STUDIES ON THE TERMINAL ELECTRON TRANSPORT SYSTEM

VI. FRAGMENTATION OF THE ELECTRON TRANSPORT PARTICLE WITH DEOXYCHOLATE

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The electron transport particle (ETP) is a submitochondrial unit which catalyzes the oxidation of succinate and DPNH by molecular oxygen without supplementation[1]. From evidence presented in this communication and to be presented in other communications it now appears likely that ETP contains approximately equal amounts of the cytochrome chains which respectively implement oxidation of succinate and DPNH and which though separable under certain conditions are functionally and structurally interlocked in the intact particle. In the present communication the fragmentation of ETP by deoxycholate into two particles is described. Under the conditions used the fragmentation leads to a red particle with all of the original succinic dehydrogenase activity and a green particle with DPNH dehydrogenase activity exclusively. The bulk of the cytochrome *a* component of ETP is localized in the green particle while cytochromes *b* and c_1 are distributed between the two particles.

METHODS

Total heme, flavin and protein were determined by procedures which have been described elsewhere[2,3]. The concentration of cytochromes *a*, *b* and *c* plus c_1 has been estimated either directly from the absorption spectra of the particle by the method of CHANCE[4], or from the absorption spectra of the individual hemes after these had been separated into two groups (*cf.* BASFORD *et al.*[3]). Spectra were recorded with a Beckman DK-2 spectrophotometer.

RESULTS

Conditions for fragmentation of ATP by deoxycholate

As the ratio of deoxycholate to protein is increased progressively an increased proportion of ETP remains soluble after ultracentrifugation under the conditions described below. At relatively low concentrations of deoxycholate, cytochrome *c* associated with some DPNH–ferricyanide activity** is extracted. Then at higher concentra-

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** The various oxidative activities of particles are expressed in terms of electron donor–electron acceptor pairs such as succinate–ferricyanide, succinate–cytochrome *c*, DPNH–oxygen, etc. The activation of the substrate is invariably the prerogative of the primary flavoprotein dehydrogenase but the electron acceptor may interact with the particle at a site other than on the primary dehydrogenase. The substrate–ferricyanide reaction is largely a measure of the primary dehydrogenase whereas the substrate–oxygen or substrate–cytochrome *c* reaction is almost exclusively a measure of the heme chain or at least of components which follow the dehydrogenase in the electron

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tions of deoxycholate, succinic–cytochrome *c* activity**, DPNH–cytochrome *c* activity** and finally cytochrome–oxidase activity** are extracted in that order***. The relationship between deoxycholate concentration and the relative amounts of DPNH and succinic–ferricyanide activity in the solubilized extract is shown in Fig. 1.

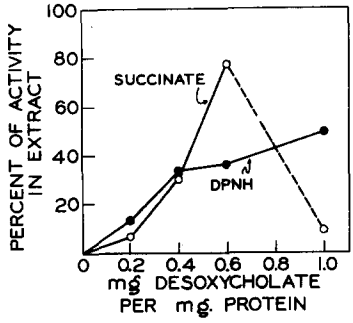

Fig. 1. Extraction from ETP of DPNH and succinic-ferricyanide activity at varying deoxycholate: protein ratios. In each individual experiment 20 ml of an ETP suspension (0.01 *M* Tris buffer of pH 8.0) containing 10.4 mg per ml was exposed to deoxycholate at the concentration indicated in the Fig. and then was centrifuged at 75,000 × *g* for 45 min. There were 925 units of DPNH-ferricyanide and 290 units of succinic-ferricyanide activity in the starting 208 mg of ETP protein.

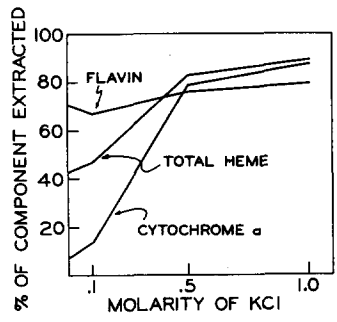

Fig. 2. Extraction of cytochromes and flavoprotein from ETP as a function of the concentration of KCl. The values on the ordinate refer to the percentages of the total of each component found in the supernate. A suspension of ETP (62 mg) was treated with deoxycholate (31 mg) in a total volume of 5.3 ml in presence of varying amounts of KCl.

The ionic strength of the suspending medium has a pronounced effect on the deoxycholate solubilization. For a given level of deoxycholate the amount of protein and heme extracted is increased as the ionic strength is raised but the amount of flavin extracted is virtually independent of the ionic strength. When the deoxycholate level is 1 mg per mg protein and the concentration of KCl is 1.0 *M* almost all of ETP is "solubilized". The small residue appears as a dull yellow precipitate. Fig. 2 shows the effect of KCl concentration on the extraction from ETP of cytochrome *a*, flavin and total cytochrome at a deoxycholate concentration of 0.5 mg per mg protein.

The following procedure for quantitative separation of succinic activity into one of two fractions has been found to be satisfactory in many repeat runs. All manipulations were carried out in the range of 0 to 3°.

A suspension of ETP in 0.25 *M* sucrose, prepared as described previously[1] is adjusted to a protein concentration of 20 mg per ml and mixed with an equal volume of 0.2 *M* Tris chloride of pH 8.0. Deoxycholate (10 % solution adjusted to pH 7.5) is then added in sufficient amount to achieve a concentration of 0.5 mg deoxycholate (expressed as deoxycholic acid) per mg of protein. The suspension which is partially clarified by the addition of deoxycholate is then centrifuged for 45 min at 105,000 × *g*

transfer sequence. It should be stressed that in referring to the succinic or DPNH dehydrogenase activity of the particle there is no implication that the dehydrogenase in question is not an intrinsic part of the particle. The dehydrogenase can be considered as a separate entity only in the restricted sense of what a particular assay system measures and not in a structural sense.

*** The pattern of cytochrome extractions found in our studies is similar in some respects to those found by STOTZ and coworkers[5,6] and EICHEL *et al.*[7] in extracts prepared by deoxycholate treatment of beef and pig heart muscle.

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(40,000 r.p.m.) in the Spinco preparative ultracentrifuge. The supernate is red while the residue is green. The green precipitate is taken up in 0.25 *M* sucrose.

The red supernate, after dialysis for 18 hours against 0.02 *M* phosphate buffer of pH 7.5, is centrifuged at 105,000 × *g* for 30 min. A red precipitate and yellow-red supernate are obtained. The red precipitate is taken up in 0.25 *M* sucrose.

Enzymic properties of the fractions

The succinic–cytochrome *c*[2] activity as well as the succinic–ferricyanide[2] activity of the red particle is 4 to 5 times higher than the succinic–oxygen[1] or succinic–ferricyanide activity[1] of the starting ETP. This in conjunction with the twin facts (1) that the bulk of the original succinic activity (*ca.* 90%) is retained in the red particle fraction, and (2) that the green particle is essentially inactive with respect to succinic dehydrogenase activity provides clear evidence that in the fragmentation of ETP under the special conditions described above succinic activity is localized in only one of the two fragment particles.

Under the same preparative conditions that lead to an all or none type of distribution of succinic activity there is approximately equipartition of DPNH–ferricyanide activity[1] between the red and green particles as shown in Table I.

TABLE I

SEPARATION OF ENZYMIC ACTIVITY BY DEOXYCHOLATE

Fraction	*Total protein*	*Ferricyanide as electron acceptor*			
		DPNH		*Succinate*	
	mg	*μmoles/min/mg**	*Units*	*μmoles/min/mg*	*Units*
ETP	1100	2.4	2640	0.87	955
Green particle	390	2.8	1100	0.20	78
Deoxycholate extract (A)	715	1.2	860	1.17	840
Red particle	143	5.8	830	4.86	696
Dialyzed supernate (A)	282	0.6	169	0.17	48
		Oxygen as electron acceptor			
ETP		3.3	3600	1.2	1320
Green particle		5.3**	2100	0.28**	110
		Cytochrome c as electron acceptor			
Deoxycholate extract (A)		1.0	715	1.5	1080
Red particle		1.55	220	5.53	765

* All activities expressed as μmoles of substrate oxidized per min per mg protein.

** Cytochrome *c* is required for oxidation of DPNH or succinate by oxygen in presence of the green particle.

The red particle corresponds closely in properties to the succinic dehydrogenase complex (SDC) which GREEN *et al.*[2] obtained by treating mitochondria with *tert.*-amyl alcohol and then retreating the light particle thus derived with *iso*butyl alcohol. The important differences are that the SDC preparation of GREEN *et al.*[2] show a lower amount of DPNH dehydrogenase activity and furthermore that this activity is demonstrable only in the ferricyanide assay and not in the cytochrome *c* reductase assay.

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By exposing ETP to a level of deoxycholate equivalent to 0.2 mg per mg of protein a red fraction can be prepared which corresponds more closely to the SDC previously described in that it shows good DPNH–ferricyanide activity but relatively low DPNH–cytochrome *c* activity (< 0.5 μmole/min/mg). At this low level of deoxycholate however a considerable amount of succinic activity remains unextracted in the green particle in the form of succinic–oxygen activity.

The green particle catalyzes the oxidation of DPNH by cytochrome *c* or ferricyanide and the oxidation of DPNH by molecular oxygen[1] in presence of added cytochrome *c*. It resembles in some respects the DPNH oxidase of MACKLER AND GREEN[8] after this unit has been "opened" with deoxycholate. Such a treated unit now shows a requirement for added cytochrome *c* in the DPNH–oxygen reaction. There are two important differences between the green particle prepared by the method described above and the DPNH oxidase of MACKLER AND GREEN. In the former both the succinic dehydrogenase and the corresponding *b* and c_1 hemes of the succinic chain have been extracted while in the latter only the dehydrogenase has been extracted without the corresponding hemes. Thus the ratio of *a* heme: c_1 hemes is roughly twice as high in the green particle as in the DPNH oxidase of MACKLER AND GREEN. It is this fact which accounts for the deeper green color of the green particle as compared to the DPNH oxidase.

Spectra of the fractions

Fig. 3 shows a comparison of the reduced spectrum of ETP, with that of the red and green particles. The almost complete disappearance of the component absorbing at 607 mμ (cytochrome *a*) in the red particle and the twofold increase in this peak relative to the 554 and 564 mμ peaks in the green particle provide an impressive demonstration of the effectiveness of the fragmentation procedure.

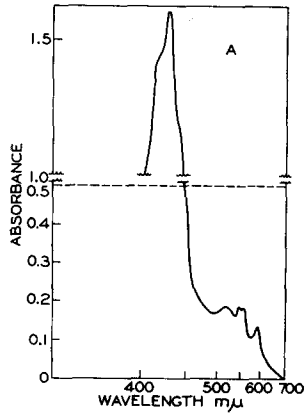

Fig. 3a

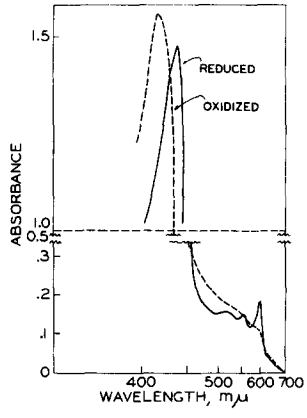

Fig. 3b

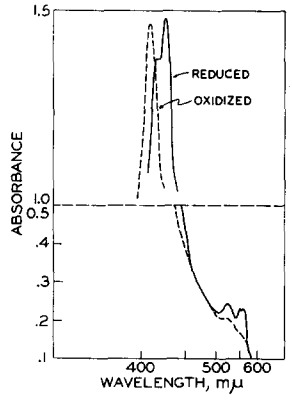

Fig. 3c

Fig. 3a. Absorption spectrum of ETP after reduction with dithionite. Conditions: 3.2 mg protein, 50 mg deoxycholate, final volume 0.75 ml. The ordinate has two separate scales for absorbance—0 to 0.5 and 1.0 to 1.5.

Fig. 3b. Absorption spectrum of the green particle in the oxidized form and after reduction with dithionite. Conditions: 3 mg protein, 100 mg deoxycholate, final volume 0.75 ml.

Fig. 3c. Absorption spectrum of the red particle in the oxidized form and after reduction with dithionite. Conditions: 1.9 mg protein 50 mg deoxycholate, final volume 0.75 ml.

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The supernate obtained after treatment of ETP with deoxycholate but before dialysis shows a spectrum similar to that of the precipitated red particle with the exception that there is more absorption in the region at 554 mμ. After precipitation of the red particle the supernate shows a component absorbing at 550 mμ which is probably cytochrome *c*.

Composition of the fraction

Table II shows the total amount of cytochromes *a*, *b*, c_1 + *c*, flavin and non-heme iron in the original ETP, in the derivative red and green particles and in the final supernate after removal of the red particle. Cytochrome *a* is localized almost exclusively in the green particle; *b* and c_1 are approximately divided between the red and green particles whereas all of the *c* remains in the final supernate.

TABLE II

COMPOSITION OF DEOXYCHOLATE FRACTIONS

Fraction	*Concentration of components in μmoles × 10⁻³ per mg protein*				
	Flavin	*Cyt. a*	*Cyt. b*	*Cyt. c + c_1*	*Total heme*
ETP	0.43	0.76	0.69	0.65	2.10
Green particle	0.35	1.40	0.63	0.68	2.80
Deoxycholate extract (A)	0.51	0.10	0.79	0.68	1.45
Red particle	0.85	0.35	2.30	1.65	4.20
Dialyzed supernate (A)	0.60	0.00	0.14	0.40	1.00

The individual hemes were estimated by the method of CHANCE[4].

Flavin is distributed between the red and green particles and the final supernate to the extent of about 30% in each. Since the final supernate contains neither succinic nor DPNH dehydrogenase activity and since the recovery of succinic and DPNH dehydrogenase activity in the two particles compared to that of the original ETP is fairly high, it is not easy to account for the relatively large fraction of total flavin in the supernate on the assumption that the bound flavin of ETP is implicated either in the succinic or DPNH dehydrogenase.

Reduction of the cytochrome components by substrates

When DPNH or succinate is added to ETP under anaerobic conditions the cytochromes are fully reduced, with the exception of cytochrome *b* which is usually reduced, to the extent of only 80%. The same result with either succinate or DPN has been found to apply to the hemes of the green particle even though 95% of the original succinic dehydrogenase activity (in ETP) has been separated from the particle. These results show very clearly the close electronic communication which must prevail not only between adjacent succinic and DPNH chains but also between one DPNH chain and another. If only one in twenty succinic chains is intact and if reduction by succinate of all the hemes whether of the succinic or DPNH chains can be observed then an elaborate arrangement of intercommunication between adjacent heme chains has to be invoked.

The reduction of cytochromes *b* and c_1 in the red fraction is never as complete with either succinate or DPNH as in ETP or the green particle. Usually succinate re-

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duces 50 to 80 % of *b* and c_1 whereas DPNH reduces 20 to 80% of c_1 and very little of *b*. The difference spectra of Fig. 5 show the reduction of the hemes of the red and green particles by DPNH and succinate respectively.

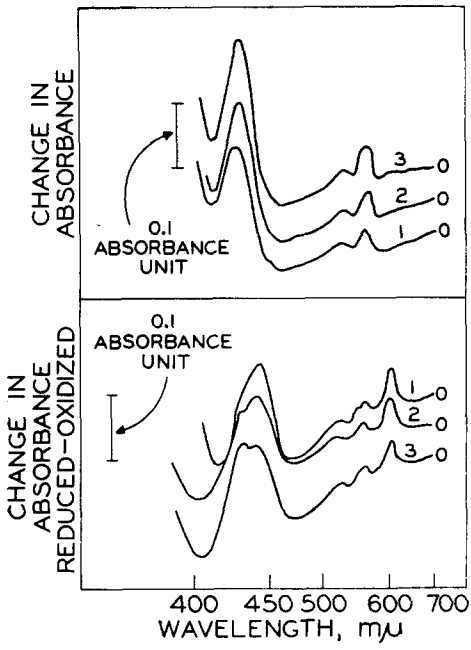

Fig. 4. Difference spectra (reduced minus oxidized) of the red particle after addition of (1) DPNH, (2) succinate and (3) dithionite respectively to each of three separate samples. The base line for the density scale is displaced from one spectrum to the next. Conditions: 1.2 mg protein, 20 μmoles phosphate buffer (pH 7.5), final volume 1.0 ml. The experiments were carried out under anerobic conditions. The samples were reduced respectively with 5 μmoles DPNH, 20 μmoles succinate or a few grains of dithionite.

Fig. 5. Difference spectra (reduced minus oxidized) of the green particle after addition of (1) DPNH (2) succinate and (3) dithionite respectively to each of three separate samples. Conditions: 3 mg protein, 20 μmoles Tris chloride (pH 7.5), 0.2 μmole KCN, final volume 1.1 ml. Other details as in legend for Fig. 4.

DPNH oxidation in the green particle

Both phosphate ions and Versene stimulate the DPNH-oxygen reaction catalyzed by ETP[1]. The same has been found to apply to the equivalent reaction catalyzed by the green particle.

The rate of the DPNH–oxygen reaction catalyzed by the green particle, which requires cytochrome *c*, is 2 to 5 times faster than the rate of the DPNH–cytochrome *c* reaction. This discrepancy suggests that some of the added cytochrome *c* is bound to the green particle and that the turnover of this bound cytochrome *c* in the DPNH oxygen reaction is much higher than that of external *i.e.* non-bound *c*. When ETP is exposed to a relatively high deoxycholate concentration under the conditions of the opening phenomenon described by MACKLER AND GREEN[8] the rate of the DPNH–cytochrome *c* reaction is identical with that of the DPNH–oxygen reaction. The chain of a particle treated in this manner is referred to as fully open. But in the light of the observations made in this communication it would appear that at this higher level of deoxycholate the DPNH chain is fragmented between the *b*, c_1 and *a* hemes. Thus opening of the chain is in this instance indeed a consequence of fragmentation. The properties of the green particle which appears to contain an unfragmented DPNH heme chain suggest a situation intermediate between the fully closed and fully open chain, *viz.* a complete requirement for added cytochrome *c* in the oxygen reaction but a greater rate for the DPNH-oxygen reaction than for the DPNH-cytochrome *c* reaction.

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Antimycin A[9] inhibits the ETP-catalyzed oxidation of DPNH and succinate by molecular oxygen. It also inhibits the green-particle catalyzed aerobic oxidation of DPNH and the red-particle catalyzed oxidation of DPNH or succinate by cytochrome *c*. To demonstrate antimycin inhibition in the cytochrome reductase assay the enzyme has to be first incubated with the reagent for 2 minutes without albumin or cytochrome *c* present.

DISCUSSION

The observations reported in the present communication are compatible with the following interpretation. ETP contains both succinic and DPNH chains in some sort of network arrangement. When the deoxycholate: protein ratio is increased to a critical value all succinic chains are cleaved and the corresponding b c_1 hemes are "solubilized" by the reagent. In addition a moiety of the DPNH chains is correspondingly cleaved. The green residue in this case contains intact DPNH chains plus the a hemes corresponding to the cleaved succinic and DPNH chains. As the deoxycholate: protein ratio is further increased all the chains become solubilized and only some as yet unidentified yellow residue resists solubilization.

There are several characteristics of the particles isolated which indicate that their electron transfer systems are not basically different from those of ETP. First the recovery of enzymic activity in the various fractions is excellent in relation to the original activity of ETP. A greater than theoretical recovery might argue that dispersion of the original particle into smaller particles had made available catalytic sites blocked off in the intact particle. This cannot be a major consideration. Secondly the integrity of the cytochrome components in ETP has not been affected at least to the extent of changes in spectral characteristics as a consequence of fragmentation.

TABLE III

RECOVERY OF COMPONENTS IN DEOXYCHOLATE FRACTIONS

Fraction	*Total component in each fraction*					
	Protein mg	*Flavin* $\mu M \times 10^{-3}$	*Cyt. a* $\mu M \times 10^{-3}$	*Cyt. b* $\mu M \times 10^{-3}$	*Cyt c + c*$_1$ $\mu M \times 10^{-3}$	*Total heme* $\mu M \times 10^{-3}$
ETP	1100	472	835	750	715	2310
Green particle (G)	390	137	546	246	258	1090
Deoxycholate extract (A)	715	365	72	565	485	1040
Red particle	143	122	49	329	236	600
Dialyzed supernate	282	170	0	40	113	282
Total recovery (G) + (A)	1105	502	618	811	743	2130

Furthermore this integrity is vouchsafed by the fact that the hemes of the green particle are rapidly reduced by succinate or DPNH. This last condition does not apply with equal force to the red particle. The cytochromes of the red particle are only partially reduced by succinate and to a lesser extent by DPNH which is either an indication of a certain amount of derangement in the electron transfer chain or a consequence of the removal of some component attached to the a heme moiety which is lost during the cleavage by deoxycholate. The fact that antimycin still inhibits the reactions catalyzed by the red as well as the green particle is particularly significant. If the

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DPNH activity of the green or red particles were a consequence of the adsorption on the particle of soluble DPNH cytochrome *c* reductase[6] then antimycin sensitivity would not be expected in either case.

ACKNOWLEDGEMENTS

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SUMMARY

At an appropriate ratio of deoxycholate to protein the electron transport particle is fragmented into a red particle similar to the succinic dehydrogenase complex and a green particle which acts as a DPNH oxidase when supplemented with cytochrome *c*. The red particle contains cytochromes *b* and c_1 (but not *a*) and catalyzes the oxidation of succinate or DPNH by ferricyanide and cytochrome *c* but not by oxygen. The green particle contains cytochrome *b*, c_1 and *a* and catalyzes the oxidation of DPNH (but not succinate) by cytochrome *c* or by oxygen in presence of cytochrome *c*. Antimycin inhibits the oxidation of succinate by cytochrome *c* (catalyzed by the red particle) or oxidation of DPNH by oxygen or cytochrome *c* (catalyzed by the green particle).

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VII. FURTHER STUDIES ON THE SUCCINIC DEHYDROGENASE COMPLEX

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The first paper of this series by GREEN, MII AND KOHOUT[1] dealt with the preparation and properties of a particulate succinic dehydrogenase complex (SDC), which catalyzes the dehydrogenation of succinate by cytochrome *c*, ferricyanide, phenazine methosulfate or 2,6-dichlorophenolindophenol.

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