Fluorescence of Nicotinamide Adenine Dinucleotides during the Active Transport of Ca²⁺ Ions in Liver Mitochondria

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Abstract

Accumulation of Ca^{2+} ions by rat liver mitochondria increases the fluorescence of the reduced pyridine nucleotides in the presence of rotenone. The fluorescence increase is sensitive to the uncouplers of the oxidative phosphorylation and to the inhibitors of the electron transfer, providing the release of the accumulated Ca^{2+} from mitochondria. The substantial change of the fluorescence occurs in the presence of acetate, when the accumulated Ca^{2+} is present in the intramitochondrial space as soluble salt. Addition of Ca^{2+} to water solution of NADH and NADPH is followed by the slight increase of the fluorescence, while the same nucleotides dissolved in the more hydrophobic medium (methanol) considerably increase the fluorescence level by addition of Ca^{2+}. The increase of the fluorescence of NAD(P)H in the mitochondria due to the accumulation of Ca^{2+} is considered not to be caused by the alkalinization of the intramitochondrial space but by the formation of the nucleotide-metal complex, localized at least partly at a hydrophobic phase.

Introduction

Changes of the reduction degree of NAD and NADP in the mitochondria can be traced either by measurement of the absorption at 340 nm or of the fluorescence at the region of 440-460 nm. This method found wide application after the apparatus of high sensitivity had been created, which made it possible to measure the reduction of the endogenous

Abbreviations used: FCCP, *p*-trifluoromethoxycarbonylcyanidephenylhydrazone; TMPD, tetramethyl-*p*-phenylenediamine.

NAD(P) in the mitochondrial suspension as well as *in vivo* [1, 2]. Considerable oxidation of NAD(P)H by addition to mitochondria ADP in aerobic conditions has been shown [3]. Similar responses have been observed at the energy-linked accumulation of Ca^{2+} in the mitochondria [4]. Chance and Azzi [5] observed the increase of the NAD(P)H absorption at 345 nm which correlated with the increase of the fluorescence caused by addition of Ca^{2+} to the mitochondria in the presence of rotenone and succinate. Having estimated the relative quantum yield of the absorption and fluorescence of the reduced pyridine nucleotides, the authors have come to the conclusion that this increase of the fluorescence reflects either the reduction or the alteration of the quantity of bound form of pyridine nucleotides [5, 6], possessing the high quantum yield of fluorescence. In this paper we have studied changes of the endogenous pyridine nucleotides fluorescence during the transport of Ca^{2+} in the mitochondria in various conditions. It was discovered that the changes of the fluorescence reflect the concentration of Ca^{2+} in intramitochondrial space and do not depend on its alkalinization. Besides, we have confirmed the results obtained by Lowry *et al.* [7] concerning changes of the NAD(P)H fluorescence in the presence of bivalent cations and we believe that the increase of the fluorescence in the presence of rotenone is due to the nucleotide-calcium complex formation localized in a hydrophobic phase of mitochondria.

Methods

Rat liver mitochondria were isolated in a medium containing 0.25 M sucrose and 1 mM EDTA (pH 7.4) as described by Johnson and Lardy [8]. The fluorescence of endogenous NAD(P)H was measured with the microfluorimeter of our construction [9]. The fluorescence was excited with monochromatic light at 366 nm and measured at 450 nm from the same surface of the cuvette. The angle between the exciting light beam and the light beam being measured makes $180°$, which excludes the influences of light scattering on the intensity of the fluorescence. The rate of oxygen consumption was measured polarographically using a rotating platinum electrode, pH value of the incubation media with a glass electrode [9]. All functions (fluorescence, proton transfer and respiration) had been studied simultaneously at $20°C$ in a sample, volume of 2 ml. The composition of the incubation media is given in the legends to the figures. Protein was determined according to Gornall *et al.* [10].

Results and Discussion

Figure 1 gives the record of the fluorescence changes of the mitochondria by addition of different compounds influencing the degree
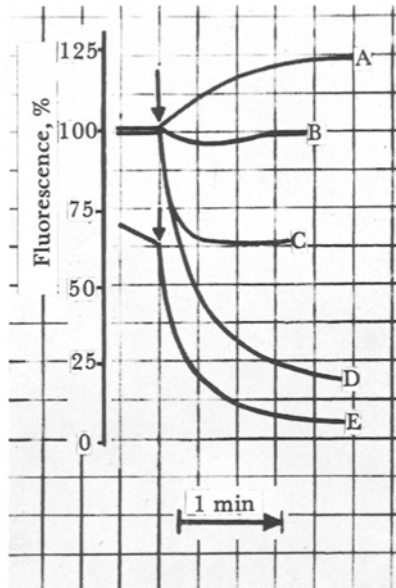
Figure 1. Fluorescence intensity of reduced pyridine nucleotides of mitochondria at different metabolic states. Mitochondria (2.6 mg protein/ml) were incubated in a media containing 0.15 M sucrose, 0.075 M KCl, 2.5 mM MgCl$_2$, 5 mM KH$_2$PO$_4$ (pH 7.4) and 10 mM succinate. The arrow indicates the addition of: A, 406 μM CaCl$_2$ in the presence of rotenone (5 μM); B, 400 μM ADP or 4 μM FCCP or 10^{-7} M valinomycin in the presence of rotenone (5 μM); C, 400 μM ADP; D, 10^{-7} M valinomycin; E, 4 μM FCCP in the presence of 4 mM malonate (without succinate). The level of fluorescence of pyridine nucleotides during the steady state 4 is taken to be 100% [11].

of NAD(P) reduction. Addition of Ca^{2+} in the presence of rotenone (curve A) induces the increase of fluorescence, which makes 20% of the total intensity (the value corresponding to full oxidation was taken for the level of fluorescence in the presence of malonate and uncoupler, curve D). In the presence of rotenone neither ADP, nor the uncoupler, nor valinomycin induce any fluorescence changes. Without rotenone, these compounds induce a decrease of fluorescence due to their effect on the energy state of mitochondria. Thus, the increase of fluorescence by addition of Ca^{2+} is highly specific and cannot be explained by a redistribution of the NAD(P)/NAD(P)H reduction level, caused by alkalinization of intramitochondrial space or by the decrease in energization of mitochondria. Another indication that the alteration of the fluorescence does not depend on alkalinization of intramitochondrial space, has been obtained in the experiments with addition of Ca^{2+} in the presence of different anions (Fig. 2). It is seen that the increase of the fluorescence takes place in the medium without penetrating anion
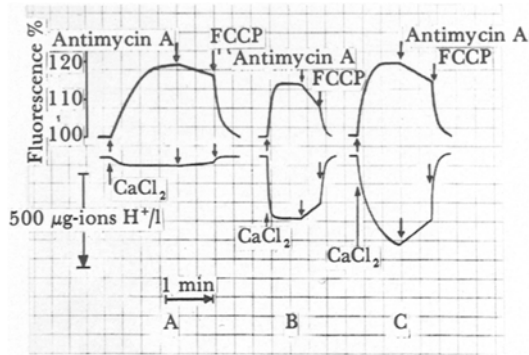
Figure 2. Changes of fluorescence and pH during active transport of Ca^{2+}. Mitochondria (2.6 mg protein/ml) were incubated in the media containing: A, 0.15 M sucrose, 0.075 M KCl, 2.5 mM $MgCl_2$, 5 μM rotenone, 10 mM succinate, 5 mM Tris-HCl (pH 7.4) and 20 mM acetate; B, as in the legend to Fig. 1, but with 5 μM rotenone; C, as in A, but without acetate. Arrows indicate the addition of 406 μM $CaCl_2$, 2 μg/ml antimycin A and 5 μM FCCP.

($H^+/Ca^{2+} \approx 2$) as well as in the medium with phosphate which decreases alkalinization of intramitochondrial space ($H^+/Ca^{2+} \approx 1$) or with acetate when transport of Ca^{2+} does not induce significant change of pH value of intramitochondrial space ($H^+/Ca^{2+} \approx 0.2$) [12, 13]. In all the cases when antimycin A was added the slow decrease of fluorescence was induced, which was followed by a slow alkalinization of the incubation medium. The results of these experiments show that the increase of fluorescence of NAD(P)H relates to the Ca^{2+} concentration in intramitochondrial space more than to alkalinization of the inner medium. The maximum change of fluorescence is found in the presence of acetate, when the accumulated Ca^{2+} is present in intramitochondrial space as a soluble salt.

The increase of mitochondrial fluorescence depends on the energy and is completely reversed at deenergization (Fig. 3). In the presence of antimycin A there occurs a slow efflux of Ca^{2+} into the incubation medium and a decrease of fluorescence. Under these conditions the addition of TMPD and ascorbate provides the accumulation of released Ca^{2+} on account of energy supplied by the terminal part of the respiratory chain and a secondary increase of fluorescence. After exhaustion of oxygen in the reaction mixture Ca^{2+} releases again from the mitochondria and the fluorescence decreases. The following addition of 4 mM ATP (not shown in the figure) providing the accumulation of Ca^{2+} under anaerobic conditions increases again the level of fluorescence.

Lowry *et al.* [7] have found that the fluorescence of NADH and NADPH depends on the presence of bivalent cations. On the basis of these facts one might think that the increase of fluorescence is caused by the formation of nucleotide-metal complexes having their spectral
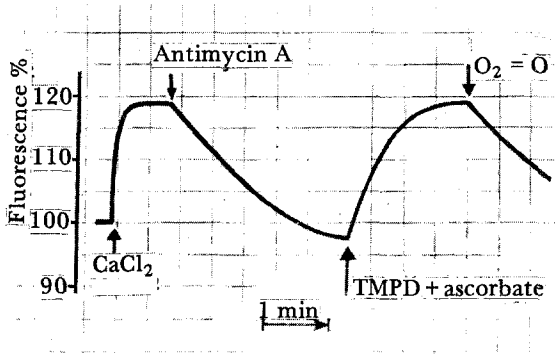
Figure 3. Changes of NAD(P)H fluorescence during transport of Ca²⁺. Medium and concentration of mitochondrial protein as in Fig. 2C. Arrows indicate additions of 406 μM CaCl$_2$, 2 μg/ml antimycin A, 200 μM TMPD and 10 mM ascorbate.

characteristics different from unbound NADH and NADPH. In order to prove this assumption we have measured the NAD(P)H fluorescence in solution by addition of Ca²⁺ under different circumstances. These results are shown in Fig. 4. In accordance with the results obtained by Lowry *et al.* [7] concerning the fluorescence of the reduced pyridine nucleotides it was found that addition of Ca²⁺ to aqueous solutions of NADH and NADPH and their mixture slightly influences the fluorescence intensity (the increase makes not more than 5%). The decrease of polarity of the medium causes a pronounced response of fluorescence at the addition of
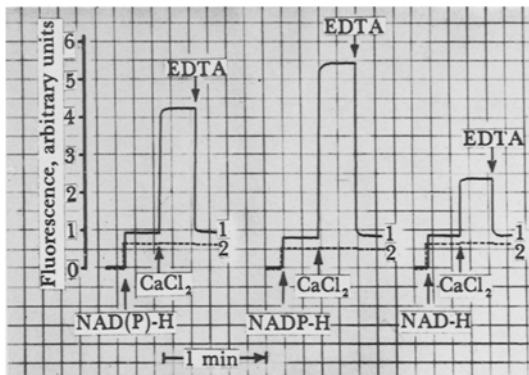

Figure 4. Effect of Ca²⁺ on fluorescence intensity of pyridine nucleotides in solution. Fluorescence was measured in a medium containing 10 mM Tris-HCl (pH 7.5) and 50 μM EDTA in water solution (trace 2) and in 90% methanol (trace 1). Arrows indicate additions of NADH (50 μM) + NADPH (50 μM), NADPH (100 μM), NADH (100 μM), CaCl$_2$ (1 mM) and EDTA (1 mM).
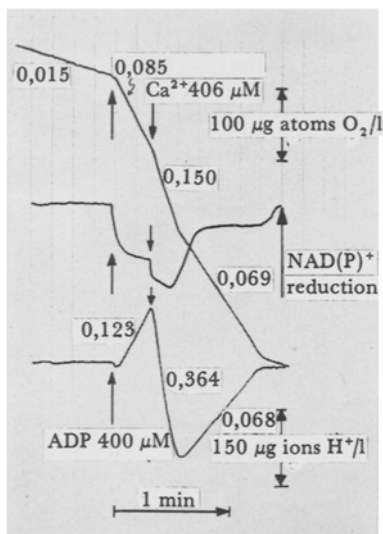
Figure 5. Changes of NAD(P)H fluorescence of mitochondria accumulating Ca^{2+} during phosphorylation of ADP. Mitochondria (2.6 mg protein/ml) were incubated in a medium containing 0.15 M sucrose, 0.75 M KCl, 2.5 mM $MgCl_2$, 5 mM KH_2PO_4 (pH 7.4) and 10 mM succinate.

Ca^{2+}. In this case the effect makes $\approx 600\%$. In both cases the alterations of fluorescence are reversible and they disappear after addition of EDTA. The results of the experiments given in Fig. 3 enable us to suggest the following explanation of the increase of mitochondrial fluorescence by addition of Ca^{2+} in the presence of rotenone.

Accumulation of Ca^{2+} gives a significant rise of its concentration in intramitochondrial space (approximately calculated concentration of calcium acetate for the experiment given in Fig. 2A makes 0.1 moles/litre intramitochondrial water). Even in the case of the small K_a value of nucleotide-metal complex formation the concentration of this complex can be very high. If a part of this complex is localized in hydrophobic medium the increase of fluorescence can be easily explained by the results given in Fig. 4.

The increase of mitochondrial fluorescence in the presence of Ca^{2+} can be found not only in the presence of rotenone, but also under conditions when a considerable part of endogenous NAD(P)H is oxidized. Figure 5 gives the record of fluorescence, respiration and pH after addition of Ca^{2+} during the process of oxidative phosphorylation. In these conditions the addition of Ca^{2+} ceases phosphorylation (as may be followed from the cessation of alkalinization of the incubation medium). After the uptake of Ca^{2+} was completed the level of fluorescence turns

to be much higher than initial despite the fact the mitochondria are in the steady state 3, which is proved by the high rate of respiration and the subsequent alkalinization of the incubation medium.

The problem of NAD and NADP localization in mitochondria is not very clear up to now. On the basis of fluorescence spectra of intramitochondrial pyridine nucleotides Chance and Baltscheffsky [6] postulated the existence of a bound form of NADH. Examining the results of the chemical analysis of mitochondrial pyridine nucleotides, Purvis [14] has concluded the existence of an unusual form of NADH in mitochondria. The nature of this compound is not clear. Taking into consideration the high content of Ca^{2+} in the mitochondria as well as the results of this paper it is possible to conclude that the bound NADH corresponds to nucleotide-metal complex localized in the hydrophobic region of the intramitochondrial space.

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