Peptides from Complex II Active in Reconstitution of Succinate-Ubiquinone Reductase*

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A preparation has been made from Complex II of beef heart mitochondria which contains in purified form two peptides, designated CII-3 and CII-4, with molecular weights of 13,500 and 7,000, respectively. Recombination of soluble succinate dehydrogenase with the peptides elicits ubiquinone reductase activity and, with Complex III, antimycin-sensitive cytochrome c reductase activity, while the "low $K_m$"-ferricyanide reductase activity (Vinogradov, A. D., Gavrikova, E. V., and Goloveshkina, V. G. (1975) Biochem. Biophys. Res. Commun. 65, 1264-1269) is lost. A ratio of 6 to 7 mol of the peptides/mol of succinate dehydrogenase is required for maximal ubiquinone reductase activity in the reconstituted system. The characteristics of the recombined mixture are those of Complex II, rather than ETP, in respect to the turnover number in the phenazine methosulfate reductase assay (38°C) and the pattern of inhibition by thenoyl trifluoroacetone.

Direct evidence for physical association of the enzyme and peptides during reconstitution has been obtained by immunoprecipitation of the mixture with antibody specific for the 70,000 dalton subunit of succinate dehydrogenase.

Chymotryptic digestion causes destruction of Peptide CII-3 and loss of ability to restore ubiquinone reductase activity, without apparent effect on Peptide CII-4. Thus an essential role of CII-3 in ubiquinone reductase activity may be inferred. Peptide CII-4 after this treatment is still able to combine with succinate dehydrogenase, as shown by immunoprecipitation. It is suggested that CII-4 may serve to bind the enzyme in the membrane or to bind and orient CII-3 for function in ubiquinone reduction.

An intermediate stage where activity is retained is detected in the chymotryptic breakdown of CII-3 at > 7000 daltons. Similarly, chymotryptic digestion of intact Complex II results in breakdown only of CII-3, and stops at an intermediate stage of ~ 9000 daltons, with no loss of activity. The rest of the molecule of CII-3, as well as CII-4, thus appears to be protected from chymotryptic attack in Complex II.

Cardiac succinate dehydrogenase is firmly attached to and partially buried in the mitochondrial inner membrane (2). In this environment it is stabilized against oxygen (3, 4) and it is able to reduce ubiquinone; this reaction is completely inhibited by TTF* and carbonamides (5, 6). These properties, characteristic of the membrane-bound enzyme, are lost when the enzyme is removed from the membrane, even when the soluble enzyme so obtained retains all the characteristics needed to be reincorporated in the membrane. Along with the loss of these properties, a new catalytic activity appears, a succinate-ferricyanide reductase that is functional at low concentrations of ferricyanide (referred to usually as "low $K_m$"-ferricyanide reductase) (7). Presumably, this activity is apparent while the enzyme is membrane-bound because the reaction site is inaccessible to ferricyanide, an impermeant anion. These differences, stability to oxygen, Q reductase activity, sensitivity to and binding of TTF, and absence of low $K_m$-ferricyanide reductase activity may be taken as definitive features of the membrane-bound enzyme.

The questions that naturally follow are how these properties are conferred on the enzyme and what components in the membrane are involved in binding the enzyme to the membrane. Since even the simplest of these membrane preparations, Complex II, contains, besides succinate dehydrogenase, two major polypeptides designated as CII-3 and CII-4 (8), cytochrome $b$, lesser amounts of other peptides from Complex III including cytochrome $c_1$, and a substantial quantity of lipid (9), it is not readily apparent which of these components might be active in these respects.

Complex II, however, contains Peptides CII-3 and CII-4, in amounts roughly equimolar to succinate dehydrogenase, as judged from their known molecular weights and relative staining intensities on polyacrylamide gels, and these peptides are not seen in gel patterns of Complexes III, IV, or V, except to the extent that succinate dehydrogenase is also present as a contaminant in the sample (8). These data, coupled with the fact that the two peptides are also present in immunoprecipitates obtained by treating detergent-dispersed submitochondrial particles with antibodies specific for succinate dehydrogenase (2), suggest that either CII-3 or CII-4, or both, could function in binding the enzyme to the membrane, or bringing about the changes in its properties, or both.

In continuing studies to define the native binding site of the enzyme, we decided to isolate the Peptides CII-3 and CII-4, and determine whether they could confer on succinate dehydrogenase the characteristic properties of the membrane-bound form of the enzyme. The present paper describes the isolation

-1 The abbreviations used are: TTF, thienoyl trifluoroacetone; DCIP, 2,6-dichlorophenolindophenol; Q, ubiquinone; DFB, 2,3-dimeroxy-5-methyl-6-pentyl-1,4-benzoquinone; DDB, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; PMS, phenazine methosulfate; SDS, sodium dodecyl sulfate; DOC, sodium deoxycholate; ETP, a nonphosphorylating inner membrane preparation from beef heart mitochondria; Complex II, succinate-ubiquinone reductase; Complex III, reduced ubiquinone-cytochrome $c$ reductase.
of these two polypeptides and their function in the reconstitution of Complex II, succinate-Q, and succinate-cytochrome c reductase activities.

EXPERIMENTAL PROCEDURES

Succinate dehydrogenase was isolated according to the method of Ackrell et al. (10) and stored after precipitation with ammonium sulfate as pellets in liquid N2. The pellets were dissolved anaerobically in 50 mM Tris/SO4 containing 0.1 mM EDTA and 20 mM succinate, pH 7.6 (0°C) (Tris/EDTA/succinate), and the enzyme maintained under a stream of oxygen-free argon until used. Complex II was prepared according to the method of Baginsky and Hatefi (11). Succinate dehydrogenase flavin contents were determined as in previous work (12). For the chymotrypsin experiments, the DOC-dispersed Complex II was not subjected to the final steps of the procedure, namely, Sephadex chromatography and centrifugation, but was instead dialyzed overnight under anaerobic conditions against 250 mM sucrose, 25 mM sodium phosphate, 20 mM succinate, pH 7.5 (4°C).

The preparation remained dispersed after this treatment, indicating the presence of residual DOC. Complex III was prepared by the method described by Rieske (13).

Succinate-phenazine methosulfate reductase activity was determined spectrophotometrically at 38°C in Tris/EDTA/succinate (pH 7.6 at 38°C) with DCIP as terminal electron acceptor (3). Succinate-ferricyanide reductase activity at the low Ks site (7) was assayed spectrophotometrically at 38°C in the same buffer, as described previously (3). Succinate-Q reductase activities referred to in this paper are actually measured with either the 8-pentyl- or 6-decyldervative of 2,3-dimethoxy-5-methyl-1,4-benzoquinone in lieu of ubi-quinone (14), and with 52 mM DCIP as the terminal electron acceptor, at 38°C in Tris/EDTA/succinate. Deactivated preparations of the enzyme were activated prior to assay by incubation with 20 mM succinate, at a protein concentration of 1.0 mg/ml, for 7 min at 38°C under anaerobic conditions. Protein was determined by the method of Lowry et al. (15) or Gornall et al. (16).

Reconstitution experiments were routinely carried out anaerobi- cally in O2-free argon in 50 mM Tris/phosphate, pH 7.0, containing 20 mM succinate and 0.5% (w/v) Triton X-100 or DOC, with 10 mM glucose, glucose oxidase, and catalase added to ensure anaerobiosis, at 22-25°C.

Antibody against the flavoprotein subunit of succinate dehydro- genase isolated from the enzyme by the method of Davis et al. (17) was generated in rabbits and partially purified by ammonium sulfate fractionation as previously described (2). Immunoprecipitations were generally carried out with the equivalent of 0.1 mg of succinate dehydrogenase/ml of antibody or control y-globulin fraction (from serum of the rabbit prior to immunization) in a medium containing 1% (w/v) Triton X-100, 100 mM NaCl, 125 mM sodium phosphate, pH 7.5, 25 mM sucrose, at a protein concentration of 1.0 mg/ml, for 7 min at 38°C, and stored on ice until used.

Isolation of Peptides CIII-3 and CIII-4—The source of the two peptides was Complex II, from which succinate dehydrogenase had been extracted with 0.8 mM sodium perchlorate in the presence of succinate and dithiothreitol (26). This material, suspended in 50 mM Tris/phosphate, pH 7, at a concentration of 10 mg of protein/ml, was twice washed with 2 mM sodium perchlorate for 30 min at 0°C to remove residual traces of succinate dehydrogenase and a significant fraction of compo- nents originating from contaminating Complex III. The residue, thus enriched in CIII-3 and CIII-4, was twice washed with Tris/phosphate to remove perchlorate, suspended in the same buffer at 5 to 10 mg of protein/ml, and extracted by stirring with 0.5% (w/v) Triton X-100 or DOC for 30 min at 0°C. The suspension was centrifuged for 40 min at 144,000 g, yielding a supernatant solution containing CIII-3 and CIII-4 which was stored on ice until used.

Fig. 1 compares the electrophoretic patterns of this peptide preparation, Complex II, and the soluble enzyme on SDS-
The gel pattern of the peptide preparation (Fig. 1C) indicates considerable purification with respect to Complex II, with Cn-3 and Cn-4, the only major components. The peptide preparation contained also cytochrome b and phospholipid (Table I). Cytochrome b was usually present at less than 5% of the mole ratio of Cn-3 to Cn-4, cytochrome c1 was usually absent, but occasionally it was found in amounts of 2.0 to 3.0 nmol/mg of protein. Most preparations of Complex II have not contained cytochrome c1. The phospholipid content of the preparation was determined as total phosphorus, as preparations of Complex II have not contained significant trichloroacetic acid-extractable phosphorus, other than that contributed by the succinate dehydrogenase flavin. The predominant phospholipid present was lecithin, as judged from thin layer chromatography; no cardiolipin was evident. On the basis of a molecular weight of 800 to 900 for lecithin, the phospholipid content may be calculated as ~ 20% in Complex II, and ~ 50% in the peptide preparation.

The peptides appear to be in the form of aggregates in the detergent extracts since they are excluded from columns of Sephadex G-200 equilibrated with 0.5% (w/v) DOC and fail to enter 7% (w/v) polyacrylamide gels run under nondenaturing conditions. Freezing, dilution, or, in the case of extracts made with DOC, extensive dialysis of the preparation resulted in precipitation of Cn-3 and Cn-4 and loss of reconstitutive function.

Reconstitution of Succinate-Q and Succinate-Cytochrome b Reductase Activities—Table II documents that on adding highly purified, soluble succinate dehydrogenase to an excess of the peptide preparation, Q reductase activity appears, succinate-PMS reductase activity is increased, and low Kₚ-ferricyanide reductase activity is lost. The changes thus resemble those occurring on reincorporation of succinate dehydrogenase into alkali-treated inner membranes (7, 27). The ratio of activities with PMS and DPB in the succinate dehydrogenase peptide mixture is higher than that in Complex II, but about the same as that obtained when Complex II is pre-treated with Triton X-100. Absolute activities are not directly comparable as the data are expressed on different bases, i.e. activity per mg of protein in the case of Complex II, activity per mg of added succinate dehydrogenase in the samples with the added peptides. The similarity in ratios of activities, however, implies that all of the succinate dehydrogenase added entered into functional reconstitution of an active Q reductase, since any soluble enzyme could only have contributed PMS reductase activity to the ratio. No loss of the reconstituted Q reductase activity occurred on extensive dilution of the sample, a fact that further implies physical combination (i.e. nondissociating), as well as functional association of the enzyme with the peptides.

The increased succinate-PMS reductase activity of the recombined enzyme and peptides, which was consistently 13 to 30% more than the activity of the uncombined enzyme, was accompanied by an increased Kₚ for PMS (Fig. 2A) much the same as in experiments where the enzyme is reconstituted with alkali-treated ETP (27). The combination of enzyme and peptides behaved more like Complex II than ETP, however, in the maximal level of activity reached, with a turnover number of 14,000 to 15,000 at 38°C, to be compared with 10,000 to 15,000 for Complex II, and 20,000 to 22,000 for ETP (6, 28). Moreover, the pattern of inhibition of the PMS reductase of the reconstituted complex by TTF was solely competitive, like that seen with Complex II and unlike that seen with ETP (6). Q reductase activity was inhibited noncompetitively, as in both Complex II and ETP, but the Kₛ was larger, ~ 70 μM (Fig. 2B) that than determined by us for Complex II (Kₛ ~ 10 μM at 38°C). The presence of Triton X-100 may be in part responsible for the higher Kₛ value, since pretreatment of Complex II with comparable concentrations of the detergent raised the Kₛ to ~ 40 μM.

Recombination of succinate dehydrogenase with the peptides also had the effect of stabilizing the enzyme, as documented in Table III.

Thus, in all these respects the reconstituted system behaved like unresolved Complex II, from which both the dehydrogenase and the peptides were originally isolated.

### Table I

<table>
<thead>
<tr>
<th>Component</th>
<th>Molar ratio of component to Cn-3 and Cn-4</th>
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<tbody>
<tr>
<td>Succinate dehydrogenase flavin</td>
<td>0 0</td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>1250 25</td>
</tr>
<tr>
<td>Cytochrome b</td>
<td>2 0.04</td>
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<tr>
<td>Cytochrome c₁</td>
<td>0 0</td>
</tr>
<tr>
<td>Ubiquinone*</td>
<td>0 0</td>
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* Molar ratio estimated in Ref. 8.

### Table II

<table>
<thead>
<tr>
<th>Sample</th>
<th>PMS</th>
<th>Fe(CN)₃⁺</th>
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<th>PMS:DPB</th>
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<tr>
<td>SDH*</td>
<td>73</td>
<td>24</td>
<td>0</td>
<td></td>
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<tr>
<td>SDH + Triton X-100</td>
<td>68</td>
<td>20</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>SDH + peptides</td>
<td>90</td>
<td>2-3⁺</td>
<td>40</td>
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<tr>
<td>Complex II</td>
<td>36</td>
<td>3⁺</td>
<td>40</td>
<td>0.9</td>
</tr>
<tr>
<td>Complex II + Triton X-100</td>
<td>31</td>
<td>3⁺</td>
<td>18</td>
<td>1.7</td>
</tr>
<tr>
<td>Complex II + Triton X-100 + SDH</td>
<td>20</td>
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</table>

* SDH = soluble succinate dehydrogenase.

Ferricyanide reductase activity is not reduced to 0 by loss of the low Kₛ reaction; these low activities may be attributable to reaction at the high Kₛ site (7).
The inclusion of DPB in the recombination mixtures did not change the stoichiometry of binding.

Reconstitution of Succinate-Cytochrome c Reductase Activity—The peptide preparation was necessary for interaction of succinate dehydrogenase with Complex III to generate succinate-cytochrome c reductase activity, as shown in Table IV. Lack of interaction between the soluble enzyme and Complex III alone has been previously reported (29) and is further documented by the data in Table IV. No loss of low $K_a$-ferricyanide reductase activity occurred, no Q reductase activity was generated, and only very small reactions with cytochrome c were observed even when extra quinone was added. Stoichiometry of Recombination—The specific activity of the peptide preparation cannot be directly determined since the peptides have no measurable activity by themselves. Addition of the peptide preparation to succinate dehydrogenase, however, as shown above, elicits Q reductase activity. The activity elicited is directly proportional to the amount of the peptides in a concentration range of 0.5 to 5 mg/ml of protein.

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added. Inclusion of the peptides, however, masked the low $K_m$-ferricyanide reductase activity, and elicited not only the Q reductase activity seen without Complex III, but also antimycin-sensitive cytochrome $c$ reductase activity. The activity in the absence of added DPB, which is almost completely antimycin-sensitive, is attributable to a direct reaction between the reconstituted "Complex II" and Complex III, facilitated by endogenous Q in the Complex III preparation (1.0 nmol of Q/mg of protein by analysis), and may imply physical association of the components. When DPB was also added, antimycin-sensitive cytochrome $c$ reduction was essentially doubled, and an antimycin-insensitive succinate-DPB-cytochrome $c$ direct reaction (14) also occurred, to the same extent as in a mixture of enzyme and peptides, with DPB and cytochrome $c$, without Complex III. Thus, for maximal cytochrome $c$ reductase activity, succinate dehydrogenase, the C$_{III}$ + C$_{IV}$ peptide preparation, DPB, and Complex III were all required.

Effect of Chymotrypsin on Peptides C$_{III}$- and C$_{III}$-—Since separation of Peptides C$_{III}$ and C$_{IV}$ had not been accomplished, indirect approaches were required to try and decide which of the two is involved in binding succinate dehydrogenase and which might be required for reconstituting Q reductase activity. An opportunity to test the importance of the C$_{III}$ component of the preparation in reconstitution was provided by the finding that it appears to be preferentially digested by chymotrypsin. As shown in Fig. 4, treatment of the purified peptide preparation with chymotrypsin resulted in essentially complete disappearance of the characteristic peak of Peptide C$_{III}$- which was accompanied, although not in a parallel fashion, by loss of the ability to elicit Q reductase on recombination with succinate dehydrogenase (Figs. 4 and 5), while at the same time, the low $K_m$-ferricyanide reductase activity remained unmasked (Table V).

![Diagram](image_url)

**Fig. 4. Effect of chymotrypsin on the composition of preparations of C$_{III}$- and C$_{III}$-** A preparation of C$_{III}$ and C$_{IV}$ (2.4 mg/ml) in 50 mM Tris/phosphate, pH 7.0, containing 0.5% (w/v) Triton X-100 was treated at 22-25°C with chymotrypsin (1 mg/ml in 50 mM Tris/HCl, pH 8.0) at a ratio of 1 mg of chymotrypsin/50 mg of peptide protein. Aliquots containing ~170 µg of peptides were removed from the incubation mixture at various time intervals and treated immediately with phenylmethylsulfonyl fluoride (1 mg/ml in ethanol) to give 0.9 mM concentration and then tested for reconstitutive activity as in Table II. Assays of activity after treatment were as under "Experimental Procedures," except that single concentrations of acceptors were used: 1.08 mM PMS, 200 µM Fe(CN)$_6^{3-}$, or 36 µM DDB + 50 µM DCIP. Activities are expressed as micromoles of succinate oxidized per min per mg of succinate dehydrogenase.

<table>
<thead>
<tr>
<th>Components added</th>
<th>Reductase activities</th>
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<tr>
<td></td>
<td>PMS</td>
</tr>
<tr>
<td>SDH* + peptides, untreated</td>
<td>80</td>
</tr>
<tr>
<td>SDH + peptides, chymotrypsin treated</td>
<td>86.3</td>
</tr>
<tr>
<td>SDH + peptides, chymotrypsin-treated, + untreated peptides</td>
<td>20.5</td>
</tr>
</tbody>
</table>

$^a$ SDH = soluble succinate dehydrogenase.

![Table V](image_url)

The ferricyanide reductase activity of succinate dehydrogenase without the peptides was 24. See also Table II.

The staining intensity of the C$_{III}$ peak increased during the digestion, probably through the contribution of the digestion products of C$_{III}$, but returned to the original peak height, as digestion continued (Fig. 4, ×—×). The absence of net change in the C$_{III}$ peak after activity had been lost during proteolysis is taken as evidence that this peptide was not attacked by chymotrypsin and that the failure of the chymotrypsin-treated peptide preparation to reconstitute Q reductase activity with succinate dehydrogenase resulted from degradation of C$_{III}$. The loss of activity was not due to the presence of inhibitory products, since normal reconstitution was observed if a sample of undigested peptide was added to a similar mixture, in the experiment documented in Table V.

Inspection of Fig. 4 reveals that during the early phase of digestion (1 min), where only 5% of the activity had been lost (Fig 5), the original peak of C$_{III}$ had almost disappeared, while a new component(s) became evident with molecular weight greater than 7,000, appearing as a broadening of the high molecular weight side of the C$_{III}$ peak (Fig. 4, dashed line). Thus, it appears that a limited digestion of C$_{III}$ does not prevent appearance of Q reductase activity on combina-

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**Fig. 5. Loss of activity of peptide preparation during chymotrypsin treatment.** Samples reserved as described under Fig. 4 were mixed with succinate dehydrogenase and tested for Q reductase activity as described in the legend of Fig. 3. The proportion of peptide to succinate dehydrogenase in the recombined mixtures is the same as that indicated by the arrow in Fig. 3, where peptides are in limiting amounts. In a control incubation of the peptide preparation with phenylmethylsulfonyl fluoride ± chymotrypsin, the peptide preparation retained over 75% of the starting activity over a 60-min period.
tion of the peptides with succinate dehydrogenase, although further degradation is accompanied by loss of activity. Corroboration of these findings comes from chymotryptic digestion of Complex II (Fig. 6). Apparently, CII-4 was the only component digested in this experiment, and the process stopped with the formation of an intermediate of ~9,000 daltons, in contrast to the experiment with the purified peptide (Fig. 4) where the intermediate was only transient. Apparently, in Complex II, Peptide CII-3 is combined with the dehydrogenase in such a way that it is protected from extensive proteolytic digestion. Of particular interest is the fact (not shown) that the Complex II preparation of Fig. 6 retained full endogenous Q reductase activity and sensitivity to TTF even after digestion with chymotrypsin for 2 h. Subsequent extraction of the succinate dehydrogenase from this chymotrypsin-treated Complex II preparation, followed by detergent extraction of the residue as described above, produced a peptide preparation containing CII-4 and the partially digested form of CII-3. The preparation had the same reconstitutive powers as a preparation containing CII-4 and unmodified CII-3. Further treatment of this preparation with chymotrypsin, however, where there was no longer the protective effect of the native Complex II structure, resulted in the rapid and complete digestion of the partially digested form of CII-3 and loss of the preparation’s ability to restore Q reductase activity.

Enzyme Binding by CII-3 and CII-4—The evidence thus far presented, attesting to complex formation between CII-3 and CII-4 and added succinate dehydrogenase, has been indirect in nature and predicated largely on the masking of the low K_m ferricyanide reaction site of the enzyme and concomitant production of Q reductase activity. An immunological approach was used, therefore, to try to provide direct evidence for a physical association of the enzyme with the peptides, and also to determine whether CII-4 could independently bind the enzyme after proteolytic destruction of CII-3. Even though CII-3 appears to be required for the transformation in catalytic activities that occurs on recombination of the enzyme with the intact peptide preparation.

A stoichiometric amount of enzyme (about 1 mol of enzyme/30 mol of CII-4 or CII-3) was recombined with the purified peptide preparation under the conditions used for reconstitution of Q reductase activity (see “Experimental Procedures”) and the mixture was assayed to ensure that DPB reductase activity was present and that the low K_m ferricyanide reductase activity was lost. Antibody to the 70,000-dalton subunit of the enzyme was then added, plus extra Triton X-100 to bring its concentration to 1%, and the mixture was left at 4°C overnight. Mixtures of antibody with enzyme alone, with peptides alone, and mixtures of control γ-globulin fraction with enzyme alone, peptides alone, and with enzyme plus peptides, served as controls. Significant precipitation occurred only in mixtures containing both enzyme or enzyme plus peptides and antibody; only traces of protein residues were obtained after centrifugation of all other mixtures. All precipitates were subjected in toto to SDS-polyacrylamide gel electrophoresis (9%, Swank-Munkres (18)) in order to establish their respective peptide compositions. Fig. 7A shows the peptide band pattern of the immunoprecipitate obtained by treating precombined enzyme and peptides with the antibody. Present in the precipitate were the 70,000-dalton subunit of the enzyme, γ-globulin, a band in the 30,000 molecular weight region corresponding to both globulin and the small subunit of the enzyme, and Peptides CII-3 and CII-4. When the peptide preparation was treated with the antibody in the absence of succinate dehydrogenase only a trace precipitate was obtained. The densitometric scan for this precipitate shows some high molecular weight bands, but none corresponding to the two subunits of the enzyme or CII-3 and CII-4 (Fig. 7B). Since the high molecular weight bands were observed also in roughly equivalent amounts in the band patterns of minor precipitates obtained with control γ-globulin (data not shown), they were attributed to nonspecific precipitation of serum proteins. Thus, whereas CII-3 and CII-4 were not themselves precipitated by the antibody specific to the 70,000-dalton subunit of succinate dehydrogenase, they were carried down in the immunoprecipitate formed when the enzyme was also present (Fig. 7A) and, therefore, must have been in tight association with the enzyme.

A complementary series of experiments was done with the peptide preparation depleted by chymotrypsin treatment of CII-3 and also the capacity (<10% of normal) to confer Q reductase activity on the enzyme. Extensive co-precipitation of CII-4 with the enzyme occurred in the presence of antibody, demonstrating that CII-4 was able to bind to the enzyme in the absence of CII-3. The densitometric tracing for the immunoprecipitate is shown in Fig. 7C, and shows clearly both succinate dehydrogenase subunits and CII-4.

Smaller precipitates were obtained also in the control mixtures that were run, namely, mixtures of chymotrypsin-treated peptide preparation with antibody or control γ-globulin fraction, as well as chymotrypsin-treated peptide preparation plus enzyme plus control γ-globulin fraction, for which the gel scan is also shown in Fig. 7C. In all the controls, the small precipitates contained the high molecular weight serum proteins referred to above, with significant amounts of CII-4. The
amount of CII-4 precipitating, however, was constant as judged from staining intensities in gel scans of the respective precipitates, and was thus independent of the presence of either the antibody or control γ-globulin. The amount of this nonspecifically precipitating CII-4 was considerably less than that obtained in the presence of enzyme and antibody, a point which is illustrated in Fig. 7D, which depicts the difference computed between the staining profiles shown in Fig. 7C. Calculations for the high molecular weight portion of the gel scans are not represented due to lack of resolution of the bands and the fact that the excessive amounts of protein in this range caused the staining intensities of the high molecular weight bands of the immunoprecipitate to deviate from Beer’s law.

DISCUSSION

The data presented in this paper show that preparations containing Peptides CII-3 and CII-4 enable succinate dehydrogenase to interact with Q analogs and Complex III and, consequently, to transfer electrons to cytochrome c via the antimycin-sensitive pathway. The recombined mixture resembled closely Complex II in reactivity with PMS and DPB, in the characteristics of TTF inhibition of these reactions, and in relative stabilization of the dehydrogenase.

To generate maximal levels of Q reductase activity approximately 6 to 7 eq of CII-3 and CII-4 were needed/mol of succinate dehydrogenase, a stoichiometry in contrast with the 1:1 proportions evident in Complex II as isolated, and perhaps suggestive of only a loose association, if any, between the enzyme and the peptides. The interaction, however, also resulted in masking of the low K_m-ferricyanide reaction site of the enzyme and it was not affected by extensive dilution of the mixture; these facts implied complex formation. Direct evidence of a tight association of these components was obtained immunologically, with the demonstration that the antibody specific for the 70,000-dalton subunit of succinate dehydrogenase, and thus unreactive toward Peptides CII-3 and CII-4, precipitated both enzyme and peptides from detergent solution. Thus, the excess of peptides required in reconstitution experiments is not readily explained by lack of physical association of the peptides with the enzyme, with consequently greater concentration requirements. At this time, explanations most consistent with all the data are that some inactive or denatured peptides might be present in the preparations, or, since the peptides are in aggregated form as isolated, that peptides within the aggregates are unavailable for recombination.

While a requirement for a peptide component is demonstrated by the destructive effect of chymotryptic action, additional components such as phospholipid and cytochrome b may also be needed, as observed by Bruni and Racker (30) and McPhail and Cunningham (31), and these, if limiting, could contribute to the high peptide/enzyme ratio required for full activity. Phospholipids, however, mainly lecithin, were present in the peptide preparation, amounting to 150 to 175 mol (as P)/mol of succinate dehydrogenase in the recombined mixture when enough peptide preparation was added to generate full Q reductase activity. (This may be compared with the value of 52 mol of P/mol of enzyme in Complex II). The addition of further phospholipid (as asolectin or beef lecithin) neither increased the maximal activity obtainable nor decreased the excess of peptides required in reconstitution experiments.

We have noted that our preparation contains cytochrome b, but in low amounts (Table I). When enough of the peptide preparation was added to achieve full Q reductase activity, the cytochrome b was still not stoichiometric with the succinate dehydrogenase present in the recombined mixture, but only about 0.3:1. This is not too different, however, from the

FIG. 7. Immunoprecipitation of enzyme/peptide mixtures before and after chymotryptic digestion of the peptides. Treatment of the peptide preparation with chymotrypsin was as described for Fig. 4, except that a single time interval of 2 h was used. After digestion and addition of phenylmethylsulfonyl fluoride the peptides were mixed with succinate dehydrogenase under the conditions used for reconstitution (see "Experimental Procedures"), at a ratio of 80 μg of succinate dehydrogenase/480 μg of peptides. The mixtures were treated with antibody and the immunoprecipitates analyzed on disc gels as described under "Experimental Procedures." A, untreated peptide preparation plus succinate dehydrogenase plus antibody; B, untreated peptide preparation plus antibody (no succinate dehydrogenase); C, chymotrypsin-treated peptide preparation plus succinate dehydrogenase, plus antibody (—) or control γ-globulin fraction (—). D, scan obtained by subtracting the dashed line from the solid line in C. The figures in parentheses in A and C are mobilities of the components relative to the dye marker.
cytochrome b/enzyme ratio in our preparations of Complex II (0.5:1 in the preparation in Table I), which also have about the same activity as the recombined mixture when assayed under the same conditions (turnover numbers ~ 5500 and 5600, respectively, in the succinate-Q reductase assay at 38°C). In either case, the low content of cytochrome b makes it difficult to rationalize a catalytic role for the cytochrome b component in Q reductase activity. Moreover, it has been determined that the cytochrome b component of Complex II is reduced by succinate only at a slow rate, much less than Q reduction, and only to the extent of ~ 40%, although the preparation used contained cytochrome b in 1:1 stoichiometry with succinate dehydrogenase (11).

A possible structural role for cytochrome b in the organization of the succinate-Q reductase complex of the mitochondrial electron transport system has been suggested by the work of Bruni and Racker (30) and McPhail and Cunningham (31). While it is possible that their preparations of cytochrome b, admittedly impure, could have derived their activity from contamination with peptides such as those described here, it cannot be eliminated that the converse may be true, that one of our peptides may be derived from cytochrome b, having lost part of the heme, i.e. that it is a cytochrome b apoprotein. Were this true, then the cytochrome b apoprotein would have to be of lower molecular weight than the value of 28,000 ordinarily estimated for the cytochrome. 'lost part of the heme,' the mitochondrial electron transport system has been suggested by the work of Bruni and Racker (30) and McPhail and Cunningham (31) as a possible role for cytochrome b, which would be consistent with the findings of Thomas P. Singer for helpful discussions and to Ms. Marianne Gallup for her excellent technical assistance.

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