Synergetic inhibition of photophosphorylation and uncoupled electron transport by *N,N'*-dicyclohexylcarbodiimide and alcohols in pea chloroplasts

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Abstract It is shown that the inhibitory effect of *N,N'*-dicyclohexylcarbodiimide (DCCD) on photophosphorylation and uncoupled electron transfer from H_2O to methylviologen (MV) in pea chloroplasts depends upon solvent concentration. Being applied as a solution in dimethyl sulfoxide (DMSO) DCCD did not suppress uncoupled electron transfer and inhibited photophosphorylation independently from DMSO concentration. If DCCD was applied as methanolic or ethanolic solution its concentration sufficient for half-maximum inhibition $[I]_{50}$ of both photophosphorylation and uncoupled electron transfer decreased at increasing alcohol content. The data suggest that the synergistic effect of DCCD and alcohols is connected with DCCD-catalyzed etherification of some carboxylic groups which are important for chloroplast electron transfer.

Key words: *N,N'*-Dicyclohexylcarbodiimide; Chloroplast; Photophosphorylation; Electron transfer

1. Introduction

N,N'-Dicyclohexylcarbodiimide is a known hydrophobic reagent used for peptide synthesis [1]. In the absence of a nucleophile, DCCD is a so-called energy transfer inhibitor, blocking oxidative photophosphorylation in mitochondria and bacteria as well as photosynthetic photophosphorylation in chloroplasts [2,4]. The effect of DCCD on chloroplast ATP-synthase (CF_0CF_1) is well documented to be connected with covalent modification of a single glutamic acid residue in subunit III of the hydrophobic part (CF_0) [5,6]. DCCD can also interact with the β-subunit of the catalytic part of the complex (CF_1) [7]. Although DCCD is known to be the most classical CF_0 inhibitor, it also suppresses linear electron transfer in chloroplasts [3,8] and binding with LHCII inhibits qE [9,10]. It was also shown that DCCD inhibits the protonic reactions around photosystem II without blocking electron transport, which requires higher concentrations of DCCD [11]. The present paper describes an unexpected synergetic inhibition of photophosphorylation and uncoupled electron transfer from H_2O to MV by DCCD and low concentration of alcohols. It also shows that the solution of DCCD in DMSO has no effect on uncoupled electron transport and that inhibition of photophosphorylation by DCCD is independent of the DMSO concentration.

Taking into account that the alcohols are weak nucleophiles, we suggest that the synergetic effect is caused by DCCD-catalyzed modification of COOH groups by alcohols.

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2. Materials and methods

Chloroplasts (class II) were isolated according to [12] from leaves of 2-week-old pea seedlings and resuspended in the isotonic storage buffer (pH 8.0) containing 10 mM tricine-NaOH, 200 mM sorbitol, 10 mM KCl, 10 mM NaCl, 2.5 mM $MgCl_2$. The dense preparation was kept at a concentration of 4 mM Chl on ice in darkness. The Chl concentration was measured according to Arnon [13].

Just before the experiment an aliquot of chloroplasts was diluted to 16 μM Chl with the reaction medium and put into a 5 ml reaction vessel stirred and thermostated at 20°C. Oxygen consumption in the reaction of uncoupled electron transfer from H_2O to MV was measured with a covered platinum electrode in the presence of 10 mM NH_4Cl and 0.1 mM MV. The photophosphorylation rate was estimated from the consumption of 'scalar' protons [14] measured with a glass electrode in the medium containing 0.05 mM phenazine methosulfate or 0.1 mM MV as electron acceptor.

The chloroplasts were illuminated for 1.5 min with a tungsten halogen lamp through a heat filter. DCCD (Serva) was purified using the procedure described by Fieser and Fieser [15]. 0.15 M solution in methanol (Sigma), ethanol for UV-spectroscopy grade (Fluka) and DMSO (Sigma) were made from freshly purified DCCD and employed to subsequent dilutions.

Details of reagent concentrations are given in the appropriate figure legends.

3. Results

Results presented in Fig. 1 show the dependencies of DCCD inhibition of photophosphorylation and uncoupled electron transfer in the presence of 0.25% (v/v) of methanol (Fig. 1a), ethanol (Fig. 1b) or DMSO (Fig. 1c) which were used for preparation of DCCD solutions. It is clear from Fig. 1 that the same concentrations of DCCD induce very different levels of suppression of cyclic, uncyclic photophosphorylation and uncoupled electron transfer from H_2O to MV depending on the type of the solvent applied for preparation of inhibitor solution. So, if DCCD was added to the reaction mixture as a solution in DMSO, then (i) the uncoupled electron transfer is not inhibited; (ii) the curves of inhibition of cyclic and uncyclic photophosphorylation practically coincide. These facts indicate that the mechanism of the DCCD inhibition of both types of photophosphorylation reactions is the same in the presence of 0.25% DMSO and is apparently connected only with ATP-synthase blockage without visible damage of the electron transport.

In contrast with the DMSO effect, alcoholic solutions of DCCD: (i) inhibit uncoupled electron transfer; (ii) induce a higher level of inhibition of uncyclic than cyclic photophosphorylation. The results suggest that in the presence of 0.25% methanol or ethanol, DCCD not only inhibits the ATP-synthase but also damages some site(s) of the electron transfer chain.

It should be noted also that the level of inhibition of photo-

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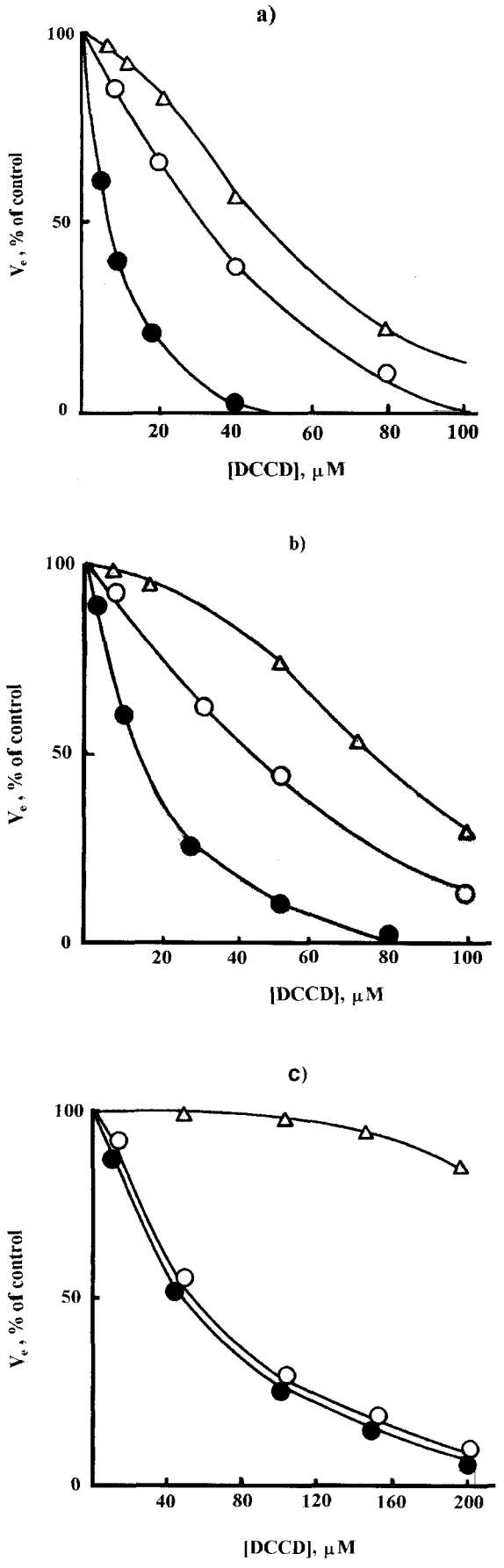

Fig. 1. Inhibition of photophosphorylation and uncoupled electron transfer from H_2O to MV by DCCD dissolved in alcohols or DMSO: (a) in the presence of 0.25% methanol; (b) in the presence of 0.25% ethanol; (c) in the presence of 0.25% DMSO. (●) Cyclic photophosphorylation (with PMS); (○) uncyclic photophosphorylation (with MV); (△) uncoupled electron transfer. The reaction mixture for determination of photophosphorylation rate contained 0.5 mM ADP, 3 mM K_2HPO_4, 1.5 mM tricine-NaOH (pH 8.0), 0.05 mM PMS or 0.1 mM MV, 10 mM KCl, 10 mM NaCl, 2.5 mM $MgCl_2$, 200 mM sorbitol, 0.1 mM MV and chloroplasts (16 μM Chl). The reaction mixture for determination of uncoupled electron transfer rate contained 10 mM NH_4Cl and 25 mM tricine-NaOH (pH 7.8), 10 mM KCl, 10 mM NaCl, 2.5 mM $MgCl_2$, 200 mM sorbitol, 0.1 mM MV and chloroplasts (16 μM Chl). Before each series of experiments, a set of fresh solutions of DCCD in methanol, ethanol or DMSO (0.005, 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 M) was prepared. DCCD was added so that the content of solvent in the reaction media was 0.25%.
←

phosphorylation and uncoupled electron transfer in the presence of 0.25% methanol is higher than that in the presence of 0.25% ethanol. According to Fig. 1 data, the concentrations of DCCD sufficient for 50% inhibition of the reaction ($[I]_{50}$) are 7 μM and 32 μM in the presence of methanol; 13 μM and 42 μM in the presence of ethanol for uncyclic and cyclic photophosphorylation respectively. In the presence of DMSO, $[I]_{50}$ for uncyclic and cyclic photophosphorylation was the same, about 60 μM.

Fig. 2 shows the effect of methanol or ethanol on the electron transfer rate in uncoupled thylakoid membranes pretreated by DCCD dissolved in DMSO. We can see that DMSO or alcohols themselves as well as DMSO+alcohols and DMSO+DCCD combinations do not suppress the oxygen consumption in the light-induced reaction from H_2O to MV up to at least 0.5% of the solvents and 0.1 mM DCCD. The addition of the alcohols up to 0.1, 0.2 and 0.5% induces increasing levels of suppression depending on the DCCD concentration. The data of Fig. 2 show that it is the DCCD and alcohol combination which induces uncoupled electron transfer inhibition because DCCD solution in DMSO, as well as alcohols themselves, do not block the reaction. Thus we have experimental evidences of a synergetic effect of ethyl or methyl alcohol and DCCD on the uncoupled electron transfer rate from H_2O to MV.

A more complete picture of the dependence of DCCD inhibition of the uncoupled electron transfer rate on molar concentration of solvents is given in Fig. 3. We can see that DCCD inhibits electron transfer in the presence of all tested solvents, but in the case of DMSO there is a range of its concentrations which does not affect the inhibition characteristics. $[I]_{50}$ is constant if DMSO concentration does not exceed 0.03 M (0.6%) Contrary to DMSO, even very low concentrations of alcohols have a synergetic effect on the DCCD inhibition of uncoupled electron transfer. The higher the solvent concentration, the lower is the concentration of DCCD sufficient for 50% inhibition of the reaction.

According to the data of Fig. 3, in which the concentrations of the solvents are expressed in molar units, the level of synergetic effect of methanol and ethanol on the DCCD inhibition of electron transport is practically the same and characteristics of the inhibition are close, in contrast with data of Fig. 1 in which solvent concentrations are expressed in percent units, as usually in the biochemical literature. In Fig. 3, under the molar scale, there are three scales of percent concentration

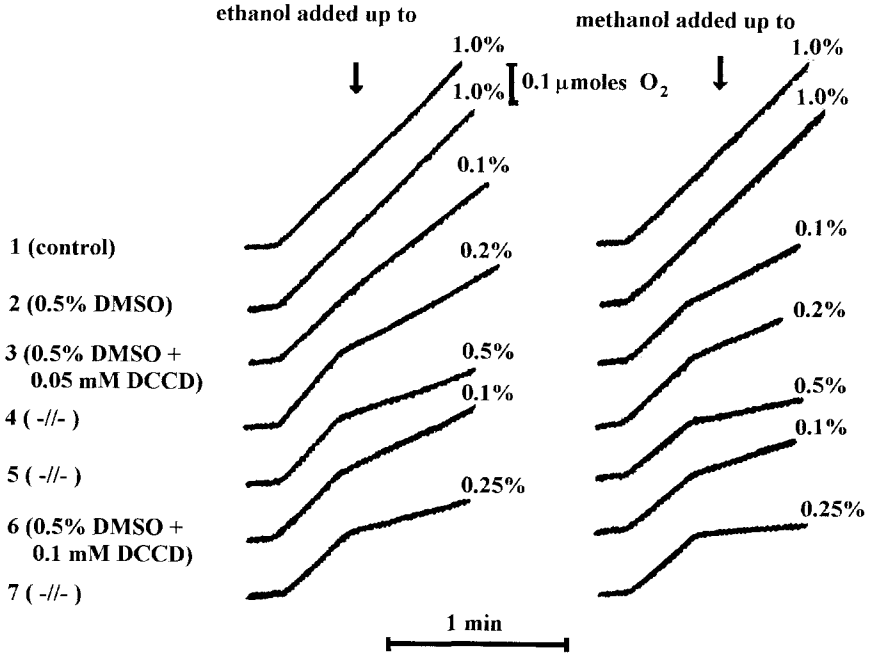

Fig. 2. The effect of solvents and DCCD on O_2 evolution in the presence of MV. Left traces: effect of ethanol; right traces: effect of methanol. Curves 1: control (reaction mixture as in Fig. 1); curves 2: reaction mixture contains 0.5% DMSO; curves 3, 4, 5: reaction mixture contains 0.5% DMSO + 0.05 mM DCCD; curves 6, 7: reaction mixture contains 0.5% DMSO + 0.1 mM DCCD.

of methanol, ethanol and DMSO respectively. We can see that the picture would be rather different if the concentrations of the solvent were expressed in percent units. These findings indicate that the characteristics of the DCCD inhibition of photophosphorylation and electron transfer processes presented earlier in many publications should be reconsidered taking into account the nature and concentration of the solvent used for DCCD solution preparation.

Finally it should be noted that the data were obtained with thylakoid membrane suspensions containing 16 μM Chl. Control experiments have demonstrated that the inhibitory level in the studied reactions depended also on chloroplast concentration. The change of chloroplast concentration led to different DCCD inhibition characteristics (not shown).

4. Discussion

The results of our research clearly show an increase of the inhibitory effect of DCCD on chloroplasts in the presence of even very low concentrations of alcohols. At the same time it has been determined that DMSO, when used to dissolve and to introduce DCCD in the reaction media, had no amplifying (synergetic) effect on the studied reactions if its amount did not exceed 0.5%. Alcohols induced the inhibitory action of DCCD if the latter was added to the reaction mixture as a solution in DMSO. This fact shows that the effect of any combination of DCCD with other inhibitors should be interpreted in taking into account the total concentration of solvents in the solution. We suggest that the observed effects are due to the induction by alcohols of a larger vulnerability of functional important sites of the electron transfer chain components to damaging attack by DCCD. It seems to us that the most likely explanation of the present results is the participation of the functional COOH groups of electron transfer proteins in the reaction of condensation with alcohols catalyzed by DCCD. It is known that DCCD reacts with carboxylic groups of proteins forming an unstable product: dicyclo-*O*-acylisourea [1]. The latter can transform into dicyclo-*N*-acylurea in a very hydrophobic medium. For example, such a process takes place in hydrophobic CF_0 where DCCD covalently modifies carboxylic groups of the subunits III. In the

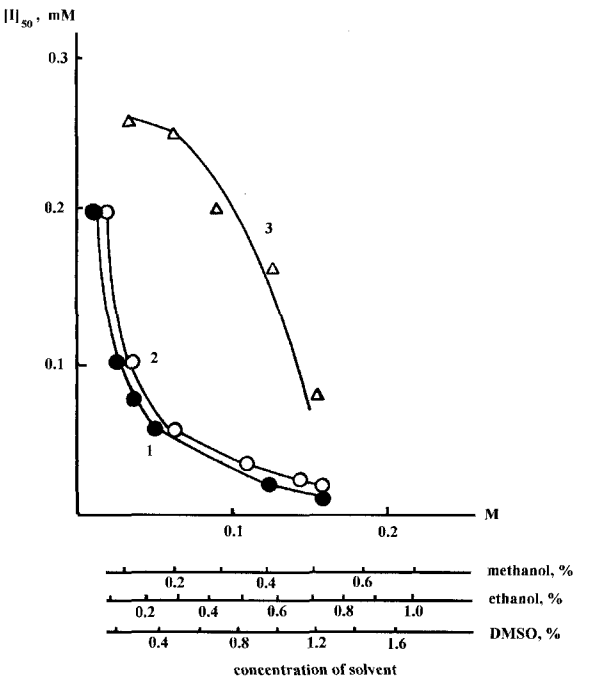

Fig. 3. Dependence of the $[I]_{50}$ of uncoupled electron transfer from MV to H_2O on molar and percent concentration of DCCD in the presence of methanol (1), ethanol (2) or DMSO (3). Conditions as in Fig. 1.

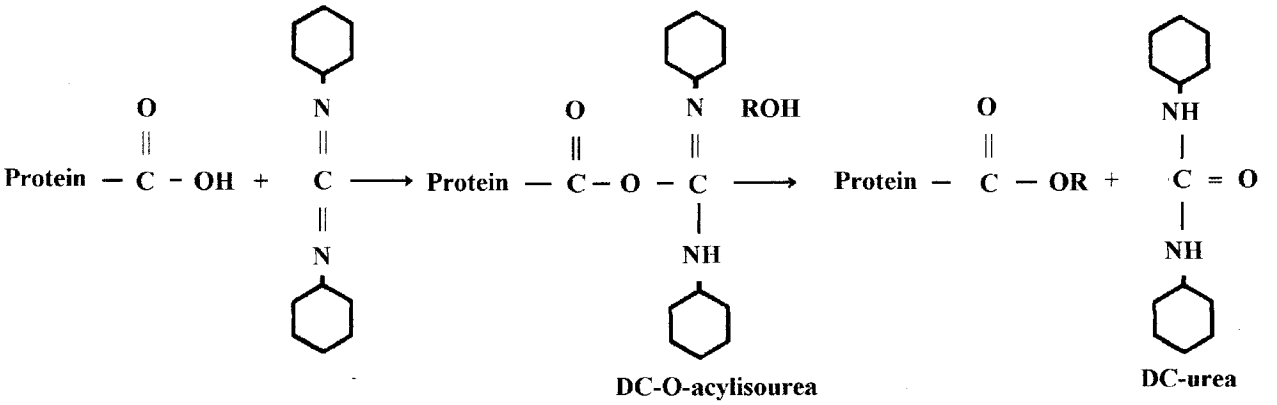

Fig. 4. Scheme of possible interaction of DCCD and alcohols with COOH groups of proteins.

presence of a nucleophile the unstable dicyclo-*O*-acylisourea can react with it, releasing *N,N'*-dicyclohexylurea and a derivative of a former COOH group. In the presence of alcohols which are weak nucleophiles, this reaction can take place as shown in Fig. 4. The scheme shows that the sites of chloroplast proteins which could be modified by alcohols in the reaction catalyzed by DCCD cannot be identified by ^{14}C-labelled DCCD because after reaction with the COOH group and alcohol the label will be released into the medium. To locate the alcohol-modified sites in the chloroplasts it will be necessary to use ^{14}C-labelled alcohols.

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