Succinate Dehydrogenase. II. Enzymatic Properties*

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ABSTRACT: Succinate dehydrogenase from beef heart has been shown to have a molecular weight of approximately 97,000 ± 5%, and to contain 1 mole of covalently bound flavin, 7–8 g-atoms of iron, and 7–8 moles of acid-labile sulfide per mole. The enzyme reduces phenazine methosulfate at V_{max}^{PMS} = 100–110 μmoles × min^{-1} × mg^{-1} of protein ($K_m^{Succ} \simeq 0.3$ mM, $K_m^{PMS} \simeq 0.48$ mM), and ferricyanide (3 mM) at 27 μmoles × min^{-1} × mg^{-1} of protein. It restores full activity to electron transport particles or complex II (succinate–coenzyme Q reductase) preparations whose succinate dehydrogenases have been selectively destroyed at pH 9.3. Experiments with complex II have shown that such reconstitution results from incorporation into the particles of 1 equiv of added succinate dehydrogenase. Both the dye reductase and reconstitution activities of succinate dehydrogenase are perfectly stable for months at −70° or below. The binding of succinate dehydrogenase in complex II appears to be mainly hydrophobic. This association can be weakened in a controlled manner by chaotropic agents, resulting in the release of soluble succinate dehydrogenase from complex II particles. Treatment of succinate dehydrogenase with the more potent chaotropic agents (trichloroacetate, thiocyanate, guanidine hydrochloride, and perchlorate), followed by low-temperature freezing and thawing, results in resolution of the enzyme into its "subunits." The resolution of succinate dehydrogenase, as monitored by the loss of dehydrogenase activity, is a function of (a) the concentration and potency of the chaotropic agent used, and (b) the number of freeze-thawing steps.

The molecular weight, composition, and substructure of an essentially pure preparation of succinate dehydrogenase from beef heart mitochondria have been presented in the preceding communication (Davis and Hatefi, 1971) and elsewhere (Hatefi *et al.*, 1970). The present communication describes the enzymatic properties of succinate dehydrogenase with respect to (a) reduction of artificial dyes and (b) reconstitution of succinate oxidase and succinate–coenzyme Q (ubiquinone) reductase activities in the presence of appropriate segments of the respiratory chain. It is important to document these characteristics of succinate dehydrogenase, because previous studies were carried out with considerably less pure preparations. Indeed, the possibility could not be discounted that the difference between the inability of the Singer-type succinate dehydrogenase (~50% pure) for reconstitution and the King-type enzyme (~30% pure), which is capable of reconstitution, might be due to the presence of additional proteins in the latter preparation. Our results clarify this question and examine the characteristics of such reconstitution in detail. It will also be shown that, in agreement with our previous work (Baginsky and Hatefi, 1969), the reconstitution activity of succinate dehydrogenase appears to depend on preserving the iron–labile sulfide system of the enzyme.

In addition, the resolution of succinate–coenzyme Q reductase particles (complex II) and of purified succinate dehydrogenase with chaotropic agents will be discussed in relation to the activity of each system. Results on complex II have shown that the resolution of this system with respect to succinate dehydrogenase is an equilibrium process (K. A. Davis, and Y. Hatefi, in preparation), and that the extent of resolution is a function of the degree of disorder imposed on the medium water by chaotropic agents.

Methods and Materials

Phenazine methosulfate and coenzyme Q reductase assays were performed at 38° as described previously (Baginsky and Hatefi, 1969), except that the concentration of succinate in the reaction mixture was 10 mM. Ferricyanide reductase assays were performed at 38° according to the method of King (1963) in the presence of 3 mM potassium ferricyanide, and succinoxidase assays at 30° according to King (1963) as described previously (Baginsky and Hatefi, 1969). Complex II and succinate dehydrogenase were prepared according to Baginsky and Hatefi (1969) and Davis and Hatefi (1971), respectively. Since they had essentially the same enzymatic properties, succinate dehydrogenase preparations A and B (see preceding communication, Davis and Hatefi, 1971) were used interchangeably in the studies described here. Unless otherwise specified, preparations of succinate dehydrogenase were stored as ammonium sulfate precipitated pellets at −70° (Davis and Hatefi, 1971) and dissolved when needed in a solution containing 50 mM sodium phosphate (pH 7.5), 20 mM succinate, and 5 mM dithiothreitol. Since dithiothreitol can reduce 2,6-dichlorophenolindophenol, which is th efinal electron acceptor in both the succinate dehydrogenase and succinate–coenzyme Q reductase assays, the concentration of the enzyme solution was chosen such that the amount of dithiothreitol added with enzyme to the reaction mixture would cause a ΔA_{600} of not more than 0.1. This initial bleaching of the dye was discounted during rate measurements. Control assays in the absence of dithiothreitol were also conducted with freshly dissolved enzyme to make certain that rates in the presence of dithiothreitol were not overestimated. For the effect of dithiothreitol on the stability of succinate dehydrogenase activity when in solution, see section 3 of

* From the Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received October 7, 1970.* Supported by U. S. Public Health Service Grant AM-08126.

† Recipient of U. S. Public Health Service Career Development Award 1-K4-GM-38291.

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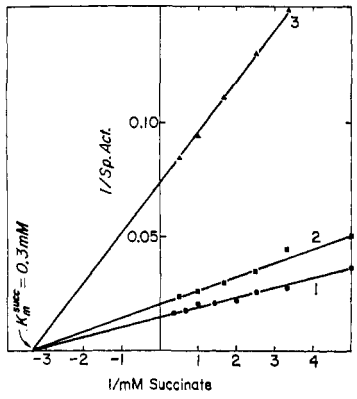

FIGURE 1: Effect of aging on PMS reductase activity and K_m^{Succ} of succinate dehydrogenase. Curve 1, succinate dehydrogenase freshly dissolved in 50 mM phosphate (pH 7.5), containing 20 mM succinate, but no dithiothreitol; curve 2, same succinate dehydrogenase solution after standing 6 hr in ice bath; curve 3, same succinate dehydrogenase solution frozen 3 days at −20°.

Results. Protein was estimated as before (Davis and Hatefi, 1971).

All chemicals were reagent grade or obtained from the sources described previously (Davis and Hatefi, 1971; Baginsky and Hatefi, 1969). Coenzyme Q_2 was the generous gift of Dr. O. Isler, Hoffman La Roche.

Results

1. Phenazine Methosulfate and Ferricyanide Reductase Activities of Succinate Dehydrogenase. Preparations of succinate dehydrogenase catalyze the oxidation of succinate by PMS[1] at a rate of 67–78 μmoles × min^{-1} × mg^{-1} of protein (Table I). This rate at V_{max}^{PMS} is 100–110, which corresponds to a turnover number of about 10,000/mole of succinate dehydrogenase. This activity is stable for months when the enzyme is kept frozen at −70° either as an ammonium sulfate precipitated pellet (see section 1 of Results in Davis and Hatefi, 1971) or in a solution containing 50 mM sodium phosphate (pH 7.5), 20 mM succinate, and 5 mM dithiothreitol. It is also stable for days at −20° in the buffer solution given above. The turnover number of complex II preparations from which succinate dehydrogenase is isolated is also about 10,000 (moles of PMS or coenzyme Q reduced per mole of succinate dehydrogenase flavin). Therefore, it appears that succinate dehydrogenase suffers no loss of activity during its isolation and purification from complex II. The ferricyanide reductase activity of the present preparation of succinate dehydrogenase, tested according to the method of King (1963), corresponds to 13.5 μmoles of succinate oxidized × min^{-1} × mg^{-1} of protein at 38°. The enzyme showed no dehydrogenase activity when either *d*- or *l*-malate was used as substrate. In agreement with the results published by King (1963), the K_m^{PMS} of succinate dehydrogenase was found to be approximately 0.48 mM, and ferricyanide above 3 mM was strongly inhibitory.

The K_m^{Succ} for the dehydrogenase preparation of Singer is reported to be 1.3 mM (Singer, 1966). However, we found that K_m^{Succ} for the bound succinate dehydrogenase of ETP or complex II was only about 0.25 mM. This difference suggested a possibility similar to the allotopic change of mitochondrial DPNH dehydrogenase, the soluble form of which exhibits a K_m^{DPNH} approximately ten times that of the particle-bound form (Hatefi and Stempel, 1969). Estimation of the K_m^{Succ} of our preparation of succinate dehydrogenase, using the same conditions as reported by Singer, gave, however, a value of 0.3 mM (Figure 1), which is very close to the K_m^{Succ} of ETP and complex II. Since the Singer-type preparation has a low content of iron and labile sulfide and is incapable of reconstitition, the possibility was considered that structural changes following the loss of iron and labile sulfide might be responsible for the four fold increase in K_m^{Succ}. This possibility was tested by prolonged aging of a solution of our succinate dehydrogenase in the absence of dithiothreitol, which results in complete loss of reconstitution activity and partial loss of sulfide and of PMS reductase activity. However, as seen in Figure 1, this treatment had no effect on the value of K_m^{Succ}. It might also be added that in these experiments succinate concentrations above 15 mM were slightly inhibitory.

TABLE I: Dye Reductase Properties of Succinate Dehydrogenase.

Parameter	Value
Succ ⟶ PMS[a] activity	67–78[b]
Succ ⟶ $K_3Fe(CN)_6$ activity	13.5[b]
Turnover number at V_{max}^{PMS}	10,000[c]
V_{max}^{PMS}	100–110[b]
K_m^{PMS} (mM)	~0.48
K_m^{Succ} (mM)	~0.3[a]

[a] at 1.65 mM PMS. [b] μmoles of succinate oxidized × min^{-1} × mg^{-1} of protein at 38°. [c] Moles of succinate oxidized × min^{-1} × $mole^{-1}$ of enzyme at 38°.

2. Reconstitution Activity of Succinate Dehydrogenase. A. RECONSTITUTION OF SUCCINATE OXIDASE ACTIVITY. It was shown by Keilin and King (1958), and subsequently in greater detail by King (1963, 1966), that incubation of heart muscle Keilin-Hartree preparations for 60 min at 38° and pH 9.3 resulted in destruction of succinate oxidase activity of the particles without apparent damage to their cytochrome system. Succinate oxidase activity could be partially restored to these particles (Keilin and King, 1958) by addition of a partially purified, soluble succinate dehydrogenase, the molecular characteristics of which are summarized in the accompanying paper (Davis and Hatefi, 1971). As mentioned above, the more purified succinate dehydrogenase preparation of Singer was devoid of such reconstitution activity.

Figure 2 shows data on the titration of alkali-treated ETP with succinate dehydrogenase. The curve marked by small, open circles (without letters) is a plot of μmoles of succinate oxidized × min^{-1} × mg^{-1} alk-ETP plus succinate dehydrogenase protein *vs.* the protein weight ratio of succinate dehydrogenase/alk-ETP. It is seen that the activity of the reconstituted system per milligram of *total* protein increases up to a value of succinate dehydrogenase/alk-ETP ≃ 0.11, remains nearly constant up to succinate dehydrogenase/alk-ETP ≃ 0.25, and declines as the succinate dehydrogenase/alk-ETP value increases further. The *apparent* lowered specific activity at succinate dehydrogenase/alk-ETP ≃ 0.25 is, of

[1] Abbreviations used are: PMS, phenazine methosulfate; Q, coenzyme Q (ubiquinone); ETP, electron transport particles prepared from mitochondria by sonication and differential centrifugation; alk-ETP, ETP particles treated at pH 9.3 and 38° as described by King (1963); DTT, dithiothreitol.

course, due to the presence of succinate dehydrogenase protein, which is in excess of that needed for reconstitution with the amount of alk-ETP present in the mixture. Since alkali treatment of ETP does not involve the *removal* of the inactivated succinate dehydrogenase protein, this type of plot (*i.e.*, per milligram of *total* protein) has also the disadvantage that the maximal specific activity calculated will perforce be lower than that of the untreated ETP.

A more interesting and realistic plot of the data is that shown in Figure 2 by the curve marked with letters. This curve actually represents two plots which are superimposed. One is a plot of μmoles succinate oxidized $\times$ min^{-1} $\times$ mg^{-1} of alk-ETP protein (the left-hand ordinate) *vs.* the protein weight ratio of succinate dehydrogenase/alk-ETP (top abscissa). This curve is marked by squares lettered from A to M. The other is a plot of μmoles of succinate oxidized $\times$ min^{-1} $\times$ mg^{-1} of succinate dehydrogenase protein (right-hand ordinate) *vs.* the protein weight ratio of alk-ETP/succinate dehydrogenase (bottom abscissa). This curve is marked by circles lettered also from A to M. The same letter in a square and in a circle indicates that the result of the same experiment was calculated and plotted once in terms of the right-hand ordinate and bottom abscissa and a second time in terms of the left-hand ordinate and top abscissa. The ordinate scales were chosen such that the maximal activity per milligram of alk-ETP would fall at the same height from the origin as maximal activity per milligram of succinate dehydrogenase, and the abscissa scales were selected such that extrapolation of the two branches of the curve (see dotted lines) would fall at the same distance from the origin at the top and the bottom abscissae. The results, as seen in Figure 2, are most interesting. (a) At any ratio of the two components, specific activity per milligram of alk-ETP or per milligram of succinate dehydrogenase is easily read from the left-hand or the right-hand ordinate, respectively. Maximum activity per milligram of alk-ETP is 1.47, which compares favorably with the original activity of 1.64 of the ETP preparation before alkali treatment. (The reason that only 90% of the original ETP activity is restored in these reconstitution experiments is discussed in section 3 below.) Maximum activity per mg succinate dehydrogenase protein as seen on the right-hand ordinate is 13.0. More accurately, the former maximum activity can be derived from the slope of the ascending branch of the curve as determined from the right-hand ordinate and bottom abscissa (slope 1.46), and the latter maximum activity f.om the same slope as determined from the left-hand ordinate and top abscissa (slope 13.1). (b) The alk-ETP/succinate dehydrogenase equivalence point as determined by extrapolation of the two branches of the curve (dotted lines in Figure 2) occurs at a mg protein ratio of alk-ETP/succinate dehydrogenase of 8.9:1. As might be expected, this value is the same as the ratio of the two maximal activities, namely 13.0/1.47 = 8.84. (c) The most interesting aspect of the lettered curve of Figure 2 is that the two inverse plots coincide throughout, especially in the ascending branch of the curve where either succinate dehydrogenase or alk-ETP is present in limiting amounts. The fact that this ascending portion of the curve extrapolates to zero activity is in agreement with the finding that alone neither succinate dehydrogenase nor alk-ETP has any succinoxidase activity. Furthermore, the fact that this branch of the curve is a straight line and passes through the origin indicates that the active succinoxidase species, regardless of the ratio of succinate dehydrogenase to alk-ETP in the mixture, is most probably composed of only one unit of each reacting component. Since essentially all the original activity of ETP is

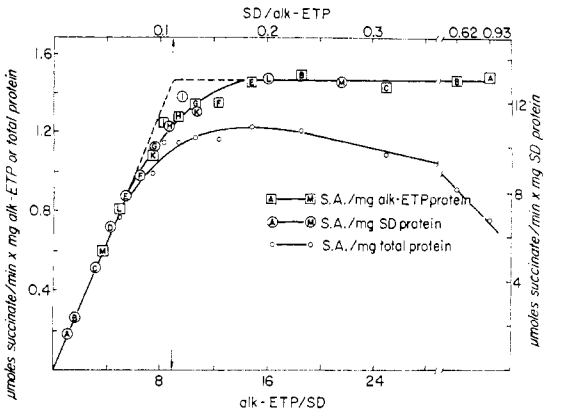

FIGURE 2: Reconstitution of succinate oxidase activity. Fresh succinate dehydrogenase was dissolved at a concentration of 7.8 mg/ml in 50 mM phosphate (pH 7.5), containing 20 mM succinate and 5 mM dithiothreitol, then immediately divided in 50-μl quantities in small test tubes, frozen in liquid nitrogen, and stored at $-70°$. It was used 2 days later for the reconstitution experiments reported here. ETP preparations stored as centrifuged pellets were suspended at a concentration of 9.1 mg/ml in 50 mM Tris (pH 8.0), containing 0.66 M sucrose. They were inactivated at pH 9.3 essentially according to the procedure of King (1963), readjusted to pH 7.7, and supplied with 20 mM succinate (protein concentration at this point was 8.35 mg/ml). Appropriate amounts of the alkali-treated ETP were then added to test tubes containing frozen succinate dehydrogenase. The components were mixed at room temperature with the help of a stirring rod, incubated 2.5 min at 30°, and assayed in the oxygraph at 30° for succinate oxidase activity according to the procedure of King (1963).

recovered after reconstitution, the unit of alk-ETP in reconstitution is probably the same as the active unit of ETP before alkali treatment; and since succinate dehydrogenase in solution appears to be monomeric (see preceding communication, Davis and Hatefi, 1971), one unit of succinate dehydrogenase in reconstitution is probably equivalent to one molecule (see also the Discussion). Both 2-thenoyltrifluoroacetone (3 mM) and antimycin A (6 μM) completely inhibited the reconstituted succinate oxidase system.

B. RECONSTITUTION OF SUCCINATE–COENZYME Q REDUCTASE ACTIVITY. Similar to ETP, alkali treatment of complex II also leads to destruction of its bound succinate dehydrogenase and the loss of both PMS reductase and coenzyme Q reductase activities. Addition of succinate dehydrogenase to these particles restored both activities. Titration of alkali-treated complex II with succinate dehydrogenase, in the same manner as the experiments of Figure 2, showed that complete restoration of coenzyme Q reductase activity (42.5 μmoles $\times$ min^{-1} $\times$ mg^{-1} of complex II) could be achieved when the protein ratio succinate dehydrogenase/complex II was approximately 5. In these experiments, the extrapolated succinate dehydrogenase/complex II equivalence point was 2.5. The reconstituted succinate–coenzyme Q reductase activity was completely inhibited in the presence of 2-thenoyltrifluoroacetone.

The experiments described above clearly indicate that succinate dehydrogenase restores succinate–coenzyme Q reductase activity to alkali-treated complex II particles. They do not show, however, a physical recombination between the two partners. Demonstration of physical reconstitution is important and necessary, especially when one of the interacting partners is a water-soluble enzyme such as succinate dehydrogenase. Evidence for such a reconstitution between succinate dehydrogenase and alkali-treated complex II particles is given in Table II. A suspension of complex II was brought to pH

TABLE II: Reconstitution of Succinate–Coenzyme Q Reductase Activity.[a]

Preparation	Protein (mg)	Flavin (nmoles/mg)	Succ → PMS (Per mg of Total Protein)	Succ → Q (Per mg of Total Protein)
Complex II, pH 9.3, 0 min at 38°	5.6		22.6	22.2
Complex II, pH 9.3, 20 min at 38°	5.6	4.92	<0.9	<0.8
Complex II + succinate dehydrogenase	5.6 + 12.6	8.65[b]	43.0	7.7
Complex II + succinate dehydrogenase, spun 60 min:				
Supernatant	10.0		48.8	<1.0
Pellet	7.9	6.7	24.4	23.9

[a] Conditions: complex II was inactivated by 20-min incubation at pH 9.3 and readjusted to pH 7.6 (protein 11.25 mg/ml). Succinate dehydrogenase was prepared and stored as an ammonium sulfate precipitated pellet for 1 day at −70°. It was dissolved in 50 mM Tris-HCl (pH 8.0) containing 20 mM succinate and 5 mM dithiothreitol before using (protein 21.1 mg/ml). Alkali-treated complex II (0.5 ml) and succinate dehydrogenase (0.6 ml) were then mixed together, assayed, and centrifuged for 60 min at 49,000 rpm. The supernatant and the pellet were, then, separated, the latter was suspended in Tris-succinate-dithiothreitol buffer, and both fractions were assayed as indicated. Activities shown are expressed as μmoles of succinate oxidized × min^{-1} × mg^{-1} of total protein at 38°. [b] Calculated.

9.3 at 38°, and its PMS reductase and coenzyme Q reductase activities followed as a function of time. After adjustment to pH 9.3 the PMS reductase activity of complex II was 22.6 and its Q reductase activity 22.2. Both activities declined further upon incubation of complex II at pH 9.3 and 38°, and reached a minimum after 20-min incubation. These activities and the flavin content of the preparation at this point are shown in Table II. Then, 12.6 mg of succinate dehydrogenase protein was added to 5.6 mg of the alkali-inactivated complex II, and the PMS and coenzyme Q reductase activities measured. The PMS reductase activity of the mixture per mg of total protein was 43, and its coenzyme Q reductase activity per mg of total protein was 7.7. The latter activity per mg of complex II protein was 24.5. The mixture was then centrifuged at 0–4° for 60 min at 49,000 rpm, and the supernatant and the pellet were separated. The supernatant (10.0 mg protein) was succinate dehydrogenase as evidenced from the fact that it had high PMS reductase activity and negligible coenzyme Q reductase activity. The pellet (7.9 mg of protein) was a physically reconstituted complex II with a PMS reductase activity of 24.4 and a coenzyme Q reductase activity of 23.9. The flavin content of the reconstituted complex II was 6.7 nmoles/mg of protein.

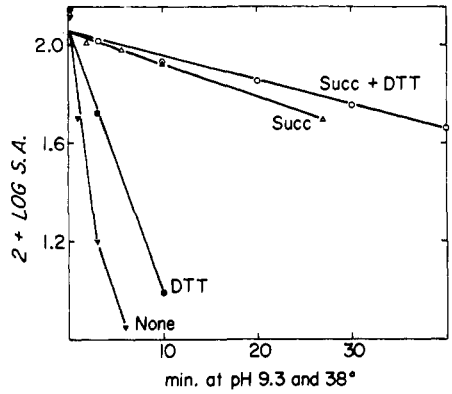

FIGURE 3: Kinetics of inactivation of succinate oxidase activity of ETP at pH 9.3 and 38°. ETP at 18.2 mg/ml of deoxygenated 0.25 M sucrose plus 10 mM Tris-HCl (pH 8.0) was brought to pH 9.3 at 38° with a predetermined amount of 1 N NaOH, incubated under an atmosphere of argon and sampled for activity as indicated. Where present in the incubation mixture, succinate was 20 mM and dithiothreitol (DTT) 5 mM. Succinoxidase assay was conducted at 30° according to the method of King (1963). All samples were neutralized with one volume of 100 mM sodium phosphate (pH 7.3), containing 20 mM succinate and incubated 3 min at 38° before assay. To those not containing succinate in the original mixture 40 mM succinate was added along with phosphate buffer at the time of neutralization, so that the final succinate concentration in all samples was 20 mM.

These results clearly show that succinate dehydrogenase combines both physically and functionally with alkali-inactivated complex II preparations, resulting in a particle with restored activity and increased flavin content. The reason for increased flavin content is that alkali treatment does not remove the succinate dehydrogenase of complex II, but only inactivates it. The extent of flavin increase in the reconstituted complex II indicates that the amount of added succinate dehydrogenase, which has combined with the particles, is equivalent to that which was originally present in complex II and subsequently inactivated by alkali treatment. This is because complex II has approximately 2.1 times as much protein per mole of flavin as the purified succinate dehydrogenase. Therefore, if 1 mole of succinate dehydrogenase (flavin = 10.3 nmoles/mg) should combine with complex II per mole of the bound and inactivated succinate dehydrogenase of complex II, then the theoretical flavin content of the reconstituted pellet of Table II would have to be

$$\frac{(2.1 \times 4.92) + (1 \times 10.3)}{2.1 + 1} = 6.66 \text{ nmoles/mg of protein}$$

As seen in this table, our measured flavin content of the reconstituted pellet is 6.7 nmoles/mg of protein. It is also seen in Table II that the loss of protein in the succinate dehydrogenase fraction (12.6 − 10.0 = 2.6 mg) is in excellent agreement with the above conclusion, because the amount of added succinate dehydrogenase which has combined with the alkali-inactivated complex II would have to be equivalent to 48.5% of the protein of complex II (5.6/2.1 = 2.7 mg). The recovered protein in the reconstituted pellet is 7.9 mg, which again is in

excellent agreement with the expected amount of (5.6 + 2.6) = 8.2 mg. With regard to incorporation of 1 mole of added succinate dehydrogenase per mole of inactivated succinate dehydrogenase present in the alkali-treated particles, it might be mentioned that similar conclusions were reached by Kimura *et al.* (1968) when they titrated alkali-treated Keilin–Hartree particles with a Keilin–King type of succinate dehydrogenase preparation.

Repeated extraction of complex II preparations with chaotropic agents also yields a particulate product, enriched in cytochrome *b* and impoverished in active succinate dehydrogenase, which can combine with added succinate dehydrogenase. However, such excessive treatment appears to damage the particles somewhat, because the reconstituted activities upon addition of succinate dehydrogenase are not as high as those obtained when alkali-inactivated complex II is employed.

Finally, it might be added that Bruni and Racker (1968) have also attempted the reconstitution of the succinate–coenzyme Q reductase system by combining succinate dehydrogenase prepared according to Keilin and King with a sodium dodecyl sulfate solubilized cytochrome *b* preparation in the presence of phospholipids. The highest activity of their reconstituted system was reported to be about 0.9 μmole of succinate oxidized (or coenzyme Q reduced) × min^{-1} × mg^{-1} of protein. As mentioned above, in preparations of complex II, or in reconstituted complex II, the molar ratio of cytochrome *b* to active succinate dehydrogenase is approximately 1:1; in the preparation of Bruni and Racker, this ratio is approximately 32:1.

3. Factors Affecting the Activities of Bound and Isolated Succinate Dehydrogenase. A. ETP AND COMPLEX II. Figure 3 shows data on the stability of the bound succinate dehydrogenase of ETP preparations at pH 9.3 and 38°. It is seen that the succinoxidase activity of ETP preparations rapidly deteriorates with first-order kinetics during incubation of the particles at pH 9.3 and 38°. Addition of dithiothreitol inhibits this process slightly, succinate is considerably more effective, and succinate plus dithiothreitol even more. In all these cases, whatever deterioration that takes place is also first-order throughout the duration of incubation at alkaline pH. Similar results are obtained with respect to the succinate dehydrogenase of complex II when the latter preparation is subjected to the conditions described in Figure 3.

As seen in Figure 3, all the inactivation curves extrapolate to a zero-time point corresponding to a succinoxidase activity of 1.39, whereas the succinoxidase activity of ETP prior to addition of alkali was 1.64. The DPNH oxidase activity of the ETP preparation used in the experiments of Figure 3 also decreased by exactly the same factor (15.5%) from 2.33 to 1.97 upon alkali treatment. These results suggest that in addition to destruction of succinate dehydrogenase, alkali treatment also has an additional deleterious effect, which is relatively small, however. It may be recalled from Figure 2 that in reconstitution of the succinoxidase system of ETP, only 90% of the original activity was recovered. The results discussed above with respect to Figure 3, might be an explanation for the 10% unrecovered activity in the experiments of Figure 2.

B. SUCCINATE DEHYDROGENASE. As pointed out in the preceding communication (Davis and Hatefi, 1971), extraction and purification of succinate dehydrogenase were performed in the presence of 5 mM dithiothreitol and 20 mM succinate. Omission of succinate resulted in a preparation of succinate dehydrogenase with about 80% of the PMS reductase activity and 90% of the reconstitution activity (in the succinoxidase assay) of the dehydrogenase isolated in the presence of both succinate and dithiothreitol. However, omission of dithiothreitol caused nearly 60% loss of PMS reductase activity and *complete* loss of reconstitution activity. It is seen, therefore, that the presence of dithiothreitol during isolation and purification is essential for preserving the activities of succinate dehydrogenase. The addition of a suitable thiol during isolation and purification of succinate dehydrogenase was suggested by the earlier studies of Baginsky and Hatefi (1968, 1969). They found that succinate dehydrogenase preparations lacking reconstitution activity could be activated in this respect by treatment with Na_2S, ferrous ions, and mercaptoethanol under conditions that appropriate apoproteins are converted back to iron–sulfur proteins. They also showed that addition of dithiothreitol to the reactivated enzyme had a stabilizing effect on its reconstitution activity. These results indicated, therefore, that damage to the iron–sulfur protein system of succinate dehydrogenase could be one reason for its loss of reconstitution activity.

The reconstitution activity of succinate dehydrogenase deteriorates in solution even in the presence of succinate and dithiothreitol. This process also obeys first-order kinetics similar to that shown in Figure 3 for the bound succinate dehydrogenase of ETP. At 0°, 50% of the reconstitution activity of succinate dehydrogenase is lost in about 75 min. However, when alkali-treated ETP is added to succinate dehydrogenase under conditions that either the dehydrogenase (succinate dehydrogenase/ETP = 0.1) or the alk-ETP (succinate dehydrogenase/ETP = 0.3) is limiting (see Figure 2), then the reconstituted activity of the system remains completely stable during the period of time that succinate dehydrogenase alone deteriorates to zero reconstitution activity. Under the conditions of these experiments (*i.e.*, in the presence of 20 mM succinate and 5 mM dithiothreitol), the PMS reductase activity of succinate dehydrogenase remains unchanged when kept at 0° for several hours. The reconstitution activity of succinate dehydrogenase can also be kept stable for months if preparations of the enzyme are frozen at −70° or below as described in section 1.

Another point with regard to the activity of succinate dehydrogenase concerns the "activation" phenomenon (Kearney, 1957; Singer, 1966). It has been demonstrated that preparations of succinate dehydrogenase show very little activity unless the enzyme is incubated for several minutes (*e.g.*, between 20 and 40°) in the presence of succinate. Our preparation of succinate dehydrogenase did not require activation in the PMS reductase assay, and showed only a short lag in reconstituted complex II systems when assayed for succinate–coenzyme Q reductase activity at 38°. However, in the reconstituted succinoxidase systems, the succinate dehydrogenase/alk-ETP mixture had to be incubated for 2.5–3 min at 30° in the presence of 20 mM succinate before maximal succinoxidase activity could be attained. Perhaps these differences are related to the fact that succinate dehydrogenase is prepared in the presence of succinate and is dissolved in a solution containing 20 mM succinate before assay, and that only in the reconstituted succinoxidase mixture (succinate dehydrogenase + alk-ETP) can the enzyme turn over to any appreciable extent before the actual assay is performed.

4. Activity Changes During Resolution of Complex II and Succinate Dehydrogenase. A. COMPLEX II. It was pointed out in the preceding communication (Davis and Hatefi, 1971) that addition of chaotropic agents to complex II preparations results in the resolution of the complex and solubilization of succinate dehydrogenase. The kinetics of this process at 0°

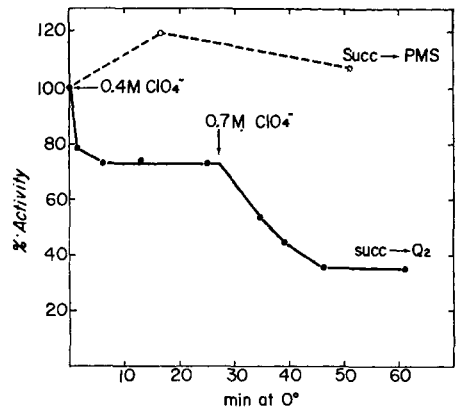

FIGURE 4: Kinetics of the resolution of complex II with 0.4 and 0.7 M $NaClO_4$. Conditions were the same as described in the accompanying communication (Davis and Hatefi, 1971) for the preparation of succinate dehydrogenase. The mixture of complex II (10 mg/ml) plus perchlorate was assayed at the times indicated for PMS and Q_2 reduction.

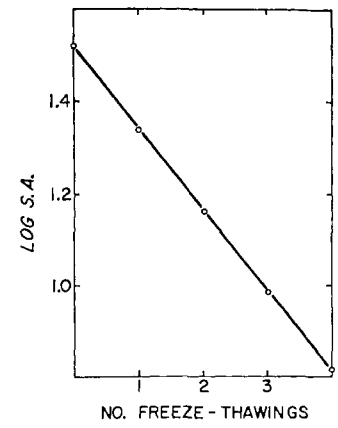

FIGURE 6: Effect of freeze-thawing on the chaotrope-induced resolution of succinate dehydrogenase. The enzyme in 50 mM sodium phosphate (pH 8.0), 20 mM succinate, and 5 mM dithiothreitol (protein 5.3 mg/ml) was treated with 0.3 M sodium trichloroacetate, frozen in liquid nitrogen, thawed at room temperature, and assayed for reduction of PMS as indicated.

are demonstrated in Figure 4. It is seen that upon addition of 0.4 M $NaClO_4$ to a suspension of complex II, the coenzyme Q reductase activity of the system decreases as a function of time to about 75% of the original activity, which remains constant thereafter. An increase in the perchlorate concentration from 0.4 to 0.7 M results in a further decrease in the Q reductase activity, which after about 20 min reaches a stable level corresponding to approximately 35% of the original Q reductase activity of complex II. By contrast, the PMS reductase activity of the system increases slightly upon addition of perchlorate and remains relatively constant thereafter. In other words, the integrated activity of complex II as demonstrated by its Q reductase activity is destroyed as a function of the concentration of $NaClO_4$, which causes the resolution of the system and solubilization of succinate dehydrogenase, whereas the activity of succinate dehydrogenase itself as measured by reduction of PMS is not so affected. These results also suggest that, unlike complex I whose resolution by chaotropic agents goes to completion (Davis and Hatefi, 1969), the resolution of complex II by $NaClO_4$ is an equilibrium process which can be shifted in the direction of greater resolution by increasing the concentration of $NaClO_4$.[2] The destabilizing effect of chaotropic agents on membranes and enzyme complexes as related to the ordered structure of medium water has been discussed elsewhere by Hatefi and Hanstein (1969, 1970), and the effect of D_2O, as aqueous solvent of greater structure than water, on the resolution of complex II has been mentioned in the preceding communication (Davis and Hatefi, 1971).

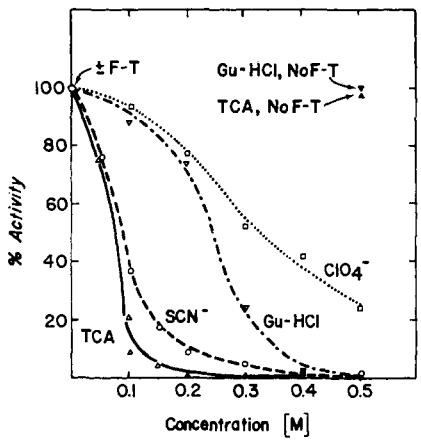

FIGURE 5: Effect of chaotropic agents and freeze-thawing on the resolution of succinate dehydrogenase. The enzyme at a protein concentration of 1.48 mg/ml of 50 mM sodium phosphate (pH 7.0) and 20 mM succinate, was treated with various chaotropic agents as shown in the figure, and frozen in liquid nitrogen and thawed at room temperature three times. Since in the presence of higher concentrations of chaotropes FP precipitated after freeze-thawing, all samples were centrifuged for 1 min at 35,000 rpm, and only the clear supernatant was assayed for PMS reductase activity. TCA, sodium trichloroacetate; GU-HCl, guanidine hydrochloride; F-T, freeze-thawing.

B. SUCCINATE DEHYDROGENASE. It was shown in the preceding communication (Davis and Hatefi, 1971) that treatment of purified preparations of succinate dehydrogenase with chaotropic agents, followed by repeated freezing and thawing in liquid nitrogen, results in resolution of the enzyme into a flavoprotein and an iron–sulfur protein fraction. Figure 5 demonstrates this resolution process as monitored by disappearance of the PMS reductase activity of succinate dehydrogenase. It is seen in Figure 5 that the PMS reductase activity of the enzyme in the absence of chaotropic agents is not affected by repeated freeze-thawing of the solution in liquid nitrogen. Nor is this activity influenced without freeze-thawing (No F-T) in the presence of 0.5 M sodium trichloroacetate or 0.5 M guanidine hydrochloride. However, the combination of chaotrope treatment followed by freeze-thawing results in the loss of PMS reductase activity as a function of the potency and the concentration of the chaotropic agent used. The shapes of the curves suggest a cooperative effect with respect to the concentration of chaotropes, and such cooperativity curves, as pointed out elsewhere (Hanstein and Hatefi, 1970), are consistent with the resolution of the enzyme system into its "subunits." Figure 6 shows the effect of the number of freeze-thawings on the resolution of succinate dehydrogenase monitored in the same manner as in the experiments of Figure 5. The experiment was conducted in the presence of only 0.3 M sodium trichloroacetate and at pH 8.0 in order to slow down the resolution process

[2] Recent unpublished studies have shown that this is indeed the case, because the equilibrium can be reversed either by removing the ClO_4^- ion with K^+ or by subsequent addition of water structure forming ions.

and be able to follow the disappearance of PMS reductase activity through several freeze-thawing steps. It is seen that the resolution of succinate dehydrogenase under these conditions proceeds exponentially with respect to the number of freeze-thawing events. Whether the effect of such freeze-thawing is more due to the known weakening of hydrophobic bonds at lower temperatures (Kauzmann, 1959; Scheraga *et al.*, 1962), or due to increased concentration of trichloroacetate ions near the protein molecules is yet to be investigated.

Discussion

1. Enzymatic Properties of Succinate Dehydrogenase. It has been shown that an essentially pure succinate dehydrogenase can be prepared from submitochondrial particles, which is stable under appropriate conditions and retains full enzymatic activity with respect to reduction of PMS and interaction with the electron transport system. Moreover, K_m^{Succ} of the purified enzyme is essentially unaltered during transition from membrane-bound to soluble form. With regard to enzymatic and reconstitution activities, the data presented here and elsewhere (Baginsky and Hatefi, 1969) suggest that the integrity of the iron–labile sulfide system of succinate dehydrogenase is essential for proper functioning of the enzyme. A slight damage to this system appears to be sufficient to cause a substantial decrease in the PMS reductase and complete destruction of the reconstitution activities. These considerations are crucial for study of the mechanism and sequence of electron transfer in succinate dehydrogenase, and point up the need for reevaluation of previous attempts with less intact preparations.

It has been shown in Figures 5 and 6 that the PMS reductase activity of succinate dehydrogenase is lost upon resolution of the enzyme even when both subunits, FP and IP, are present in the same mixture. These results may mean that the subunits, or at least FP, become denatured during resolution of succinate dehydrogenase by chaotropic agents. On the other hand, it is possible that PMS reduction requires the integrated action of both FP and IP, and that resolution has destroyed electronic communication between them. This thought brings to mind the fumarate reductase activity of succinate dehydrogenase. With the heart enzyme, fumarate reduction (electron donor: added $FMNH_2$) is much slower than succinate oxidation (electron acceptor: PMS). However, in yeast Singer and his colleagues (Hauber and Singer, 1967; Tisdale *et al.*, 1968) have shown the existence of a fumarate reductase, which contains FAD, has a molecular weight of 62,000–63,000, and no succinate–dye reductase activity. It is possible that mammalian FP, if it can be obtained without denaturation, might have fumarate reductase activity, and that IP might be responsible for changing the equilibrium of the system (succinate + oxidized FP-IP $\rightleftharpoons$ fumarate + reduced FP-IP) in the direction of succinate oxidation.

2. Stoichiometry of the Reconstitution Systems. Reconstitution studies involving succinate dehydrogenase and alkali-inactivated complex II, in which active complex II was isolated after differential centrifugation and removal of excess succinate dehydrogenase, showed that the amount of added succinate dehydrogenase now bound to complex II was exactly equal to the original amount of inactivated succinate dehydrogenase of alkali-treated complex II. Thus, the reactivated complex II contained one equivalent of active and one of inactivated succinate dehydrogenase. Whether the newly added complement of succinate dehydrogenase displaces the inactivated enzyme on complex II or simply adds onto a site which also bears the inactivated enzyme is now known. However, the results of preliminary experiments with ETP appear to favor the former possibility. A preparation of *active* ETP was mixed with severalfold excess of an aged succinate dehydrogenase preparation which was devoid of reconstitution activity. The mixture was centrifuged, and the ETP fraction was isolated and assayed for succinoxidase activity. This material had lost considerable succinoxidase activity as compared to a control ETP sample to which only buffer was added, thus indicating possible displacement of the *active* succinate dehydrogenase of ETP by the added, *inactive* succinate dehydrogenase. It might also be added here that addition of active succinate dehydrogenase to active ETP does not increase the succinoxidase activity of the latter.

It was shown in Figure 2 that the equivalence point of succinate dehydrogenase/alk-ETP mixtures as judged from attainment of maximal succinoxidase activity corresponded to a protein weight ratio of alk-ETP:SD of 8.9:1. The acid-unextractable flavin of ETP preparations used in these experiments was approximately 0.20–0.25 nmole/mg of protein. Assuming all such flavin in ETP is succinate dehydrogenase flavin, then the equivalence ratio of 8.9:1 in reconstitution experiments of the type shown in Figure 2 (*i.e.*, without centrifugation and recovery of the reconstituted ETP) would mean that 10.3 nmoles of succinate dehydrogenase flavin was added per 8.9 mg of total protein of ETP in the reconstitution mixture. In other words, saturation of alkali-treated ETP with active succinate dehydrogenase for restoration of full succinoxidase activity required the addition of at least 4.6–5.8 times as much succinate dehydrogenase as is normally present in ETP. A similar calculation as above for the succinate dehydrogenase/alkali-treated complex II indicates that restoration of full Q reductase activity to alkali-treated complex II preparations requires the addition of at least five times as much succinate dehydrogenase as is present in inactivated complex II preparations.

It is seen that the values for ETP and complex II reconstitution systems are very close. Combined with the results of Table II discussed above, these data suggest that incorporation of one equivalent of active succinate dehydrogenase into alkali-treated ETP or complex II particles requires the addition of approximately five times as much succinate dehydrogenase when both the soluble enzyme and the particle are present in the mixture. Unfortunately, as such this simple conclusion does not agree with the titration experiments shown in Figure 2, because in this figure extrapolation of the segment of the curve with limiting succinate dehydrogenase to zero activity goes through the origin and not through a point in the abscissa beyond zero succinate dehydrogenase. (The latter possibility would be expected from a cooperative binding.) It is not difficult, of course, to conceive of a picture that would agree with all the results, but in the absence of evidence we shall not belabor the point further.

3. Electron Transfer Pathway of Complex II. The electron transfer pathway from succinate to coenzyme Q remains an intriguing problem. It was shown earlier that in complex II both succinate dehydrogenase and cytochrome *b* can be reduced by succinate, that both the rate and the extent of cytochrome *b* reduction was increased in the presence of added coenzyme Q_2, and that cytochrome *b* reduction in the presence or absence of added Q_2 was inhibited by 2-thenoyltrifluoroacetone. However, the reduction rate of the bulk of cytochrome *b* was only a fraction of the rate at which coenzyme Q was reduced by complex II. No other component has yet been found in complex II, which could be considered as a possible electron carrier acting between succinate dehydrogenase and

coenzyme Q. Therefore, in the absence of such a component, the available evidence on complex II (Baginsky and Hatefi, 1969) and other preparations with succinate–coenzyme Q reductase activity (Bruni and Racker, 1968) permit of the following possibilities. (a) That cytochrome *b* of complex II is an intermediate electron carrier from succinate dehydrogenase to coenzyme Q, but that its redox potential in complex II is such that under steady-state conditions the concentration of reduced cytochrome *b* is very small. (b) That in the soluble state, succinate dehydrogenase is incapable of reducing coenzyme Q, but that it can do so without the intervention of any other intermediate when it is bound to an appropriate lipid–protein matrix. An essential component of this matrix might well be the cytochrome *b* of complex II.

That any preparation of cytochrome *b* will not replace the cytochrome *b* fraction of complex II has been tested with the use of complex III (reduced coenzyme Q–cytochrome *c* reductase) (Hatefi *et al.*, 1962). These preparations contain about 8 nmoles of cytochrome *b* and 4 nmoles of cytochrome c_1 per mg of protein. However, addition of succinate dehydrogenase to highly purified preparations of complex III failed to elicit any reconstituted activity. It might be added in this connection that recent evidence (Chance *et al.*, 1970) suggests the possible existence of more than one type of cytochrome *b* in the electron transport system.

Acknowledgments

The authors thank Mr. C. Munoz and Mr. P. Trejada for their most valuable technical assistance.

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