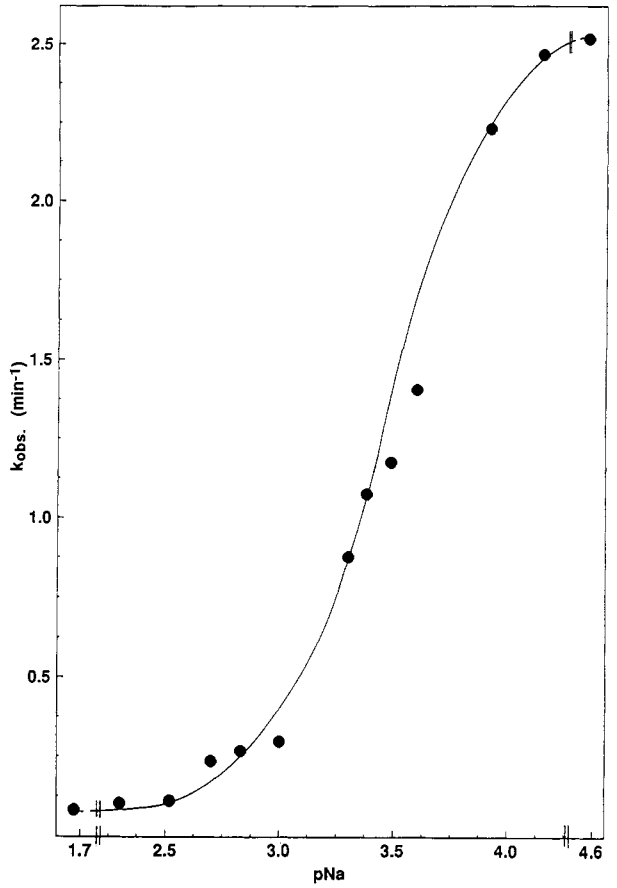


FIGURE 3: Effect of Na^+ concentration on the rate constant of ATPase inactivation by DCCD. The incubation mixtures contained at 25 °C 1 μM ATPase, 20 mM each of K-MES, K-MOPS, and K-TRICINE, pH 6.5, 0.025–20 mM NaCl, and 10 μM DCCD. The rate constants for inactivation, k_{obs}, were determined as described under Experimental Procedures.

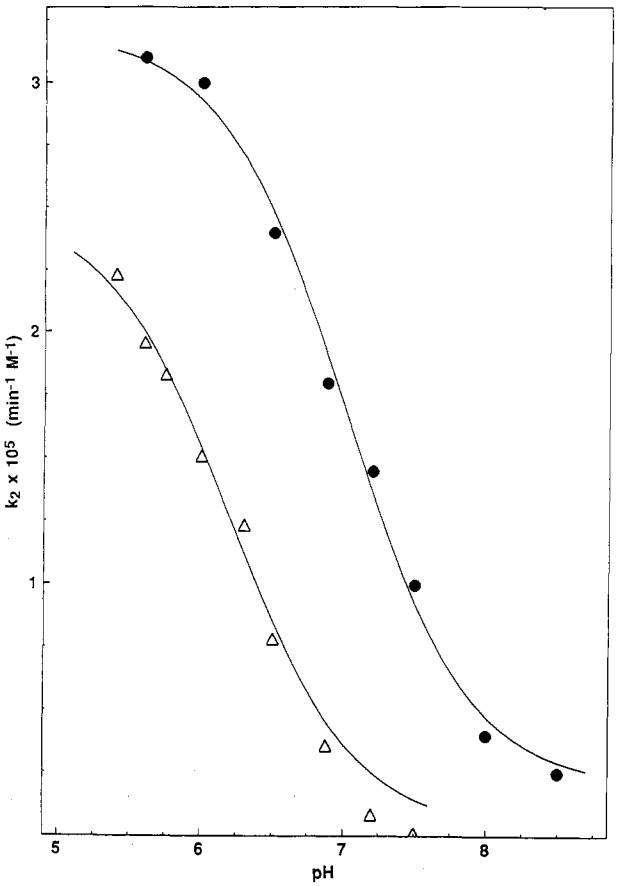


FIGURE 4: Second-order rate constants for ATPase inactivation by DCCD as a function of pH without (●) or with (△) 0.5 mM NaCl added. The F_1F_o ATPase was incubated at 25 °C with 2 μM DCCD in a buffer system containing 20 mM each of MES, MOPS, and TRICINE, adjusted with KOH to the appropriate pH values. The endogenous Na^+ concentration was 25–30 μM. Samples (0.02 mL) were taken at various times (8–180 s), the reactions were stopped by adding the aliquots to 0.98 mL of the assay mixture, and the samples were used immediately afterward for measuring ATPase activities. The lines were drawn according to eq 2 with pK = 7.0 for reactions without NaCl addition and with pK = 6.2 for reactions with 0.5 mM NaCl. For details of the data analysis, see the Experimental Procedures.

half-maximal activation of ATPase activity also decreased with increasing pH (see below).

Effect of pH. The rate constant for inactivation of the ATPase by DCCD was highly pH-dependent. The data depicted in Figure 4 are measured k_2 values, which followed a calculated titration curve, as shown by the line drawn. The values of k_2 increased more than an order of magnitude in going from pH 8.5 to pH 5.5, apparently leveling off in the acidic as well as in the alkaline pH range. The midpoint of the transition in the absence of Na^+ occurred near pH 7.0, indicating that the DCCD-reactive amino acid residue (Glu-65 of subunit c) has a pK of 7.0. Also shown in Figure 4 is the effect of Na^+ ions on the rate of ATPase inhibition by DCCD in relationship to pH. The data indicate nearly complete protection from inhibition by DCCD in the presence of 0.5 mM Na^+ at pH values above 7.0. The rate of inactivation increased sharply in going from pH 7.0 to pH 5.5, the lowest pH value where measurements could be made without denaturation of the ATPase. Nevertheless, within the measurable range the shape of the curve obtained with 0.5 mM Na^+ appears to be the same as that obtained in the absence of Na^+ but the pK becomes 0.8 unit more acidic. The curve of k_2 against pH in the presence of 1 mM NaCl ran left of those shown in Figure 4 with the midpoint of transition at about pH 5.8 (data not shown). In summary, these data indicate that protonation of the carboxylic acid side chain of glutamate-65 of subunit c is essential for its reaction with DCCD. The apparent shift in the pK of this group by Na^+ toward more acidic values is indicative for a Na^+-dependent shift of the Glu-H $\rightleftharpoons$ Glu^- + H^+ equilibrium to the right. These results are to be expected if Na^+ ions bind to the dissociated Glu^- residue.

Effect of EIPA on ATPase Activity and on Inactivation of ATPase by DCCD. Amiloride and its derivatives are known inhibitors of various membrane transport proteins, e.g., Na^+ channels, Na^+/H^+ exchangers, and Na^+/Ca^{2+} exchangers (Kleyman & Cragoe, 1988). It was shown previously that 0.5 mM amiloride had no effect on Na^+ transport into proteoliposomes containing the *P. modestum* ATPase (Laubinger & Dimroth, 1988). As more hydrophobic derivatives such as EIPA are often more inhibitory than amiloride (Kleyman & Cragoe, 1988), we investigated the effect of EIPA on ATPase activity. In the presence of a 50 or 100 μM concentration of this compound ATPase was inhibited 30% or 50%, respectively (not shown).

We also studied the effect of EIPA on inactivation of the ATPase by DCCD (Figure 5). ATPase was incubated with EIPA and DCCD for various times, and inactivations were terminated by 50-fold dilution of the incubation mixtures into the ATPase assay mixture. The dilution resulted in a dissociation of the reversible inhibitor EIPA from the enzyme, relieving inhibition by this compound, while the enzyme

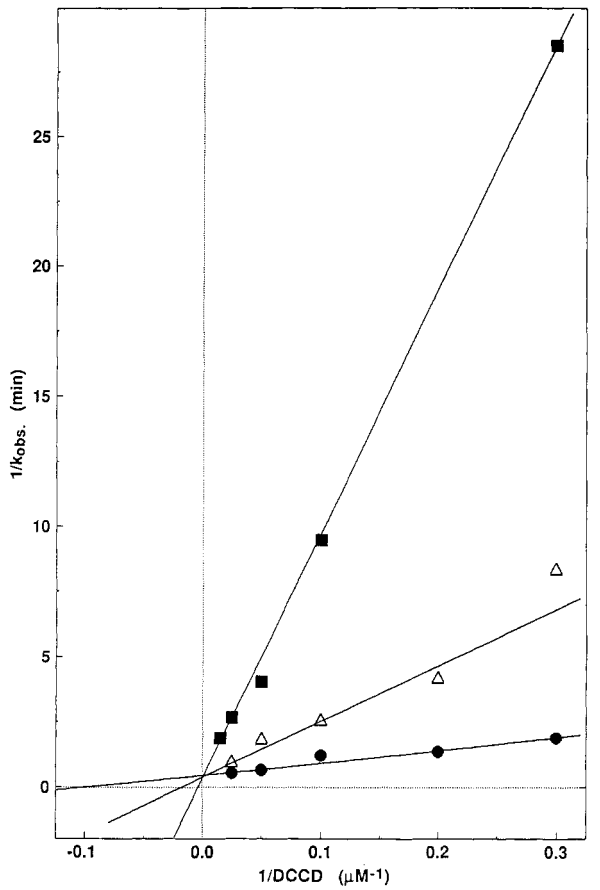


FIGURE 5: Dependence of ATPase inactivation rate constants from DCCD concentrations at the following fixed concentrations of EIPA: 0.1 mM (●), 0.5 mM (▲), 1 mM (■) (double reciprocal plot). The ATPase was preincubated for 3 min with the respective EIPA concentrations before inactivation was initiated by DCCD addition. The other conditions were the same as described in the legend to Figure 2. EIPA has been dissolved in dimethyl sulfoxide. ATPase was not affected by the solvent in the concentration applied (1%).

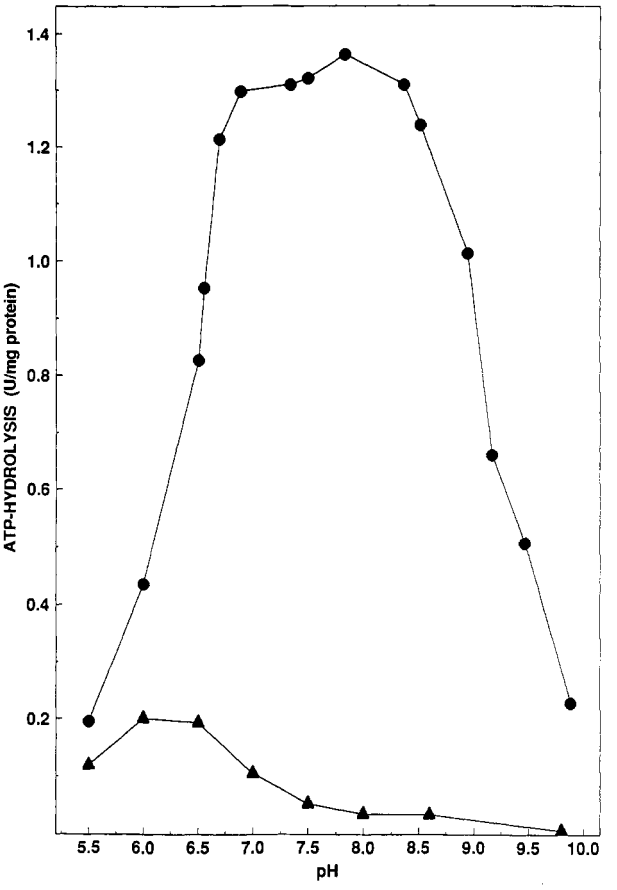


FIGURE 6: pH dependence of ATPase activity in the presence of saturating Na^+ concentrations (8 mM NaCl) (●) or without NaCl addition (endogenous Na^+ concentration 0.03 mM) (▲). Data are the means of two determinations. ATPase was not inactivated under the conditions used.

modified by DCCD stayed inactive. The data clearly indicate that EIPA in a concentration range of 0.1–1 mM protected the ATPase from inactivation by DCCD; i.e., at a fixed DCCD concentration the inactivation rate constant decreased with increasing EIPA concentrations. Double reciprocal plots of inactivation rate constants *versus* DCCD concentrations yielded straight lines for each fixed EIPA concentration that intersected at the $1/k_{obs}$ axis. These data indicate that EIPA acts as a competitive inhibitor *versus* modification of the enzyme by DCCD. The EIPA binding site is therefore probably at or close to the DCCD-reative glutamate residue of subunit c.

Effects of Na^+ and H^+ Concentration on ATPase Activity. In previous studies it has been shown that the *P. modestum* ATPase switched from Na^+ to H^+ pumping as the Na^+ concentration dropped below ~1 mM (Laubinger & Dimroth, 1989). Similarly, F_o liposomes catalyzed $\Delta\psi$-driven Na^+ translocation that switched to H^+ translocation at low (<2 mM) Na^+ concentrations (Kluge & Dimroth, 1992). These results indicated a common transport mechanism for the two cations and thus competition for a common binding site on the enzyme. To accommodate the recognition of either Na^+ or H^+ by such a site, it has been suggested that proton translocation proceeds with H_3O^+ as the transported species, because Na^+ and H_3O^+ form very similar coordination complexes with crown ethers (Boyer, 1988).

To further pursue this idea, we carefully examined the effect of Na^+ concentration on ATPase activity at different pH values. The maximal ATPase activity observed with saturating Na^+ concentrations followed a bell shaped curve with a broad pH optimum around pH 7.5 and rather sharp decreases below pH 7.0 and above pH 8.5 (Figure 6). In contrast, ATPase activity in the absence of Na^+ ions was much smaller, and significant activities were observed only at pH values below neutrality. The pH optimum of the Na^+-independent ATPase was at pH 6.0–6.5, and at increasing pH values the activity dropped continuously to reach nondetectable levels at pH 9.7. The degree of activation by Na^+ ions increased continuously from about 1.5-fold at pH 5.5 to about 20-fold at pH 7.5 and above. This remarkable activation of the enzyme by Na^+ ions indicates that a reaction step involving binding of Na^+ ions to the F_o moiety is rate limiting for the ATPase activity. The pH profile of maximal ATPase activity at saturating Na^+ therefore reflects the pH profile of the Na^+-binding active site on F_o, not the pH dependence of the ATP-hydrolyzing catalytic center on F_1. A plot of log v_{max} against pH (Tipton & Dixon, 1979) indicated pK values of 6.8 and 8.7 that may therefore be the pK values of ionizing groups at the Na^+ binding site.

The dependence of ATPase activity on Na^+ concentration is shown in Figure 7 for pH 6.5 and 9.0. Although the maximal activity was about the same at these two pH values, about 10 times higher Na^+ concentrations were required at the acidic pH to saturate the enzyme. Na^+ concentrations of 1.3 and 0.5 mM produced half-maximal activation at pH 6.5 and 9.0, respectively. Noteworthy is a marked change in the shape of

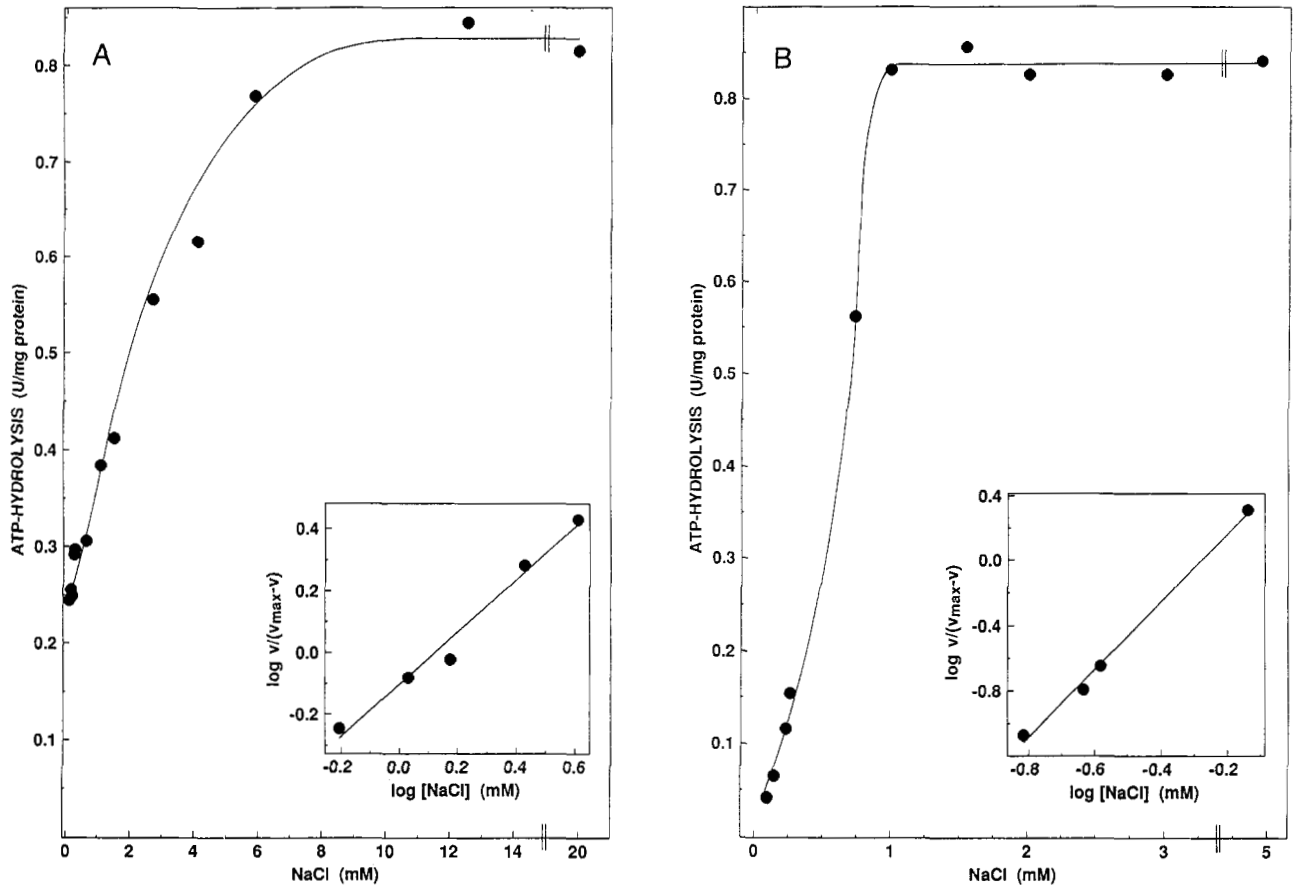


FIGURE 7: Activation profiles of the *P. modestum* ATPase by NaCl at pH 6.5 (A) and 9.0 (B). Data are the means of two measurements. Insets: Hill plots.

the activation profile in going from acidic to alkaline pH values. Curves obeying usual Michaelis–Menten kinetics were found only for pH values around 7.5. At significantly higher or lower pH values the curves showed various degrees of cooperativity as revealed from Hill-plot analyses. Negative cooperativity (n_H = 0.6) was found at pH 6.5 and strong positive cooperativity (n_H = 2.6) was observed at pH 9.0 (insets of Figure 7). Increasing the pH from 6.5 to 9.0 caused a continuous increase of n_H and decrease of $[Na^+]_{0.5}$ (data not shown). Similar profiles of initial rates *versus* Na^+ concentration as observed for the ATPase were found for the transport of Na^+ ions into reconstituted proteoliposomes (not shown). In this system, the following values were obtained: at pH 6.5, $[Na^+]_{0.5}$ = 1.5 mM, n_H = 0.6; at pH 9.0, $[Na^+]_{0.5}$ = 0.47 mM, n_H = 2.5. These results strongly support the view that the rate of ATP hydrolysis is controlled by a specific step of the Na^+ transport cycle. Measurements of ATP hydrolysis can therefore conveniently be used even with the soluble F_1F_o ATPase to monitor the rate-limiting step of Na^+ translocation, i.e., a reaction taking place quite a distance from the catalytic center of F_1 at the Na^+-binding active site of the F_o moiety.

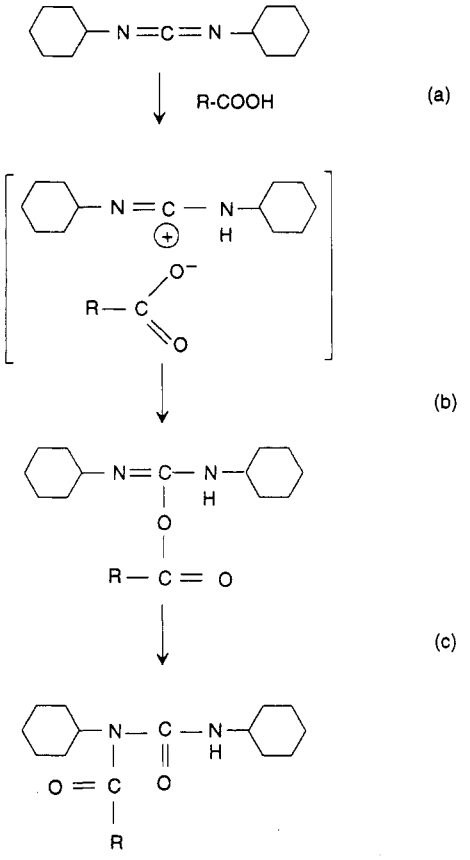
The strong positive cooperativity observed in Na^+ transport or Na^+-activated ATPase activity (n_H = 2.6 at pH 9.0) indicates that at least three Na^+ binding sites interact with each other in the enzyme. This is consistent with a Na^+ to ATP stoichiometry of 3 or higher and indicates further that Na^+ is bound at least to three sites at the same time. As subunit c is the only one present in a sufficient copy number to account for the binding of three Na^+ ions, this result is additional evidence for the binding of Na^+ to subunit c. The negative cooperativity observed at pH 6.5 (n_H = 0.6) may indicate that binding of the first Na^+ decreases the Na^+-binding affinities of the vacant sites. It may also mean, however, that more than one binding site must be occupied with Na^+ for catalytic activity and that Na^+ binding at pH 6.5 is impeded by protonation of part of the active-site glutamate residues.

DISCUSSION

The specific reaction of DCCD with an acidic amino acid residue of the highly hydrophobic c subunit is a characteristic of F_1F_o ATPases [for reviews see Fillingame (1990), Penefsky and Cross (1991), and Dimroth et al. (1992)]. The resulting modification leads to a blockade of proton translocation (H^+ or Na^+ translocation in the case of the *P. modestum* ATPase) and of coupled ATPase activity. Subunit c is supposed to form a hairpin-like structure with two membrane-spanning α-helices that are connected by a hydrophilic loop. A conserved aspartic or glutamic acid residue in the middle of the C-terminal α-helix has been shown to be the target for the reaction with DCCD and has been suggested to play an important role in proton translocation [for a review see Fillingame (1990)]. In spite of its different cation specificity, subunit c of the *P. modestum* ATPase contains the conserved acidic amino acid in the middle of the C-terminal membrane-spanning α-helix (Glu-65) (Ludwig et al., 1990) and becomes specifically labeled upon incubation with [^{14}C]DCCD (Laubinger & Dimroth, 1988; Kluge & Dimroth, 1993). This modification by DCCD is accompanied by the loss of ATP-coupled Na^+ or H^+ translocation and ATPase activity of F_1F_o (Laubinger & Dimroth, 1988) and with the inhibition of Na^+ or H^+ translocation through the reconstituted F_o moiety (Kluge & Dimroth, 1992).

Because of its hydrophobicity, DCCD appears to be well suited to react with a carboxylic acid within a hydrophobic

Scheme I: Hypothetical Reaction of DCCD with the Protonated Carboxyl Group of Glu-65 of Subunit c



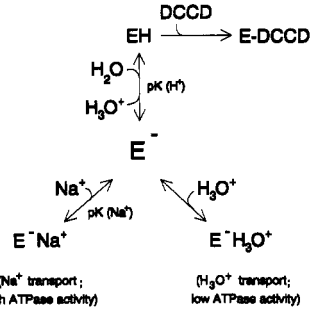
environment. The chemical events of the reaction are supposed to involve the steps shown in Scheme I (Azzi et al., 1984). Proton transfer from the acid to one of the imide nitrogens of DCCD (a) followed by nucleophilic attack of the carboxylate at the resulting carbonium ion (b) yields the *O*-acylisourea derivative which subsequently rearranges into the more stable *N*-acylurea component (c).

The reaction of F_1F_o from *P. modestum* with DCCD was determined by the inhibition of ATPase activity. These studies were not performed with the membrane-embedded ATPase as usual, but with the detergent-solubilized enzyme which has obvious advantages for measuring the inactivation kinetics. The results revealed a rate for the inactivation of ATPase by DCCD with a second-order rate constant of 1×10^5 M^{-1} min^{-1} at pH 5.6 and 0 °C, which is in the range of the second-order rate constants (k_{cat}/K_m) of many enzymes. The reaction is roughly 10^7 times faster than the reaction of DCCD with acetic acid at the same pH and temperature (deTer & Silverstein, 1966) and is about 10^3 times faster than the reaction of DCCD with membrane-bound Ca^{2+}–ATPase of sarcoplasmic reticulum (Murphy, 1981). The high rate of inactivation is in accord with the remarkable specificity for modification of a single amino acid residue in the protein (Glu-65 of subunit c). Therefore, DCCD must bind very specifically close to this site. The rate of the reaction of DCCD with subunit c at pH 6.5 is about 3 orders of magnitude faster than the reaction of DCCD with the β-subunits of various F_1F_0 ATPases (Satre et al., 1979; Esch et al., 1981). The modification of the β-subunits has therefore been performed at considerable longer incubations and higher DCCD concentrations than used in the experiments described here. Under our experimental conditions labeling was exclusively at the c subunit (Kluge & Dimroth, 1993). The inactivation rate constants reported are therefore not disturbed by partial modification of the β-subunits but clearly reflect the reaction of DCCD with subunit c. An interesting observation is the more than 10-fold increased reactivity of DCCD with the soluble ATPase as compared to the membrane-bound enzyme. This indicates that the DCCD binding site is accessible from the aqueous phase rather than from the lipid phase, where most of the hydrophobic carbodiimide will dissolve if the reaction is carried out with membrane-bound enzyme.

The rate constant for inactivation of the ATPase by DCCD was highly pH-dependent. It increased about an order of magnitude in going from pH 8.5 to pH 5.5 and followed a titration curve of a group with $pK = 7.0$ in the absence of Na^+. A similar pK (6.8) was derived from the pH dependence of proton translocation through F_o from a thermophilic bacterium (Okamoto et al., 1977). The pH dependence is in accord with the reaction mechanism shown in Scheme I and is also in accord with the pH dependence for inactivation of other membrane-bound enzymes by DCCD (Azzi et al., 1984). We conclude from these results that the carboxyl group of Glu-65 of subunit c must be protonated in order to react with DCCD and that this carboxyl group has a pK of 7.0. The apparent increase in pK by about 3 units over glutamate in aqueous solution is in accord with the assumed location of the reactive glutamate residue in a hydrophobic environment within the middle of the membrane. An alternative mechanism might be a general-acid-catalyzed concerted protonation of a DCCD nitrogen atom and attack of the Glu-65 carboxylate at the central C atom. This would be related to the suggested mechanism of the reaction of carbodiimides with acetic acid in aqueous solution (Williams & Ibrahim, 1981). If this mechanism is valid for the ATPase, the pH–rate profile should reflect two dissociation constants: one for protonation of DCCD with an increase of reactivity upon acidification and one for protonation of the carboxylate with a decrease of reactivity upon acidification. As only a single dissociation constant was observed and as the reactivity increased and did not decrease upon acidification, we conclude that this alternative mechanism is probably not valid for the reaction of DCCD with the ATPase c subunit.

A crucial result of these studies is the protection of ATPase from DCCD inactivation by Na^+ ions, which suggests that the reagent reacts at the Na^+ binding site. We therefore hypothesize that the Na^+ binding site is in close proximity to the DCCD-reactive glutamate residue of subunit c. Of considerable importance are the effects of Na^+ concentration and pH on the inactivation rate constant of the ATPase by DCCD. Half-maximal protection from inactivation was observed with 0.3 mM Na^+ at pH 6.5 and with 0.05 mM Na^+ at pH 8.0. As these values should represent dissociation constants ($K(Na^+)$) of the E^-Na^+ complex, they indicate that binding of Na^+ to the enzyme is pH-dependent, with $pK(Na^+)$ increasing from 2.5 to 3.3, as the pH rises from 6.5 to 8.0. These results can be explained by the model shown in Scheme II, where E^- represents ATPase with Glu-65 of subunit c in its dissociated state. This residue is supposed to be competent for Na^+ or H_3O^+ binding or may become protonated if the pH decreases. On H^+ or Na^+ binding to E^- the associated equilibrium $E^-Na^+ \rightleftharpoons E^- + Na^+$ or $EH \rightleftharpoons E^- + H^+$, respectively, will be shifted to the right (cf. Scheme II). Complementary to the increase of $pK(Na^+)$ at increasing pH was a decrease of $pK(H^+)$ from 7.0 in the absence of Na^+ to 6.2 or 5.8 in the presence of 0.5 or 1 mM Na^+, respectively (cf. Figure 4). Please also note that higher Na^+ concentrations (about 10 mM and above) gave almost complete protection

Scheme II: Model for the Interaction of Na^+, H_3O^+, and DCCD with the Active-Site Glutamate Residue of Subunit c[a]



[a] Na^+ and H_3O^+ are assumed to compete for binding at the carboxylate group of Glu-65 (symbolized as E^-). This competition causes a Na^+-dependent shift of the $EH \rightleftharpoons E^- + H^+$ equilibrium to the right ($pK(H^+)$ decreases) and a H^+-dependent shift of the $E^-Na^+ \rightleftharpoons E^- + Na^+$ equilibrium to the right ($pK(Na^+)$ decreases). DCCD reacts with undissociated glutamic acid (EH), while glutamate (E^-) is the active-site residue for Na^+ (H_3O^+) binding and associated reactions, i.e., ATP-coupled Na^+ (H_3O^+) transport or Na^+ (H_3O^+)-activated ATPase activity. Enzyme forms E^- and EH are supposed to be catalytically inactive.

from inactivation by DCCD even at pH 6.5 (Figure 3). These results therefore support the model of competition between Na^+ and H^+ for binding to the Glu^- residue at the active site (Scheme II). Occupation of the Na^+ binding site(s) on E^- by Na^+ is obviously required for ATP-coupled Na^+ transport, and also for triggering ATPase activity of soluble F_1F_o. By analogy, "proton" translocation may involve binding of H_3O^+ to E^- which also could cause some activation of ATPase activity. The undissociated form of the enzyme (EH) is on the other hand supposed to be inactive in the physiological reactions but highly reactive toward modifiction by DCCD (see above).

The cation binding model shown in Scheme II is also in accord with the pH–rate profile of the ATPase. As stated in the Results, the Na^+-dependent ATPase activity provides a measure for the rate-determining step at the Na^+ binding site and may thus provide important information about the mechanism at this site. From the dependence of v_{max} on pH, (kinetic) pK values of 6.8 and 8.7 were obtained that may reflect the pK values of ionizing groups at the Na^+ binding site. Catalytic activity thus seems to be generated upon dissociation of a group with pK = 6.8 and to dissipate upon dissociation of a second group with pK = 8.7. The lower (kinetic) pK value is in the range of the DCCD-reactive glutamate residue and may therefore be related to the dissociation of this group. In comparing the pK values derived from enzyme kinetic measurements with those derived from inactivation rate constants with DCCD, one should take into account that the kinetic pK values may be distorted by rate constants and may contain contributions from more than one group (Tipton & Dixon, 1979), whereas the pK values derived from inactivation rates more likely represent the true pK's of the DCCD-reactive active-site glutamate residues. Also interesting is the pH dependence of the Na^+-independent activity of the enzyme. In the acidic range, the activity increased to reach its optimum at pH 6 and then decreased again to undetectable levels in the alkaline pH range (above pH 8). This activity may reflect partial activation of the ATPase by H_3O^+, if this cation replaces Na^+ at its binding site.

Intrigued by the very high rate and specificity of the reaction of DCCD with Glu-65 of subunit c we considered the possibility that parts of the DCCD structure might resemble the structure of an amino acid side chain that could come into close contact with Glu-65 during the catalytic cycle. As shown in Scheme I, proton transfer from the glutamic acid to DCCD produces a protonated species that comprises structural elements of an arginine. The rationale for the DCCD reactivity and specificity could thus be the resemblence at the active site of protonated DCCD and arginine forming a salt bridge to glutamate-65. Some observations related to this hypothesis are important: First, all ATPases contain a conserved arginine in the polar loop region connecting the two membrane-spanning helices of subunit c that is absolutely essential for function. Another conserved and functional important arginine is on subunit a. Second, the structure of the two helices of subunit c could be well-defined by nuclear magnetic resonance data, while the region around the conserved arginine appeared to be relatively mobile (Norwood et al., 1992). Third, EIPA, a guanidinium group-containing hydrophobic compound, protected the enzyme competitively from the modification by DCCD (Figure 5). Fourth, orientation of the conserved active-site carboxyl group toward the opposite helix is in accord with the binding of DCCD from the aqueous phase and would also be in accord with the binding of the conserved arginine through movement of the polar loop into the space between the two helices. Fifth, formation of a salt bridge between the conserved arginine and glutamate residues by this movement of the polar loop immediately suggests a reasonable mechanism of Na^+ or H_3O^+ transport and the coupled conformational changes of the enzyme molecule. Na^+ or H_3O^+ ions bound to Glu^- may thus be displaced from this binding site by ion exchange with arginine. Sixth, the catalysis would not proceed through an energetically unfavorable species with a negative charge in the very hydrophobic environment of the membrane, because this charge would be balanced by binding Na^+, H_3O^+, or arginine.

In summary, the data presented are consistent with the DCCD-reactive glutamate residue of subunit c being the binding site for Na^+ (or H_3O^+). Such evidence includes the high reactivity of this group with DCCD in a pH-dependent manner, specific protection from inactivation by DCCD with Na^+, a Na^+-dependent downshift in the p$K(H^+)$ of the reactive glutamate residue, and a corresponding H^+-dependent downshift of p$K(Na^+)$. Also in accordance is the pH-rate profile of ATPase activity from which a similar pK was derived as from the pH dependence of inactivation by DCCD. Although this wealth of information is strong support for our conclusion, definite proof of the Na^+-binding ligands on the enzyme has to await a high-resolution structure.

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