FLAVOPROTEINS OF THE ELECTRON TRANSPORT SYSTEM AND THE SITE OF ACTION OF AMYTAL, ROTENONE, AND PIERICIDIN A

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Previous studies[1-4] have shown that the mitochondrial electron transport system can be divided into four enzyme complexes: DPNH-coenzyme Q reductase (complex I),[5] succinate-coenzyme Q reductase (complex II), reduced coenzyme Q-cyt. *c* reductase (complex III), and cyt. *c* oxidase (complex IV). When mixed together in the presence of cyt. *c*, these complexes recombined in the sequence and the approximate ratio of components found in intact mitochondria, and thus reconstituted a particulate unit with all the apparent catalytic and inhibitor-response properties of intact electron transfer particles (ETP). On the basis of these findings, the tentative structure shown in Figure 1 was proposed for the electron transport system. Subsequently, it was shown[4] that complex I could be resolved into three fractions: a DPNH dehydrogenase ferroflavoprotein (FP) (mol wt $\simeq$ 70,000) containing FMN, iron, and labile sulfide in an approximate ratio of 1:4:4; an iron-sulfur protein (IP) containing equimolar amounts of iron and labile sulfide but no flavin; and a third protein fraction with properties similar to the mitochondrial "structural" proteins. Moreover, it was demonstrated that electron transfer between the resolved components of complex I can occur as shown in reaction (1):

$$\text{DPNH} \rightarrow \text{FP} \rightarrow \text{IP} \rightarrow \text{Q}. \qquad (1)$$

Meanwhile, the laboratories of Singer and Sanadi and recently those of Chance, Ernster, and their colleagues have published material which fully agrees with our results, but each has advanced a different interpretation of the structure of the segment of the respiratory chain represented by complex I. In this communication, these results will be separately examined, and, in the light of our recent studies, an explanation encompassing the results obtained by ourselves and the above investigators will be offered.

Materials and Methods.—Particulate DPNH-cyt. *c* reductase (the binary I–III complex) was prepared according to the method of Hatefi *et al.*;[6] cytochrome oxidase according to that of Fowler *et al.*;[7] complex I according to the procedure of Hatefi *et al.*;[1] and FP and IP according to that of Hatefi and Stempel.[4] Iron was estimated by the procedure of Doeg and Ziegler,[8] and labile sulfide according to that of Fogo and Popowsky.[9] Piericidin A was a gift from Dr. K. Folkers.

Results and Discussion.—(1) By treatment of mitochondrial particles with phospholipase, Singer and his colleagues have isolated a segment of the respiratory chain which contains FMN, iron, and labile sulfide and catalyzes the reduction of ferricyanide by DPNH.[10] Except for the lack of coenzyme Q reductase activity, the Singer preparation is very similar to complex I (Table 1). Both preparations have high ferricyanide reductase activity and comparable amounts

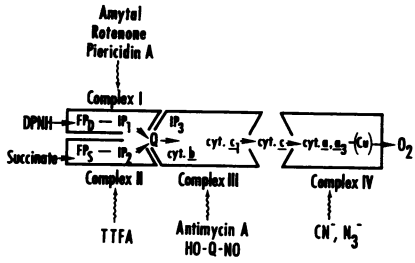

FIG. 1.—Schematic representation of the functional arrangement of complexes, I, II, III, and IV in the mitochondrial electron transport system (*IP* denotes nonheme iron protein; FP_D, DPNH dehydrogenase; and FP_S, succinic dehydrogenase).

of FMN, iron, and labile sulfide. Both exhibit a substrate-inducible electron paramagnetic resonance (EPR) signal at $g = 1.94$,[1, 11, 12] and their DPNH-ferricyanide reductase activity is characteristically inhibited at high DPNH concentrations.[4, 13] However, contrary to our experience with complex I, Singer and his colleagues maintain that their preparation (mol wt 550,000 according to ref. 14; 890,000 per mole of flavin according to ref. 11) is a homogeneous protein and represents the DPNH dehydrogenase of the electron transport system.[10, 11] Moreover, they regard the small-molecular-weight DPNH dehydrogenases of mitochondrial origin as a degradation product of their enzyme.[10, 11] Since phospholipase treatment destroys the Q reductase activity of complex I without affecting its ferricyanide reductase activity,[1] and since the composition and the ferricyanide reductase activity of the Singer preparation and complex I are very similar, it is possible that the Singer preparation is not a single protein, but rather an integrated complex of FP and IP. As will be seen later, substrate reduction and oxidation of complex I in the presence and absence of rotenone and Amytal clearly establish the involvement of two distinct redox species in this system.

(2) Sanadi and his colleagues have isolated a DPNH dehydrogenase from sonicated mitochondria by treatment of the particles with 11 per cent ethanol at 45° and pH 5.3.[15, 16] Earlier preparations of DPNH dehydrogenase by application of heat-acid-ethanol are those of Mahler *et al.*,[17] deBernard,[18] and Mackler.[19] These preparations, especially the Sanadi enzyme, resemble FP both in composition and catalytic activity, but the fact that among its diaphorase-like activities (ferric chelates and quinones can serve as electron acceptors) DPNH dehydrogenase can reduce coenzymes Q and that this reaction is partially inhibited by rotenone has led Sanadi *et al.* to equate their enzyme with the DPNH-Q reductase system of intact mitochondria and to assign an ectopic position to the iron-protein of complex I.[20] However, similar to its other reductase activities (cyt. *c*,[21] indophenol, menadione), the Q reductase activity of the dehydrogenase

TABLE 1. *Similarities in the properties of complex I and the Singer dehydrogenase.*

Property	Complex I	Singer dehydrogenase
FMN (mμmoles/mg)	1.4–1.5	1.12*
FMN:Fe:labile sulfide	1:26:26	1:17.5:27.8*
DPNH-induced EPR signal at $g = 1.94$	+	+
Turnover no., ferricyanide	6.5×10^5*	$8–15 \times 10^5$*
Inhibition of ferricyanide reduction at high [DPNH]	+	+

* Data of T. P. Singer.[11]

TABLE 2. *Differences in the reductase properties of complex I and FP.*

Conditions	Complex I	FP
Q_1 reduction		
Amytal (3 mM)	Inhibition 85%	Inhibition 10%
Demerol (0.5 mM)	Inhibition 95%	No inhibition
Rotenone (1 μM)	Inhibition 100%	Inhibition 20%
Piericidin A (1 μM)	Inhibition 95%	Inhibition 15%
Guanidine (50 mM)	Inhibition 70%	*Activation* 75%
Ferricyanide reduction		
DPNH (2 mM)	Inhibition 80%	No inhibition
Mersalyl (0.1 mM)	No inhibition	Inhibition 100%
K_m (*M* DPNH)	7×10^{-6}	6×10^{-5}
Moles DPNH oxidized per mole of flavin	75×10^3	14×10^3

is a property which is absent in mitochondria or ETP.[1, 4] These reductase properties, along with certain other modifications suggestive of conformational change, appear as the enzyme is detached from particles.[22] Table 2 shows a comparison of the Q-reductase and the ferricyanide-reductase properties of FP and complex I. It is seen that concentrations of Amytal, Demerol, rotenone, and Piericidin A, which produce 85–100 per cent inhibition of Q reductase activity with complex I, cause little or no inhibition with FP. Moreover, 50 mM guanidine, which inhibits the Q reductase activity of complex I by 70 per cent, actually activates FP by 75 percent. The ferricyanide reductase activities of the two preparations also show marked differences. Thus, the complex I activity is sharply inhibited at DPNH concentrations above 0.1 mM, whereas the FP activity is not. An active sulfhydryl group, which is unavailable (or uninvolved) in the segment of complex I concerned with ferricyanide reduction, becomes inhibitable by mercurials in FP. The K_m of the reaction for DPNH increases severalfold, and the activity per mole of flavin diminishes to a similar degree. Although not shown in Table 2, the Q-reductase and ferricyanide-reductase properties of ETP parallel those of complex I and differ from those of FP. Therefore, it appears that, similar to FP, the comparable preparation of Sanadi *et al.* is *only a component* of the rotenone-, Amytal-sensitive DPNH-Q reductase system of mitochondria (for other flavoproteins capable of reducing cyt. *c* and quinones, including coenzymes Q, see refs. 3 and 23).

Another point with respect to the low activity and the low iron-labile sulfide content of the Mahler-type preparations (see, for example, ref. 24) requires explanation. As pointed out earlier, all these dehydrogenases were extracted from mitochondrial particles by treatment with acid (pH 4.8–5.3) at temperatures between 40 and 45°. As seen in Figure 2*B* and *C*, incubation of FP at 38° and pH 4.8 results in the loss of enzymatic activity and a comparable loss of labile sulfide, while at pH 8.0 the enzyme is relatively stable (Fig. 2*A* and *C*).

(3) On the basis of absorption (475 minus 510 mμ) and fluorescence spectroscopy of sonicated and whole mitochondria in the presence and absence of rotenone, Chance, Ernster, and their colleagues[25] have recently differentiated two oxidation-reduction components in the region of the electron transport system between DPNH and Q-cyt. *b* (equivalent to the region represented by complex I). They have concluded that these two components are two flavoproteins acting in sequence: DPN $\rightarrow$ FP_{D_1} $\rightarrow$ FP_{D_2} $\rightarrow$ (cyt. *b*, UQ). The site of action of rotenone

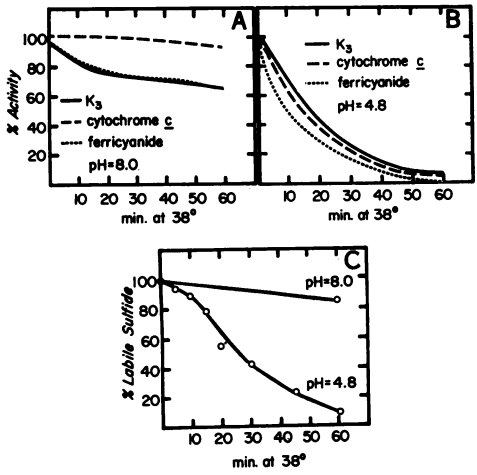

FIG. 2.—Inactivation and release of labile sulfide from FP by incubation at pH 4.8 and 38°. (*A*, *B*) Loss of menadione (K_3), cyt. *c*, and ferricyanide reductase activities (for assay conditions, see ref. 4) of FP at pH 8.0 (*A*) and at pH 4.8 (*B*). (*C*) Loss of labile sulfide at pH 8.0 and pH 4.8.

and Amytal, as well as the first site of energy conservation, has been placed between FP_{D_1} and FP_{D_2}. Once again, although we fully agree with these experimental data, we find that their identification of the component immediately on the oxygen side of the rotenone inhibition site as a second flavoprotein is subject to a different interpretation. As will be seen below, this absorbing species is in all probability not a flavoprotein; rather, it appears to be the IP component of complex I.

It was shown several years ago by Hatefi *et al.*[3, 6] that in the 460-mμ region of particulate DPNH-cyt. *c* reductase (the binary I–III complex), two different species could be identified by graded DPNH reduction of the enzyme preparation, as well as by DPNH reduction of the Amytal-treated particles.[6] The bleaching of the enzyme preparation at 460 mμ was also considerably greater than that expected from reduction of its total flavin content. Subsequent preparations of complex I, FP, and IP further confirmed these early findings. It was found that in complex I the absorbancy change at 450 mμ after DPNH reduction was twice the change expected from its flavin content (Δ_{450} of complex I/μmole of flavin/ml = 24.3),[26] whereas in FP this change was exactly equal to its flavin content (see also Table 2 of ref. 3). The oxidized minus reduced spectrum of IP (maximum absorbancy change at about 450 mμ) plus its reducibility by DPNH-reduced FP[4] further indicated that the excess 450–460-mμ bleaching of complex I might be due to reduction of IP.

Direct evidence for the oxido-reduction of FP and IP in the presence and absence of inhibitors has now been obtained by the use of the double-beam spectrophotometer. These results confirm our earlier findings and establish IP as a component of complex I immediately on the oxygen side of the rotenone inhibition site. Figure 3 shows the absorbancy change at 460 minus 510 mμ in particulate DPNH-cyt. *c* reductase (the binary I–III complex). It is seen that in the presence of rotenone or Demerol only about 50 per cent of the total reduction induced by DPNH has occurred. In agreement with our earlier findings[6] and the recent data of Chance *et al.*,[25] these results indicate a reduction of FP and an inhibition of

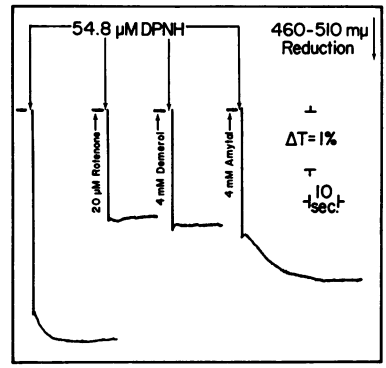

FIG. 3.—Reduction of the binary I-III complex by DPNH in the presence and absence of rotenone, Demerol, and Amytal at 460 minus 510 mμ. The binary I-III complex (0.828 mg/ml) was suspended in a solution containing 0.65 *M* sucrose, 1 mM histidine, and 30 mM potassium phosphate, pH 8.0. The temperature of the reaction mixture was 3–5°. The measurements were made by the Aminco-Chance dual-wavelength/split-beam spectrophotometer.

reduction of a 460-mμ absorbing component by rotenone and Demerol. More comprehensive results on the oxido-reduction of FP and IP and the site of action of Amytal, Demerol, rotenone, and Piericidin A were obtained with complex I. To study both the reduction and oxidation of FP and IP in complex I, advantage was taken of the fact that preparations of complex I are usually contaminated with about 0.5 to 1 per cent complex III (total cyt. *b* plus cyt. c_1 content < 0.1 mμmole/mg).[27] As a result, preparations of complex I exhibit a small antimycin-sensitive DPNH-cyt. *c* reductase activity (about 4 μmoles cyt. *c* reduced/min/mg of complex I, as compared to 60 μmoles cyt. *c* reduced/min/mg of the binary I–III complex). Therefore, in the experiments with complex I, catalytic amounts of cyt. *c* and purified cytochrome oxidase were also added in order to link the electron transfer system of complex I to oxygen and thereby to establish a slow oxidation of complex I after reduction with DPNH. Thus, as seen in Figure 4*A*, addition of DPNH to complex I resulted in a rapid reduction at 460 mμ. This level of reduction was maintained for about 70 seconds until the added DPNH was exhausted. Then the reduced components of complex I were slowly re-oxidized. This cycle of reduction and oxidation could be repeated by addition of a second increment of DPNH. When rotenone or Piericidin A was added just after DPNH reduction, a rapid oxidation of 50–60 per cent of the total reducible material took place, and addition of a second increment of DPNH 80 seconds after addition of the inhibitor did not cause further reduction of the rapidly oxidized component. Essentially similar results were obtained with Demerol and Amytal, although, as expected, the inhibitory effect of Amytal was not as complete as the others (see below). These results indicate that rotenone, Piericidin A, Demerol, and Amytal interrupt electron flow between the two components of complex I. As a result, the component on the oxygen side of the inhibited point is rapidly reoxidized and cannot be reduced by further addition of DPNH.

When complex I was pretreated with rotenone, Piericidin A, or Demerol and then DPNH was added, only about half of the total reducible material in complex I was reduced (Fig. 4*B*). Pretreatment with Amytal did not result in complete inhibition of electron flow beyond FP and, depending on the concentration of Amytal added, the steady-state level of reduced IP changed accordingly. Moreover, the electron leak through the Amytal inhibition site permitted a slow DPNH oxidation, which eventually led to the oxidation of reduced FP as well.

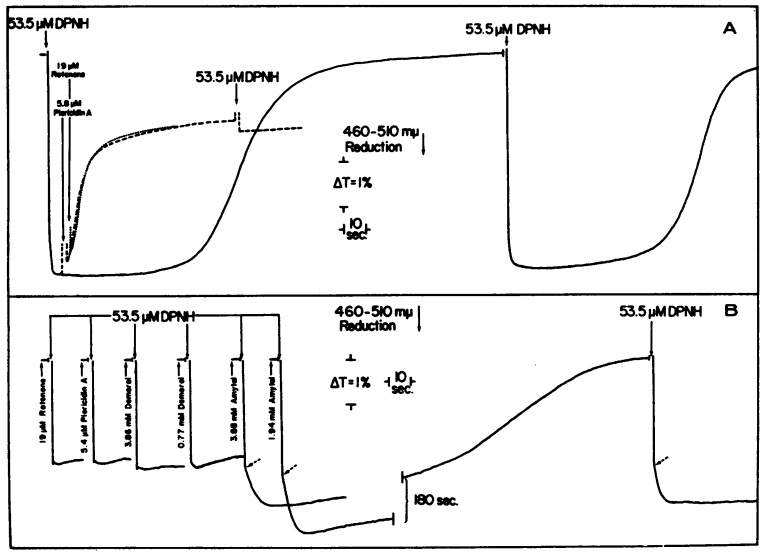

FIG. 4.—Reduction and oxidation of complex I in the presence and absence of inhibitors at 460 minus 510 mμ. The reaction mixture contained 0.61 mg complex I, 0.027 mg cytochrome *c*, and 0.06 mg cytochrome oxidase per milliliter of the solution described in Fig. 3. Other conditions were also the same as in Fig. 3.

As compared to the rate of complex I reoxidation shown in Figure 4*A*, the reoxidation of complex I treated with 1.94 mM Amytal was 72 per cent inhibited (Fig. 4*B*), and that with 3.88 mM Amytal (not shown in Fig. 4*B*) was more than 90 per cent inhibited. Amytal experiments of Figure 4*B* also show that after DPNH addition, a rapid reduction of FP equivalent to that obtained with rotenone-, Piericidin A-, and Demerol-treated complex I took place; then there was an abrupt change in the rate (marked by broken arrows) as electrons leaked through the Amytal-inhibited point to reduce IP (see also Fig. 3).

The above results are in full agreement with the data of Chance, Ernster, and their colleagues,[25] but make their conclusion regarding a second flavoprotein highly questionable. Since the composition of complex I in terms of flavin, iron, and labile sulfide is known and since the presence in this system of a ferroflavoprotein and a reducible iron-protein has been demonstrated by resolution of the complex and characterization of these components, the above results are much more consistent with the following scheme than with the scheme of Chance *et al.* with two sequential flavoproteins:

$$\text{DPNH} \rightarrow \text{FP} \rightarrow \text{IP} \rightarrow \text{Q}$$
$$\uparrow\!\!\sim$$
Rotenone, Piericidin A, Amytal, Demerol

Two points still remain to be clarified:

(1) In addition to absorption changes at the flavin region, Chance *et al.*[25] observed parallel fluorescence changes (436 → 570 mμ) in the presence and absence of

rotenone indicative of two fluorescing redox species. Since the isolated FP fluoresces strongly when excited at 440 mμ but IP does not, the nature of the second fluorescing species observed by Chance *et al.* in ETP still remains obscure.

(2) On the basis of EPR spectroscopy of ETP preparations, Palmer *et al.*[28] concluded that rotenone and Piericidin A inhibit electron transfer at two sites in the respiratory chain: (*a*) immediately on the substrate side of coenzyme Q, and (*b*) immediately on the substrate side of cyt. c_1. The results communicated in this paper show clearly that in complex I the site of rotenone and Piericidin A inhibition is between FP and IP, and not between IP and Q. Moreover, our recent studies indicate that at inhibitor concentrations comparable to those used by Palmer *et al.*,[28] neither Piericidin A nor rotenone exerts an appreciable inhibition on complex III.

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Abbreviations: Q and UQ, coenzyme Q (ubiquinone); cyt., cytochrome; TTFA, 2-thenoyltrifluoroacetone; HO-Q-NO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; K_3, menadione; FMN, riboflavin 5′-phosphate; and DPNH, reduced diphosphopyridine nucleotide. For definitions of flavoproteins FP_{D_1} and FP_{D_2}, see ref. 25.

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[5] Technological assay difficulties experienced earlier[1] for reduction of water-insoluble coenzymes Q_6 and Q_{10} by complex I have now been overcome. The V_{max} with Q_6 as electron acceptor (V_{max} = 20 μmoles Q_6 reduced/min/mg of complex I protein) is in good agreement with that obtained for the water-soluble Q_1 (V_{max} = 25).[3]

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[12] Both FP and IP exhibit an EPR signal at g = 1.94 after reduction with dithionite. However, the details of the two EPR signals are completely different.

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[21] Unlike the cyt. *c* reductase activities of ETP and the binary I-III complex, the cyt. *c* reductase activity of FP is not inhibited by Amytal, rotenone, and antimycin A. Moreover, the FP activity has a very high K_m ($\sim 6 \times 10^{-4}$ *M* cyt. *c*)[4] as compared to the K_m of antimycin-sensitive systems ($\sim 12 \times 10^{-6}$ *M* cyt. *c*).

[22] Changes accompanying dislocation of integrated enzymes have been observed also in the case of other mitochondrial components such as the redox potential of cyt. *b*, the K_m of malate dehydrogenase, and the activity and oligomycin sensitivity of adenosine 5'-triphosphatase.

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