Spectral properties of fluorescent flavoproteins of isolated rat liver mitochondria

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The different flavoproteins contributing to flavin fluorescence of isolated rat liver mitochondria have distinct excitation and emission spectra. The NAD-linked flavin component was identified as α-lipoamide dehydrogenase, while the non-NAD-linked component was found to be electron transfer flavoprotein. The differences in excitation and emission properties of the mitochondrial flavoproteins permit selective recording of their redox state changes in isolated mitochondria.

Flavoprotein fluorescence *α-Lipoamide dehydrogenase* *Electron transfer flavoprotein*

1. INTRODUCTION

Tissue flavin fluorescence has been widely used as an indicator of the redox state of the mitochondrial NADH/NAD^+ system [1–3,16,18]. These investigations were based on the assumption that about 90% of the tissue flavin fluorescence is of mitochondrial origin [1] and this mitochondrial flavin fluorescence is mainly caused by α-lipoamide dehydrogenase flavoprotein [4,5]. In [6], however, it was reported that 3 distinct fluorescent flavoprotein species can be detected in isolated rat liver mitochondria. These were α-lipoamide dehydrogenase ($E_{m7.4} = -283$ mV), electron transfer flavoprotein ($E_{m7.4} = -52$ mV), and an unknown, only dithionite-reducible, flavin compound. Other mitochondrial flavoenzymes did not contribute to flavin fluorescence of mitochondria. This paper presents the emission and excitation spectra of these 3 fluorescent flavoproteins which showed considerable differences, thus permitting spectral discrimination between these flavin components. Therefore, selective measurement of redox state changes in α-lipoamide dehydrogenase and electron transfer flavoprotein flavins is possible in intact mitochondria by choosing appropriate excitation and emission wavelengths of fluorescence.

Abbreviation: TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole

2. MATERIALS AND METHODS

DL-β-Hydroxybutyrate, oxoglutarate, NADH and ADP were purchased from Boehringer (FRG), rotenone, malonate, L-(−)-palmitoylcarnitine and palmitoyl-CoA from Serva (FRG), and sodium dithionite from Ferak (West Berlin). A medium consisting of 0.11 M sucrose, 60 mM Tris-HCl, 60 mM KCl, 10 mM potassium phosphate, 5 mM $MgCl_2$, 0.5 mM Na_2EDTA and 15 mM glucose (pH 7.4) was used throughout the experiments. Myxothiazol [9] was a kind gift from Dr Trowitzsch (Braunschweig, FRG), and the uncoupler TTFB was a generous gift from Dr Beechey (Sittingbourne, England). Rat liver mitochondria were isolated from female Wistar rats as described by Steinbrecht and Kunz [7]. Special care was exercised in removing lipid layers after each centrifugation step. With 10 mM oxoglutarate (+ 1 mM malonate) as substrate, the respiratory control index was routinely better than 4. Time-dependent changes in flavin and

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NAD(P)H fluorescence were recorded by means of a multifunctional optical device [8] equipped with light guides in a light-screened, stirred oxygraph vessel. For flavin fluorescence measurements, the excitation light of 436 nm was filtered out with an RSIF 436 Hg interference filter (VEB Carl Zeiss, Jena, GDR), while a GG 14 cut-off filter ($\lambda \geqslant$ 500 nm; VEB Schott, Jena, GDR) was used for emission light. Simultaneous NAD(P)H fluorescence measurement was performed while using 366 nm excitation light and monitoring emitted light at 400 nm. Fluorescence spectra were recorded on a JY-3 CS spectrofluorimeter (Jobin Yvon, France). Difference spectra were obtained by numerical subtraction on the connected Silex computer unit.

3. RESULTS AND DISCUSSION

In fig.1 the redox behaviour of the mitochondrial NAD(P) system and fluorescent flavoproteins is shown in a typical experiment. Isolated rat liver mitochondria were incubated in the presence of ADP and uncoupler to deplete them of endogenous substrates. This procedure yielded full oxidation of the NAD(P) system and the fluorescent flavoproteins (state I). To reduce selectively the NAD-dependent flavin of α-lipoamide dehydrogenase (cf. [6]), rotenone and β-hydroxybutyrate were added. A stable reduced state of flavoproteins and NAD(P)H was then achieved (state II). Myxothiazol and palmitoylcarnitine were added to reduce the non-NAD-linked fluorescent flavoprotein which was assumed to be the electron transfer flavoprotein [6]. A marked response was seen in the flavin fluorescence signal, while the redox state of the NAD(P) system remained unchanged. This state of reduction (state III) was stable too since myxothiazol, a highly effective inhibitor of the b-c_1 complex [9,10], was employed. Addition of sodium dithionite produced another decrease in flavin fluorescence due to an unknown flavin component (cf. [6]) (state IV). The effect observed in the NAD(P)H fluorescence signal on dithionite addition was obviously an artefact attributable to its own absorption of the 366 nm excitation light. The flavoprotein trace clearly demonstrates that stable states of reduction can be reached by choosing nearly complete blocking inhibitors. We recorded the excitation and

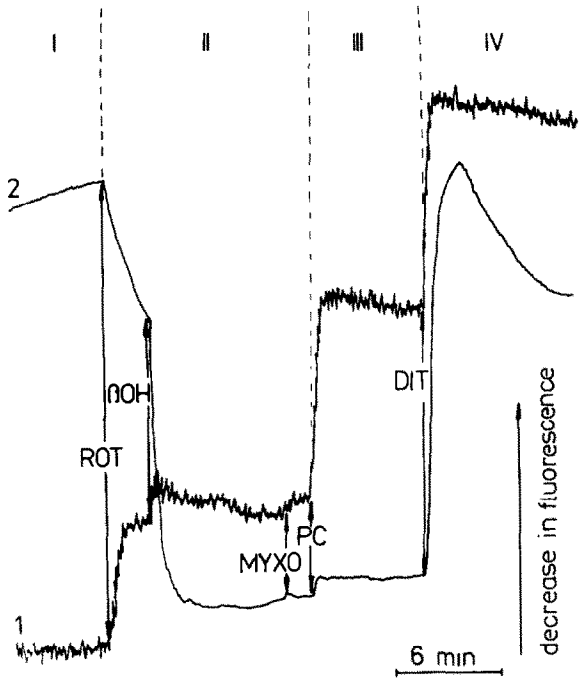

Fig.1. Redox behaviour of fluorescent flavoproteins (1) and NAD(P)H (2) in isolated rat liver mitochondria. Rat liver mitochondria (3.47 mg/ml) were incubated under the conditions described in section 2. Additionally, 0.25 mM ADP and 3 μM TTFB were present. Additions: 3 μM rotenone (ROT); 10 mM β-hydroxybutyrate (βOH); 2.3 μg/ml myxothiazol (MYXO); 50 μM palmitoylcarnitine (PC); a few crystals sodium dithionite (DIT). Conditions of fluorimetry as described in section 2. The vertical dotted lines divide the different states of flavin reduction (I–IV).

emission spectra of mitochondria in the stable states I–IV described to investigate the exact spectral properties of the different fluorescent flavoprotein species observable in isolated rat liver mitochondria. Obviously, the pure spectrum of the individual component can then be obtained simply by numerical subtraction of the different spectra. The result of this operation is shown in figs 2 and 3. Spectra A are the differences between the spectra scanned in the fully oxidized state (TTFB and ADP present) and those of the partially reduced state of flavoproteins in the presence of β-hydroxybutyrate and rotenone (I–II), thus representing the excitation and emission spectra of the NAD-linked flavoprotein identified as α-lipoamide dehydrogenase [4–6]. The excitation

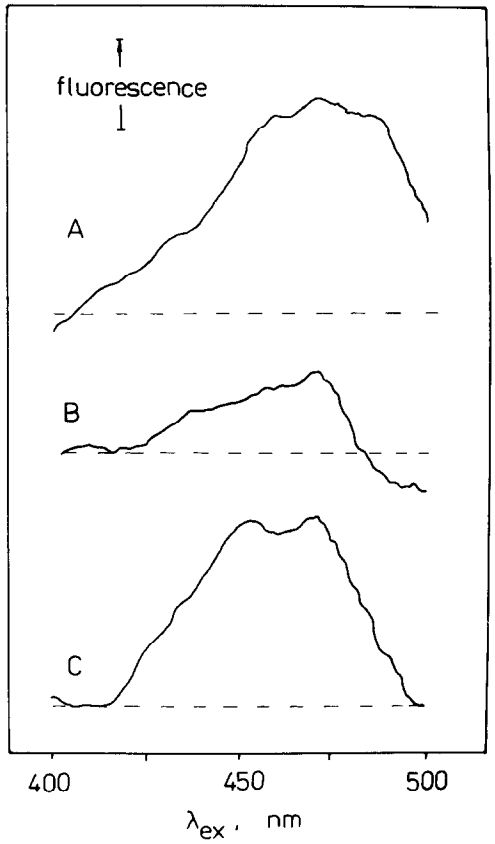

Fig.2. Excitation spectra of the mitochondrial fluorescent flavoprotein components. Rat liver mitochondria (4.44 mg/ml) were incubated in the medium described in section 2 in the presence of 0.25 mM ADP and 3 μM TTFB. After 5 min the fully oxidized excitation spectrum was run and stored on diskette using 520 nm as emission wavelength (to increase suppression of excitation light an OG 4 $\lambda \geqslant$ 515 nm cut-off filter [VEB Schott, Jena] was placed before the emission monochromator). Then, 3 μM rotenone and 10 mM β-hydroxybutyrate were added and the second spectrum run and stored. Thereafter, 2.3 μg/ml myxothiazol and 50 μM palmitoylcarnitine were added and the third spectrum run and stored. At least a few crystals of sodium dithionite were added and the fourth spectrum run and stored. Spectra: A, difference between spectra in states I and II; B, difference between spectra in states II and III; C, difference between spectra in states III and IV. Dashed lines, baselines of spectra.

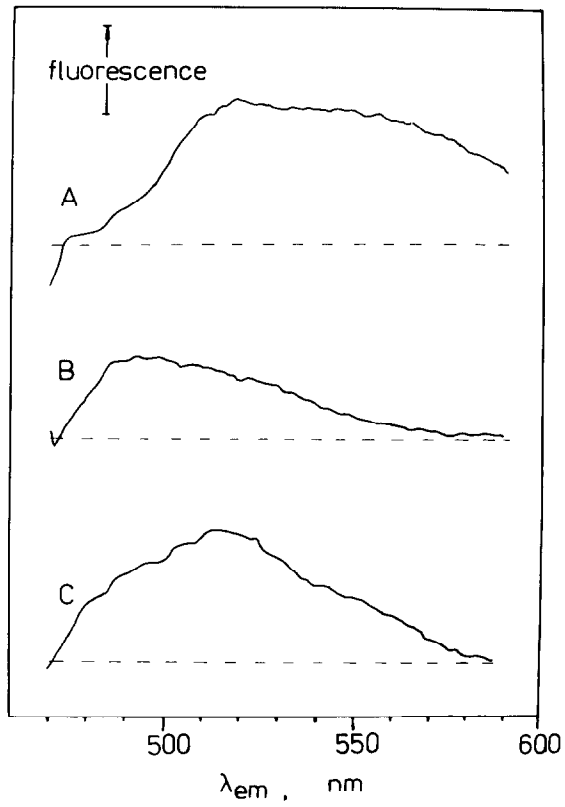

Fig.3. Emission spectra of the mitochondrial fluorescent flavoprotein components. The experiment was performed as described in fig.2 using 435 nm as the excitation wavelength (without any filters for emitted light). A, B, C are the differences of the emission spectra recorded in the states I, II, III, IV as outlined under fig.2.

spectrum in fig.2A has a broad maximum at about 470 nm and a small shoulder at 432 nm. The emission spectrum exhibits a maximum at 520 nm with a shoulder at 550 nm (fig.3A). Having a fairly broad pattern too, this spectrum is very similar to the emission spectrum of the isolated pyruvate dehydrogenase complex [11] and isolated α-lipoamide dehydrogenase [12], which can provide additional independent evidence of its origin.

Apparently, the difference spectra (II–III) between the state in the presence of β-hydroxybutyrate and rotenone and the state after myxothiazol and palmitoylcarnitine addition can be attributed to electron transfer flavoprotein (figs 2, 3; spectra B). The excitation spectrum showed 2 maxima: a small one at 435 nm and a large one at 468 nm. The emission spectrum had its maximum at 490 nm. Again, these spectra were very similar

to the fluorescence spectra of isolated electron transfer flavoprotein as reported by Hall and Kamin [13,14] who described an excitation maximum at 438 nm and 462 nm and an emission peak at 485–490 nm. These data yield further evidence that the fluorescent non-NAD-linked flavin component having an $E_{m7.4} = -52$ mV as described in [6] is really identical with electron transfer flavoprotein.

Isolated rat liver mitochondria contain in addition an exclusively dithionite-reducible fluorescent flavoprotein [6]. It could not be reduced by addition of either NADH or palmitoyl-CoA, thus ruling out the possibility that this component may be identical with cytochrome-b_5 reductase or a flavoprotein of the peroxisomal β-oxidation system as suggested recently [6] (not shown). The finding that the fraction of this flavin compound increased slightly during 2 h storage of mitochondria implies that at least part of this component might be flavin freed flavoproteins as a result of lytic processes during storage. The fluorescence spectra (III–IV) shown in figs 2,3 (spectrum C) are the differences between the state of mitochondria fully reduced by substrate addition (III) (rotenone, β-hydroxybutyrate, myxothiazol and palmitoylcarnitine present) and the dithionite-reduced state (IV). The excitation spectrum exhibits 2 maxima at 453 nm and 470 nm, while the emission spectrum has a pek at 514 nm. These maxima are nearly the same as those described for free flavins in heterogeneous environments [15], but differ from those of free FAD or FMN in aqueous solution having the typical emission peak at 525 nm. However, one cannot rule out the possibility that the fluorescent flavin compound observed which was only dithionite reducible is a still unidentified flavoprotein not in redox contact with the respiratory chain.

Careful examination of the excitation spectra shown in fig.2 reveals that, while showing typical bands of the corresponding flavoproteins, they differ from the spectra of the isolated enzymes with respect to the relative heights of peaks. This is clearly seen when the electron transfer flavoprotein excitation spectrum reported by Hall and Kamin [13] is compared with our data. The main peak at 438 nm is in fig.2 merely seen as a shoulder of the spectrum. Similarly, the 2 peaks of the excitation spectrum of the flavin compound reducible by dithionite only were equally high in our spectra, but it is well known that the main absorption band of flavins is in the region of 450 nm and only a small shoulder appears at abut 470 nm. The discrepancies observed are obviously due to the Soret bands of the cytochromes which produce a pronounced inner filter effect of the excitation light in the range 410–450 nm. These effects were not seen in the low-temperature fluorescence excitation spectra recorded by Chance et al. [16] for fully reduced and fully oxidized pigeon heart mitochondria. However, those workers noted that, at low temperature, background pigments interfered to a lesser extent.

From the data presented it can be concluded that it is possible to monitor selectively redox changes of α-lipoamide dehydrogenase flavin (λ_{ex} = 480 nm, λ_{em} = 545 nm) and of electron transfer flavoprotein (λ_{ex} = 435 nm, λ_{em} = 485 nm) in intact mitochondria. The fluorescence characteristics described of the individual flavoproteins must be taken into consideration when adopting tissue flavin fluorescence measurements to estimate mitochondrial NAD redox state [1–3]. The differences in the fluorescence spectra of flavoproteins also account for the controversy in the literature concerning the respective contributions of the various flavoenzymes to flavin fluorescence of mitochondria [4–6,17] which obviously depend on the excitation and emission wavelengths used.

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