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References

Bartholeyns, J., and Moore, S. (1974), *Science 186,* 444-445.
Crestfield, A. M., Stein, W. H., and Moore, S. (1962), *Arch. Biochem. Biophys. Suppl. 1,* 217.
D'Alessio, G., Floridi, A., De Prisco, R., Pignero, A., and Leone, E. (1972a), *Eur. J. Biochem. 26,* 153.
D'Alessio, G., Parente, A., Guida, C., and Leone, E. (1972b), *FEBS Lett. 27,* 285.
Di Donato, A., and D'Alessio, G. (1973), *Biochem. Biophys. Res. Commun. 55,* 919.
Dostál, J., and Matoušek, J. (1972), *J. Reprod. Fertil. 31,* 273-275.
Dunker, A. K., and Ruekert, R. R. (1969), *J. Biol. Chem. 244,* 5074.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70.
Floridi, A., D'Alessio, G., and Leone, E. (1972), *Eur. J. Biochem. 26,* 162.
Forlani, L., Chiancone, E., Vecchini, P., Floridi, A., D'Alessio, G., and Leone, E. (1967), *Biochim. Biophys. Acta 140,* 170.
Guy, O., Shapanka, R., and Greene, L. J. (1971), *J. Biol. Chem. 246,* 7740.
Kawahara, K., and Tanford, C. (1966), *J. Biol. Chem. 241,* 3228.
Leone, E., Greco, L., Rastogi, R. K., and Iela, L. (1973), *J. Reprod. Fertil. 34,* 197.
Leone, E., Suzuki, H., Greco, L., Parente, A., Farina, B., and La Montagna, R. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Proc. Meet. 8th,* Abstract 259.
Libonati, M., and Floridi, A. (1969), *Eur. J. Biochem. 8,* 81.
Malorni, M. C., Di Donato, A., and D'Alessio, G. (1972), *Boll. Soc. Ital. Biol. Sper. 48,* 606.
Matoušek, J. (1973), *Experientia 29,* 858.
Mills, J. N., and Tang, J. (1967), *J. Biol. Chem. 242,* 3093.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6,* 819.
Richards, F. M., and Wyckoff, H. W. (1971), *Enzymes, 3rd Ed., 4,* 647.
Schachman, H. K. (1957), *Methods Enzymol. 4,* 32.
Taniguchi, T., and Libonati, M. (1974), *Biochem. Biophys. Res. Commun. 58,* 280.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Zahler, W. L., and Cleland, W. W. (1968), *J. Biol. Chem. 243,* 716.

The Role of Protein and Lipids in Stabilizing the Activity of Bovine Heart Succinate Dehydrogenase†

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ABSTRACT: When incubated in an air atmosphere, solubilized succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) quickly loses the capability to recombine with membrane components to catalyze mitochondrial related electron transport activities. At 0° the loss in reconstitution capability is a first-order process; the half-life of the enzyme is 1.6 hr at this temperature. The enzyme is stabilized by recombining it with submitochondrial particles or with a cytochrome *b* preparation-phospholipid mixture. The presence of the cytochrome *b* preparation in the succinate dehydrogenase-cytochrome *b*-phospholipid complex is obligatory, indicating that protein-protein interactions between succinate dehydrogenase and other membrane components are important in stabilizing the capability of the flavoprotein to transfer electrons to other respiratory components. Treatment of this complex with phospholipase C results in loss of most of the succinate-dichlorophenolindophenol reductase activity and almost complete hydrolysis of phospholipid. Succinate dehydrogenase maintains its capability to participate in mitochondrial electron transport for several hours if the phospholipase treated complex is reconstituted with lysolecithin at the time of assay. Phospholipids are therefore not required for the stabilization process, but rather for formation of an active reductase complex. A lipophilic environment, if required for stabilization, can be provided by diglycerides. Diglycerides also can provide an environment conducive to electron transfer from succinate to ubiquinone but do so less efficiently than intact phospholipids.

The properties of membrane associated enzymes are controlled or modified by interaction with other components of the membrane. A notable example is the mitochondrial ATPase which requires phospholipid for activity and is oligomycin sensitive when associated with the membrane (Racker, 1963; Kagawa and Racker, 1966). This enzyme when solubilized from the membrane is not inhibited by oligomycin and does not require phospholipid for activity (Pullman et al., 1960). The importance of both protein-protein and protein-lipid interactions in conferring the mem-

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brane associated properties to this enzyme has been demonstrated by several studies (Kagawa and Racker, 1966; Bulos and Racker, 1968a,b; Knowles et al., 1971; Cunningham and George, 1975). The observations with this enzyme emphasize that a more complete understanding of membrane function requires a knowledge of the influence of protein–protein and protein–lipid interactions on the properties of membrane components.

Another membrane associated protein which is well suited for a study of protein–protein and protein–lipid interactions in membranes is succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC 1.3.99.1). This enzyme has been isolated and purified from the mitochondrial membrane by several procedures and has different properties depending on the method of preparation (Singer et al., 1973). The flavoprotein, solubilized and purified by the method of King (1963) in the presence of succinate and absence of oxygen, can be recombined with alkali-treated submitochondrial particles or other membrane components to yield either succinate ubiquinone reductase (Bruni and Racker, 1968) or succinate oxidase (King, 1963; Yamashita and Racker, 1969) activities. The capability of this enzyme to combine with the membrane components and catalyze respiratory chain activities has been termed the reconstitution property of succinate dehydrogenase (King, 1963). The solubilized enzyme loses its ability very quickly to participate in reconstituted mitochondrial electron transport systems, whereas the mitochondrial associated enzyme maintains its biological viability indefinitely. These observations indicate that the stability of succinate dehydrogenase is a membrane conferred property, but do not indicate the relative contributions of protein–protein and protein–lipid interactions in stabilizing the enzyme. In this paper we have investigated the participation of protein and lipids in stabilizing the reconstitution activity of succinate dehydrogenase. Furthermore, the phospholipid requirement for transfer of electrons from succinate dehydrogenase to its membrane associated acceptor has been reevaluated.

Experimental Procedure

Materials. Sodium dodecyl sulfate, cholic acid, and deoxycholic acid were obtained from Matheson Coleman and Bell and were recrystallized from 95, 70, and 70% ethanol, respectively. Sigma supplied cytochrome *c* (type II), UQ_{10},[1] *Clostridium welchii* phospholipase C (type I), egg lecithin (type III), egg lysolecithin (grade II), DCPIP,[1] and antimycin A. Fisher Scientific furnished TTB.[1] Crystalline bovine serum albumin was obtained from Pentex Chemicals, asolectin from Associated Concentrates, Inc., and sodium succinate from Boehringer Mannheim. A sample of 2-[1-^{14}C]linoleoylphosphatidylcholine was kindly supplied by Dr. Moseley Waite. All other chemicals used were of reagent grade.

Analytical Methods. Protein concentrations of mitochondria and alkali-treated submitochondrial particles were measured by the biuret determination (Gornall et al., 1949) in the presence of 0.3% sodium deoxycholate. The protein contents of cytochrome *b* and succinate dehydrogenase preparations, and the reconstituted complexes, were measured by the method of Lowry et al. (1951) in the presence of 0.17% sodium deoxycholate. For quantitative determination of lipid phosphorus the method of Chalvardjian and Rudnicki (1970) was utilized. Flavine analyses were carried out by the method of Cerletti et al. (1963).

Preparations. Liposomes of asolectin were prepared by the method of Conover et al. (1963) with the following modifications. Monothioglycerol was left out of the liposome buffer and sonication was performed with a Heat Systems Model W-185E sonifier at a power output of 80 W under an argon atmosphere. The resulting liposomes were stored at 4° under argon at a concentration of 80–100 mg of phospholipid/ml. Liposomes of lecithin were prepared as follows. The hexane present in 500 mg of egg lecithin was removed under a stream of argon. The lecithin was redissolved in 5 ml of ether which was then evaporated under a stream of argon. The last step was repeated. The solid lecithin was then suspended in 5 ml of 0.25 *M* sucrose–0.01 *M* Tris-SO_4–0.5 m*M* EDTA (pH 7.4) and sonicated for 20 min with the sonifier at a power output of 50 W and the sample maintained at 10° in a salt–ice bath under an argon atmosphere. The resulting liposomes were dialyzed overnight at 4° against the suspension buffer and centrifuged at 21,000*g* for 10 min to remove particulate material. The resulting liposomes (80 mg of phospholipid/ml) were stored at 4° under argon. Liposomes of radioactive lecithin were prepared from 50 mg of egg lecithin and 2 mg of 2-[1-^{14}C]linoleoyllecithin (specific activity, 2.8×10^6 cpm/μmol) in a volume of 2 ml by the procedure outlined above for lecithin liposomes with the following modifications. EDTA was omitted from the suspension buffer, sonication was carried out for 30 min at 0°, and the dialysis step was omitted. The sonicated material was centrifuged for 5 min at 2000*g* to remove particulate material and then used immediately.

Beef heart mitochondria were prepared by the procedure of Blair (1967) as described earlier (Cunningham and George, 1975). Succinate dehydrogenase was prepared by the method of King (1963) as modified by Bruni and Racker (1968). Argon was used to exclude oxygen during the preparation and 1 m*M* dithiothreitol was included in elution and storage buffers for the enzyme. The fraction that sediments between 0.45 and 0.65 saturation with respect to ammonium sulfate in the final step of purification was utilized in these studies. This preparation was stable for several months if stored in liquid N_2.

The method of Rieske and Tisdale (1967) as modified by Bruni and Racker (1968) was utilized to prepare a cytochrome *b* enriched fraction. The last ammonium sulfate fractionation step was omitted to minimize loss of cytochrome *b*. The heme content of these preparations ranged from 4 to 11 nmol/mg, corresponding to purities from 11 to 39% assuming the Rieske and Tisdale (1967) preparation is pure. No cytochrome c_1 was detectable in the dithionite reduced minus ferricyanide oxidized difference spectrum of this preparation. This fraction had a phosphorus content of 11 nmol/mg of protein, indicating very low residual amounts of phospholipid. Ubiquinone analysis carried out by the following procedure indicated the presence of a small amount of the quinone. The cyt *b* was extracted by the method of Redfearn (1967) to remove residual ubiquinone. The extract was dissolved in a small volume of ethanol and applied to a thin-layer plate which was then developed in petroleum ether (30–60°)–ethyl ether–formic acid (80:20:

[1] The abbreviations used are: UQ_{10}, ubiquinone with ten isoprenoid units; DCPIP, 2,6-dichlorophenolindophenol; TTB, 4,4,4-trifluoro-1-(2-thienyl-1,3-butanedione; SDH, succinate dehydrogenase; cyt *b*, a mitochondrial preparation enriched with respect to cytochrome *b*; PLC, phospholipase C from *Clostridium welchii*; BSA, bovine serum albumin.

1.5). The plate was allowed to dry and then developed in benzene–chloroform (1:1). The spots were located in iodine vapor. The identity of ubiquinone was verified first by comparing its R_f to that of an authentic standard developed on the same thin-layer plate, and secondly, by an oxidized–$NaBH_4$ reduced spectrum of the material extracted from the thin-layer plate. The relatively low level of the ubiquinone precluded measuring its concentration in the cytochrome *b* preparations. For the sake of brevity the cytochrome *b* enriched fraction will be referred to as cyt *b* in this paper.[2]

The submitochondrial particles in which succinate dehydrogenase was inactivated were prepared from mitochondria by the procedure outlined by King (1967). This preparation will be referred to as alkali-treated particles in this paper. Defatted bovine serum albumin was prepared by the method of Arion and Racker (1970). The Sigma preparation of *Cl. welchii* phospholipase C was further purified as described by Cunningham and George (1975). The purified enzyme had no EDTA insensitive egg yolk coagulation activity when assayed by the procedure of Kushner (1957).

ASSAY FOR SUCCINATE DEHYDROGENASE ACTIVITY. Succinate dehydrogenase activity was measured by its ability to recombine with alkali-treated particles to catalyze succinate driven respiration. The assay utilized is a modification of the procedure described by King (1967). Activity was measured at 30° with an oxygen electrode in an assay medium containing 40 m*M* NaP_i (pH 7.4), 20 m*M* sodium succinate, 1 m*M* EDTA, 1.5 mg of cytochrome *c*, and 2 mg of alkali-treated particles in a final volume of 1.8 ml. The assay was initiated by addition to the assay mix an amount of SDH that catalyzed the uptake of 1–10 μg-atoms of oxygen per minute. This assay is designated the succinoxidase assay. Deviations from the above procedure will be noted in the figure legends.

The activities of complexes containing SDH, cyt *b*, and phospholipid were measured by the succinate–DCPIP reductase assay described by Bruni and Racker (1968). In this assay ubiquinone mediates the transfer of electrons from succinate dehydrogenase to DCPIP.

Procedures. FORMATION OF SDH–CYTOCHROME *b*–PHOSPHOLIPID COMPLEXES. These complexes were prepared as described by Bruni and Racker (1968). The preparation consisted essentially of incubating SDH, cyt *b*, and phospholipid together and then washing the resulting particulate complex to remove any material that did not incorporate into the complex. In experiments involving phospholipase C treatment of the complex, several modifications were necessary. The EDTA was eliminated from all the buffers to prevent inhibition of the phospholipase. In the wash procedure the first resuspension buffer was changed to 0.1 *M* Tris-maleate–20 m*M* sodium succinate (pH 6.5) and the second buffer to 20 m*M* sodium succinate–0.05 *M* Tris-Cl (pH 7.4). The pH was changed in the final resuspension buffer to the value for optimal activity of phospholipase C. Furthermore, these changes eliminated phosphate from the complex medium which interfered with phosphorus determinations made on the complexes. These changes resulted in only a slight decrease in the succinate–DCPIP reductase activity of the complexes.

PHOSPHOLIPASE C TREATMENT OF THE SDH–CYTOCHROME *b*–PHOSPHOLIPID COMPLEXES. Phospholipase C was added to the complex at a concentration of 120 μg/mg of protein. Calcium chloride was added at a level of 5 m*M* and incubation was carried out at 23° for 90 min. At that time 0.2 mg of defatted BSA/mg of complex was added and the mixture was centrifuged 10 min at 30,000 rpm in a Beckman 40 rotor. The pellet was resuspended with 20 m*M* succinate–0.05 *M* Tris-Cl (pH 7.4) in a volume equal to that of the complex before centrifugation. In some experiments aliquots of the treated complex were removed for lipid phosphorus analysis and SDH–DCPIP reductase activity measurements at times during the incubation. In these samples EDTA was added to a final concentration of 3.3 m*M* to inactivate phospholipase C. Before phosphorus analyses were performed the aliquots were washed as described above. In each experiment, a portion of the complex was subjected to the same procedures as the phospholipase C treated sample except 50% glycerol was added to the incubation mixture in place of phospholipase C.

In one experiment the complex was prepared with liposomes containing 2-[1-^{14}C]linoleoyllecithin. After phospholipase treatment the lipids remaining after the wash procedure were extracted from both the phospholipase C treated and the untreated, washed samples by the method of Newkirk and Waite (1971) and analyzed by their procedure to determine the conversion of lecithin to diglyceride by phospholipase treatment.

Results

SDH plus Alkali-Treated Particles. Succinate dehydrogenase quickly loses its ability to recombine with alkali-treated particles if incubated in an air atmosphere. This observation was made earlier by King (1963) and is reaffirmed in this study. As shown in Figure 1 the reconstitution activity of SDH decreases to about 10% of its original activity within 6 hr if the enzyme is incubated alone at 0°. Under these conditions the activity drop is first order and the half-life of the reconstitution activity is 1.6 hr. In contrast, the capability of SDH to participate in the reconstituted succinoxidase system is maintained for several hours if the solubilized enzyme is recombined with alkali-treated particles. As shown in Figure 1, succinoxidase activity increases about 10% and then drops slightly over a period of several hours when SDH is incubated at 0° in combination with alkali-treated particles. These observations confirm that the membrane or components therein stabilize the form of SDH that participates in mitochondrial associated electron transport reactions.

SDH plus Phospholipid Alone. Upon demonstrating the capability of alkali-treated particles to stabilize the reconstitution activity of SDH, studies were carried out to determine which components of the membrane were responsible for the stabilizing effect seen with the particles. In these experiments we have attempted to separate the relative contributions of protein–protein and protein–lipid interactions in stabilizing the flavoprotein. The investigations of previous workers (Bruni and Racker, 1968; Cerletti et al., 1970) suggested that phospholipids might stabilize SDH. Accordingly, the flavoprotein was incubated with phospholipids and assayed at intervals for its ability to catalyze succinoxidase activity in combination with alkali-treated particles. The phosphatides used in this experiment were not obtained from mitochondria but are representative of the phospholip-

[2] For purposes of brevity the term cyt *b* is used in the text, but the authors acknowledge that the protein–protein associations occurring with SDH could be with proteins other than cytochrome *b* in the cytochrome *b* enriched fraction.

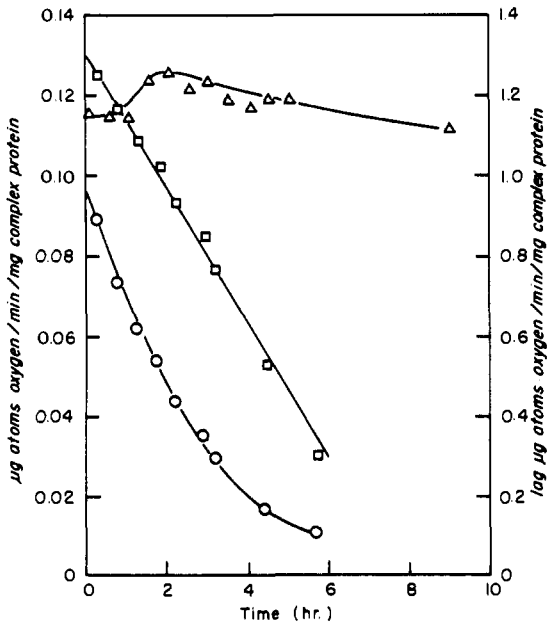

FIGURE 1: Activity of SDH in the absence and presence of alkali-treated particles. SDH was incubated at 0° in the absence of any membrane particles (○). At the time designated 0.036-mg aliquots of the enzyme were assayed by the succinoxidase assay. The log of activity loss in the absence of particles is indicated by (□). (△) indicates the activity of SDH in the presence of alkali-treated particles with time. In this part of the experiment a mixture containing 1.5 ml of alkali-treated particles (20 mg/ml), 1.5 ml of a buffer containing 1 m*M* sodium succinate, 40 m*M* NaP_i (pH 7.4), 1 m*M* EDTA, and 1.1 mg of SDH was allowed to incubate 5 min at room temperature and then centrifuged for 45 min at 105,000*g* and resuspended in 2 ml of the above buffer. The sample was centrifuged at 105,000*g* for 20 min, resuspended as before, and centrifuged again. It was then resuspended in a volume of 1.5 ml with the above buffer. Aliquots of the washed complex (1.4 mg of protein) were assayed by the succinoxidase assay, but with no additional alkali-treated particles. The specific activities reported for both portions of the experiment are in terms of total protein comprised of both SDH and alkali-treated particles.

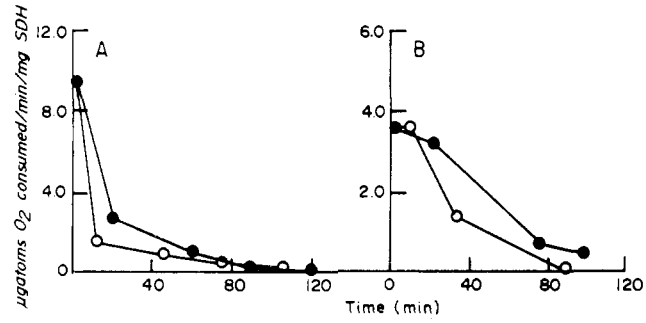

FIGURE 2: Stability of SDH in the presence of phospholipids only. (A) Asolectin liposomes were incubated with SDH at 30° at a concentration of 7 mg of lipid/mg of SDH. As a control, soluble SDH was incubated in a volume of SDH storage buffer (see Experimental Procedure) equal to the volume of phospholipid added in the above sample. At the times indicated 0.015 mg of SDH in the presence or absence of asolectin was assayed by the succinoxidase assay. (B) Lecithin liposomes were incubated with SDH in a ratio of 4.8 mg/mg of SDH at 22°. At the times indicated aliquots containing 0.05 mg of SDH were assayed by the succinoxidase assay. The lecithin-SDH ratio is the same as that in the SDH-cyt *b*-lecithin complex described later: (●) SDH incubated in the absence of phospholipid; (○) SDH incubated with phospholipid.

ids known to exist in the mitochondrial membrane. The results shown in Figure 2 indicate that phospholipid has no effect on the stability of the reconstitution activity of SDH. In fact, in the presence of either a phospholipid mixture (asolectin, part A) or lecithin (part B) the reconstitution activity of the enzyme appears to decay slightly more rapidly than if the flavoprotein is incubated in the absence of lipid. Furthermore, mixtures of phospholipid and ubiquinone exerted no stabilizing effect on the reconstitution activity of SDH (data not shown), demonstrating that the quinone did not exert a protective effect on the flavoprotein. These results indicate that protein-lipid interactions alone are not sufficient to confer the membrane related property of stability to SDH.

SDH plus the Cytochrome b Enriched Fraction Alone. Several studies (Ziegler and Doeg, 1962; Tisdale et al., 1963; Bruni and Racker, 1968; Davis and Hatefi, 1971) have suggested an interaction between SDH and cytochrome *b* in the succinate-ubiquinone reductase segment of the electron transport chain. The observations of these investigators suggested the possibility that cytochrome *b* might stabilize the reconstitution activity of SDH. When SDH was mixed with cyt *b*,[2] however, a precipitate formed immediately which had no observable activity in the succinoxidase assay. The loss in reconstitution activity of SDH occurred immediately and suggested that the cyt *b* bound SDH in a manner such that the enzyme could no longer reconstitute with alkali-treated particles. Attempts to separate the SDH from cyt *b* in an active form were unsuccessful using solubilizing systems such as 1-butanol (alkaline pH), cholate-ammonium sulfate, urea, guanidine-HCl, and sodium dodecyl sulfate, suggesting that SDH was bound very tightly to the cyt *b* fraction.

SDH plus Combinations of Phospholipid and Cytochrome b. Since no succinoxidase activity was detected when SDH-cyt *b* mixtures were recombined with alkali-treated particles, another assay system was employed to assess the effects of cyt *b* on the reconstitution activity of SDH. Bruni and Racker (1968) had previously demonstrated that a reconstituted complex containing SDH, cytochrome *b*, and phospholipid catalyzed a uniquinone-dependent succinate-DCPIP reductase activity which was sensitive to the iron chelator, TTB. Presumably the electrons are being transferred from SDH to ubiquinone and then to DCPIP in this system. This activity is distinguishable from a diaphorase (succinate-DCPIP reductase or succinate phenazine methosulfate reductase) activity which is not sensitive to TTB and which only requires the presence of SDH for activity (King, 1963). Moreover, in contrast to the succinoxidase assay, the alkali-treated particles are not required. We have employed this reconstituted complex and accompanying assay to assess the effectiveness of combinations of cyt *b* and phospholipid in stabilizing the reconstitution activity of SDH. As demonstrated in Figure 3, the succinate-DCPIP reductase activity of a complex containing SDH, cyt *b*, and asolectin is stable for several hours. In this experiment SDH was incubated either complexed with cyt *b* and phospholipid or in their absence. At times during the experiment the SDH being incubated alone was combined with cyt *b* and asolectin to form a succinate-DCPIP reductase complex and then assayed. The times indicated on the figure represent the time of incubation alone plus the time required for preparation of the complex. Therefore, a 4-hr point for SDH incubated in the absence of other membrane components also includes the time required for complex preparation (approximately 2 hr). The enzyme in the presence of cyt *b* and asolectin was much more stable than when incubated alone. As shown in the figure, SDH incubated alone before being added to cyt *b* and phospholipid

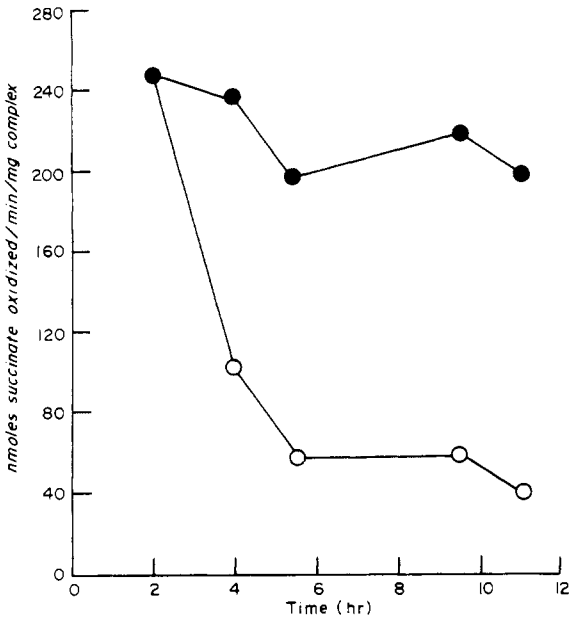

FIGURE 3: Stability of SDH in the SDH-cyt *b*-asolectin complex. SDH was incubated at 0° either in the absence of membrane components or complexed with cyt *b* and asolectin. The complex was prepared as described in the Experimental Procedure section with 1.15 mg of cyt *b*, 3.6 mg of asolectin, and 0.2 mg of SDH. The 6 to 1 ratio of cyt *b* to SDH was used instead of Bruni's 2 to 1 because of the less pure cyt *b* fractions utilized in the experiments. To measure the activity of SDH incubated alone a 0.04-mg aliquot of the enzyme was added to 0.23 mg of cyt *b* and 0.72 mg of asolectin with complex formation being achieved as described in the Experimental Procedure section. A 0.047-mg aliquot of the initial complex or the complex prepared with aged SDH was assayed by the succinate-DCPIP reductase assay: (○) activity of the aged SDH; (●) activity of the SDH initially complexed with cyt *b* and asolectin. The 2-hr lag before the first point represents the time lapse between removing SDH from liquid N_2 and the first assay of the reconstituted complex. The time indicated in the abscissa is time of incubation of SDH and represents both the time of incubation at 0° and the time for preparation of the complex.

quickly lost the capability to recombine to form an active succinate-DCPIP reductase complex. These observations indicate that the capability of SDH to participate in the reconstituted succinate-ubiquinone segment of the electron transport chain is stabilized in the presence of cyt *b* and phospholipid. Furthermore, the reconstituted succinate-DCPIP reductase system provides a convenient assay for the reconstitution activity and also more clearly defines the components involved in stabilizing SDH. In addition, these results suggest that the immediate loss in succinoxidase activity seen with SDH-cyt *b* mixtures is not due to denaturation of the flavoprotein because under these latter conditions SDH is stable for several hours in the succinate-DCPIP reductase where it is associated with the cytochrome *b* enriched fraction.

Characteristics of the Reconstituted Succinate-DCPIP Reductase Complex. The complex described above which stabilizes the activity of succinate dehydrogenase is quite similar to the succinate-ubiquinone reductase complex described by Bruni and Racker (1968). The succinate-DCPIP reductase activity of the complex described here is not strictly dependent on the addition of exogenous quinone. With complexes reconstituted with some freshly prepared samples of cyt *b*, ubiquinone was required for TTB sensitive succinate-DCPIP reductase activity (Table I, preparation 1). In complexes made with other preparations, however, the quinone was not required (Table I, preparation 2). Moreover, the age of the cyt *b* preparation appears to affect whether ubiquinone is required for stimulation of activity of the succinate-DCPIP reductase complexes. Complexes prepared with preparation 1 required ubiquinone when the cyt *b* was freshly prepared. However, within 36 days the activity in the absence of added ubiquinone (ethanol added) had risen to that seen in the presence of exogenous quinone. If no dependence on ubiquinone was noted in complexes prepared with fresh cyt *b* preparations, aging the cyt *b* had no effect on the dependence for ubiquinone. This is seen with preparation 2 in which there was no requirement for added quinone either at 2 days or 5 months. The results in Table I suggest that the succinate-DCPIP reductase activity of the complex in the absence of added ubiquinone is probably due to small amounts of endogenous quinone remaining in the cyt *b* preparations. Analysis for ubiquinone, described in the Experimental Procedure section, established that, indeed, small amounts of the quinone are present which could presumably participate in electron transfer from SDH to DCPIP. It is possible that with some preparations the endogenous ubiquinone is not accessible until cyt *b* is stored for several days. Succinate-DCPIP reductase activity of complexes prepared from those preparations would be dependent on added ubiquinone until the endogenous ubiquinone was rendered more accessible through the changes occurring during storage. In any event, the data in Table I support the supposition that the activity measured is DCPIP reduction mediated by ubiquinone.

Further evidence which strongly supports the idea that the succinate-DCPIP reductase complex is a reconstituted succinate-ubiquinone reductase complex comes from the observation that it will combine with complex III (reduced ubiquinone-cytochrome *c* reductase) to catalyze a TTB and antimycin A sensitive succinate-cyt *c* reductase activity (Cunningham and Spach, unpublished results). Moreover, the reconstituted complex is very sensitive to TTB. Addition of 2.5 μM TTB results in 50% inhibition of the reductase activity. This value is actually lower than the 10 μM TTB required for 50% inhibition of succinate-coenzyme Q reductase in mitochondria (Tappel, 1960). We have routinely utilized the higher concentrations of TTB used by Bruni and Racker (1968) in their study. The lower inhibition by TTB when UQ_{10} was added (Table I) has been noted previously by Takemori and King (1964). The UQ_{10} in the concentrations utilized apparently interferes with TTB in this system.

The data in Table I also demonstrate that the activity of a SDH-cyt *b*-phospholipid complex reconstituted with lecithin is only slightly lower than that obtained with asolectin, demonstrating that purified phosphatides suffice for the phospholipid requirement. Furthermore, the succinate-DCPIP reductase activity is relatively insensitive to inhibition with antimycin A. Inhibiton from 16 to 30% was obtained with antimycin at levels of 240 μg/mg of protein. In contrast, antimycin A at levels of 0.05 μg/mg of protein inhibited reconstituted succinoxidase and NADH oxidase activities 100 and 93%, respectively (Nishibayashi-Yamashita et al., 1972). These observations suggest that the cytochrome *b*-cytochrome c_1 segment of the electron transport chain is not involved in the succinate-DCPIP reductase activity measured in these experiments.

A representative succinate-DCPIP reductase complex contains 0.55 nmol of flavine and 4.15 nmol of cytochrome *b* per mg of protein, corresponding to a molar ratio of SDH to cytochrome *b* of 1:6.7. Complexes prepared with asolec-

Table I: Characteristics of the Succinate—DCPIP Reductase Activity of the Cyt *b*—SDH—Phospholipid Complex.[a]

Cyt *b*, Age	Lipid	Additions[b]	nmol of Succinate Oxidized/min per mg of Protein: TTB Absent	nmol of Succinate Oxidized/min per mg of Protein: 0.5 m*M* TTB	Fold Stimulation by UQ_{10}	% Inhibition by TTB
			Preparation 1			
3 days	Asolectin	Ethanol	49	5		90
3 days	Asolectin	UQ_{10}	162	59	3.3	64
36 days	Asolectin	Ethanol	208	18		91
36 days	Asolectin	UQ_{10}	222	54	1.1	76
36 days	Lecithin	Ethanol	186	4		98
36 days	Lecithin	UQ_{10}	180	29	0.97	84
			Preparation 2			
2 days	Asolectin	None	185	15		92
2 days	Asolectin	Ethanol	143	19		87
2 days	Asolectin	UQ_{10}	161	62	0.87, 1.1[c]	61
5 months	Asolectin	None	230	33		86
5 months	Asolectin	Ethanol	154	15		90
5 months	Asolectin	UQ_{10}	232	118	1.01, 1.51[c]	49

[a] The complexes containing either asolectin or lecithin were prepared as described for Figure 3 and in the Experimental Procedure section. Aliquots (0.04 mg) were assayed by the succinate—DCPIP reductase assay. [b] UQ_{10} was added to a final concentration of 30 μM from an ethanolic solution. Ethanol was added in a volume equal to that of the UQ_{10} (50 μl). [c] The first number compares the value obtained in the presence of UQ_{10} to that obtained when nothing is added. The second refers to the UQ_{10} values as compared to the activity in the presence of ethanol.

tin contain an average of 1.7 mg of phospholipid/mg of protein and those prepared with lecithin contain 0.8 mg of phosphatide/mg of protein.

Effect of BSA on SDH. The following experiment suggests that SDH is somewhat specific for the proteins that participate in stabilizing its reconstitution activity. As shown in Figure 4A the ability of SDH to reconstitute with alkali-treated particles is not stabilized in the presence of BSA which is known to bind with hydrophobic moieties such as lipids or aromatic dyes. Furthermore, as shown in Figure 4B, BSA–phospholipid mixtures would not maintain the property of SDH to combine with cyt *b* to form the succinate-DCPIP reductase activity. In contrast to the effects noted when cyt *b* alone was added, the reconstitution activity in the presence of BSA did not drop immediately as shown in both the succinoxidase and succinate-DCPIP reductase experiments. This observation further indicates that the interaction of SDH with membrane proteins is more specific than that with proteins such as BSA that interact with hydrophobic ligands.

Phospholipase C Treatment of the Succinate-DCPIP Reductase Complex. The experiments described thus far are consistent with the idea that both mitochondrial proteins and phospholipids are required for stabilizing the reconstitution activity of SDH. In order to assess the relative contributions of protein and phospholipid in stabilization of the flavoprotein, experiments were carried out to alter the lipid complement of the complex once it was formed from SDH, cyt *b*, and phospholipid. Phospholipase C was utilized in these experiments to hydrolyze the phospholipids incorporated into the succinate-DCPIP reductase complex. Initially several experiments were carried out to establish optimal conditions for hydrolyzing the phosphatides within the complex in which both phospholipid phosphorus release and loss in succinate-DCPIP reductase activity were followed. The conditions established as optimal are described in the Experimental Procedure section and are comprised of a purified phospholipase C treatment of a complex reconstituted with lecithin. The kinetics of phospholipase C treatment of the reconstituted complex are shown in Figure 5. In this

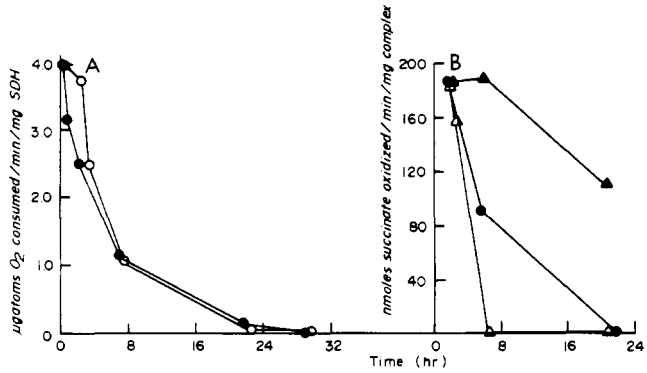

FIGURE 4: Effects of BSA on the stability of SDH. All incubations of SDH, in the presence or absence of other components, were carried out at 0°. (A) Succinoxidase activity: SDH (0.2 mg) was incubated alone or with 1.15 mg of BSA; succinoxidase activity was measured on aliquots containing 0.034 mg of SDH protein; (●) SDH incubated alone; (○) SDH incubated with BSA. (B) Succinate-DCPIP reductase activity: SDH was incubated separately, complexed with cyt *b*-asolectin, or complexed with BSA-asolectin. The cyt *b*-SDH-asolectin complex was formed using 2.3 mg of cyt *b*, 0.4 mg of SDH, and 7.2 mg of asolectin. In the incubation of SDH with BSA-asolectin, 0.4 mg of SDH was mixed with 2.3 mg of BSA and 7.2 mg of asolectin. To assay the SDH incubated alone, a 0.08-mg aliquot was first complexed with 0.46 mg of cyt *b* and 1.44 mg of asolectin. To assay the SDH-BSA-asolectin complex, an aliquot was first complexed with cyt *b* at a level such that the SDH:cyt *b*:asolectin ratio was the same as that in the control SDH-cyt *b*-asolectin complex. After addition of SDH or SDH-BSA-asolectin to cyt *b* at the times indicated the normal procedure for complex formation and assay for succinate-DCPIP reductase activity was followed as described in the Experimental Procedure section. All complexes were assayed using 0.075 mg of complex protein: (●) SDH incubated alone; (▲) SDH incubated with cyt *b*-asolectin; (△) SDH incubated with BSA-asolectin.

particular experiment phospholipid hydrolysis was essentially completed within the first hour. Activity loss parallels loss in phospholipid phosphorus which substantiates that the decrease in activity occurs due to the hydrolysis of phospholipid in the complex. After terminating phospholipase C activity (90 min) further loss in succinate-DCPIP reduc-

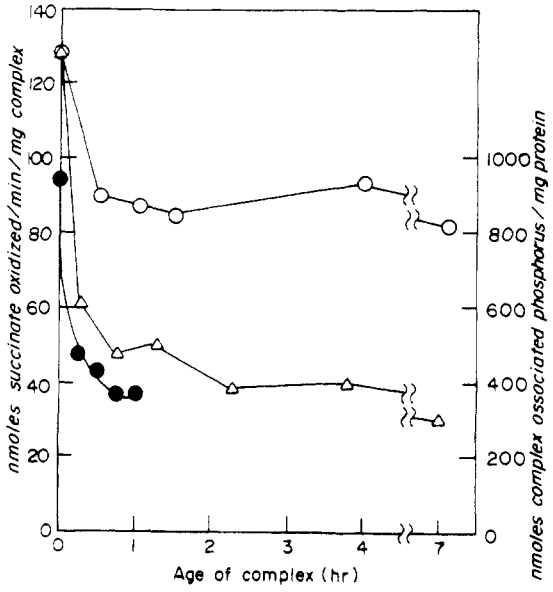

FIGURE 5: Activity and lipid phosphorus content of untreated and phospholipase C treated complexes vs. age of the complex. Complex formation was carried out as described in the Experimental Procedure section. The complex was divided, with one portion treated with phospholipase C as outlined in the Experimental Procedure section. At 15-min intervals during the first hour of phospholipase incubation, an aliquot of the treated complex was removed and EDTA added to stop the action of phospholipase C. These aliquots were washed as described in the Experimental Procedures section and assayed for their lipid phosphorus content. The DCPIP reductase activities measured early were obtained from aliquots of the control and phospholipase C treated sample. At 90 min the activity of phospholipase C was terminated by addition of EDTA. Both the untreated and phospholipase C treated samples were washed above and utilized for further assays of succinate-DCPIP reductase activity: (○) activity of the untreated complex; (△) activity of the PLC-treated complex; (●) lipid phosphorus content of washed aliquots of the treated complex.

Table II: Reconstitution of Succinate—DCPIP Reductase Activity to PLC Treated with Lysolecithin.[a]

Sample No.	Content	nmol of Succinate Oxidized/min per mg of Protein	nmol of Complex Phosphorus/mg of Protein
1	Control	129	1070
2	Control + 4 hr aging	93	1070
3	Control + 7 hr aging	82	1070
4	PLC treated + 7 hr aging	29	365
5	PLC treated + 7 hr aging + lysolecithin	139	
6	No. 5 washed	82	1500

[a] Formation of the complex using lecithin, its assay, phospholipase C treatment, and lipid phosphorus analysis were carried out as outlined in the Experimental Procedure section. In this experiment the complex was split into 2 parts, one of which was treated with phospholipase C. After washing both the control and treated samples to remove phosphorus and PLC the complexes were assayed at the times indicated, which are the times from addition of PLC to the complex. After aging the PLC treated sample at 23° for 7 hr, an aliquot was incubated with lysolecithin at a level of 1 mg/mg of complex protein for 5 min at 30°. The sample was assayed and then centrifuged and resuspended in the 20 m*M* sodium succinate—0.05 *M* Tris-Cl (pH 7.4), and assayed again.

tase occurs very slowly indicating that activity remaining is relatively stable.

To assess the requirement for the intact phospholipid for maintaining the stability of the enzyme, the phospholipase C treated complexes were allowed to sit several hours and then reconstituted with phospholipid. After addition of phospholipid the succinate-DCPIP reductase activity was measured. In initial experiments reconstitution with lecithin was attempted unsuccessfully; no succinate-DCPIP reductase activity was noted after addition of this phosphatide. This result suggested, among other possibilities, that some constituent of the complex was blocking access of lecithin to the components with which it normally interacted. If diglyceride was interfering, then a more detergent-like phosphatide might facilitate reconstitution of the complex. In Table II reactivation of the phospholipase C treated complex with lysolecithin is seen. In this experiment the phospholipase C treated sample was allowed to sit at room temperature for several hours after addition of phospholipase C. After phospholipase treatment and aging for 7 hr the activity had dropped to 22% of that of the original complex. At this time excess lysolecithin was incubated with the phospholipase C treated complex. In the presence of lysolecithin the activity was increased to a level comparable to that of the original complex. The activity of the complex reconstituted with lysolecithin drops to a value equal to that of the aged control when it is washed to remove uncomplexed lipid. This is noted by comparing sample 6 with sample 2 in Table II. This observation suggests that loss of activity in control samples is not due to loss in activity of SDH, but rather to loss in easily removed phospholipid which can contribute to succinate-DCPIP reductase activity of the complex. The complex cannot be formed initially by using lysolecithin. Evidently once the complex is formed the lysolecithin plus products of phospholipase digestion can substitute for diacyl phosphatides.

As shown in Table III, the succinate-DCPIP reductase activity remaining within the complex after phospholipase C treatment is TTB sensitive, as is the activity from the lysolecithin reconstituted complex. Solubilized succinate dehydrogenase does have succinate-DCPIP reductase activity, but it is not sensitive to TTB. None of the other components of the complex have the reductase activity. The activity remaining after phospholipase treatment may be due to small amounts of phospholipid in the complex or, alternatively, could be maintained by products of phospholipase C treatment. To more clearly differentiate between these possibilities the following experiment was carried out. A complex was prepared which included 2-[1-^{14}C]linoleoyllecithin and was treated with phospholipase C. An analysis by thin-layer chromatography of the radioactive products extracted from the phospholipase treated particles revealed that very little phospholipid was left after enzymatic digestion (Table IV). In this particular experiment succinate-DCPIP reductase activity dropped slightly below 50%, but only 4% of the lecithin remained after phospholipase C treatment. Furthermore, the diglyceride product remained with the complex and was not removed as readily as was phospholipid from the untreated sample (see total counts per minute per milligram of protein). These observations suggest the possibility that the remaining diglyceride can facilitate some succinate-DCPIP reductase activity in the complex, but does so less well than phospholipid. Furthermore, it is obvious that the phosphoryl base portion of the

Table III: TTB Sensitivity of PLC Treated and Lysolecithin Reconstituted Complex.[a]

Sample	Additions	Specific Act.[c] TTB Absent	Specific Act.[c] 0.5 m*M* TTB	% Inhibition by TTB
Control		170	1	99
Control	UQ_{10}[b]	170	41	76
PLC treated		48	4	92
PLC treated	UQ_{10}	41	10	77
PLC treated + lysolecithin		87	1	99
PLC treated + lysolecithin	UQ_{10}	78	14	82
Cytochrome *b*		0	0	
SDH		53	64	0
Lecithin		0	0	
Lysolecithin		0	0	
UQ_{10}		0	0	

[a] Formation of the complex, its assay, and phospholipase C treatment were carried out as outlined in the Experimental Procedure section. After removal of phospholipase C from the complex and subsequent assay for DCPIP reductase activity, 0.04 mg of the complex was incubated with lysolecithin at the level of 1 mg of lysolecithin/mg of complex protein for 5 min at 30°. The lysolecithin treated complex was then assayed for DCPIP reductase activity. The components of the complex were assayed at levels equivalent to their concentration in an assay aliquot of the complex. [b] Final concentration = 30 μM. [c] Nanomoles of succinate oxidized per minute per milligram of complex protein.

Table IV: Lipid Content of Phospholipase C Treated Succinate—DCPIP Reductase Complex.[a]

Sample	Sp Act.[b]	Protein (mg)[c]	cpm Lecithin	cpm Diglyceride	cpm Total/mg of Protein
Untreated, unwashed	126				
Untreated, washed	110	0.039	12,150	716	3.3×10^5
PLC treated	53	0.037	577	16,017	4.5×10^5

[a] Liposomes of 2-[1-^{14}C]linoleoyllecithin (see Experimental Procedure section) were utilized to prepare the succinate—DCPIP reductase complex. Assay of the complex and phospholipase C treatment were carried out as before. After phospholipase C treatment aliquots of both the untreated, washed, and the PLC-treated complex were extracted as described in the Experimental Procedure section. An aliquot of the lipids extracted from each of the complexes was analyzed by the procedure of Newkirk and Waite (1971) to determine the lipids remaining after phospholipase treatment. [b] Nanomoles of succinate oxidized/minute per milligram of complex protein. [c] The values reported represent the amounts of protein associated with the quantity of lipid which was added to the thin-layer plates for resolution by chromatography.

phospholipid molecule is not required for stabilization of SDH.

In the experiments with radioactive lecithin, which were carried out three times, analysis of radioactive products indicated that hydrolysis was essentially complete whereas lipid phosphorus determinations indicated less hydrolysis by phospholipase C. In one experiment only 4% of the lecithin remained as indicated by analysis of radioactive products, whereas the lipid phosphorus determination indicated only 83% hydrolysis of phospholipid. These observations indicate that the wash procedure utilized did not completely remove all phosphorylcholine formed from enzymatic hydrolysis of the phosphatides, and suggest that analysis of radioactive products is a more accurate method to evaluate the effect of the phospholipase C on the complex. Moreover, this finding indicates that in experiments described in Figure 5 and Table III phospholipid hydrolysis was more complete than is indicated by the phosphorus analyses carried out. These results also reinforce the idea that the complete phospholipid molecule is not required for complete stabilization of SDH. The incomplete loss of phosphorylcholine is difficult to explain with the information available, but the possibility exists that it remains associated via ionic interactions with some component(s) of the complex.

Discussion

The reconstitution activity of SDH is stabilized by mitochondrial membrane components. Protein-protein interactions appear to be obligatory in the stabilization process, since phospholipids alone do not prevent loss of reconstitution activity. The lack of stability of SDH in the presence of phospholipids noted in this study is in contrast to the observations of Cerletti et al. (1970) who reported that succinate-phenazine methosulfate activity was stabilized by phosphatidylserine. The difference in the effect of phospholipids on these two activities further emphasizes the difference in activities exhibited by soluble SDH. The phenazine methosulfate activity demonstrated by solubilized SDH is not TTB sensitive, even when SDH is recombined with other membrane components (Bruni and Racker, 1968). In contrast, reconstitution of SDH–ubiquinone reductase (Bruni and Racker, 1968) or SDH–DCPIP reductase described in this paper is very sensitive to low levels of TTB.

The succinate-DCPIP reductase activity reconstituted in this study was not always dependent on addition of ubiquinone. The observation, however, that small amounts of ubiquinone were present in the cyt *b* preparations suggests strongly that the reconstituted system was indeed a succinate-ubiquinone reductase system with the endogenous ubiquinone facilitating electron flow to DCPIP. The TTB sensitivity of the reductase activity further suggests that electrons are transferred via the natural electron transfer process to DCPIP. Presumably in this system either exogenous or endogenous ubiquinone can facilitate electron transfer in the lipophilic milieu of the complex which is provided by the phospholipids.

As mentioned above, this study establishes clearly that the requirements for stabilizing the reconstitution activity of SDH are more stringent than those for maintaining the phenazine methosulfate reductase activity. Possibly interaction of SDH with the cyt *b* fraction stabilizes the readily oxidizable iron-sulfur moiety of the enzyme (Baginsky and Hatefi, 1969) which is required for reconstitution (King, 1963; Hanstein et al., 1971). Support for this idea comes from the observation that stabilization of SDH reconstitution activity appears to require the presence of mitochondrial membrane proteins. Bovine serum albumin when substituted for cyt *b* in the stabilization experiments exerted no discernible effects. It neither stabilized the enzyme nor caused the immediate loss in succinoxidase activity seen

with addition of cyt *b*. Clearly the effects of other mitochondrial proteins on the stability of SDH have to be assessed to determine if the flavoprotein resides within the mitochondrial membrane bound to a specific protein which protects the labile iron–sulfur moiety.

The requirement for phospholipid in stabilization of the reconstitution activity of SDH is more difficultly interpreted. It is clear the phospholipid has to be present in order to measure any type of activity when cyt *b* is added. Several observations in this study suggest, however, that phospholipids as such are not required for stabilization of the reconstitution activity of SDH. Phospholipase C treated succinate–DCPIP reductase complexes can be reactivated to original activity with lysolecithin several hours after enzymatic hydrolysis of phospholipids. This result suggests that either the diglyceride remaining stabilizes the enzyme or that protein–lipid interactions are not important in maintaining the activity of the flavoprotein. It is possible that phospholipid is only required to facilitate formation of the succinate–ubiquinone reductase complex. Bruni and Racker (1968) noted that activity of the succinate–ubiquinone reductase complex was lowered if SDH was added to cytochrome *b* before phospholipid. This suggests that phospholipid provides the proper environment in which the cyt *b* fraction and SDH can interact to form an active complex. In this regard, it is notable that in the reconstitution of the succinoxidase complex from respiratory components, omission of phospholipid results in formation of an inactive complex which can be activated only by a prolonged incubation with phospholipid and then only to an activity which is 50% of control (Nishibayashi-Yamashita et al., 1972). These studies suggest that phospholipid facilitates the proper alignment between electron transport components which allows respiratory activity to be expressed. Diglycerides will not substitute for phospholipids in facilitating the formation of the complex. Attempts to substitute diolein for phospholipid in the original complexes were unsuccessful.

If a lipophilic environment is required for maintaining the stability of SDH, then it is obvious that it can be provided by diglyceride. Experiments with the 2-[1-^{14}C]linoleoyllecithin indicate that very small amounts of phospholipid remain after phospholipase C treatment. Under these conditions diglyceride also appears to maintain some TTB sensitive succinate–DCPIP reductase activity. These experiments also suggest that reductase activity does not require phospholipids specifically, but rather the hydrophobic environment which can also be provided to some extent by diglycerides.

From this study and those of others (Bruni and Racker, 1968; Baginsky and Hatefi, 1969; Hanstein et al., 1971; Davis and Hatefi, 1971) a model for the succinate–ubiquinone reductase segment of the electron-transport chain emerges. The multicomponent SDH molecule interacts specifically with membrane proteins, possibly cytochrome *b*, in a manner which protects the labile iron–sulfur moiety of the flavoprotein. This protein complex resides within a lipophilic environment provided physiologically by phospholipids. The enzymatic properties of this complex are undoubtedly affected by the presence of phospholipids (Cerletti et al., 1967), as are the activities of many other membrane proteins. The lipophilic environment also facilitates the interaction between ubiquinone and its immediate electron donor which may be either SDH or cytochrome *b*. The ubiquinone may be rather mobile, as has been suggested earlier (Klingenberg, 1968) and by the observation in this study that either endogenous or exogenous quinone can give rise to a TTB sensitive activity.

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References

Arion, W., and Racker, E. (1970), *J. Biol. Chem. 245,* 5186.

Baginsky, M., and Hatefi, Y. (1969), *J. Biol. Chem. 244,* 5313.

Blair, P. (1967), *Methods Enzymol. 10,* 78.

Bruni, A., and Racker, E. (1968), *J. Biol. Chem. 243,* 962.

Bulos, B., and Racker, E. (1968a), *J. Biol. Chem. 243,* 3891.

Bulos, B., and Racker, E. (1968b), *J. Biol. Chem. 243,* 3901.

Cerletti, P., Caiafa, P., Giordano, M., and Testolin, G. (1970), *Lipids 5,* 953.

Cerletti, P., Giovenco, M., Giordano, M., Giovenco, S., and Strom, R. (1967), *Biochim. Biophys. Acta 146,* 380.

Cerletti, P., Strom, R., and Giordano, M. (1963), *Arch. Biochem. Biophys. 101,* 423.

Chalvardjian, A., and Rudnicki, E. (1970), *Anal. Biochem. 36,* 225.

Conover, T., Prairie, R., and Racker, E. (1963), *J. Biol. Chem. 238,* 2831.

Cunningham, C., and George, D. (1975), *J. Biol. Chem.* in press.

Davis, K., and Hatefi, Y. (1971), *Biochem. Biophys. Res. Commun. 44,* 1338.

Fleischer, S., and Fleischer, B. (1967), *Methods Enzymol. 10,* 406.

Gornall, A., Bardawill, C., and David, M. (1949), *J. Biol. Chem. 177,* 751.

Hanstein, W., Davis, K., Ghalambor, M., and Hatefi, Y. (1971), *Biochemistry 10,* 2517.

Kagawa, Y., and Racker, E. (1966), *J. Biol. Chem. 241,* 2467.

Kagawa, Y., and Racker, E. (1971), *J. Biol. Chem. 246,* 5477.

King, T. (1963), *J. Biol. Chem. 238,* 4037.

King, T. (1967), *Methods Enzymol. 10,* 322.

Klingenberg, M. (1968), in Biological Oxidations, Singer, T., Ed., New York, N.Y., Interscience, p 3.

Knowles, A., Guillory, R., and Racker, E. (1971), *J. Biol. Chem. 246,* 2672.

Kushner, D. (1957), *J. Bacteriol. 73,* 297.

Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951), *J. Biol. Chem. 193,* 265.

Newkirk, J., and Waite, M. (1971), *Biochim. Biophys. Acta 225,* 224.

Nishibayashi-Yamashita, H., Cunningham, C., and Racker, E. (1972), *J. Biol. Chem. 247,* 698.

Pullman, M., Penefsky, H., Datta, A., and Racker, E. (1960), *J. Biol. Chem. 235,* 3322.

Racker, E. (1963), *Biochem. Biophys. Res. Commun. 10,* 435.

Redfearn, E. (1967), *Methods Enzymol. 10,* 381.

Rieske, J., and Tisdale, H. (1967), *Methods Enzymol. 10,* 353.

Singer, T., Kearney, E., and Kenney, W. (1973), *Adv. En-*

zymol. Relat. Areas Mol. Biol. 37, 189.
Takemori, S., and King, T. (1964), *J. Biol. Chem. 239,* 3546.
Tappel, A., (1960), *Biochem. Pharmacol. 3,* 289.
Tisdale, H., Wharton, D., and Green, D. (1963), *Arch. Biochem. Biophys. 102,* 114.
Yamashita, S., and Racker, E. (1969), *J. Biol. Chem. 244,* 1220.
Ziegler, D., and Doeg, K. (1962), *Arch. Biochem. Biophys. 97,* 41.

Mandelate Racemase from *Pseudomonas putida.* Magnetic Resonance and Kinetic Studies of the Mechanism of Catalysis†

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ABSTRACT: The interactions of mandelate racemase with divalent metal ion, substrate, and competitive inhibitors were investigated. The enzyme was found by electron paramagnetic resonance (EPR) to bind 0.9 Mn^{2+} ion per subunit with a dissociation constant of 8 μM, in agreement with its kinetically determined activator constant. Also, six additional Mn^{2+} ions were found to bind to the enzyme, much more weakly, with a dissociation constant of 1.5 mM. Binding to the enzyme at the tight site enhances the effect of Mn^{2+} on the longitudinal relaxation rate ($1/T_{1p}$) of water protons by a factor of 11.9 at 24.3 MHz. From the frequency dependence of $1/T_{1p}$, it was determined that there are ~3 water ligands on enzyme-bound Mn^{2+} which exchange at a rate $\geq 10^7$ sec^{-1}. The correlation time for enzyme-bound Mn^{2+}-water interaction is frequency-dependent, indicating it to be dominated by the electron spin relaxation time of Mn^{2+}. Formation of the ternary enzyme-Mn^{2+}-mandelate complex decreases the number of fast exchanging water ligands by ~1, but does not affect τ_c, suggesting the displacement or occlusion of a water ligand. The competitive inhibitors D,L-α-phenylglycerate and salicylate produce little or no change in the enzyme-Mn^{2+}-H_2O interaction, but ternary complexes are detected indirectly by changes in the dissociation constant of the enzyme-Mn^{2+} complex and by mutual competition experiments. In all cases the dissociation constants of substrates and competitive inhibitors from ternary complexes determined by magnetic resonance titrations agree with K_M and K_i values determined kinetically and therefore reflect kinetically active complexes. From the paramagnetic effects of Mn^{2+} on $1/T_1$ and $1/T_2$ of the ^{13}C-enriched carbons of 1-[^{13}C]-D,L-mandelate and 2-[^{13}C]-D,L-mandelate, Mn^{2+} to carboxylate carbon and Mn^{2+} to carbinol carbon distances of 2.93 ± 0.04 and 2.71 ± 0.04 Å, respectively, were calculated, indicating bidentate chelation in the binary Mn^{2+}-mandelate complex. In the active ternary complex of enzyme, Mn^{2+}, and D,L-mandelate, these distances increase to 5.5 ± 0.2 and 7.2 ± 0.2 Å, respectively, indicating the presence of at least 98.9% of a second sphere complex in which Mn^{2+}, and C_1 and C_2 carbon atoms are in a linear array. The water relaxation data suggest that a water ligand is immobilized between the enzyme-bound Mn^{2+} and the carboxylate of the bound substrate. This intervening water ligand may polarize or protonate the carboxyl group. From $1/T_{2p}$ the rate of dissociation of the substrate from this ternary complex ($\geq 5.6 \times 10^4$ sec^{-1}) is at least 52 times greater than the maximal turnover number of the enzyme (1070 sec^{-1}), indicating that the complex detected by nuclear magnetic resonance (NMR) is kinetically competent to participate in catalysis. Relationships among the microscopic rate constants are considered.

Mandelate racemase (EC 5.1.2.2), a tetramer of molecular weight 278,000, catalyzes the racemization of mandelic acid. This "one substrate" enzyme has recently been shown to exhibit an absolute requirement for divalent metal ions for activity (Fee et al., 1974a); thus it represents one of the simplest examples of metal ion activation of enzymatic catalysis. Activation of the apoenzyme by divalent metal ions may result from stabilization of a catalytically active enzyme conformation, by facilitation and directional coordination of the binding of substrate at the active site of the enzyme, or by direct participation in the catalytic mechanism *via* the ability of electron-withdrawing divalent cations to stabilize carbanions. A possible carbanion intermediate for mandelate racemase has previously been proposed (Kenyon and Hegeman, 1970).

The present study was undertaken to determine the spatial relationship of enzyme, cofactor, and substrate in the fully active ternary enzyme complex and to help elucidate

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