OXIDATION-REDUCTION PROPERTIES OF THE MITOCHONDRIAL FLAVOPROTEIN CHAIN*

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The adaptation of spectrophotometric and fluorometric methods for studies of terminal oxidations recently led to the discovery of a new component of the flavoprotein region of the mitochondrial respiratory chain (Chance et al. 1967). The NADH-ubiquinone reductase system has flavoprotein components (FpD1 and FpD2) on both sides of the first phosphorylation site. An apparent dissimilarity was found between the fluorescence characteristics of the NADH dehydrogenase in intact mitochondria and in particles derived from mitochondria by sonic irradiation (Chance et al. 1967). In the present study the fluorescent component of FpD1 in intact mitochondria has been identified as a low potential flavoprotein located in the matrix space. Evidence is also presented of the existence of another flavoprotein before the first phosphorylation site, having a low fluorescence and a higher redox potential.

MATERIAL AND METHODS

Rat liver mitochondria were isolated by conventional methods (Schneider 1948) in 0.25 M sucrose containing 1 mM EDTA. Submitochondrial particles from rat liver were made essentially according to Kielley and Bronk (1958). Lithium acetoacetate was prepared from methyl acetoacetate (Sigma Chemical Co.)

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according to Hall (1962), and crystallized twice from ethyl ether/methanol. In some experiments the lithium salt was converted to sodium salt on a Dowex 50 column. The method of simultaneous fluorometric and spectrophotometric measurement of flavins has been published previously (Chance et al. 1967). Simultaneous measurements of flavin and NADH fluorescences were made by means of a two channel, alternating filter fluorometer. Simultaneous measurements of flavin absorbance and NADH fluorescence were made in a dual wavelength spectrophotometer-fluorometer combination. Flavin absorbance was measured at 475-510 nm and NADH fluorescence at 412 nm measured by using the 366 nm mercury line, modulated at 120 Hz as exciting light. NADH-dehydrogenase activity was assayed according to Minakami et al. (1962) and lipoamide dehydrogenase essentially as described by Veeger and Massey (1962).

RESULTS AND DISCUSSION

When mitochondria were uncoupled with FCCP (= carbonyl cyanide p-trifluoro methoxyphenylhydzazone) and malonate added to inhibit the succinic dehydrogenase, the addition of rotenone caused a reduction of the flavoproteins by endogenous substrates (Fig. 1). A further reduction could be achieved by addition of β-hydroxybutyrate (β-OHB). Subsequent addition of acetoacetate (AcAc) caused a reoxidation of the fluorometrically measurable flavins. However, little reoxidation of flavins was observed spectrophotometrically.

Redox potentials of the β-OHB/AcAc pair were calculated by assuming $E_{m\ 7} = -266$ mV at 25° (Krebs et al. 1962), and corrected to pH 7.4 (Clark 1960). It was assumed that in these experimental conditions the redox potential was mainly determined by the D-(-)-isomer of β-OHB (Lehninger and Greville 1953). The data shows an $E_{m\ 7.4} = -305$ mV for the high fluorescence flavoprotein before the first phosphorylation site. The potential of the low fluorescence component of FpD1 was too high to be determined in intact mitochondria with the redox couple in question.

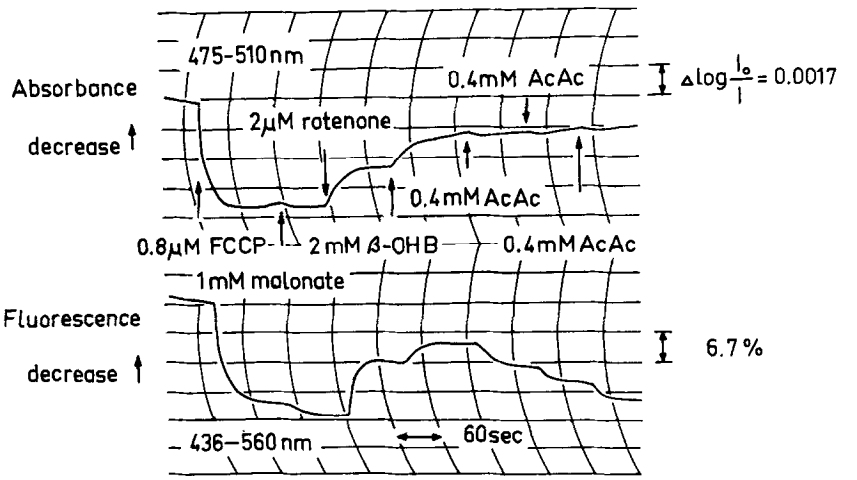

Fig. 1. Titration of the mitochondrial flavoproteins before the first phosphorylation site with β-OHB/AcAc. Conditions: 50 mM KCl, 75 mM mannitol, 67 mM sucrose, 23 mM tris chloride, 0.16 mM EDTA, pH 7.4,.3.9 mg of protein/ml.

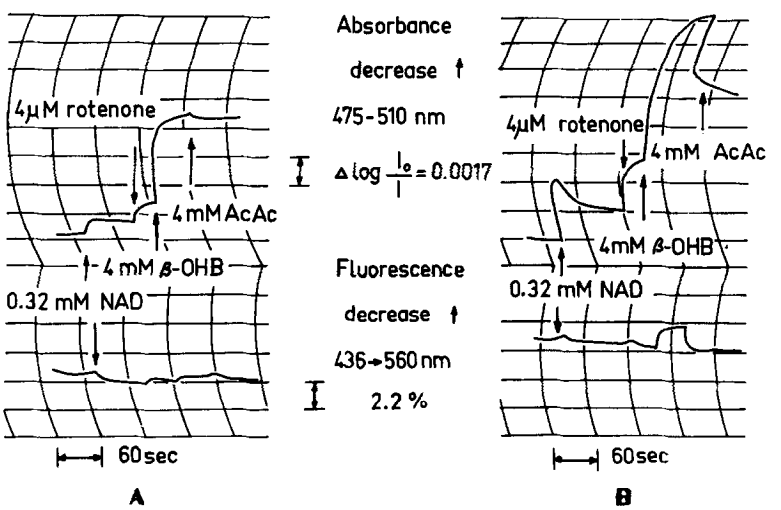

Fig. 2. Redox properties of the particulate and soluble flavoproteins of rat liver mitochondria. A. Submitochondrial particles, 2.3 mg of protein/ml, 30 mM potassium phosphate, pH 7.4, 1mM EDTA. B. Sonic supernatant and submitochondrial particles, 30 mM potassium phosphate, pH 7.4, 1mM EDTA, 2.3 mg of particle protein/ml, 4.6 mg of soluble protein/ml.

Osmotic and sonic rupturing of the mitochondria is known to result a fractionation of the mitochondria into a soluble phase, representing the matrix space of mitochondria (Frisell et al. 1965, Sottocasa et al. 1967), and into a particulate phase, mostly composed of vesicles originating from the cristae. A low potential, high fluorescence flavoprotein was recovered in the soluble phase. The redox potential of this flavoprotein could be titrated with the β-OHB/AcAc pair by using particles as a source of the dehydrogenase. In Figure 2, however, there is evidence that the submitochondrial particles contained a rotenone sensitive NADH oxidase with a low fluorescence flavoprotein on the NADH side of the rotenone sensitive site. The flavin reduction observed after the addition of NAD (Fig. 2B) is considered to be due to traces of endogenous substrates in the sonic supernatant. A titration of the rotenone inhibited system with the lactate/ryruvate couple and added lactate dehydrogenase and by direct measurement of NADH by fluorometry gave $E_{m\ 7.4} = -250$ mV for the low fluorescence flavoprotein. However, because of the rotenone leak and the low NADH/flavin ratio needed for the reoxidation of the flavin, this value rather is a lower limit of the redox potential of the flavoprotein.

The reoxidation of the highly fluorescent component in mitochondria was sensitive to high concentrations (2.5 mM) of arsenite when AcAc was employed as an oxidant of NADH in a rotenone inhibited system. That the phenomenon was not due to an inhibition of the β-OHB dehydrogenase enzyme, was demonstrated in experiments with simultaneous measurement of flavin and NADH fluorescence. Essentially the same results were obtained with the supernatant phase reduced by NADH when lactate dehydrogenase and pyruvate were used to reoxidize the flavins. The reoxidation of the particulate flavoprotein in the same conditions and in the presence of rotenone was not sensitive to arsenite.

Only circumstantial evidence for the identity of the high fluorescence flavoprotein can be given. The redox potential of this flavoprotein (-305 mV) is near to the -340 mV given for purified lipoamide dehydrogenase (Searls and Sanadi 1959). The relative insensitivity of the flavoprotein-NADH reaction to

arsenite is compatible with the insensitivity of the diaphorase moiety of lipoamide dehydrogenase to arsenite, as has been found in purified preparations of the enzyme (Sanadi 1963). The effects of high concentrations of arsenite on oxidation-reduction properties of the high fluorescence component could be explained by a conformational chance of the enzyme, causing a shift of the redox potential of the flavin and an apparent stabilization of the reduced state.

TABLE 1

Distribution of NADH dehydrogenase and lipoamide dehydrogenase activities in submitochondrial fractions

	particles		soluble	
total protein	57 mg		151 mg	
	μmole·min^{-1}		μmole·min^{-1}	
	mg^{-1} protein	nmole^{-1} flavin ***)	mg^{-1} protein	nmole^{-1} flavin ***)
NADH dehydrogenase *)	13.2	80.0	0.79	8.26
$Lip(SH)_2NH_2$ dehydr. **)	0.018	0.106	4.25	44.7
NADH oxidase	0.68	4.22	-	-

*) V_{max} $(Fe(CN)_6)^{3-}$ at 0.1 mM NADH

**) V_{max} $LipS_2NH_2$ at 0.1 mM NADH

***) NADH reducible flavin in the presence of rotenone, based on ϵ = 10 mM^{-1}cm^{-1} (475-510 nm)

The distribution of NADH dehydrogenase and lipoamide dehydrogenase activities in the soluble and membranous fractions of the mitochondria were determined (Table 1.). A high specific activity of lipoamide dehydrogenase was found in the soluble phase. The NADH dehydrogenase was located mainly in the particulate phase.

The fluorometric data suggest that the fluorescence of the low potential flavin pool is mostly displayed by a single component. From a careful analysis of the spectrophotometric data of the AcAc oxidizable flavoprotein of intact mitochondria it can be estimated that the high fluorescence component accounts for 20 per cent of the total NADH-linked flavins. From the fractionation experiments as presented in Table 1, the soluble flavoproteins contribute to

60 per cent of the NADH-linked flavoproteins of mitochondria. One third of the soluble flavoproteins could be reduced by reduced lipoamide, which is in agreement with the data from the high fluorescence component of intact mitochondria. Reduction of the soluble flavoproteins by NADH was not sensitive to 9 μM dicumarol. The role of the high fluorescence flavoprotein as a component of the NADH oxidase system cannot be assessed with certainty on basis of the present data. The results emphasize the importance of using multiple techniques in studies on the energy-linked oxidation-reduction changes of the mitochondrial flavoproteins. As is shown, the flavoprotein pool before the rotenone site cannot be interpreted as a homogeneous entity.

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