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Inhibition of the Membrane-Bound Adenosine Triphosphatase of *Escherichia coli* by Dicyclohexylcarbodi-imide

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The inhibition of the membrane-bound adenosine triphosphatase of *Escherichia coli* by DCCD (dicyclohexylcarbodi-imide) is studied under conditions of varying KCl concentration. An increase in K^+ concentration and in other cations causes an increase in the DCCD sensitivity of the enzyme, as well as significant changes in the kinetic parameters.

The membrane-bound ATPase† of *Escherichia coli*, although insensitive to the usual inhibitors, such as oligomycin, rutamycin, ossamycin and venturicidin (R. J. Fisher, unpublished work), has been shown to be inhibited by DCCD (Beechey *et al.*, 1967; Fisher & Sanadi, 1971; Roison & Kepes, 1973). A thorough study of conditions optimum for inhibition has not yet been carried out. Beechey *et al.* (1967) pointed out that DCCD inhibition in ox heart mitochondria is time-dependent, and found that a value of 0.5nmol of DCCD/mg of protein was sufficient to give 50% inhibition of the ATPase after 24h of preincubation. Harold & Baarda (1969) reported an I_{50} of 1.0nmol of DCCD/mg of protein for the ATPase of *Streptococcus faecalis* if membranes are incubated for 18h with the inhibitor. In contrast with this, the reported values for the inhibition of the *E. coli* enzyme by DCCD are 10–20 times higher (Evans, 1975; Fisher & Sanadi, 1971; Roison & Kepes, 1973, Fillingame, 1975; Altendorf & Zitzmann, 1975; Hare, 1975). The membrane sector of the ATPase (Roison & Kepes, 1973; Fillingame, 1975) has been shown to be the site of DCCD action; however, DCCD has been shown to cause non-specific inhibition of the isolated ATPase from ox heart mitochondria at concentrations of 17nmol of DCCD/mg of protein (Beechey *et al.*, 1975). For these reasons, it is important to maintain conditions of inhibition that give the lowest possible titres of DCCD, thereby minimizing non-specific effects. We show below that, in the presence of 0.2M-(added) K^+, the I_{50} for DCCD, K_m for MgATP and $V_{max.}$ of the *E. coli* ATPase are all significantly lowered. Since the physiological concentration of K^+ in growing cells of *E. coli* is 0.2M (Lubin & Ennis, 1964), measurements of the ATPase activity done in the absence of this cation probably do not reflect the behaviour of the enzyme in the cell.

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† Abbreviations: ATPase, adenosine triphosphatase; DCCD, dicyclohexylcarbodi-imide; Mops, 4-morpholinepropanesulphonic acid; I_{50}, amount of inhibitor per mg of protein to give a 50% inhibition.

Materials and Methods

E. coli K12 was obtained in bulk from Miles Laboratories, Elkhart, IN, U.S.A. *E. coli* W6 was grown aerobically on glucose/yeast extract as described (Fisher & Sanadi, 1971), and the membranes were prepared by using a French pressure cell (Houghton *et al.*, 1975). The buffer used in the preparation and storage of the membrane particles was 50mM-Tris/H_2SO_4/10mM-$MgSO_4$, pH 7.8.

The ATPase activity was determined by a pH assay similar to that described by Nishimura *et al.* (1962): H^+ release was initiated by addition of 1.0mg of *E. coli* membranes to a 5ml water-jacketed cell containing 5mM-Mops, MgATP and KCl where designated. The total background cation concentration was between 10 and 20mM total in K^+, Na^+ and Mg^{2+}. The pH of the reaction mixture was adjusted to 8 with KOH. The change in H^+ concentration was monitored with a Radiometer pH-meter (model pHM26), calibrated with standard 100mM-HCl at each concentration of MgATP and KCl used. H^+ release was linear with time for 15–30s under these conditions, and initial rates were estimated from this portion of the curve.

Protein was determined by the biuret prodedure as described by Jacobs *et al.* (1956), with bovine serum albumin as standard.

All DCCD used was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A., and was recrystallized from ethanol, and dissolved in methanol for use. Methanol (to 5mM) alone had no

effect on ATPase activity, and the final concentration was always kept lower than 5mM. For DCCD titres and kinetics, membranes were preincubated with the inhibitor for 21 h on ice. Controls were left on ice for 21 h to account for any cold-labile loss of activity.

Results

K_m and $V_{max.}$ values for MgATP

The apparent K_m value for MgATP, as well as the $V_{max.}$ for the ATPase, is considerably decreased in the presence of added 0.2M-KCl, as shown in Table 1. The apparent K_m for MgATP decreased continuously from 0.91 mM in the absence of added KCl, to 0.23 mM at 0.2M-KCl. No further lowering of the K_m was observed at higher K^+ concentrations. The $V_{max.}$ of the ATPase, however, increased with added K^+, reaching a value about 2-fold higher at 30–50mM-K^+. The $V_{max.}$ then decreased, and, as with the K_m, no further decrease was noted above 0.2M-K^+.

The K_i for ADP decreased 6-fold in 0.2M-K^+, but the K_i for adenyl-5′-yl imidodiphosphate remained essentially the same. The effect of K^+ may be on the association of the enzyme with the membrane, such that in the absence of KCl there is a dissociation from the membrane and concomitant increase in kinetic constants (Roison & Kepes, 1973). This, however, is not the case, because if the membranes are incubated for 20h at 0°C in either high- or low-salt conditions and then concentrated by centrifugation (250000g for 1h) there is a 95% recovery of the ATPase activity.

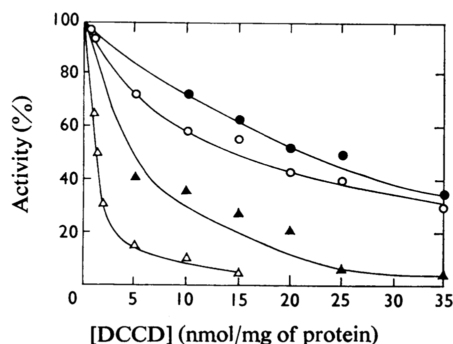

Fig. 1. *Time-dependence of inhibition by DCCD*
Appropriate amounts of DCCD were added to membranes (37mg of protein/ml) in 0.2M-KCl and incubated at 0°C for the specified times: ●, 15min; ○, 20min; ▲, 60min; △ 21h. The ATPase activity at the 100% level was 0.47 µmol of H^+/min per mg.

Time-dependence of DCCD inhibition

The data in Fig. 1 demonstrate the time-dependence of the DCCD inhibition of the *E. coli* ATPase, in added 0.2M-KCl. This dependence is analogous to that found in ox heart mitochondria by Beechey *et al.* (1967), which is predictable if the inhibition is dependent on a covalent-bond formation. The maximum inhibition is found after 20h of preincubation on ice.

I_{50} values for DCCD

To determine the effect of KCl on the membrane-bound DCCD-sensitive ATPase, DCCD titrations were done at various KCl concentrations. In fresh membranes, the controls showed an initial increase in ATPase activity, then a decrease, as KCl increased. In older membranes, no initial activation was seen. At a constant concentration of DCCD, the amount of inhibition increased with KCl. The I_{50} for DCCD in added 0.2M-KCl was approx. 5% of that in the low-salt (no added KCl) system, and greater than 95% inhibition was obtained, compared with approx. 80% in the low-salt system (Table 1).

The presence of KCl creates a DCCD-sensitive ATPase that was previously insensitive. Inhibition in low-salt conditions must result from another reaction of the DCCD, such as activation of an inhibitory protein in the solubilized ATPase (Vogel & Steinhart, 1976), or a non-specific inhibition at high concentrations of DCCD (Beechey *et al.*, 1975).

Table 1. *Kinetic constants for the membrane-bound ATPase in high- and low-salt conditions*
The K_m, K_i, (ADP), K_i [adenyl-5′-yl imidodiphosphate (p[NH]ppA)] and $V_{max.}$ values were determined in low- and high-salt conditions by varying the MgATP concentration with and without fixed values of ADP or p[NH]ppA. The values were taken from double-reciprocal plots of the data. The I_{50} for DCCD was determined after incubation for 21h at 0°C with different concentrations of inhibitor in the presence or absence of 0.2M-KCl. The other details of the assays are as described in the Materials and Methods section.

Experiment	K_m (MgATP) (mM)	$V_{max.}$ (µmol of H^+/min per mg)	K_i (ADP) (µM)	K_i (p[NH]ppA) (µM)	I_{50} (DCCD) (nmol/mg of protein)	Inhibition in the presence of DCCD (%)
Low-salt	0.91	0.62	275	1.4	24.0	80
High-salt	0.23	0.47	43	1.0	1.4	95

Discussion

The presence of added 0.2M-KCl causes an increased sensitivity to DCCD, resulting in a lower I_{50} value and an increased maximum inhibition. An explanation for this may be that the KCl is creating easier access of the DCCD to the inhibitor site, so that 1.4nmol of DCCD/mg of protein in a high-salt system binds as many sites as 24nmol of DCCD/mg of protein in low-salt conditions. This is not the case, because, when membranes were incubated in low-salt conditions for 20h at 0°C with 5nmol of DCCD/mg of protein, there was only slight inhibition when assayed in the low-salt system. However, when assayed in 0.2M-KCl, inhibition was greater than 95%. This indicates that all the DCCD-binding sites are available in low-salt as well as high-salt conditions.

Another reason is that the KCl causes a change in the ATPase complex, leading to increased sensitivity. This may be similar to the poor coupling that Roison & Kepes (1973) postulated at pH9.0, at which point the ATPase inhibition is abolished. The KCl may be creating a better-coupled system, increasing the number of membrane-bound DCCD-sensitive ATPases that in low-salt conditions were insensitive. This is substantiated by the change in K_m (MgATP) and K_i (ADP) observed during a change from low- to high-salt conditions, indicating a conformational change.

The lack of change in the K_i for adenyl-5′-yl imidodiphosphate suggests that the effect of the cation may be localized to certain portions of the enzyme. If there exist distinct regulatory and catalytic binding sites for MgATP on the enzyme, as postulated by Schuster *et al.* (1975), the site of adenyl-5′-yl imidodiphosphate binding (the regulatory site) may remain unaffected by the KCl. It is important to note that uncouplers had no affect on kinetic parameters in either high- or low-salt conditions.

The effect of KCl is not specific for K^+, since the same results were obtained with CsCl, LiCl and NaCl. Vogel & Steinhart (1976) suggested the importance of ionic strength in the reversible dissociation of *E. coli* ATPase, and a similar effect may be occurring here. Alternatively, the univalent cations may neutralize charges in the phospholipids, leading to changes in the ATPase association with the membrane. Choline chloride produced the same effect, but longer periods of incubation were necessary. This indicates that the chloride anion is less important than the cation. The larger choline ion has less accessibility to the active site than the K^+ cation, and therefore the lag is observed. Sucrose (0.2M) did not produce any effect, thus ruling out the possibility of an osmolarity effect.

The effect of high ionic strength is of considerable importance in studying the membrane-bound ATPase, in that it more accurately reflects the physiological milieu of the ATPase. The I_{50} value reported here, 1.4nmol of DCCD/mg of protein, is close to the values reported in other systems (Beechey *et al.*, 1967; Harold & Baarda, 1969; Abrams & Baron, 1970), indicating some similarity between the ATPases. In addition, a regulatory action by K^+ on ATPase activity would be a way of maintaining specific concentrations of that cation in the cell.

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