# *FLAVOPROTEINS OF THE MITOCHONDRIAL RESPIRATORY CHAIN**

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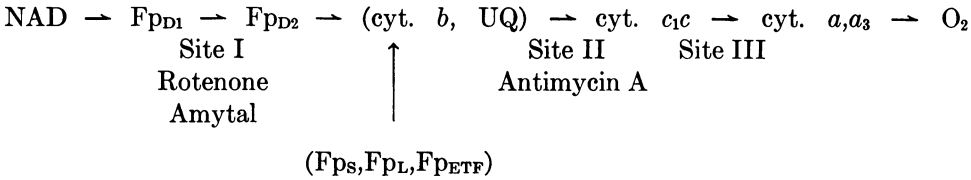
One approach to the location of the first coupling site of oxidative phosphorylation (Site I) and the site at which the inhibitors, rotenone and amytal, interrupt electron transport in the respiratory chain of avian and mammalian mitochondria has been based upon a comparison of the steady-state responses of the respiratory carriers nicotinamide adenine dinucleotide (NAD) and flavoprotein ($Fp_D$) to uncoupling agents and to amytal or rotenone.[1] On this basis, Site I and the rotenone site have been assigned to point *A* in the sequence: $NAD \overset{A}{\rightarrow} Fp_D \overset{B}{\rightarrow} (UQ,b)$. Discrepancies in this assignment have been noted in a number of laboratories: Several workers (cf. ref. 2) assign Site I to *A* but the rotenone site to *B;* Klingenberg[3] proposes the opposite; Schatz and Racker[4] assign both Site I and the rotenone site to *B*. More recent studies of reversed electron transport have indicated an additional component, identical with neither the nonheme iron nor the sulfhydryl component of $Fp_D$, at *B*.[5] A reconciliation of these viewpoints is now based on the finding that the NAD dehydrogenase portion of the respiratory chain contains two flavoproteins operating in sequence, with both the rotenone site and the coupling Site I between them, as in Scheme I below:




*Materials and Methods.*—Changes of flavoprotein absorbance and fluorescence[6] in mitochondria and submitochondrial particles were measured simultaneously with a double-beam spectrophotometer[7] and an attached fluorometer. Suitable filters guarded each photomultiplier against light from the opposite system. Similar procedures were used for simultaneous recording of flavoprotein fluorescence and pyridine nucleotide absorbance. Mitochondria were prepared from rat liver and kidney,[8] pigeon heart,[9] beef heart,[10] *Saccharomyces cerevisiae*,[11] *S. carlsbergensis* (NCYC 74S),[12] and *Torulopsis utilis* (NCYC 193).[12] Submitochondrial particles were prepared from beef heart mitochondria after sonication in the presence of EDTA;[13] depletion of succinic dehydrogenase was done by cyanide treatment.[14]

*Results.—Submitochondrial particles:* The flavoproteins of the respiratory chain in submitochondrial particles are retained by the vesicles in spite of sonication, while pyruvate and oxoglutarate dehydrogenases ($Fp_L$)[15] DT-diaphorase,[16] and electron transfer flavoprotein ($Fp_{ETF}$)[17] are released in soluble form. The experiment of Figure 1 illustrates the oxidation-reduction changes of flavoprotein in beef

heart submitochondrial particles following additions of antimycin A, NADH, rotenone, and succinate. The addition of NADH causes a rapid reduction of flavoprotein that is partially reversed by the subsequent addition of 2.1 $\mu$M rotenone (Fig. 1*A*). In these circumstances, the partial oxidation of flavoprotein caused by the addition of rotenone is due to a "leak"[18–20] through the antimycin A-sensitive site and the cytochrome chain to oxygen. The subsequent addition of succinate restores the flavoprotein reduction. This is better illustrated in Figure 1*B*, in which the addition of succinate not only causes a smaller reduction of flavoprotein than does NADH, but also diminishes the reduction obtained when NADH is added subsequently. Transhydrogenase activity causes a slow reduction of $Fp_{D1}$ following addition of NADPH, if NAD is added or bound NAD is present.[5] No evidence for a direct reaction of $Fp_{D1}$ and NADPH has been obtained.

These experiments divide the flavoproteins of submitochondrial particles into two categories: firstly, a flavoprotein on the NAD side of the rotenone site, reducible by NADH but not by succinate; secondly, flavoproteins on the antimycin A side of the rotenone site, reducible by succinate or, when rotenone is absent, by NADH. The first category may be equated with $Fp_{D1}$ and the second with $Fp_S$ and $Fp_{D2}$ of Scheme I.[21]

Evidence that $Fp_{D2}$ is responsible for the fluorescence and absorbancy changes following rotenone addition to the NADH-supplemented system is included in Figures 1*C* and *D*. Particles devoid of succinic dehydrogenase activity[14, 22] show no reduction of flavin on succinate addition. (The small absorbancy increase in Fig. 1*C* on succinate addition is not completely understood.) Thereafter, re-

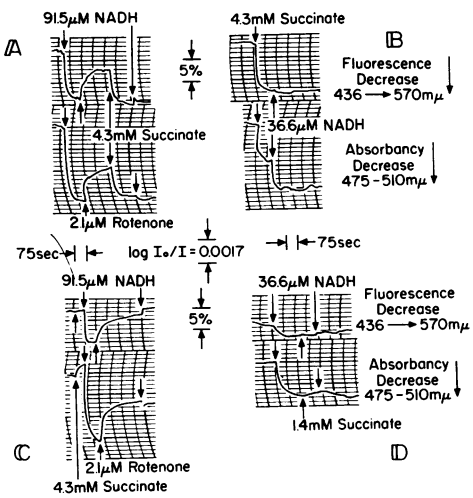



FIG. 1.—Reduction and oxidation of flavoproteins in beef heart submitochondrial particles. Fluorometric (436 → 570 mμ) and spectrophotometric (475–510 mμ) measurements. (*A*) 1.4 mg protein/ml of control particles suspended in 50 mM Tris-acetate, pH 7.4, 10 μg antimycin A in 3.5 ml, 25°. (*B*) Control particles as in (*A*), 2.1 μM rotenone added. (*C*), (*D*) Parallel experiments to (*A*) and (*B*), respectively, with KCN-treated particles at 1.6 mg protein/ml.

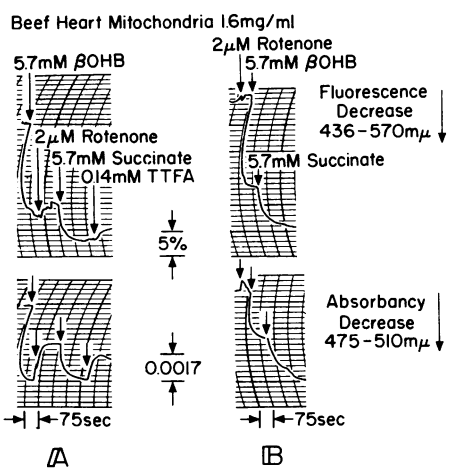



FIG. 2.—Reduction and oxidation of flavoproteins in beef heart mitochondria. Fluorometric and spectrophotometric measurements as in Fig. 1. Protein (1.6 mg)/ml, suspended in 50 mM Tris-acetate, 100 mM KCl, pH 7.4, treated with 3 μM PCP, 0.6 mM $AsO_3^-$, 0.7 mM $AsO_2^-$, 0.6 mM malonate, 5 μg antimycin A in 3.5 ml, 25°.

sponses to rotenone and NADH confirm those of Figure 1*A*. Similarly, in Figure 1*D*, succinate addition following NADH causes no perceptible changes of absorption or fluorescence. Thus, the major contribution to the NADH and rotenone responses is made by $Fp_{D2}$, which is reducible by succinate or NADH but which cannot be identical to $Fp_S$.

*Intact mitochondria.*—In Figure 2, the reduction of flavoprotein in intact beef heart mitochondria is achieved by the addition of β-hydroxybutyrate. The addition of rotenone then causes a partial oxidation of flavoprotein, again confirming the two types of flavoprotein that were found in the submitochondrial particles, i.e., $Fp_{D1}$ on the NAD side and $Fp_{D2} + Fp_S$ on the cytochrome side of the rotenone site. The reduction of $Fp_{D2}$ and $Fp_S$ is restored by the subsequent addition of succinate. The specific reduction of $Fp_{D1}$ can be obtained by adding substrate to the rotenone-treated mitochondria (Fig. 2*B*), an experiment analogous to that of Figure 1*B*. However, here the fluorescence increase on adding the NAD-linked substrate is large, while in Figure 1*B* it was very small. We attribute this to the possibility that water has entered Site I of the submitochondrial particles, with a consequent decrease of the fluorescence efficiency of the bound flavin.[23] Alternatively, a water-stabilized complex may be formed in the submitochondrial particles.[24]

Evidence for the presence of $Fp_{D2}$ in intact mitochondria is further provided by the oxidation of flavoprotein by thenoyltrifluoroacetone (TTFA) (Fig. 2*A*), which inhibits ubiquinone (UQ) reductase activity.[25] TTFA addition shows that $Fp_S$ contributes very little fluorescence and does not account for the succinate-reducible flavoprotein that is also reducible by NADH, i.e., $Fp_{D2}$. In other experiments (not shown), it has been found that $Fp_{ETF}$ of rat liver mitochondria is not reduced by succinate or by NAD-linked substrates.[26] Similar behavior has been observed in rat liver (cf. Fig. 5*B*), pigeon heart, and rat kidney (not shown).

*Energy-linked reduction of $Fp_{D1}$:* An energy requirement for maximal reduction of flavoprotein by succinate in rat liver mitochondria is shown in the experiments of Figure 3. In Figure 3*A*, the addition of antimycin A, followed by succinate, to energy- and substrate-depleted mitochondria causes a flavoprotein reduction that is equated with $Fp_{D2}$. More extensive flavoprotein reduction (i.e., $Fp_{D1}$) is obtained when the uncoupling effect of pentachlorophenol is reversed with bovine serum albumin (BSA)[27] and ATP (Figs. 3*A* and *B*). The ATP effect is inhibited by oligomycin, rotenone, or atractylate. Alternatively, energy can be provided by the oxidation of ascorbate-TMPD (NNN'N tetramethyl-*p*-phenylenediamine) in a reaction insensitive to oligomycin or atractylate but still sensitive to rotenone (Fig. 3*C*). The flavoprotein reducible by succinate in an energy-dependent manner ($Fp_{D1}$) can also be reduced by NAD-linked substrates. Separate experiments show that if, at the conclusion of any of the traces of Figure 3, rotenone and then oxoglutarate and $NH_3$ are added, the energy-linked reduction of $Fp_{D1}$ is reversed, suggesting that $Fp_{D1}$ is at the oxidation-reduction level of NADH (Fig. 4). Thus, the presence of the first energy-conservation site (Site I) between $Fp_{D1}$ and $Fp_{D2}$ is both experimentally demonstrable and thermodynamically necessary, in view of the widely differing redox potentials of the two flavoproteins.

*Kinetic responses of $Fp_{D1}$ and $Fp_{D2}$:* Kinetic experiments have been undertaken to provide a more certain location for $Fp_{D1}$ and $Fp_{D2}$ in the pathway of hydrogen and

electron transfer between NAD and cytochrome *b*-UQ. In the experiment of Figure 4*A*, the addition of rotenone to rat kidney mitochondria oxidizing malate-glutamate at 5° clearly shows that the reduction of a flavoprotein ($Fp_{D1}$) precedes that of NAD. At 20° the transients are more rapid (Fig. 4*B*), and the further addition of arsenite and $NH_3$ results in complete reoxidation of both $Fp_{D1}$ and NAD. If we assume that the changes of NAD and $Fp_{D1}$ in Figure 4*B* are from the extremes of fully oxidized to fully reduced (transient after rotenone) and back again (transient after $NH_3$), it is possible to calculate a value for the apparent equilibrium constant $K_{eq} = [NAD]\,[Fp_{D1}{}^{red}]/[NADH_2]\,[Fp_{D1}{}^{ox}]$. At the point where $Fp_{D1}$ was 50 per cent reduced, the value for the equilibrium constant is 0.33 for the transient following rotenone and 0.95 for the transient following ammonia. The true $K_{eq}$ value lies between the two. The relationship between the rates of reduction of $Fp_{D1}$ and NAD in Figure 4*A* is therefore explicable only in terms of the sequence of Scheme I.

*Energy-dependent oxidation of $Fp_{D2}$:* An energy-dependence for the oxidation of $Fp_{D2}$ by oxoglutarate and $NH_3$ is demonstrated in experiments of the type shown in Figure 5*A*. After depletion with energy and substrates, antimycin A and oxoglutarate are added to reduce both $Fp_{D1}$ and $Fp_{D2}$. The further addition of $NH_3$ oxidizes $Fp_{D1}$, whereas $Fp_{D2}$ is not oxidized until oxidative phosphorylation is recoupled and ATP added. Similar experiments show that the absorbancy changes following addition of $NH_3$ in Figure 5*B* are only 30 per cent complete and that the remaining 70 per cent is obtained only with BSA and ATP supplements. This is in accord with the data of Figure 2, which show $Fp_{D2}$ of intact mitochondria to have a relatively low fluorescence compared to that of $Fp_{D1}$. In other experiments, the

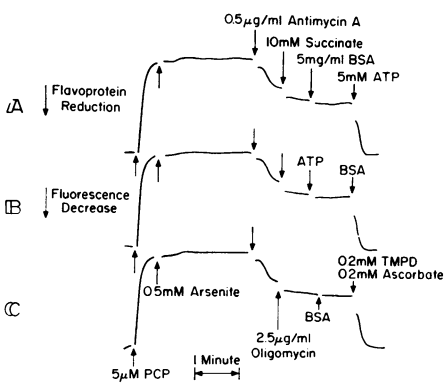



FIG. 3.—Energy-dependent reduction of flavoprotein in rat liver mitochondria. Fluorometric measurements with excitation at 450–460 mμ and emission measured at wavelengths above 510 mμ. Six mg mitochondrial protein/2.0 ml of 80 mM KCl, 20 mM Tris-Cl, pH 7.2, 1 mM EDTA, 20°. Additions as indicated. In each experiment the flavoprotein reduced by succinate is considered to be $Fp_{D2}$ and the further energy-dependent reduction is considered to be $Fp_{D1}$. Appropriate controls demonstrated that succinate was required.

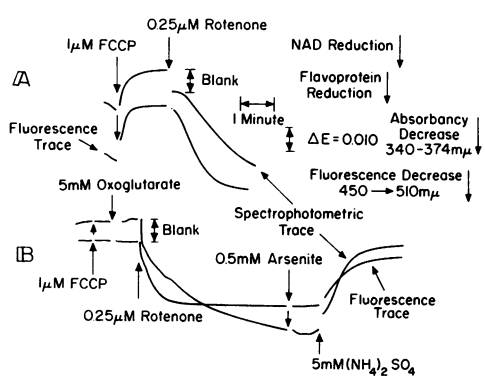



FIG. 4.—Kinetics of reduction and oxidation of NAD and $Fp_{D1}$ in rat kidney mitochondria. Fluorometric measurements as in Fig. 3, with higher sensitivity in (*A*) than in (*B*); absorption photomultiplier guarded with Wratten 18A filter; response time 0.3 sec. 2.0 ml 80 mM KCl, 20 mM Tris-Cl, pH 7.2, 1 mM EDTA. (*A*) Supplemented with 5 mM L-malate, 2.5 mM L-glutamate; 4 mg mitochondrial protein/ml, 5°. (*B*) 4.6 mg mitochondrial protein/ml, 20°. The flavoprotein reduced on addition of rotenone is considered to be $Fp_{D1}$.

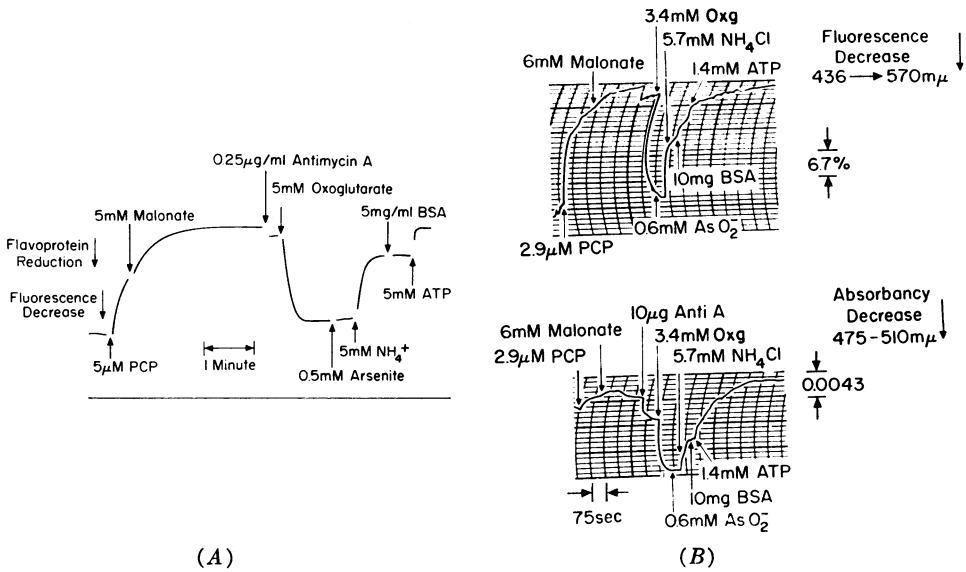



FIG. 5.—Energy-dependent oxidation of $Fp_{D2}$ by oxoglutarate and $NH_3$ in rat liver mitochondria. (*A*) Fluorometric measurements and experimental conditions as in Fig. 3. It is considered that both $Fp_{D1}$ and $Fp_{D2}$ are reduced following the addition of 2-oxoglutarate and antimycin A, whereas only $Fp_{D1}$ is reoxidized by oxoglutarate and $NH_3$ in the uncoupled state. The ratio of BSA to antimycin A was not sufficient to release the antimycin A-inhibition of respiration. (*B*) Fluorometric (436 → 570 mμ) and spectrophotometric (475–510 mμ) measurements. Mitochondrial protein (3.6 mg)/ml suspended in 20 mM triethanolamine, pH 7.4, 80 mM KCl in 3.5 ml, 25°.

relative rates of $Fp_{D2}$ and cytochrome *b* oxidation have been compared following ATP addition under conditions similar to those of Figure 5*B*. No prior oxidation of cytochrome *b* was recorded. Thus it is concluded that $Fp_{D2}$ lies between Site I and cytochrome *b*.

*Yeast flavoproteins:* The organization of the respiratory chain between NAD and cytochrome *b*-UQ is of particular interest in yeast mitochondria, since Site I and the rotenone-sensitive site are apparently absent[11, 28, 29] from *S. cerevisiae* and *carlsbergensis*, but present[30–32] in *T. utilis*. Figure 6 affords a comparison of the responses of flavoprotein fluorescence of mitochondria prepared from the rotenone-sensitive *T. utilis* (*A*) and the rotenone-insensitive *S. carlsbergensis* (*B*). Following uncoupling, rotenone addition causes an immediate flavoprotein reduction due to endogenous substrate in *T. utilis*, while in *S. carlsbergensis* no effect is observed until electron transport is interrupted by antimycin A. A low-potential flavo-

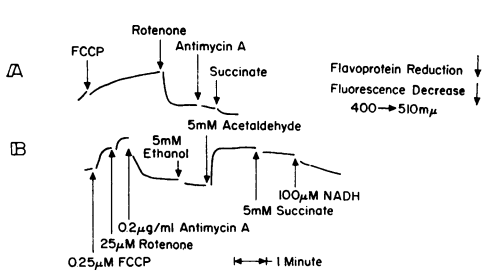



FIG. 6.—Flavoproteins of yeast mitochondria. Fluorometric recordings as for Fig. 3. 2.0 ml of 0.5 *M* sorbitol, 10 mM $K_2HPO_4$, pH 6.5, 20 mM Tris-malate pH 6.5, 0.1 mM EDTA, 10 mM KCl. (*A*) *T. utilis* mitochondria, 1.4 mg protein/ml. (*B*) *S. carlsbergensis* mitochondria, 1.5 mg protein/ml.

protein involved in *S. carlsbergensis* is identified by its oxidation by acetaldehyde, a property shared by the flavoprotein of *T. utilis* (not shown). Thus, both flavoproteins are of the $Fp_{D1}$ type. A flavoprotein similar to $Fp_{D2}$ remains reduced in the presence of acetaldehyde (Fig. 6*B*). A flavoprotein similar to $Fp_S$ is reduced in both materials on addition of succinate (Figs. 6*A* and *B*) and is not oxidized by acetaldehyde. A flavoprotein reduced by added NADH ($Fp_{D2X}$) is associated with the external pathway of NADH oxidation and represents still another flavoprotein component of yeast. Although these results are preliminary, they are consistent with the data on mammalian systems and indicate that flavoproteins of types $Fp_{D1}$ and $Fp_{D2}$ are present in both the rotenone-sensitive and -insensitive oxidation pathways of yeast.

*Discussion.*—These data are best explained in terms of Scheme I, although further confirmation is required from a demonstration of the ability of the flavoproteins that we have termed $Fp_{D1}$ and $Fp_{D2}$ to be oxidized and reduced at rates consistent with their proposed location in the respiratory chain. The presence of two flavoproteins between NAD and cytochrome *b*-UQ suggests a reconsideration of experimental data that was originally interpreted on the basis that there was only one such flavoprotein. Thus it may now be possible to reconcile conflicting reports[1,29,33] concerning the location of Site I and the rotenone and amytal site in various preparations. It is evident that absorbancy measurements of a State 3- to -4 transition involving a crossover point between $Fp_{D1}$ and $Fp_{D2}$ would show a change of flavoprotein absorbance that represents the difference between the opposing changes occurring in $Fp_{D1}$ on the one side and $Fp_{D2}$ (and possibly $Fp_S$ and $Fp_{ETF}$) on the other. Thus a reinterpretation of previous experiments[1] locates the energy-coupling site between NADH and $Fp_{D2}$. In the absence of experimental data for a crossover point between NADH and $Fp_{D1}$, and in view of the low potential of $Fp_{D1}$, we tentatively locate the energy-coupling site between the two flavoproteins $Fp_{D1}$ and $Fp_{D2}$. This assignment leads us to attribute properties previously assigned to NAD and NADH[34-37] to flavoprotein.[38-40]

The association of a considerable amount of iron protein with the NAD side of the rotenone site is clearly established.[41] More recent studies employing the submitochondrial particles used in these experiments show that a large part of the iron protein ESR signal that can be obtained with NADH as substrate in the presence of antimycin A can also be obtained in the presence of rotenone.[42] At present, it is not possible to assign any iron protein ESR signal specifically to $Fp_{D2}$, since the observed signals could be associated as well with $Fp_S$. The fact that in *Saccharomyces* electron transport through $Fp_{D1}$ and $Fp_{D2}$ occurs in the absence of both rotenone-sensitivity and an iron protein ESR signal suggests that neither of these properties is necessary for electron flow;[43] they seem to be associated with the energy-coupling reaction.

In addition to reconciling different viewpoints on the nature of energy-coupling sites in the respiratory chain and on the components essential to the energy-conservation at Site I, these studies may lead to a re-evaluation of the nature of solubilized NADH dehydrogenases. Firstly, the observed large decrease of the fluorescence efficiency of $Fp_{D1}$ consequent to the formation of the submitochondrial particles reconciles the inconsistency between the presence of highly fluorescent flavoproteins in mitochondria and nonfluorescent flavoproteins in purified NADH

dehydrogenases.[6] The fluorescence changes observed in nonphosphorylating preparations are largely due to $Fp_{D_2}$, which is apparently absent in the NADH dehydrogenases which reduce ferricyanide.[44, 45] The apparent lack of $Fp_{D_2}$ in the DPNH—UQ reductase[46–48] raises the possibility that the physiological function of this segment of the respiratory chain would be restored by the inclusion of $Fp_{D_2}$.

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