Vol. 179, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1991 Pages 507-511

INHIBITORY EFFECTS OF DICYCLOHEXYLCARBODIIMIDE ON SPINACH CYTOCHROME b_6-f COMPLEX

Liang-Bi Li, Linda Yu, and Chang-An Yu

Department of Biochemistry, OAES, Oklahoma State University
Stillwater, OK 74078

Received July 15, 1991

The electron transfer activity of purified cytochrome b_6-f complex of spinach chloroplast is inhibited by dicyclohexylcarbodiimide (DCCD) in a concentration and incubation time dependent manner. The maximum inhibition of 75 % is observed when 300 mole of DCCD per mole of protein (based on cytochrome f) is incubated with cytochrome b_6-f complex at room temperature for 40 min. The inhibition of the complex is not due to the formation of cross links between subunits but due to the modification of carboxyls. The amount of DCCD incorporation is directly proportional to the activity loss, suggesting that some carboxyl groups in the complex are directly or indirectly involved in the catalytic function. The incorporated DCCD is located mainly at cytochrome b_6 protein. The partially inhibited complex shows the same H^+/e- ratio as that of the intact complex when embedded in phospholipid vesicles.

The cytochrome b_6-f complex, which catalyzes electron transfer from plastoquinol to plastocyanin, is a segment of the photosynthetic electron transfer chain [1-7]. It is similar to the cytochrome b-c_1 complex of the mitochondrial respiratory chain in both function and essential redox components [7,8]. The oxidation of plastoquinol is coupled with the generation of a proton gradient across the thylakoid membrane having a H^+/e- ratio approaching 2 [9,10], which is compatible with the Q-cycle mechanism. According to the Q-cycle hypothesis, protons are translocated by a ligand conduction mechanism in which plastoquinol is the only proton carrier. Some of the available experimental evidence argues against a Q-cycle mechanism in the cytochrome b_6-f complex. The uncertainty of the fast electron transfer between the two cytochromes b_6 and the similar redox potentials and spectral characteristics of these two cytochromes are not predicted by the Q-cycle hypothesis. Other proton transfer mechanisms, such as redox linked proton pumping, have been proposed [11]. The detection of a specific DCCD binding site in yeast cytochrome b-c_1 complex and the inhibition of proton transfer, but not of electron transfer, by DCCD seem to support the proton pumping mechanism [12]. In the case of the cytochrome b_6-f complex, DCCD inhibits both proton and electron transfer activities, and the inhibition is directly proportional to the uptake of DCCD by cytochrome b_6 protein, suggesting the involvement of a carboxyl group of the cytochrome b_6 protein in both electron and proton transport.

0006-291X/91 $1.50

MATERIALS AND METHODS

The lipid- and plastoquinone-deficient cytochrome b_6-f complex was prepared and assayed as reported [13]. This isolated complex contained, in nanmoles/milligram of protein: cytochrome f ,10.1; cytochrome b_6,19.8 and plastoquinone 6.1. The specific activity of the complex was 45 μmoles cytochrome c reduced per mg per hour after being replenished with glycolipids isolated from chloroplasts[13].

The cytochrome b_6-f complex phospholipid vesicles were prepared essentially according to the method of Leung and Hinkle [14], except a phospholipid-to-protein ratio of 50 and potassium phosphate buffer, pH 7.0, were used. The assay medium for proton ejection contained 1.35 mL of 0.1 M KCl, 50 μL cytochrome b_6-f vesicle (5 μM cyt, f), 4 μL cytochrome c (2 mM) and 2 μL valinomycin (1 mg/mL). The reaction was started by the addition of 5 μL of 10 mM PQ_2H_2. PQ_2H_2 [15] and Decanoy-N-methylglucamide (DMG) [16] were synthezied according to the reported methods .

Incorporation of [^{14}C]DCCD into cytochrome b_6-f complex protein and inhibition of enzymatic activity were determined by a method similar to that used to measure the incorporation of photoactivated azido-Q [13]. One hundred μL of cytochrome b_6-f complex, 10 μM cytochrome f, in 50 mM Tris-succinate buffer, pH 7.0, containing 0.4 % DMG, 1 % sodium cholate, and 10 % glycerol, were incubated with 5 μL of ethanolic solution containing the indicated amount of DCCD at room temperature for 40 min. After incubation, the mixtures were diluted with 3.5 volumes of 50 mM Tris-succinate buffer containing 1 % sodium cholate and one volume of asolectin solution (2 mg/mL) in 0.5 % DMG. The mixture was then further incubated at 0° C for 30 min before enzymatic activity was determined. To determine the incorporation of DCCD into protein, 5 μl of DCCD treated samples (at the end of 40 min. incubation) were withdrawn and spotted on Whatman paper No. 3. After all samples were spotted and dried, the paper was developed with chloroform:methanol (2:1) (v/v). Denatured proteins remained at the origin while unreacted DCCD moved up with the solvent. The protein spots were cut out and placed in scintillation vials containing 5 ml of Insta-gel. Radioactivity was determined in a Packard Tri-Carb Scintillation Analyzer, 1900 CA.

Distribution of radioactivity among the subunits of the cytochrome b-f complex was estimated by the reported method [13]. The [^{14}C]DCCD treated samples were dialyzed against water and extracted with organic solvent, as described previously [17]. The extracted samples were redissolved in 50 mM K/Na-phosphate buffer, pH 7.0, to a protein concentration of 1 mg/ml. SDS-PAGE and radioactivity determination were performed according to Doyle et al. [13].

RESULTS AND DISCUSSION

Inhibition of cytochrome b_6-f complex by DCCD: As shown in the Figure 1, the inhibition of the cytochrome b_6-f complex depends on the concentration of DCCD used. Maximum inhibition of 75 % is observed when 300 mole of DCCD per mole of protein (based on cytochrome f) is incubated with cytochrome b_6-f complex at room temperature for 40 min. The stock solution of DCCD was freshly made in absolute ethanol. The concentration of solvent in the samples was kept constant at 5%, while various concentrations of DCCD were used. In the control experiment, in the presence of solvent and absence of DCCD, the complex lost about 10 % activity during incubation.

Figure 2 shows the incubation time dependent inhibition of cytochrome b_6-f complex by DCCD. At a constant concentration of DCCD, the activity of the cytochrome b_6-f complex decreases in direct proportion to the incubation time. A maximum inhibition is reached at 40 min and with a $T_{1/2}$ of 10 min.

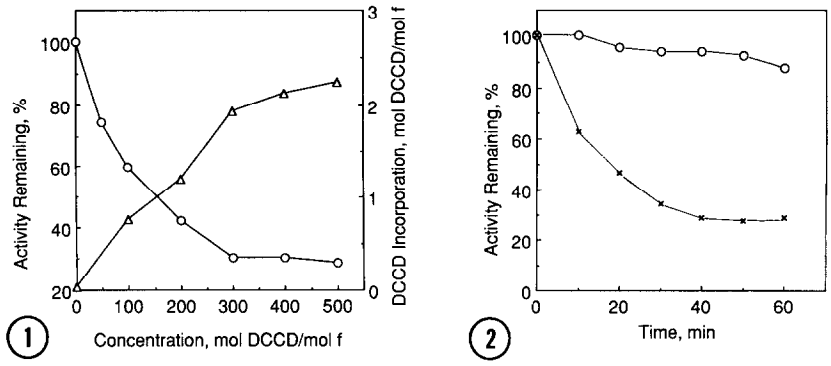

Fig. 1. DCCD concentration-dependent inhibition of the cytochrome b_6-f complex. The complex (10 μM cytochrome f), in 50 mM Tris-succinate buffer, pH 7.0, containing 1% sodium cholate and 10% glycerol, was treated with the indicated ethanolic DCCD solution for 40 min at room temperature (-O-). The sample prepared under identical conditions without DCCD was used as a reference for the calculation of the remaining activity. 100% activity represents 41 μmoles cytochrome c reduced per nmole cytochrome f per hour. The curve with triangles (Δ) represents DCCD incorporation into the cytochrome b_6-f complex when treated with [^{14}C] DCCD under the same conditions.

Fig. 2. Effect of DCCD incubation time on the inhibition of the cytochrome b_6-f complex. The experimental conditions were the same as in Fig. 1, except that a constant amount of DCCD, 300 mol/mol cytochrome b_6-f complex, was used and incubation times were varied. The cross symbols (-x-) show the sample treated with DCCD; the circles (-O-) represent control experiments in which only solvent was added to the complex .

Correlation between activity inhibition and incorporation of DCCD: When cytochrome b_6-f complex is treated with [^{14}C]DCCD at various concentrations for 40 min, the incorporation of DCCD into protein is proportional to the concentration of DCCD used. As indicated in Figure 1, the amount of DCCD incorporation is directly proportional to the activity loss, suggesting that some carboxyl groups in the hydrophobic region of the complex are directly or indirectly involved in the catalytic function of the cytochrome b_6-f complex. If the activity loss due to DCCD resulted from intra-subunit or inter-subunit cross linking, no direct correlation between DCCD uptake and activity loss would be expected. Also, the formation of an inter-subunit cross link should be readily detectable by SDS-PAGE of the DCCD-treated complex. The SDS-PAGE patterns of treated and untreated cytochrome b_6-f complex are identical (data not shown) clearly indicating that no cross-linking occurs between subunits. From the specific radioactivity of DCCD it was calculated that when 0.7 mole of DCCD is incorporated into one mole of cytochrome b_6-f complex, there is 40 % activity loss. This suggests that one or two moles of carboxyls per mole of complex are modified by DCCD.

Binding of [^{14}C]DCCD to cytochrome b_6: Figure 3 shows the distribution of radioactivity among the subunits of the [^{14}C]DCCD treated complex. DCCD is bound specifically to the cytochrome b_6 protein. Very little radioactivity is found in the other subunits. Occasionally some radioactivity is observed at the top of the gel due to aggregation of the cytochrome b_6 protein. The appearance of radioactivity at the top of the gel correlates with a decrease in the radioactivity in the cytochrome b_6 protein, suggesting that part of the latter is aggregated under the conditions used. This correlation

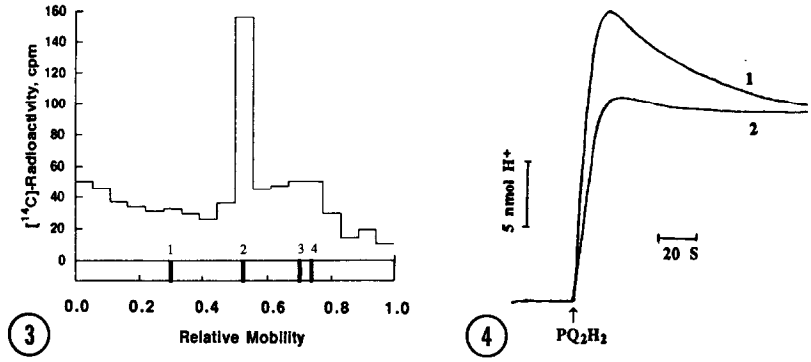

Fig. 3. The distribution of [^{14}C]DCCD among the subunits of the cytochrome b_6-f complex. The cytochrome b_6-f complex was treated with 300 molar excess of [^{14}C] labeled DCCD, 10 nCi/mol DCCD, at room temperature for 40 min under the conditions given in Fig. 1. After incubation, the sample was dialyzed extensively against water and extracted with organic solvent. The extracted sample was lyophilized, redissolved in 50 mM K/Na phosphate buffer to a protein concentration of 1 mg/mL and subjected to SDS-PAGE after treatment with 1 % SDS and 1 % β-mercaptoethanol at 37 °C for 2 hrs. The conditions for electrophoresis and the estimation of radioactivity distribution were the same as reported previously (13). The Arabic numerals 1 to 4 represent subunits I to IV of the cytochrome b_6-f complex.

Fig. 4. Proton ejection of DCCD treated and untreated cytochrome b_6-f complex inlaid in phospholipid vesicles. The preparation of cytochrome b_6-f complex-phospholipid vesicles is detailed in the text. Curve 1 is the untreated complex and curve 2 (67 % inhibition) is the DCCD treated sample.

also suggests that the loss of activity during treatment with DCCD is due to modification of carboxyl groups in the cytochrome b_6 protein, which renders cytochrome b_6 protein less soluble.

Since the only carboxyl groups that will be labeled by DCCD are those located in the hydrophobic environment lacking active hydrogen groups in the vicinity, the carboxyl groups could either be Asp-155 or Glu-166 These two amino acid residues are located in the fourth transmembrane helice of the five transmembrane helice model of cytochrome b_6. In the four transmembrane helice model, these residues are located on the lumen side of the thylakoid membrane (18).

DCCD inhibition of proton ejection from cytochrome b_6-f phospholipid vesicles: Figure 4 shows the proton ejection by cytochrome b_6-f complex embedded in phospholipid vesicles using cytochrome c as the electron acceptor and PQ_2H_2 as the electron donor. The ratio of H^+/e- for intact cytochrome b_6-f vesicles is about 1.9. The DCCD treated complex shows a similar H^+/e- ratio when incorporated into phospholipid vesicles despite the significant decrease in proton ejection rate. This decrease in proton ejection rate is proportional to the decrease in activity of the complex after treatment with DCCD. In contrast to yeast cytochrome b-c_1 complex [12], in which DCCD inhibits proton translocation but has little effect on electron transfer activity, the electron transfer activity of the cytochrome b_6-f complex is directly linked to proton translocation. It should be mentioned that in intact thylakoid membrane, the electron acceptor plastocyanin is located on the lumen side and protons are transported in. In the reconstituted vesicle system, with cytochrome c as the electron acceptor, only those complexes arranged with cytochrome f facing outward will be accessible to cytochrome c and active in electron transfer. The cytochrome b_6-f

complexes which have the opposite topological arrangement will not catalyzes electron transfer or proton translocation.

ACKNOWLEDGMENTS

Journal Article No.J-6050 of the Agricultural Experiment Station, Oklahoma State University. This work was supported by grants from the USDA (AMD 9101532) . We thank Dr. Roger Koeppe for his critical review of this manuscript.

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