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Evaluation of electron-transfer flavoprotein and α-lipoamide dehydrogenase redox states by two-channel fluorimetry and its application to the investigation of β-oxidation

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A new method permitting the simultaneous evaluation of the redox states of α-lipoamide dehydrogenase and electron-transfer flavoprotein in intact rat liver mitochondria by two-channel fluorimetry is described. It is shown that correction for the partial overlap of emission spectra can readily be introduced after a calibration procedure is performed. This method was applied to the investigation into regulation of palmitoylcarnitine oxidation. It was found that in the presence of rotenone, malonate and a redox buffer for the mitochondrial NAD-system, the β-oxidation flux was sensitive to variations in redox state of respiratory chain electron carriers at low states of NAD reduction. Therefore, the concept of β-oxidation control caused solely by the NAD redox state can no longer be sustained.

Introduction

In the earlier literature about flavoprotein fluorescence of isolated mitochondria, the flavoprotein fluorescence signal was suggested to be caused mainly by the α-lipoamide dehydrogenase [1,2]. Recently, it was shown [3,4] that it is possible to resolve this signal of isolated rat liver mitochondria into three components. Two of them responding to redox changes of respiratory chain were identified to be α-lipoamide dehydrogenase (the NAD-linked response) and electron-transfer flavoprotein (the CoQ-linked response). The remaining part of the flavoprotein fluorescence signal has its origin from flavoproteins being not in redox contact to the respiratory chain. These flavoproteins are reducible by dithionite only. In the present work, the reported differences in the excitation and emission spectra of α-lipoamide dehydrogenase and electron-transfer flavoprotein [4] were used to evaluate their redox states. This method was applied to investigation into the regulation of palmitoyl carnitine oxidation in rat liver mitochondria which is still a controversial matter [5–10]. In the presence of rotenone, malonate and a redox buffer for the intramitochondrial NAD-system, the palmitoyl-carnitine oxidation was seen to be uncoupler-stimulable at low states of NAD reduction. This was accompanied by electron-transfer flavoprotein oxidation while the NAD redox state, which can be monitored via Fp_{lip} fluorescence [1,3], remained almost unchanged.

Abbreviations: Fp_{lip}, α-lipoamide dehydrogenase flavoprotein; CoQ, coenzyme Q; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole.

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Therefore, the concept that the NAD redox state is the main regulating effector of β-oxidation [7,8,11] fails to hold up under these conditions. Preliminary results were presented elsewhere [12].

Materials and Methods

Materials. Rotenone, L(−)palmitoylcarnitine, malonate and lithium acetoacetate were purchased from Serva (F.R.G.), β-hydroxybutyrate, oxoglutarate, oxaloacetate, ADP and malate from Boehringer (F.R.G.), oligomycin from Calbiochem (U.S.A.), potassium cyanide from BDH (U.K), and antimycin and duroquinone from Sigma (U.S.A.). All other chemicals were 'analytical grade' from VEB Laborchemie Apolda (G.D.R.). Myxothiazol was a kind gift by Dr. Trowitzsch (Braunschweig, F.R.G.), the uncoupler TTFB by Dr. Beechey (Aberystwith, U.K.).

Oxaloacetate, duroquinone and cyanide were employed as fresh solutions only. Duroquinole was prepared as described in Ref. 3. The incubation medium used throughout all experiments consisted of 110 mM sucrose/60 mM Tris-HCl/10 mM KCl/10 mM potassium phosphate/5 mM $MgCl_2$/0.5 mM Na_2EDTA/15 mM glucose (pH 7.4).

Methods. Rat liver mitochondria were isolated according to Steinbrecht and Kunz [13] from 24 h starved rats. The respiratory control index when using 10 mM oxoglutarate (+ 1 mM malonate) as substrate was routinely better than 4. Flavin fluorescence and oxygen consumption were measured simultaneously in a light-screened stirred oxygraph vessel thermostatted at 25 °C. The oxygen-consumption measurements were performed with a teflon-coated Clark electrode connected to a custom-built rate meter allowing the simultaneous determination of oxygen content and rate of respiration. The fluorescence measurements were conducted with a multichannel surface fluorimeter equipped with light guides [14]. The sample was exited at 436 nm and 450 nm wavelengths by using DRSIF 436Hg and DIF 450 interference filters (VEB Carl Zeiss, Jena, G.D.R.), respectively, and an XBO 101 highly stabilized xenon high-pressure lamp (VEB Narva, G.D.R.). The emission light was filtered out at 491 nm and 525 nm, respectively, by using the DIF 491Hg and IF 525 interference filters combined with appropriate cut-off filters (450 and 500, respectively; VEB Carl Zeiss, Jena, G.D.R.) to diminish reflectance artefacts.

Results and Discussion

Evaluation of electron transfer flavoprotein and Fp_{lip} redox states by two-channel fluorimetry

Due to the partial overlap of the relatively broad emission and exitation spectra of Fp_{lip} and of electron transfer flavoprotein reported in Ref. 4 it was impossible to obtain pure fluorescence signals selectively from one of the two respiratory-chain-linked fluorescent flavoproteins of isolated rat liver mitochondria. To solve this problem we adopted two-channel fluorimetry, choosing one channel to record under optimal conditions electron-transfer flavoprotein fluorescence (436 nm → 491 nm; cf. Ref. 4) and the other one to record Fp_{lip} fluorescence (450 nm → 525 nm; cf. Ref. 4). To correct for the spectral overlap occurring we had to perform a signal calibration under conditions where, additional to fluorescence changes of both flavoproteins, a fluorescence change of only one flavoprotein was observable. Such a calibration experiment is shown in Fig. 1. ADP and the uncoupler TTFB were added to the mitochondria to completely oxidize both flavoproteins. In the Fp_{lip} channel a somewhat larger fluorescence increase was seen than in the channel choosen for optimal electron-transfer flavoprotein detection. Rotenone and β-hydroxybutyrate were added to obtain a situation in which only one flavoprotein is reduced whilst the other one remains completely oxidized. Under these conditions a large reduction of intramitochondrial NAD^+ occurred (cf. Refs. 3 and 4), while all CoQ-linked redox carriers remained oxidized. The flavin fluorescence quenching observed under these circumstances was demonstrated to be caused by α-lipoamide dehydrogenase reduction [1–4]. Therefore, this state can be used for the required intermediate calibration point of 100% Fp_{lip} reduction and 0% electron-transfer flavoprotein reduction. In fact, as can be seen in Fig. 1, a large reduction induced by rotenone and β-hydroxybutyrate addition was seen in the Fp_{lip} channel (trace 1), whereas the effect observed in the other channel (trace 2) was smaller.

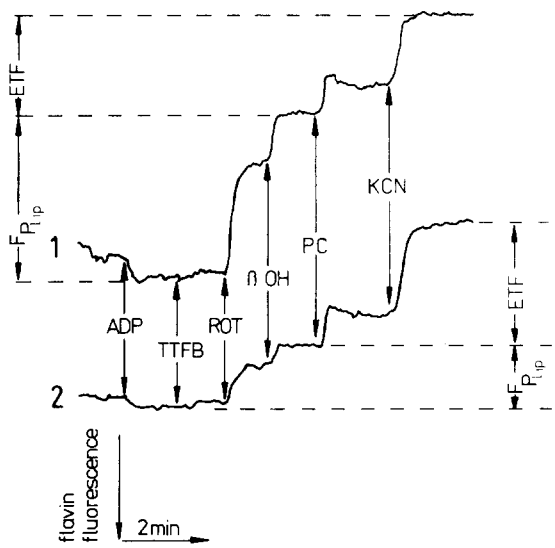


Fig. 1. Calibration of flavoprotein fluorescence at two different emission and exitation wavelength pairs. Rat liver mitochondria (4.7 mg/ml) were incubated in the standard medium. Trace 1: Flavin fluorescence 450 nm → 525 nm (Fp_{lip}-channel). Trace 2: Flavin fluorescence 436 nm → 491 nm (electron-transfer flavoprotein (ETF) channel). Additions: ADP 0.5 mM, TTFB 3 μM, rotenone (ROT) 3 μM, β-hydroxybutyrate (βOH) 10 mM, L(−) palmitoylcarnitine (PC) 50 μM, KCN 4 mM. The horizontal dashed lines mark the reference states: both flavoproteins oxidized; Fp_{lip} reduced, electron-transfer flavoprotein oxidized; both flavoproteins reduced.

This stable state of partial flavin fluorescence quenching (identified by the dashed midlines in Fig. 1) can be used to correct for the contribution of Fp_{lip} occurring in the electron-transfer flavoprotein channel. Palmitoylcarnitine and cyanide were added to reach the fully reduced state of both flavoproteins. Palmitoylcarnitine addition entailed a transient flavin reduction followed by a slow reoxidation (Fig. 1). This effect was mediated by a transient increase in respiration (not shown) and most probably a result of the sensitive control of β-oxidation flux by the NADH/NAD^+ ratio at high NAD-redox states (cf. the next sub-Section). Following cyanide addition, a larger fluorescence decrease was noted in the electron-transfer flavoprotein channel than in the Fp_{lip} channel. Due to the fact that the flavin fluorescence quenching occurring is caused by electron-transfer flavoprotein reduction (as previously demonstrated [3,4]) the effect observed in the Fp_{lip} channel must be linked to spectral overlap and can be used for data correction. To exclude artefacts caused by inner filter effects of cytochromes, myxothiazol instead of cyanide was added in control experiments, and merely a negligible difference of less than 5% of the total signals was observed (not shown). Therefore, this calibration experiment readily yielded the respective percentages of electron-transfer flavoprotein and Fp_{lip} which accounted for the flavoprotein fluorescence signals at the two wavelength pairs: in the electron-transfer flavoprotein channel, contributions of 66% electron-transfer flavoprotein and 34% Fp_{lip} were detected, whereas those seen for the Fp_{lip} channel were 62% Fp_{lip} and 38% electron-transfer flavoprotein. This calibration can be used to evaluate intermediate redox states while assuming that no spectral changes occur during oxidation-reduction and supposing that the signals consist of the above-mentioned components only. Let the actual percentage of total flavoprotein reduction measured at 450 nm → 525 nm be equal to A and that measured at 436 nm → 491 nm be equal to B. These two values are then obviously linked to the individual redox states of Fp_{lip} equal to X and of electron-transfer flavoprotein equal to Y according to the following equations:

$$0.62X + 0.38Y = A \quad (Fp_{lip}\text{ channel})$$

$$0.34X + 0.66Y = B \quad \text{(electron-transfer flavoprotein channel)}$$

Then it should be possible to evaluate intermediate redox states of Fp_{lip} and electron-transfer flavoprotein simply by solving this system of equations. To verify the validity of this method we performed redox titrations of electron-transfer flavoprotein with the duroquinol/duroquinone couple as described in Ref. 3 under conditions where the redox state of Fp_{lip} was kept constant. This was accomplished by adding rotenone and 10 mM β-hydroxybutyrate. Furthermore, myxothiazol was added to block the oxidation of the CoQ-pool via the respiratory chain. Then, duroquinol/duroquinone was added in different ratios and the redox states of Fp_{lip} and electron-transfer flavoprotein calculated by solving the above equations. The outcome of such an experiment is depicted in Fig. 2. It can be seen that the redox state

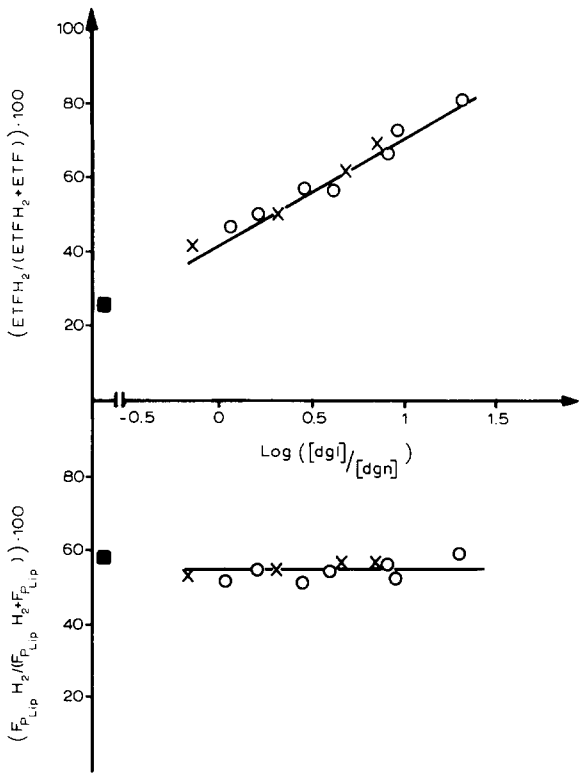


Fig. 2. Duroquinol/duroquinone titrations of fluorescent flavoproteins at two different emission and exitation wavelength pairs. Rat liver mitochondria (4.3 mg/ml) were preincubated for 10 min in the standard medium as described in Materials and Methods, containing additionally 0.5 mM ADP, 5 mM malonate, and 3 μM TTFB. Then 5 μM myxothiazol, 10 mM β-hydroxybutyrate and 3 μM rotenone were added. Oxidative titrations (×): after addition of 500 μM duroquinol (dql) small amounts of duroquinone (dqn) were added. Reductive titrations (○): after addition of 50 μM duroquinone appropriate duroquinol additions were made. Filled square: point after addition of 50 μM duroquinone. The redox states of both electron transfer flavoprotein: $(ETFH_2/(ETFH_2 + ETF))\cdot 100$ and Fp_{lip}: $(Fp_{lip}H_2/(Fp_{lip}H_2 + FP_{lip}))\cdot 100$ were calculated as described in the text. A Nernst curve with $n = 1.2$ was drawn through the data points of the upper plot. The midpoint potential of −40 mV was calculated from the inflection point, using $E_{m7.4} = -19$ mV for the dql/dqn couple [28].

of electron-transfer flavoprotein changed as a function of varying duroquinol/duroquinone ratios from about 20% to 100%, while that of Fp_{lip} remained constant at some 60% reduction. A Nernst curve with $n = 1.2$ was drawn through the data points for the electron-transfer flavoprotein redox states. The midpoint potential of the semiquinone/quinone couple (only the quinone form of electron transfer flavoprotein is fluorescent [15]) was calculated to be −40 mV at pH 7.4. This is in agreement with the data reported by Voltti and Hassinen ($E_{m7.4}$ between −40 mV and −56 mV) [16] and Gustafson et al. ($E_{m7.1} = -14$ mV; if considering a pH dependency) [17] but fails to be in good agreement with the value reported by Husain et al. ($E_{m7.5} = +4$ mV) [29].

Unexpectedly, a comparatively low redox state of Fp_{lip} was noted even in the presence of β-hydroxybutyrate and rotenone. This could be interpreted in two different ways. First, the NAD-linked flavoprotein fluorescence signal was not homogeneous and contained a duroquinone-oxidizable high-potential flavin in addition to Fp_{lip}. This possibility seems to be very unlikely due to earlier observations [3] that the NAD-linked flavin fluorescence signal is completely titrable with the β-hydroxybutyrate/acetoacetate redox couple and behaves as a single component. Second, it is reasonable to assume a slight unspecific reaction to occur, of the relatively high concentrations of duroquinone employed for redox buffering, with redox centres on the substrate side of the rotenone block. This could also explain the partial reoxidation of Fp_{lip} observed after duroquinone (50 μM) addition in the presence of β-hydroxybutyrate and rotenone alone (filled square), and therefore the relatively low redox state of Fp_{lip} during the duroquinol/duroquinone titration experiment shown in Fig. 2. Moreover, it was reported by DiVirgilio and Azzone [30] that the NADH : duroquinone reductase activity of rat-liver mitochondria is only to 18% rotenone sensitive being an additional argument in favour of this explanation.

It should be pointed out, however, that the redox state of α-lipoamide dehydrogenase determined by this method is not the true redox state of its flavin. This is due to the fact that the two-electron reduced state of α-lipoamide dehydrogenase FAD$\begin{matrix}\diagup\mathrm{SH}\\ \diagdown\mathrm{SH}\end{matrix}$ was found to have only 15% of the flavin fluorescence of the oxidized enzyme [31]. Additionally, it has been reported that NAD^+ binding to the oxidized enzyme cause considerable alterations in its fluorescence [32]. Nevertheless, it has been shown [1–3] that it is possible to use the

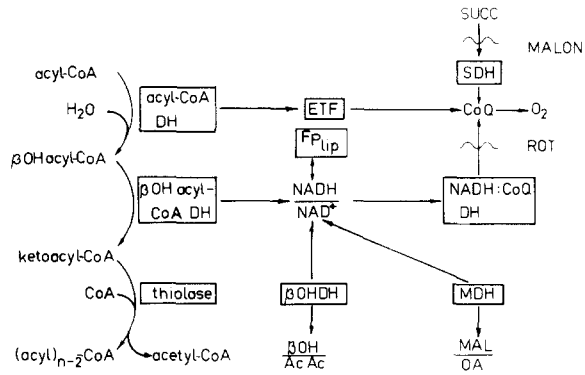
determined by fluorimetry redox state of Fp_{lip} as a marker of intramitochondrial NADH/NAD^+.

To summarize, the above-described method lends itself to evaluating the redox states of both electron-transfer flavoprotein and α-lipoamide dehydrogenase.

Application of two-channel flavin fluorimetry to investigation into the regulation of β-oxidation

It is well documented in the literature [7,8,11] that hydrogen donors from carbohydrate and amino acid metabolism lead to a marked suppression of β-oxidation. As early as in the classic paper by Bremer and Wojtczak [7] two mechanisms were discussed which explain this phenomenon observed by these workers for succinate: (1) additional hydrogen donors lead to increased NAD reduction and inhibition of β-oxidation flux via the NAD-dependent β-hydroxyacyl-CoA dehydrogenase; and (2) additional hydrogen donors lead to increased reduction of respiratory chain electron carriers (especially of CoQ), being therefore less effective acceptors of electrons from the flavin-dependent acyl-CoA dehydrogenases. The importance of the first type of redox regulation was demonstrated and a direct relation observed to exist between NAD redox state and β-oxidation flux [8,11]. However, little attention has been paid to the second mechanism, of which the contribution to redox control of β-oxidation has not yet been elucidated. Two-channel flavin fluorimetry enabled us to test directly the influence of a change in one of the discussed parameters on β-oxidation flux. This is due to the Fp_{lip} redox state being indicative of the degree of reduction of the intramitochondrial free NAD system [1–3], whereas electron-transfer flavoprotein obviously is a good marker of the redox state of respiratory chain electron carriers as can be easily deduced from Scheme I.

In order to study the effect of redox state changes of respiratory chain redox carriers on the β-oxidation rate, it was necessary to maintain the degree of NAD reduction comparatively constant. We, therefore, investigated the palmitoyl carnitine oxidation in rotenone + malonate inhibited mitochondria with a kinetically buffered NAD redox state (via β-hydroxybutyrate dehydrogenase or malate dehydrogenase; cf. Scheme I). Furthermore, this β-oxidation model system used by many other investigators [11,18,19] is highly advantageous in that it permits the β-oxidation flux to be measured directly as rate of respiration. This is possible, since only the electrons generated by acyl-CoA dehydrogenase can flow to oxygen, whereas the reducing equivalents produced by β-hydroxyacyl-CoA dehydrogenase lead to a slight redistribution of the redox buffer constituents (e.g., acetoacetate is reduced to β-hydroxybutyrate; cf. Scheme I). Due to inhibition of the citric acid cycle turnover by malonate, further NADH-producing dehydrogenases during palmitoyl carnitine oxidation should not be considered (cf. Ref. 11). Under these conditions we performed redox transitions in the respiratory chain by uncoupling of oligomycin-inhibited mitochondria. The redox state changes occurring were directly monitored by two-channel flavin fluorimetry.



Scheme I. Redox-dependent reactions of β-oxidation. Abbreviations: DH, dehydrogenase; SDH, succinate dehydrogenase; MDH, malate dehydrogenase; βOH DH, β-hydroxybutyrate dehydrogenase; SUCC, succinate; MAL, malate; OA, oxaloacetate; βOH, β-hydroxybutyrate; AcAc, acetoacetate; MALON, malonate; ROT, rotenone. Wavy lines define the site of inhibition.

The experiments adapted to simultaneously measure respiration and flavin fluorescence were conducted as shown in Fig. 3A and B. Mitochondria were first incubated in the presence of malonate and ADP to obtain a fully oxidized reference state of flavoproteins necessary for quantitiative redox state determination (uncoupler addition did not yield further flavoprotein oxidation as demonstrated in Fig. 1). Observed after

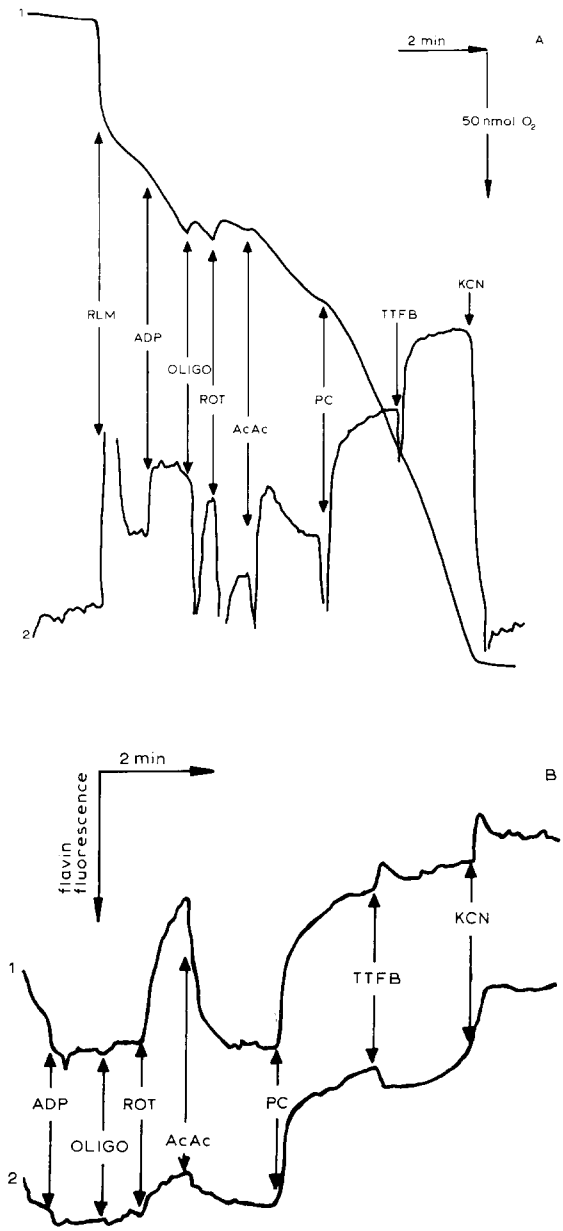


Fig. 3. Simultaneous measurements of rate of respiration (A) and flavoprotein fluorescence (B) during acetoacetate-stimulated palmitoyl carnitine oxidation in the presence of rotenone. Rat liver mitochondria (4.9 mg/ml) were incubated in the standard medium described in the methodical section, with 2 mM malonate being additionally present. A: 1, respiration trace; 2, first derivative. B: 1, flavin fluorescence 450 nm → 525 nm (Fp_{lip}-channel); 2, flavin fluorescence 436 nm → 491 nm (electron-transfer flavoprotein channel). Additions: ADP 0.5 mM, oligomycin (OLIGO) 0.25 μg/ml, rotenone (ROT) 3 μM, acetoacetate (AcAc) 10 mM, L(−) palmitoyl carnitine (PC) 50 μM, TTFB 3 μM, and KCN 4 mM.

ADP addition was an oxidation (produced mainly by Fp_{lip}) in the fluorescence traces presented in Fig. 3B; it was accompanied by a transient increase in respiration due to the presence of endogeneous substrates (Fig. 3A). Then, oligomycin was added to block the mitochondrial ATP production and, hence, utilization of $\Delta\tilde{\mu}_{H^+}$. Rotenone entailed a marked decrease in respiration and an increase in Fp_{lip} reduction also caused by the endogeneous substrates. Thereafter, acetoacetate was added as an external pool of oxidizing equivalents equilibrating with the intramitochondrial NAD system via β-hydroxybutyrate dehydrogenase. This, in turn, resulted in an almost complete oxidation of Fp_{lip} with a transient increase in oxygen consumption due to oxidation of endogeneous fatty acids [20] (Fig. 3A). L-Palmitoylcarnitine causes a remarkable further increase in respiration which was accompanied by reduction of both flavoproteins (Fig. 3A and B). The procedure described in the previous subsection was applied and electron-transfer flavoprotein and Fp_{lip} reduction of 69% and 33%, respectively, were calculated for this state. Subsequent addition of the uncoupler TTFB yielded a 1.4-fold stimulation of respiration (under these conditions a direct measure of β-oxidation flux; cf. introductory part of this section) (Fig. 3A). This acceleration of respiration was accompanied by electron-transfer flavoprotein reoxidation to 51%, while the Fp_{lip} reduction level rose to 48%. The following addition of cyanide then produced full electron-transfer flavoprotein reduction, while Fp_{lip} due to acetoacetate was oxidized to a greater extent (26% reduction). Not directly seen in the fluorescence trace in Fig. 3B, this effect was evidenced after evaluation of redox states by the procedure described in the previous section. Thus, this experiment revealed that under these conditions electron-transfer flavoprotein and Fp_{lip} behaved in opposed manners. With the flux increasing, electron-transfer flavoprotein was oxidized while Fp_{lip} was reduced. The effect noted for Fp_{lip} apparently resulted from incomplete kinetic buffering of the NAD system via β-hydroxybutyrate dehydrogenase. Thus, at high rates of β-oxidation the rate of NADH production reached a level at which the removal of reducing equivalents by dehydrogenase is limited. On the other hand, the electron transfer

flavoprotein redox transition was obviously due to an energy-linked redox state change of respiratory chain electron carriers as caused by the collapse of $\Delta\tilde{\mu}_{H^+}$ after uncoupling [21,22]. Thus, the uncoupler stimulability of palmitoylcarnitine oxidation may be attributed to a redox transition at the acceptor site of electrons coming from acyl-CoA dehydrogenase. This clearly speaks in favour of the second type of β-oxidation control mechanisms mentioned above. A regulatory effect of the NAD system under these conditions can be definitely ruled out because of its opposed redox state change.

However, it is reasonable to assume that the influence on the rate of β-oxidation as caused by the respiratory chain is a function of the stationary NAD redox state which, in turn, is the main parameter governing the maximal flux rate [11,18]. Consequently, we studied the present β-oxidation model system at various stationary states of NAD reduction. This can be done by varying the redox potential of the kinetic buffer of the intramitochondrial NAD system. Thus, we performed the same experiments as described above except that, instead of acetoacetate, various β-hydroxybutyrate/acetoacetate ratios or, in some cases, oxaloacetate were added. We then plotted the calculated redox states of Fp_{lip} and electron-transfer flavoprotein versus the measured β-oxidation rate in the oligomycin-inhibited (×) and uncoupled (○) states of palmitoylcarnitine oxidation. The result of such a plot is presented in Fig. 4A and B. It can be seen that at high β-hydroxybutyrate/acetoacetate ratios which are indicated by high Fp_{lip} reduction, low electron-transfer flavoprotein reduction levels and a low β-oxidation flux were observed (points on the left in Fig. 4A and B). Under these circumstances no uncoupler stimulation of palmitoylcarnitine oxidation was seen (○ and × coincided). This situation is therefore characterized by a single flux-governing effector: the availability of free NAD^+. When the ratio of β-hydroxybutyrate/acetoacetate was lowered to values below unity, stationary Fp_{lip} redox states of less than 80% were noted. This led to enhanced β-oxidation flux rates and the occurrence of an uncoupler stimulability. Thus, the regulatory effect induced by the respiratory chain is restricted to a range of low NAD redox states.

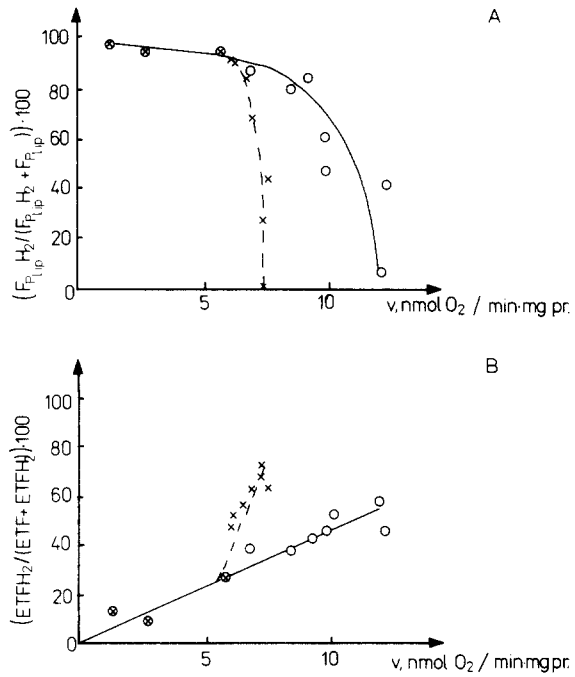


Fig. 4. Flux dependencies of Fp_{lip} (A) and electron-transfer flavoprotein (B) redox states under coupled (×) and uncoupled (○) conditions. Rat liver mitochondria (about 5 mg/ml, three different preparations) were incubated in the standard medium with 2 mM malonate being additionally present. Conditions of measurement and additions as in Fig. 3A and B. Instead of acetoacetate alone, different β-hydroxybutyrate/acetoacetate ratios or 10 mM oxaloacetate (points with approx. 12 nmol O_2/min mg pr. respiration) were added. The respective redox states of Fp_{lip} and electron-transfer flavoprotein were calculated as described in the text. ×, oligomycin-inhibited state of β-oxidation, ○, TTFB-uncoupled state of β-oxidation.

Moreoever, as can be seen in the plot in Fig. 4A, the sensitivity of flux to variations in NAD reduction in this region is much lower than at high NAD redox states. Obtained also by Latipää et al. [11] even though with a different technique of NAD redox state determination, this finding is supposed to reflect a Michaelis-Menten type behaviour for β-hydroxyacyl-CoA dehydrogenase with respect to free NAD^+, implying that the sensitivity of enzyme velocity to variations in NAD^+ concentration is high in the range of K_m and low near v_{max}. As a cause for the extreme high sensitivity of β-oxidation flux to variations of the NAD redox state at a high degree of NAD reduction the very effective product inhibition of acyl-

CoA dehydrogenase by enoyl-CoA and β-hydroxyacyl-CoA compounds should be additionally taken into consideration (cf. Ref. 11). This may have larger influence under conditions where the outer carnitine palmitoyltransferase is inhibited by malonyl-CoA and the intramitochondrial palmitoyl-CoA concentration should expected to be lower than in the above-described experiments.

The dependence of electron-transfer flavoprotein redox state on flux exhibited a different behaviour as can be seen in Fig. 4B. For the uncoupled state, a linear relationship was found between redox state and flux, reflecting most probably the behaviour of the CoQ-pool which can be described by the Kröger-Klingenberg equation [23,24]. Under oligomycin-inhibited conditions, the linear electron-transfer flavoprotein redox state to β-oxidation flux relationship was seen to have a higher slope which, again can be interpreted in terms of the Kröger-Klingenberg model for CoQ-pool behaviour while assuming a diminished CoQ oxidation rate (cf. Refs. 23 and 24) due to $\Delta\tilde{\mu}_{H^+}$ control of electron transport.

To summarize, a qualitative description of redox-dependent β-oxidation flux regulation becomes possible. At high degrees of NAD reduction, the palmitoyl carnitine oxidation rate is extremely sensitive to variations in NAD redox state, and no additional respiratory chain-induced influence should be taken into consideration. This finding is in line with earlier investigations [8,11]. At low degrees of NAD reduction, however, the β-oxidation flux becomes additionally sensitive to variations in the redox state of respiratory chain electron carriers. Therefore, under these conditions at least two rate-controlling parameters should be considered while neglecting at this stage of investigation other additional control mechanisms proposed [9,10,25]. This complex behaviour clearly outlines the limits of the classic 'rate-limiting step' concept when applied to complex metabolic pathways. For exact quantification of β-oxidation flux control, the control theory developed by Kacser and Burns [26] as well as Heinrich and Rapoport [27] should be adopted as already suggested by Latipää et al. [11].

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