Tanokura, M., Aramaki, H., Goto, K., Hashimoto, U., Toyomori, Y., & Yamada, K. (1986a) *J. Biochem. (Tokyo) 99*, 1211–1218.
Tanokura, M., Imaizumi, M., & Yamada, K. (1986b) *FEBS Lett. 209*, 77–82.
Williams, T. C., Corson, D. C., McCubbin, W. D., Oikawa, K., Kay, C. M., & Sykes, B. D. (1986) *Biochemistry 25*, 1826–1834.
Woledge, R. C., Curtin, N. A., & Homsher, E. (1985) *Energetic Aspects of Muscle Contraction*, Chapter 4, Academic, New York.
Wnuk, W., Cox, J. A., & Stein, E. A. (1982) in *Calcium and Cell Function* (Cheung, W. Y., Ed.) Vol. II, pp 243–278, Academic, New York.
Yagi, K., Matsuda, S., Nagamoto, H., Mikuni, T., & Yazawa, M. (1982) in *Calmodulin and Intracellular Ca^{2+} Receptors* (Kakiuchi, S., et al., Eds.) pp 75–91, Plenum, New York.
Yamada, K. (1978) *Biochim. Biophys. Acta 535*, 342–347.
Yamada, K., & Kometani, K. (1982) *J. Biochem. (Tokyo) 92*, 1505–1512.
Yazawa, M., Sakuma, M., & Yagi, K. (1980) *J. Biochem. (Tokyo) 87*, 1313–1320.

Involvement of a Carboxyl Group in the Interaction between Succinate Dehydrogenase and Its Membrane-Anchoring Protein (QPs) Fraction†

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ABSTRACT: The involvement of the carboxyl groups in the membrane-anchoring protein (QPs) in reconstitution of succinate dehydrogenase to form succinate–ubiquinone reductase is studied by using a carboxyl group modifying reagent, dicyclohexylcarbodiimide (DCCD). Inactivation of QPs by DCCD is found to be dependent on the temperature, pH, detergent, and DCCD concentration used. When QPs is treated with 300 molar excess DCCD at room temperature for 10 min, about 90% of the original reconstitutive activity is lost. When intact or reconstituted succinate–ubiquinone reductase formed from reconstitutively active succinate dehydrogenase and QPs is treated with DCCD under the same conditions, no loss of succinate–ubiquinone reductase activity is observed. However, when a mixture of reconstitutively inactive succinate dehydrogenase and QPs is treated with DCCD before being reconstituted with active succinate dehydrogenase, an inactivation behavior similar to that with QPs alone is observed. These results indicate that DCCD modifies the carboxyl groups of QPs which are essential for the interaction with succinate dehydrogenase to form succinate–ubiquinone reductase. Inactivation of QPs by DCCD parallels the incorporation of DCCD into QPs. About two carboxyl groups per molecule of QPs are essential for the interaction with succinate dehydrogenase. These essential carboxyl groups are located in the smaller subunit (M_r 13 000) of QPs. Modification of QPs by DCCD also alters the heme environment of cytochrome b_{560}.

Mitochondrial succinate–ubiquinone (succinate–Q)[1] reductase (Ziegler & Doeg, 1962), which catalyzes the electron transfer from succinate to Q, is composed of four protein subunits with molecular weights of 70 000, 27 000, 15 000, and 13 000. The isolated complex can be resolved into two reconstitutively active fractions: a soluble succinate dehydrogenase (Keilin & King, 1958; Davis & Hatefi, 1971; Ackrell et al., 1977; Yu & Yu, 1980a) composed of the two larger subunits and a membrane-anchoring fraction, generally known as QPs (Yu & Yu, 1980b), protein fraction (Vinogradov et al., 1980), CII-$_{3,4}$ (Ackrell et al., 1980), or a cytochrome b_{560} fraction (Hatefi & Galenta, 1980), containing the two smaller subunits. Further resolution of succinate dehydrogenase into M_r 70 000 and 27 000 subunits has not yet been achieved. To date, attempts to dissociate QPs into the M_r 15 000 and 13 000 subunits resulted in a significant loss of ability to reconstitute with succinate dehydrogenase. Isolation of the M_r 13 000 protein, practically devoid of the M_r 15 000 protein, has been reported (Yu & Yu, 1980b; Vinogradov et al., 1980), but the reconstitutive activity of this preparation is only 20% compared to that obtained with two-subunit QPs. Isolation of a single-polypeptide cytochrome b_{560} has not yet been reported.

The interaction between succinate dehydrogenase and QPs is not only functionally necessary but also structurally important. Succinate dehydrogenase is stable in its membrane-bound form, such as in submitochondrial particles, isolated succinate–cytochrome *c* reductase, or succinate–Q reductase. However, it becomes very labile when detached from its membrane-anchoring frction; the half-life is about 20 min at 0 °C under aerobic conditions. The close physical relationship between succinate dehydrogenase and its membrane-anchoring proteins has been recently demonstrated

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[1] Abbreviations: Q, ubiquinone; DCCD, dicyclohexylcarbodiimide; DCIP, dichlorophenolindophenol; PMS, phenazine methosulfate; DATA, *N,N'*-diallyltartardiamide; DMG, decanoyl-*N*-methylglucamide; SDS, sodium dodecyl sulfate; QPs, a two-subunit protein fraction that converts succinate dehydrogenase into succinate–Q reductase; EPR, electron paramagnetic resonance; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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through studies of the cytochrome b_{560} of the two-subunit QPs (Yu et al., 1987). It was observed that the redox potential, absorption properties, and EPR characteristics of cytochrome b_{560} are changed drastically by the presence of succinate dehydrogenase. The presence of cytochrome b_{560} in the succinate–Q reductase region of the mitochondrial electron-transfer chain was recently confirmed by the EPR detection of this cytochrome in a fully active succinate–cytochrome *c* reductase, although several lines of evidence have argued against the direct involvement of cytochrome b_{560} in electron transfer from succinate to Q by succinate–Q reductase. These are the following: cytochrome b_{560}, either in succinate–Q reductase or in two-subunit QPs, cannot be reduced by succinate; its presence in a substoichiometric amount, with respect to succinate dehydrogenase, in succinate–cytochrome *c* and succinate–Q reductases; and the lack of redox change in cytochrome b_{560} during the catalytic cycle of these reductases. However,the structural role of this cytochrome in succinate–Q reductase is quite well established. The fact that all isolated QPs preparations having high reconstitutive activity contain both cytochrome b_{560} and the M_r 13 000 protein (Yu & Yu, 1980b; Ackrell et al., 1980; Hatefi & Galenta, 1980) and that spectral properties of cytochrome b_{560} depend on the presence of succinate dehydrogenase (Yu et al., 1987) support the proposed structural role of cytochrome b_{560}.

Elucidation of the mode of interaction between succinate dehydrogenase and QPs is essential for understanding the reaction mechanism of mitochondrial succinate–Q reductase. The interaction between these two components has been thought to involve both ionic (electrostatic) and hydrophobic forces, as supported by the following observations: (i) succinate dehydrogenase can be solubilized from the membrane at alkaline pH (Keilin & King, 1958); (ii) the reconstitution of succinate–Q reductase from succinate dehydrogenase and QPs is ionic strength dependent (Yu & Yu, 1980c); and (iii) an amino group in the succinate dehydrogenase is essential for reconstitution (Yu & Yu, 1981). The involvement of hydrophobic interactions is suggested by the fact that succinate dehydrogenase can be detached from the membrane by chaotropic reagents (Davis & Hatefi, 1971).

Since an amino group in succinate dehydrogenase is essential for reconstitution with QPs to form succinate–Q reductase (Yu & Yu, 1981), it is likely that a negatively charged group in QPs such as the carboxy group of a glutamyl or aspartyl residue, may serve as the anion in the electrostatic interaction. To test this, we have used a carboxyl modifying reagent, dicyclohexylcarbodiimide (DCCD), to modify the carboxyls in QPs and then examined the reconstitutive properties of the DCCD-treated QPs. Herein, we report detailed experimental conditions for DCCD modification of QPs, the effect of DCCD modification on reconstitutive activity and spectral characteristics of QPs, the protecting effect of succinate dehydrogenase against DCCD inactivation of QPs, and the location of the essential carboxyl group in subunits of QPs.

Experimental Procedures

Materials. Dichlorophenolindophenol (DCIP), phenazine methosulfate, and dicyclohexylcarbodiimide (DCCD) were obtained from Sigma. [^{14}C]DCCD was from Amersham. Insta-Gel counting cocktail was from Packard Instrument Co. *N,N'*-Diallyltartardiamide (DATA) was from Bio-Rad. Decanoyl-*N*-methylglucamide (DMG) was synthesized in our laboratory according to the method reported by Hildreth (1982). Succinate–cytochrome *c* reductase (Yu & Yu, 1982), ubiquinol–cytochrome *c* reductase (Yu & Yu, 1980a), succinate–Q reductase (Yu & Yu, 1982), two-subunit QPs (Yu et al., 1987), and reconstitutively active and inactive succinate dehydrogenase (Yu & Yu, 1980a) were prepared and assayed according to methods developed in this laboratory. Isolated two-subunit QPs was stirred with Bio-Beads SM-2 (1 g, wet weight, Bio-Beads/7 mL), overnight, to remove most of the Triton X-100 present in the preparation. The purified QPs was treated with glycerol to a final concentration of 10% and stored at −80 °C until use.

Reaction of DCCD with QPs. Frozen QPs suspension in 50 mM Tris-HCl buffer, pH 7.0, containing 30 mM sucrose and 10% glycerol was thawed, the pH adjusted to 6.0 with 1 M MES, and DMG added to a final concentration of 0.2%; 0.2-mL aliquots of QPs, 0.15 mg/mL, were placed in a room temperature water bath for 5 min and then mixed with 5 μL of absolute alcohol containing various concentrations of DCCD. The stock solution of DCCD was prepared fresh in absolute ethanol pretreated with molecular sieves to completely remove water. After addition of DCCD, the mixtures were incubated at room temperature for another 10 min before cooled to 0 °C and reconstituted with excess succinate dehydrogenase (5 μL, 20 mg/mL in 50 mM sodium/potassium phosphate buffer, pH 7.8, containing 20 mM succinate). Succinate–Q reductase activity was assayed after the reconstituting mixture was incubated at 0 °C for 20 min.

Incorporation of [^{14}C]DCCD into QPs. [^{14}C]DCCD was diluted with cold DCCD to a specific activity of 5000 cpm/nmol. Reaction of [^{14}C]DCCD with QPs was carried out as described in the previous section. Two 10-μL aliquots were withdrawn from each of the DCCD-treated samples and spotted on separate paper (Whatman 3). When all the samplings were complete, one of the papers was developed with chloroform/methanol (2:1), air-dried, and redeveloped with butanol saturated with H_2O; the other paper was not developed. The original spots in these two papers were cut out, 5 mL of Insta-gel scintillation counting fluid was added, and radioactivity was determined in a Packard 400 CL/D scintillation counter. The counting efficiency was 50%. Radioactivity on the undeveloped paper for the corresponding sample was used as reference for the calculation of DCCD incorporation.

The distribution of [^{14}C]DCCD between the two subunits of QPs was determined by applying the [^{14}C]DCCD-treated sample to an SDS–DATA gel column after it was dissociated with 1% SDS and 1% β-mercaptoethanol at 37 °C for 2 h. The SDS–DATA gel column was prepared essentially according to the SDS–Bis system of Swank and Munkres (1971) except the cross-linker, Bis, was replaced with a cleavable cross-linker, *N,N'*-diallyltartardiamide (DATA), and 0.1 M Tris–H_3PO_4, pH 6.8, was replaced with 0.1 M Tris–NaH_2PO_4, pH 6.8. The electrophoretic buffer used was 0.1 M Tris–NaH_2PO_4, pH 6.8, containing 0.1% SDS; 4 mA per gel column was used, and electrophoresis was completed in 4 h. Since this SDS–PAGE system is able to separate the free DCCD from protein subunits of QPs, an organic solvent extraction procedure to remove non-protein-bound DCCD before the SDS–PAGE is not necessary. After staining and destaining processes, the gel was sliced according to the protein stain. The portion of the gel containing no protein stain was also cut in slices the same size as those of the protein bands. After the gel slices were completely dissolved with 0.3 mL of 2% periodic acid at room temperature for 1 h (Marinetti et al., 1979), 5 mL of Insta-gel was added and radioactivity determined.

Results and Discussion

Effect of Detergents on the Reconstitutive Activity of QPs. In contrast to isolated succinate dehydrogenase, isolated QPs

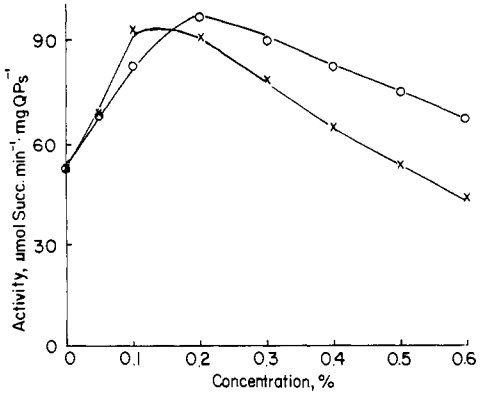

FIGURE 1: Effect of Triton X-100 and DMG concentrations on the reconstitutive activity of QPs. 0.2-mL aliquots of QPs, 0.17 mg/mL, in 50 mM Tris-HCl, pH 7.0, containing indicated concentrations of Triton X-100 (×) or DMG (○) were incubated at room temperature for 10 min, cooled down to 0 °C, and reconstituted with succinate dehydrogenase (5 μL 20 mg/mL, in 50 mM sodium/potassium phosphate buffer, pH 7.8, containing 20 mM succinate). The succinate–Q reductase activity was assayed after samples were incubated at 0 °C for 20 min.

is in the particulate form in the absence of detergent. Full reconstitution of succinate–Q reductase from isolated succinate dehydrogenase and QPs can only be achieved when the isolated QPs is predispersed in certain detergents prior to reconstituting with succinate dehydrogenase. Among detergents tested, such as deoxycholate, cholate, Tween-80, Triton X-140, Triton X-100, and decanoyl-*N*-methylglucamide (DMG), Triton X-100 and DMG are most effective. Figure 1 shows the effect of the concentration of Triton X-100 and DMG on the reconstitutive activity of QPs. Maximum reconstitution is obtained when QPs is dispersed in 0.1% Triton X-100 or in 0.2% DMG, at pH 7.0. The reconstitutive activity of isolated QPs, in the absence of added detergents, varies slightly with preparations depending on the amount of residual Triton X-100 present in the preparation. Triton X-100 used in the isolation of QPs is removed from the final preparation with Bio-Beads SM-2. There are two reasons for removing Triton X-100 from QPs preparation: first, only the Triton X-100-free sample can be stored at −80 °C for months without lost of its reconstitutive activity; second, a high concentration of Triton X-100 in isolated QPs prevents QPs from reacting effectively with DCCD.

Effect of DCCD Concentration on the Reconstitutive Activity of QPs. Since Triton X-100 and DMG are the most effective dispersing detergents for reactivating QPs reconstitutive activity, it is reasonable to test the effect of DCCD on QPs dispersed in either Triton X-100 or DMG. Figure 2 shows the DCCD concentration dependent inactivation of QPs at various concentrations of DMG (Figure 2A) and of Triton X-100 (Figure 2B). Inactivation of QPs by DCCD is both DCCD and detergent concentration dependent. When QPs is dispersed in different concentrations of DMG, at pH 6.0, and then treated with various concentrations of DCCD at room temperature (see Figure 2A), a maximal inactivation of 80% is observed when the detergent concentration is 0.2%, and the DCCD used is 250 mol/mol of QPs. When the DMG concentration is higher than 0.2%, the inactivation of QPs by DCCD, at the same DCCD concentration, decreases as the DMG concentration increases. For example, when 250 mol of DCCD/mol of QPs is used, the percent of QPs inactivation decreases from 80% to 50% as the DMG concentration increases from 0.2% to 0.8%. It should be mentioned that when QPs is dispersed in 0.8% DMG, in the absence of DCCD, it has only 70% of the reconstitutive activity compared to that

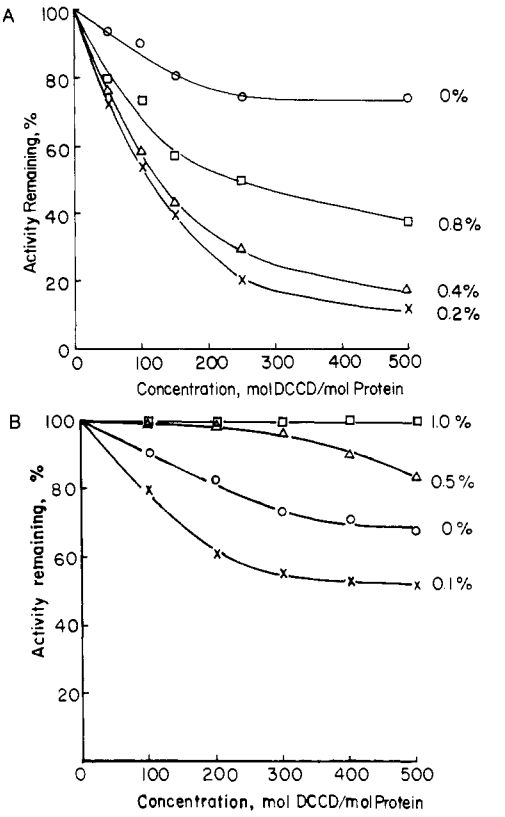

FIGURE 2: Effect of DCCD concentration on the inactivation of QPs under various concentrations of detergents. (A) Aliquots of 0.2 mL were withdrawn from each QPs sample dispersed in 50 mM Tris-HCl/MES buffer, pH 6.0, containing 0 (○), 0.2% (×), 0.4% (△), or 0.8% (□) of DMG and incubated at room temperature for 5 min before mixing with 5 μL of absolute alcohol containing the indicated concentrations of DCCD. The mixtures were incubated at room temperature for another 10 min, cooled to 0 °C, and then reconstituted with succinate dehydrogenase (5 μL, 20 mg/mL). (B) Conditions were identical with those described for (A), except QPs was dispersed in 50 mM Tris-HCl, pH 6.0, containing 0 (○), 0.1% (×), 0.5% (△), or 1% (□) Triton X-100. Succinate–Q reductase activity was assayed 20 min after the reconstitution. 100% activity represents 100 μmol of succinate oxidized per minute per milligram of QPs protein at 23 °C.

of QPs dispersed in 0.2% DMG.

When QPs is dispersed in different concentrations of Triton X-100, at pH 6.0, and then treated with various concentrations of DCCD, a maximal inactivation of 50% is observed when the detergent concentration is 0.1%, and the DCCD used is 250 mol/mol of QPs. Although the maximal inactivation of QPs by DCCD is lower in Triton X-100, the DCCD concentration required for maximal inactivation is the same in both systems. The detergent concentration effect is more pronounced in the Triton X-100 system than in the DMG system. When QPs is dispersed in 1% Triton X-100, no DCCD inactivation is observed, even when used in 500 molar excess to QPs. Possible explanations for the decrease in QPs inactivation by DCCD with increase in detergent concentration are the following: first, at higher detergent concentrations, DCCD becomes more soluble in water and therefore reacts faster with water, thus decreasing the amount of DCCD available to protein; second, at higher detergent concentrations, the DCCD–carboxyl adduct is more easily hydrolyzed. The latter explanation is supported by the observation that when the Triton X-100 concentration of a DCCD-inactivated QPs (50%, in 0.1% Triton X-100, pH 7.0) is raised to 0.2%, inactivation is completely reversed. Moreover, the extent of DCCD inactivation that can be reversed by increasing Triton X-100 concentration is pH dependent. At pH 6.0, when the con-

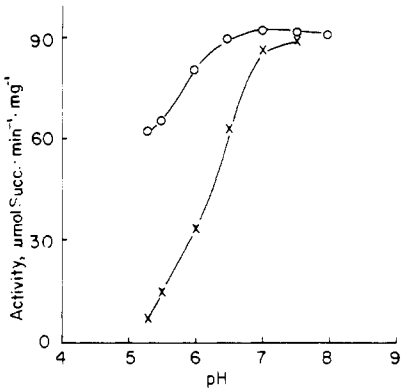

FIGURE 3: pH-dependent inactivation of QPs by DCCD. 0.2-mL aliquots of QPs, 0.17 mg/mL, in 50 mM Tris-HCl, pH 7.0, containing 10% glycerol were titrated either with 1 M Tris base or with 1 M MES to the indicated pH values. DMG was added to these samples to give the final concentrations of 0.2%. Samples were then incubated at room temperature for 5 min before being treated with 5 μL of 95% ethanolic solution with (×) or without (○) DCCD. The concentration of DCCD used was 150 mol of DCCD/mol of protein. The mixtures were further incubated at room temperature for 10 min, cooled to 0 °C, and then reconstituted with succinate dehydrogenase (5 μL, 20 mg/mL in 50 mM sodium/potassium phosphate buffer, pH 7.8, containing 20 mM succinate). Succinate–Q reductase activity was assayed 20 min after the reconstitution.

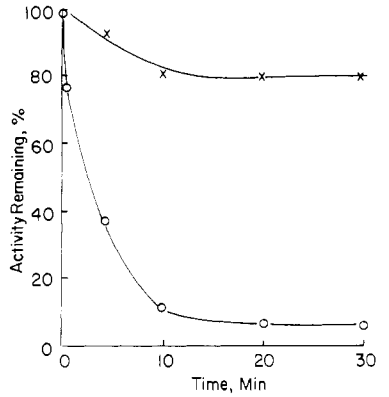

FIGURE 4: Time course of the inactivation of QPs by DCCD. To 2 mL of QPs solutions, 0.17 mg/mL, in 50 mM Tris/MES buffer, pH 6.0, containing 0.2% DMG, which have been incubated at room temperature for 5 min, were added 50 μL of absolute alcohol (×) and 50 μL of DCCD in absolute alcohol (○), respectively. The concentration of DCCD used was 300 mol/mol of QPs. The mixtures were kept at room temperature, and at indicated time intervals, 0.2-mL aliquots were withdrawn from each sample, cooled to 0 °C, and reconstituted with succinate dehydrogenase. 100% activity represents 100 μmol of succinate oxidized per minute per milligram of QPs at 23 °C.

centration of Triton X-100 is increased from 0.1% to 0.2%, only about 60% of the inactivation caused by DCCD is reversed. Although reversing DCCD inactivation by increasing detergent concentration is also observed with DMG, the effect is much smaller. At pH between 6 and 7, increasing the DMG concentration of DCCD-inactivated QPs (80%) from 0.2% to 1% reverses less than 10% of the DCCD inactivation. Thus, it is clear that DMG is the better detergent for studying DCCD inactivation of QPs because it gives a greater and more stable modification.

Effect of pH on the Inactivation of QPs by DCCD. Figure 3 shows the pH dependence of inactivation of QPs by DCCD. The optimal pH for the reconstitutive activity of QPs is between 7 and 8. Above pH 8.0 (data not shown) or below pH 7, the reconstitutive activity of QPs starts to decrease. When QPs, at various pHs, is treated with a 150 molar excess of DCCD before it is reconstituted with succinate dehydrogenase, the reconstituted succinate–Q reductase activity decreases as the pH of the solution decreases. The extent of QPs inactivation by DCCD, compared to corresponding untreated QPs samples, increases as the reacting pH decreases. This is similar to the reported observation that DCCD reacts with ATPase more effectively at acidic pH (Pougeois et al., 1979). Although the maximal inactivation of QPs by DCCD is at pH 5.3, a reacting pH of 6.0 is used in this investigation to avoid possible denaturation of succinate dehydrogenase at a pH below 6.0.

Effect of Incubation Time and Temperature on the Inactivation of QPs by DCCD. Figure 4 shows the time course for the inactivation of QPs by DCCD at room temperature and at 0 °C. A maximal inactivation of 90% (70%, compared to the corresponding untreated QPs, which suffers 20% inactivation under the same conditions) is obtained when QPs is incubated with a 300 molar excess of DCCD at room temperature (23 °C) for 10 min. When QPs is incubated with DCCD at 0 °C, a maximum inactivation of 20% is obtained (data not snown), indicating that DCCD inactivation of QPs is temperature dependent. Since QPs is reasonably stable at room temperature (only 20% loss in reconstitutive activity after 30 min, see Figure 4), the interaction between DCCD and QPs is routinely carried out at room temperature for 10 min. It should be noted that the 10-min incubation time for the optimal reaction of DCCD with QPs is shorter than that reported for the DCCD modification of yeast complex III (Beattie et al., 1984).

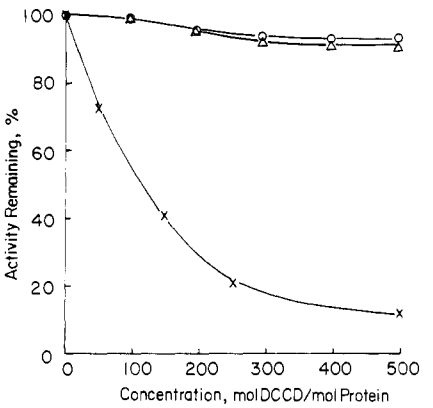

FIGURE 5: Effect of succinate dehydrogenase on the inactivation of QPs by DCCD. 0.2-mL aliquots of samples containing (1) a mixture of QPs (0.17 mg/mL) and reconstitutively inactive succinate dehydrogenase (0.5 mg/mL) (×), (2) a mixture of QPs (0.17 mg/mL) and reconstitutively active succinate dehydrogenase (0.5 mg/mL) (△), and (3) isolated succinate–Q reductase (0.5 mg/mL) (○) were mixed with 5 μL of absolute alcohol containing the indicated concentrations of DCCD. After incubation at room temperature for 10 min, samples were cooled to 0 °C, and reconstitutively active succinate dehydrogenase (5 μL, 20 mg/mL) was added. Succinate–Q reductase activity was assayed 20 min after the reconstitution. Reconstitutively inactive succinate dehydrogenase was prepared from active succinate dehydrogenase. Purified, active succinate dehydrogenase, 10 mg/mL, in 50 mM sodium/potassium phosphate buffer, pH 7.8, was incubated at 0 °C for 2 h. The sample was shaken occasionally during the incubation.

Effect of Succinate Dehydrogenase on the Inactivation of QPs by DCCD. The results described above clearly demonstrate that when QPs, dispersed in 50 mM MES, pH 6.0, containing 0.2% DMG, is treated with 300 molar excess DCCD at room temperature for 10 min, about 90% of its reconstitutive activity is lost. It is, however, not clear whether loss of reconstitutive activity results from denaturation of QPs by modifying carboxyl groups essential for catalytic activity of succinate–Q reductase or from modifying carboxyl groups essential for structural interaction with succinate dehydrogenase. One way to differentiate these two possibilities is to investigate the effect of DCCD on isolated, functionally

active succinate–Q reductase. When intact succinate–Q reductase was treated with 300 molar excess DCCD, no inactivation of succinate–Q reductase was observed (see Figure 5), suggesting that the inactivation of QPs by DCCD results from modification of carboxyl group(s) essential for interaction with succinate dehydrogenase. Such a carboxyl group is apparently covered by succinate dehydrogenase in intact succinate–Q reductase, thus inaccessible to DCCD. This deduction is further supported by the observation that inactivation of QPs by DCCD is prevented by the presence of reconstitutively active, but not reconstitutively inactive, succinate dehydrogenase.

When reconstituted succinate–Q reductase formed from reconstitutively *active* succinate dehydrogenase and QPs is treated with DCCD, no decrease in activity is observed (see Figure 5). However, when a mixture of reconstitutively *inactive* succinate dehydrogenase and QPs was treated with DCCD prior to the addition of reconstitutively *active* succinate dehydrogenase, only about 10% of the reconstituted succinate–Q reductase activity is observed. The extent of decrease in reconstituted succinate–Q reductase upon DCCD treatment of a mixture of QPs and reconstitutively inactive succinate dehydrogenase is the same as that of QPs treated with DCCD in the absence of succinate dehydrogenase, indicating that reconstitutively inactive succinate dehydrogenase does not protect QPs from DCCD modification. The loss of reconstitutive activity of QPs in a DCCD-treated mixture of QPs and inactive succinate dehydrogenase cannot be explained by assuming that the reconstitutively *inactive* dehydrogenase occupies the binding site of QPs, thus preventing reaction with active succinate dehydrogenase. The season is that, without DCCD treatment, a mixture of inactive succinate dehydrogenase and QPs shows the same reconstituted succinate–Q reductase, after the addition of active dehydrogenase, as that obtained from QPs and active succinate dehydrogenase alone. This result is in line with the previous finding (Yu & Yu, 1980c) that reconstitutively inactive succinate dehydrogenase does not form a complex with QPs. Therefore, the carboxyl group(s) in QPs which is (are) essential for reconstitution with succinate dehydrogenase is (are) not protected by inactive succinate dehydrogenase and is (are) vulnerable to DCCD treatment.

Incorporation and Distribution of DCCD in the Subunits of QPs. Figure 6 shows the correlation between the loss of reconstitutive activity of QPs and DCCD uptake by QPs. When QPs is incubated with various concentrations of [^{14}C]DCCD at room temperature for 10 min, the incorporation of DCCD into the QPs protein is directly proportional to the loss of reconstitutive activity of QPs, up to 200 molar excess of DCCD used. Beyond that point, further activity decrease is very small as is increased DCCD uptake. The break point for activity loss coincides with that for DCCD incorporation, suggesting that the specific carboxyl group(s) in QPs required for interaction with succinate dehydrogenase is (are) more DCCD reactive than other carboxyl groups in QPs.

The existence of nonessential (for binding) carboxyl groups in QPs is confirmed by the uptake of DCCD by intact or reconstituted succinate–Q reductase without impairment of activity. The amount of nonspecific DCCD uptake by QPs is calculated from the amount of DCCD uptake by reconstituted succinate–Q reductase less the amount of DCCD uptake by succinate dehydrogenase alone assuming no carboxyl group(s) in succinate dehydrogenase is (are) covered by QPs. The fact that the same amount of DCCD is incorporated into active and inactive succinate dehydrogenase and the reconstitutive activity of succinate dehydrogenase is insensitive to DCCD treatment indicate that the carboxyl groups of succinate dehydrogenase are not involved in the interaction with QPs. Assuming that the rate of DCCD modification of nonspecific carboxyl groups in QPs is roughly constant over concentrations of DCCD used, the number of carboxyl groups in QPs essential for interaction with succinate dehydrogenase to form succinate–Q reductase can be calculated from Figure 6 to be about two.

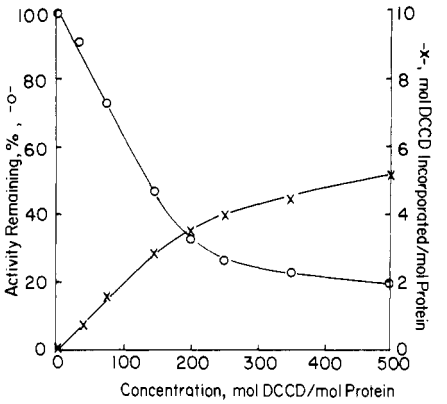

FIGURE 6: Correlation between DCCD incorporation and inactivation of QPs. To 0.2-mL aliquots of QPs, 0.18 mg/mL, in 50 mM Tris/MES, pH 6.0, containing 30 mM sucrose and 0.2% DMG was added 5 μL of absolute alcohol containing the indicated concentrations of [^{14}C]DCCD at room temperature. The specific radioactivity of DCCD used was 5000 cpm/nmol. After incubation at room temperature for 10 min, 0.1-mL aliquots were withdrawn from each tube, cooled to 0 °C, and reconstituted with succinate dehydrogenase. Succinate–Q reductase activity (O) was assayed after incubation at 0 °C for 20 min. For measurement of DCCD incorporation (×), 10-μL aliquots were withdrawn from samples and spotted on Whatman 3 paper. The conditions for the paper chromatography and the method for radioactivity determination were detailed under Experimental Procedures.

Since isolated QPs contains two protein subunits (M_r 15 000 and 13 000), it is of interest to know where the essential carboxyl groups are located. This can be determined by measuring the radioactivity distribution in the two subunits of QPs treated with various amounts of [^{14}C]DCCD followed by SDS–PAGE. Interestingly, when these [^{14}C]DCCD-treated QPs samples are subjected to SDS-PAGE, the intensity of the protein stain in the M_r 13 000 band decreases as the DCCD concentration used increases, while the intensity of the protein stain on the M_r 15 000 band remains unchanged. The decrease in the M_r 13 000 protein band is concurrent with the increase in the protein stain on the top of the gel, suggesting that the DCCD treatment causes the M_r 13 000 subunit of QPs to aggregate, thus remaining on the top of the gel under SDS–PAGE conditions. Table I shows the radioactivity distribution in the M_r 15 000 and M_r 13 000 subunits of QPs treated with DCCD. The amount of ^{14}C radioactivity uptake by the M_r 15 000 protein is rather small, indicating that modification of carboxyl groups in this protein does not cause inactivation of QPs. On the other hand, the amount of radioactivity in the top of the gel (aggregated form of the M_r 13 000 protein) increases as the DCCD concentration used increases, up to 200 mol of DCCD/mol of QPs. Beyond that, no further increase in the radioactivity in the aggregated form is observed. The rate of radioactivity uptake by the M_r 13 000 protein parallels that of QPs inactivation by DCCD, indicating that the essential carboxyl groups are located in the M_r 13 000 protein subunit.

Effect of DCCD on the Spectral Properties of Cytochrome b_{560}. From the radioactivity incorporation experiments, it is

Table I: Effect of DCCD Concentration on Radioactivity Distribution in Subunits of QPs[a]

DCCD used (mol/mol of QPs)	% inactivation	[^{14}C]DCCD uptake (cpm)	
		M_r 15 000	M_r 13 000[b]
0	0	0	0
132	49	50	420
176	61	70	650
350	75	100	750

[a] Aliquots (0.2 mL) of QPs, 0.12 mg/mL, in 50 mM Tris/MES buffer, pH 6.0, containing 0.2% DMG and 10% glycerol were added to 10 μL of absolute alcohol containing the indicated concentrations of [^{14}C]DCCD at room temperature. After incubation at room temperature for 10 min, 80-μL aliquots were withdrawn from each sample, cooled to 0 °C, reconstituted with 5 μL of succinate dehydrogenase (10 mg/mL), and assayed for succinate–Q reductase activity after the reconstituting mixtures were incubated at 0 °C for 20 min. Meanwhile, 100-μL aliquots were withdrawn from the [^{14}C]DCCD-treated samples, digested with 1% SDS and 1% β-mercaptoethanol at 37 °C for 2 h, and subjected to SDS–PAGE. After staining and destaining processes, the protein bands at M_r 15 000 and at the top of the gel (M_r 13 000) were sliced, and radioactivity was determined. [b] Protein band at the top of the gel.

Table II: Effect of DCCD Concentration of Cytochrome b_{560} Content

DCCD used (mol of DCCD/mol of QPs)	% activity remaining[a]	% cytochrome b_{560} remaining[b]
0	100	100
100	60	77
200	35	52
300	25	43

[a] 100% activity represents 90 μmol of succinate oxidized per minute per milligram of protein at 23 °C after reconstituting with excess succinate dehydrogenase. [b] Concentration of cytochrome b_{560} measurement is based on the Soret absorption of oxidized form QPs treated only with absolute ethanol, where 100% equals 20 nmol/mg of protein.

clear that the carboxyl groups in QPs that are essential for interaction with succinate dehydrogenase to form succinate–Q reductase are located on the M_r 13 000 subunit. This subunit of QPs has been reported to contain no prosthetic groups except Q while the M_r 15 000 protein subunit has been characterized as cytochrome b_{560}. The close relationship between the M_r 13 000 subunit and cytochrome b_{560} (M_r 15 000) of QPs is evident from the fact that all isolated QPs preparations having high reconstitutive activity contain both protein subunits and have a cytochrome *b* content of from 16 to 22 nmol/mg of protein. Although isolation of a M_r 13 000 protein practically devoid of cytochrome b_{560} has been reported (Yu & Yu, 1980b; Vinogradov et al., 1980), the preparation showed only partial reconstitutive activity (20%). No single peptide cytochrome b_{560} has yet been obtained. Of interest is whether or not modification of the essential carboxyl group in the M_r 13 000 protein affects the properties of cytochrome b_{560}. We find that the cytochrome b_{560} content (oxidized Soret band) in DCCD-treated QPs samples decreases as the amount of DCCD used increases (see Table II), indicating that DCCD treatment alters the heme environment of cytochrome b_{560} in QPs. We use the oxidized Soret absorption of QPs to indicate the cytochrome b_{560} content because cytochrome b_{560} is very unstable in the dithionite reduced state (Hatefi & Galenta, 1980; Yu et al., 1987).

Although the decreased cytochrome b_{560} content after DCCD treatment correlates well with QPs inactivation (see Table II), it is unlikely that this correlation is due to direct modification of cytochrome b_{560}. This is because DCCD incorporation into the M_r 15 000 protein is very small and independent of the extent of QPs inactivation.

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Registry No. Succinate dehydrogenase, 9002-02-2; succinate–ubiquinone reductase, 9028-11-9; cytochrome b_{560}, 37337-51-2.

REFERENCES

Ackrell, B. A. C., Kearney, E. B., & Coles, C. J. (1977) *J. Biol. Chem. 252*, 6963–6965.

Ackrell, B. A. C., Bethany-Ball, M., & Kearney, E. B. (1980) *J. Biol. Chem. 255*, 2716–2769.

Beattie, D. S., Cleijan, L., & Bosch, C. (1984) *J. Biol. Chem. 259*, 10526–10532.

Davis, K. A., & Hatefi, Y. (1971) *Biochemistry 10*, 2509–2516.

Hatefi, Y., & Galenta, Y. M. (1980) *J. Biol. Chem. 255*, 5530–5537.

Hildreth, J. E. K. (1982) *Biochem. J. 207*, 363–366.

Keilin, D., & King, T. E. (1958) *Nature (London) 181*, 1520–1522.

Marinetti, T. D., Okamura, M. Y., & Feher, G. (1979) *Biochemistry 18*, 3126–3133.

Pougeois, R., Statre, M., & Vignais, P. V. (1979) *Biochemistry 18*, 1408–1412.

Swank, R. T., & Munkres, K. D. (1971) *Anal. Biochem. 39*, 462–477.

Vinogradov, A. D., Gaurikov, V. G., & Gavrikova, E. V. (1980) *Biochim. Biophys. Acta 592*, 13–27.

Yu, C. A., & Yu, L. (1980a) *Biochim. Biophys. Acta 591*, 409–420.

Yu, C. A., & Yu, L. (1980b) *Biochemistry 19*, 3577–3585.

Yu, C. A., & Yu, L. (1980c) *Biochim. Biophys. Acta* 593, 24–35.

Yu, C. A., & Yu, L. (1982) *J. Biol. Chem. 257*, 2016–2021.

Yu, L., & Yu, C. A. (1981) *Biochim. Biophys. Acta 637*, 373–386.

Yu, L., Xu, J.-X., Haley, P. E., & Yu, C. A. (1987) *J. Biol. Chem. 262*, 1137–1143.

Ziegler, D. M., & Doeg, K. A. (1962) *Arch. Biochem. Biophys. 97*, 41–50.