Acta Biochimica Polonica

Vol. 40, No. 2/1993

QUARTERLY

Continuous recording of intramitochondrial pH with fluorescent pH indicators: novel probes and limitations of the method***

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Received 19 March, 1993

In addition to 2',7'-bis-(2-carboxyethyl)-5(6)- carboxyfluorescein (BCECF) used so far to monitor intramitochondrial pH, two other fluorescent pH indicators, 4',5'-dimethyl-5(6)-carboxyfluorescein (DMCF) and carboxyseminaphthofluorescein (carboxy-SNAFL-1), were applied for this purpose. These probes are taken up by isolated rat liver mitochondria in form of diacetate esters, hydrolyzed within mitochondria to free acids, and respond to changes of intramitochondrial pH by changing their fluorescence emission intensity. With all three probes energization of mitochondria by electron donors or acceptors was accompanied by fluorescence changes characteristic for alkalization, whereas deenergization by respiratory inhibitors or protonophores produced changes typical for acidification. Contrary to this, transition from State 4 to State 3, known to shift intramitochondrial pH towards acidification (equivalent to a decrease of ΔpH), was accompanied by paradoxical responses of the fluorescent pH probes used: the fluorescence of DMCF increased as if the matrix compartment became more alkaline, the fluorescence of BCECF, measured in single excitation/emission wavelength mode, did not change, and the fluorescence of carboxy-SNAFL-1 could be interpreted as either alkalization or acidification, depending on the excitation/emission wavelength pair used. It was shown that depletion of intramitochondrial Mg^{2+} and Ca^{2+} using divalent metal ionophore A23187 decreased fluorescence intensity with all three probes examined, whereas subsequent addition of Mg^{2+} or Ca^{2+} increased the fluorescence. It is therefore proposed that the atypical response of intramitochondrial pH indicators upon State 4 - State 3 transition is due to changes of intramitochondrial free Mg^{2+}, as related to different complexing abilities of ATP and ADP towards magnesium.

The pH gradient across the inner mitochondrial membrane (ΔpH)[1] is one of two components of the electrochemical proton gradient. Although its contribution to the total mitochondrial protonmotive force is usually much smaller than that of the electric potential difference, ΔpH plays an important role in mitochondrial transport processes, e.g. those of inorganic phosphate and pyruvate. The mitochondrial pH gradient has been routinely

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***This work was supported in part by the Central Programme for Basic Research under Grant No. CPBP 04.01 and in part by the State Committee for Scientific Research under grant No. KBN 4.1329.9101

determined from the distribution of a permeant week acid between the inner mitochondrial compartment and the external medium [1 - 3]. This has been performed using isotopically labelled week acids and filtration or centrifugation techniques to separate mitochondria from the medium. Since the accumulation factor of the probe in the inner mitochondrial compartment usually does not exceed 10 (equivalent to ΔpH of 1), these techniques imply rather poor precision. Moreover, they do not permit a continuous tracing of ΔpH changes. To overcome the latter drawback in measuring ΔpH changes in bacteria, Hellingwerf & van Hoorn [4] applied salicylic acid as the probe and a salicylate-sensitive electrode to continuously monitor its concentration in the medium. We have been, however, unsuccessful in adopting this method for mitochondrial suspensions (Szewczyk and Duszyński, unpublished).

During the last decade, fluorescein derivatives whose fluorescence depends on their protonation/deprotonation status have been successfully used for measuring intracellular pH [5 - 8].

Cells were first loaded with esterified, membrane-permeable, forms of the dyes which were subsequently hydrolyzed by intracellular esterases to free, non-permeable, dyes and their fluorescence was monitored either by conventional spectrofluorimetry or by fluorescence microscopy. One of the most frequently used derivatives is BCECF. This probe has the advantage of having pH-dependent and pH-independent regions in its excitation spectrum. This enables to use the ratio of fluorescence intensities at two excitation wavelengths, rather than the intensity at a fixed excitation wavelength, as the measure of pH. The ratioing procedure, though requiring special optical and electronic arrangements, permits to calibrate the system independently of the amount of the dye loaded into the cells [5, 9].

BCECF has also been used to measure intramitochondrial pH. Both the ratio mode [10 - 14] and measurement of the fluorescence intensity at a fixed excitation wavelength [15 - 16] have been applied. In the present paper we describe attempts to use other pH-sensitive fluorescein derivatives to measure intramitochondrial pH changes. We also examined the reliability of BCECF and other derivatives as mitochondrial pH probes and found that their signal may depend on intramitochondrial Mg^{2+} and Ca^{2+} concentrations and thus can be affected by compounds, like adenine nucleotides and orthophosphate, complexing these divalent metal ions.

MATERIALS AND METHODS

Rat liver mitochondria were prepared according to Johnson & Lardy [17] using 75 mM sucrose, 225 mM mannitol, 3 mM Hepes-KOH (pH 7.4) and 1 mM EGTA as the isolation medium.

Mitochondria (1 ml, 25 - 50 mg protein) suspended in the isolation medium were incubated with either 10 μM BCECF/AM, 100 μM DMCF/DA or carboxy-SNAFL-1/DA, 1 μg per mg protein, added as 1 or 10 mM stock solutions in DMSO so that the final concentration of DMSO never exceeded 1%. After 60 min incubation at 0°C[2] a 30-fold volume of the isolation medium was added and the mitochondria were sedimented by centrifugation for 10 min at 8000 × *g*. The pellet was resuspended in the same volume of the isolation medium and centrifuged again. The final pellet was suspended in 1 ml of the isolation medium. Such treatment affected only slightly mitochondrial energy-coupling functions, and preparations whose respiratory control ratio with succinate fell below 4 were rejected.

pH gradient between the mitochondrial inner compartment and the external medium was

[1]Abbreviations: BCECF, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; BCECF/AM, acetoxymethyl ester of BCECF; carboxy-SNAFL-1, carboxyseminaphthofluorescein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DMCF, 4',5'-dimethyl-5(6)-carboxyfluorescein; carboxy-SNAFL-1/DA and DMCF/DA, diacetate esters of carboxy-SNAFL-1 and DMCF; DMSO, dimethylsulphoxide; EGTA, ethyleneglycol bis(2-aminoethylether)-N,N,N',N'-tetraacetate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; ΔpH, pH difference on both sides of the inner mitochondrial membrane.

[2]In recent experiments, not included in this paper, mitochondria were loaded with these probes at 25°C for 20 min

calculated from the distribution of [^{14}C]acetate or 5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione [3].

Fluorescence was measured in LS-5B fluorescence spectrometer (Perkin-Elmer, England) in rectangular (1 × 1 cm, 3 ml) cuvettes at a constant temperature (20 or 25°C) in the medium containing 100 mM sucrose, 50 mM KCl, 10 mM Hepes-KOH, 1 mM EGTA and mitochondria corresponding to 0.1 - 0.2 mg protein/ml, pH 7.0 (for DMCF) or 7.2 - 7.4 (for BCECF and carboxy-SNAFL-1). pH of all reagents added during the assay, except for KCN, had been previously carefully adjusted to the same value as that of the medium. Changes of external pH were monitored in parallel samples using glass electrode. The wavelength pairs (excitation/emission, respectively) were 513/537 nm for DMCF and 500/530 nm for BCECF. Excitation and emission wavelengths for carboxy-SNAFL-1 were variable and are specified in the text and legends to respective figures. Fluorescence is expressed in arbitrary units, its initial intensity or its intensity at fully energized state being usually set as 100.

BCECF/AM, DMCF/DA and carboxy-SNAFL-1/DA were purchased from Molecular Probes (Eugene, Oregon, U.S.A.).

RESULTS

DMCF as a probe

Figure 1 shows a typical fluorescence trace for DMCF-loaded mitochondria. Mitochondria respiring with endogenous substrates (initial part of the trace) exhibited a high fluorescence, corresponding to high internal pH. Addition of glutamate, which is co-transported with proton, resulted in a pronounced decrease of the fluorescence. A subsequent addition of malate, promoting glutamate/malate oxidation *via* the transamination pathway and thus increasing the rate of proton ejection, partly restored the initial high pH of the matrix (increased fluorescence). Addition of rotenone, the inhibitor of NADH dehydrogenase, was followed by a slow decrease of fluorescence due to the dissipation of the proton gradient. Succinate, which feeds electrons to the respiratory chain beyond the rotenone-sensitive site, produced a rapid increase of the fluorescence until a plateau was attained. Cyanide, inhibitor of cytochrome oxidase, produced again a decrease of matrix pH due to slow dissipation of the proton gradient. The initial rapid increase

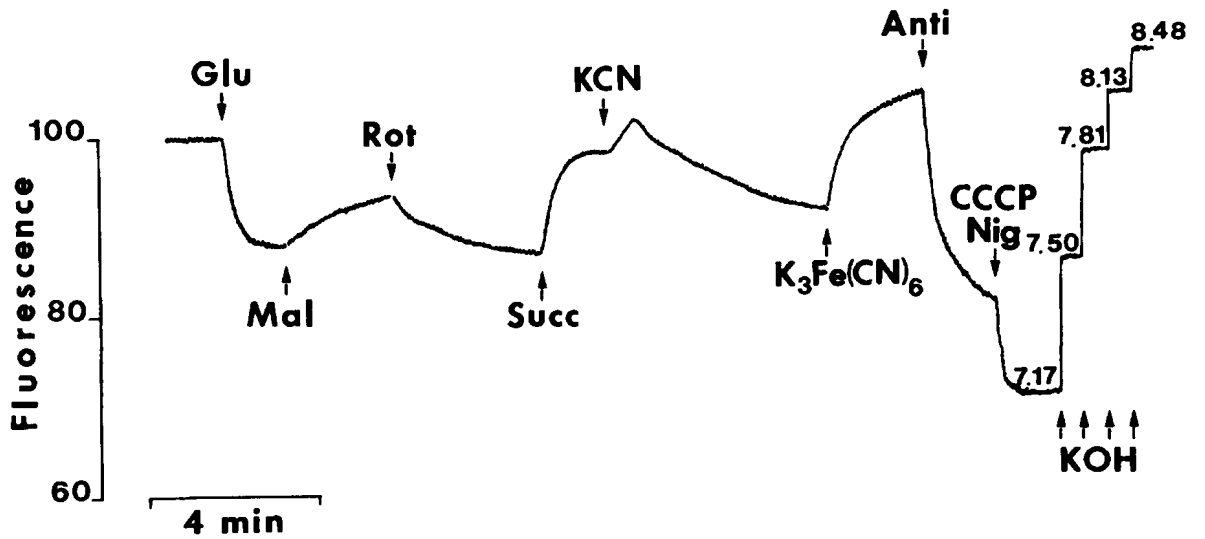

Fig. 1. Fluorescence changes of intramitochondrially entrapped DMCF upon energization and deenergization of mitochondria.

Mitochondria were suspended in the standard incubation medium (see Materials and Methods) supplemented with 3 mM $MgCl_2$, and the following additions were made where indicated: 4.9 mM glutamate (Glu), 1 mM malate (Mal), 1.6 μM rotenone (Rot), 4.8 mM succinate (Succ), 1 mM KCN, 2.4 mM ferricyanide, 2 μM antimycin A (Anti), 1.6 μM nigericin (Nig) plus 1.6 μM CCCP, and a few aliquots of 5 - 10 μl of 1 M KOH. Initial pH was 7.02 and was increased to 7.17 after addition of KCN. The numbers at the trace indicate pH values of the medium

of fluorescence after cyanide addition was due to the alkalization of the external medium by unbuffered KCN solution which then propagated to the matrix compartment. Ferricyanide, an external acceptor of electrons from cytochrome *c*, restored the flux through a fragment of the respiratory chain and again promoted proton ejection (alkalization of the matrix expressed by fluorescence increase). This was rapidly abolished by addition of antimycin A, an inhibitor of complex III of the respiratory chain[3]. CCCP plus nigericin completely collapsed the pH gradient between the mitochondrial matrix and the external medium. Thus the subsequent fluorimeter readings, together with indications of the glass electrode, could be used to calibrate the assay. This was done by a few additions of small amounts of KOH or NaOH solution.

Fluorescence changes depicted in Fig. 1, corresponding to additions of electron donors, electron acceptor and respiratory blockers agree, at least qualitatively, with expected changes of intramitochondrial pH. In contrast to this, State 4 - State 3 transition brought about an unexpected effect. Addition of ADP in the presence of phosphate and a respiratory substrate produced an increase of the fluorescence, the effect normally interpreted as alkalization of the matrix compartment (Fig. 2, trace A). Meanwhile, matrix pH calculated from the distribution of [^{14}C]acetate or [^{14}C]dimethyl-oxazolidine-dione revealed a small but measurable acidification by 0.03 - 0.07 pH unit (not shown). The effect of ADP on DMCF fluorescence could be reversed or prevented by carboxyatractyloside, a blocker of the adenine nucleotide translocase. Inorganic phosphate de-

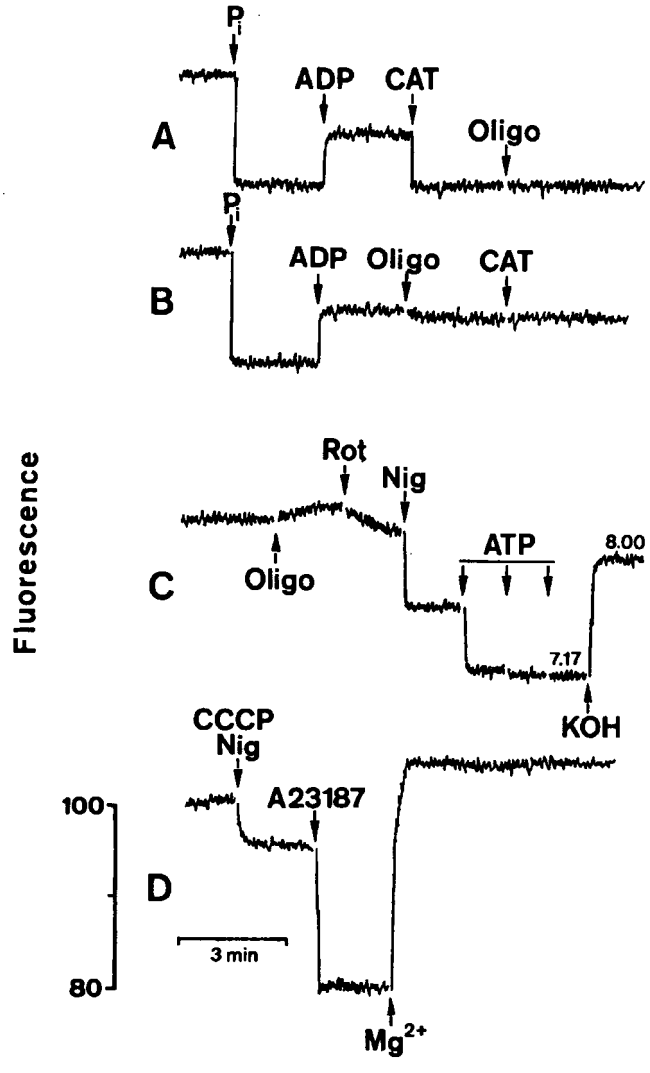

Fig. 2. DMCF fluorescence changes upon State 4 - State 3 transition and the effects of various inhibitors and ionophores. Mitochondria were suspended in the standard medium supplemented with 4.8 mM succinate and 1.6 μM rotenone (pH 7.0). The additions were (final concentrations): 5 mM phosphate, 0.17 mM ADP, 1.5 μM carboxyatractyloside (CAT), 2 μM oligomycin (Oligo), 1.6 μM nigericin, 0.33 mM ATP (each addition), 2 μM A23187 and 0.8 mM Mg^{2+}. The numbers at trace C indicate pH values of the medium before and after addition of KOH

[3]A rapid decrease of ΔpH after antimycin A, much faster than after rotenone or cyanide, can be explained by channel-opening activity of this antibiotic [18]

creased and ADP increased DMCF fluorescence even when added to mitochondria whose internal pH had been clamped with the external one by a prior addition of nigericin (not shown).

In contrast to carboxyatractyloside, oligomycin, an inhibitor of mitochondrial F_1F_o-ATPase, did not reverse the paradoxical effect of ADP and prevented its reversal by carboxyatractyloside (Fig. 2, trace B). In another protocol mitochondrial ATPase was first inhibited by oligomycin, the particles were deenergized by rotenone and their ΔpH was collapsed by addition of nigericin. The subsequent addition of ATP produced a large decrease of the fluorescence (Fig. 2, trace C), i.e. it had the opposite effect to that of ADP. In such mitochondria additions of ADP and P_i were without effect (not shown).

ADP, ATP and P_i had no effect on the fluorescence of free DMCF outside mitochondria. Nor did ADP shift the excitation and emission spectra of intramitochondrially trapped DMCF.

To check whether the paradoxical fluorescence change upon State 4 - State 3 transition could be caused by changes in intramitochondrial concentration of free Mg^{2+} the effect of the divalent cation ionophore A23187 was examined. When added to fully deenergized mitochondria, it produced a further decrease of the fluorescence (Fig. 2, trace D). This effect could not be due to matrix acidification, since pH of the inner compartment had already been clamped to that of the external medium by CCCP and nigericin. Moreover, subsequent addition of Mg^{2+} resulted in a rapid increase of the fluorescence intensity. A similar effect was also produced by Ca^{2+}. Mg^{2+} added to the mitochondrial suspension in the absence of A23187 had no effect on intramitochondrial DMCF fluorescence (not shown).

Mg^{2+} in submillimolar concentrations slightly increased the fluorescence intensity of free DMCF (obtained by acid hydrolysis of DMCF/DA) without shifting the maximum of its emission wavelength when tested at pH 7.0 and at low ionic strength (20 mM Hepes). However, at pH 8.5 (conditions when practically all DMCF is present in the ionized form) or at high ionic strength (100 mM KCl) Mg^{2+} was without effect.

Since ADP, P_i, Mg^{2+} and A23187 may produce mitochondrial swelling or contraction, it can be argued that changes of light scattering might be responsible for the observed changes of fluorescence intensity. This is, however, unlikely to be the case, as osmotic contraction of mitochondria produced by rapid injection into the fluorimeter cuvette of concentrated sucrose solution to increase its concentration by 50 mM had no visible effect (not shown).

BCECF as a probe

As shown previously [19], mitochondria loaded with BCECF and measured at the fixed excitation wavelength mode showed increased fluorescence when energized by respiratory substrates and decreased fluorescence upon deenergization with respiratory inhibitors or protonophores. These responses were, at least

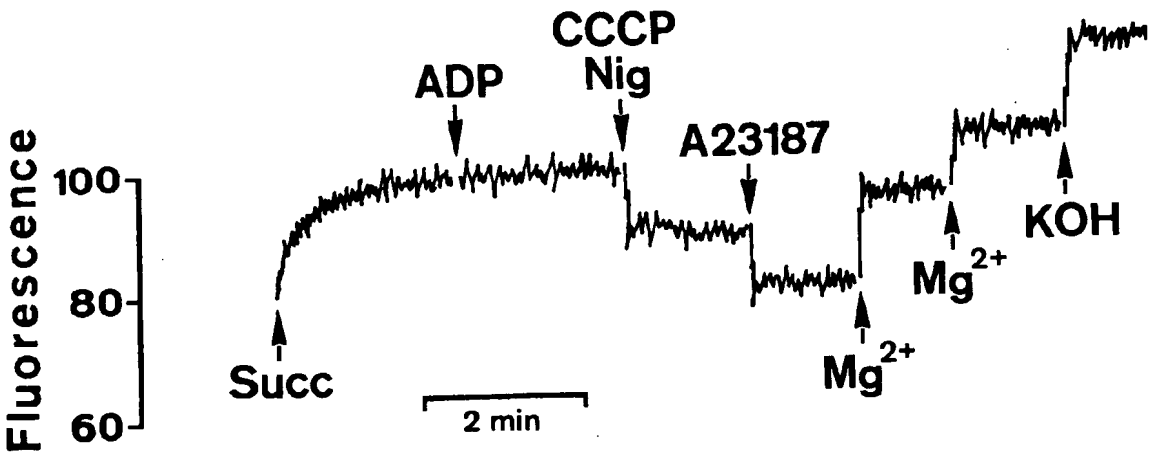

Fig. 3. Effect of ADP and A23187 on BCECF fluorescence in mitochondria.
Additions and incubation medium were as in Fig. 2, except that pH was 7.4

qualitatively, similar to those observed by other authors in the dual excitation wavelength mode [10 - 14]. However, transition from State 4 to State 3 had no observable effect on BCECF fluorescence (Fig. 3). Addition of A23187 after CCCP and nigericin produced a further decrease of the fluorescence which could be reversed by Mg^{2+} (Fig. 3). Mg^{2+} added before A23187 had no effect, but the subsequent addition of the ionophore resulted in a large fluorescence increase (not shown). Thus, in general, mitochondria loaded with BCECF exhibited similar fluorescence responses to A23187 and Mg^{2+} as those loaded with DMCF.

Carboxy-SNAFL-1 as a probe

Spectral properties of this probe seem especially suitable for measuring intracellular and intramitochondrial pH. Because of its p*K* value close to 7.8 [20] this probe exists, at physiological pH, in protonated and deprotonated forms at approximately equal quantities. Both forms are fluorescent but have different excitation (Fig. 4 A) and emission (Figs. 4 B and C) spectra. When excited at 490 nm, the protonated form emits maximally at 540 nm, whereas the deprotonated form, excited at 540 nm, emits maximally at 618 nm. pH changes in mitochondria can thus be monitored by recording the fluorescence of either the acidic or the basic forms of the dye [20].

Figure 5 shows such changes of the fluorescence of deprotonated (trace A) and protonated (trace B) forms of carboxy-SNAFL-1 entrapped in mitochondria. It might be expected that one trace should represent a mirror image of the other, since whenever the amount of one form of the dye increases, the content of the other one decreases. As can be seen, this is only partly true, namely, after addition of the respiratory substrate succinate and upon calibration of the system with KOH. In contrast, changes following addition of phosphate and nigericin plus CCCP, though oppositely directed, differ considerably in magnitude. Still more striking is the effect of ADP which produces an increase of fluorescence in both optical setups, clearly

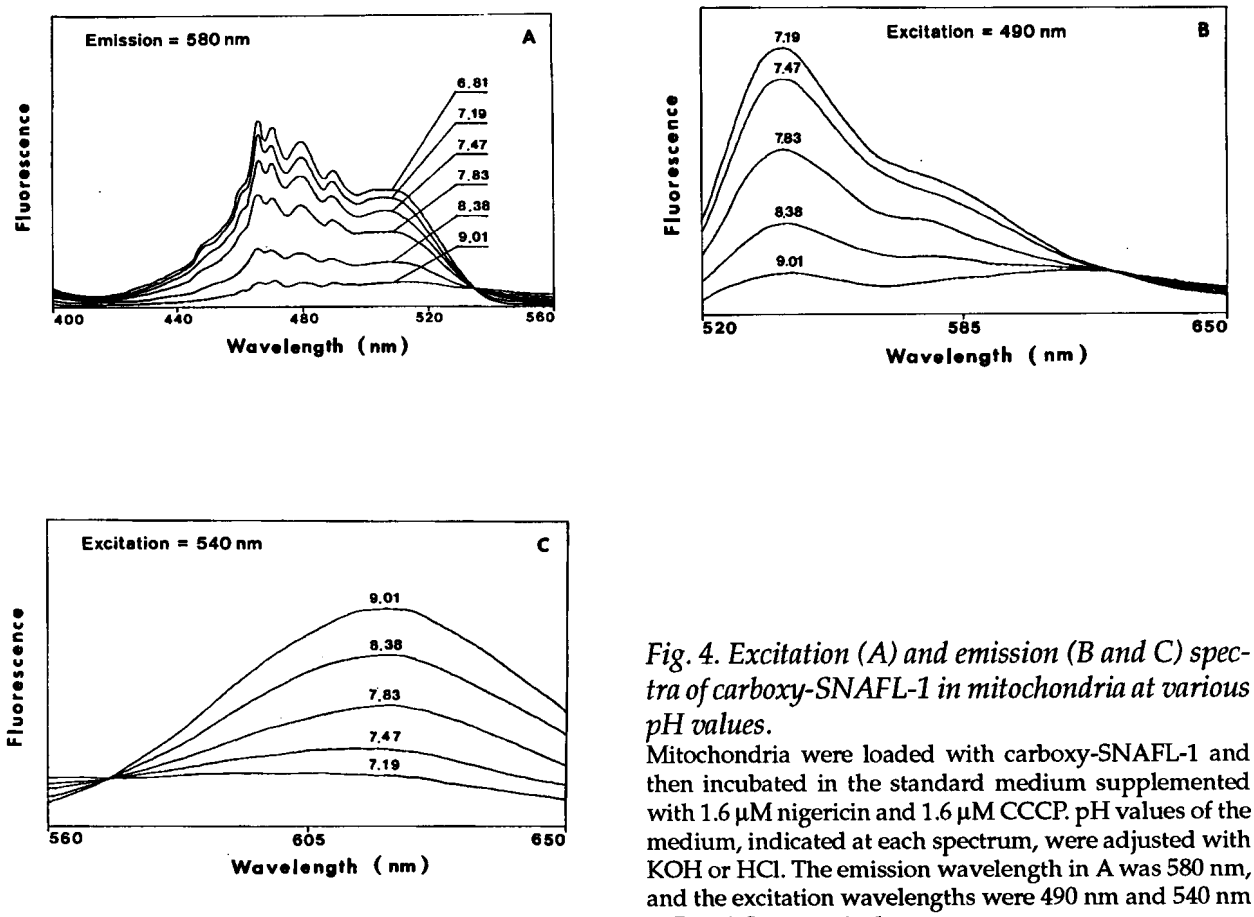

Fig. 4. Excitation (A) and emission (B and C) spectra of carboxy-SNAFL-1 in mitochondria at various pH values.
Mitochondria were loaded with carboxy-SNAFL-1 and then incubated in the standard medium supplemented with 1.6 μM nigericin and 1.6 μM CCCP. pH values of the medium, indicated at each spectrum, were adjusted with KOH or HCl. The emission wavelength in A was 580 nm, and the excitation wavelengths were 490 nm and 540 nm in B and C, respectively

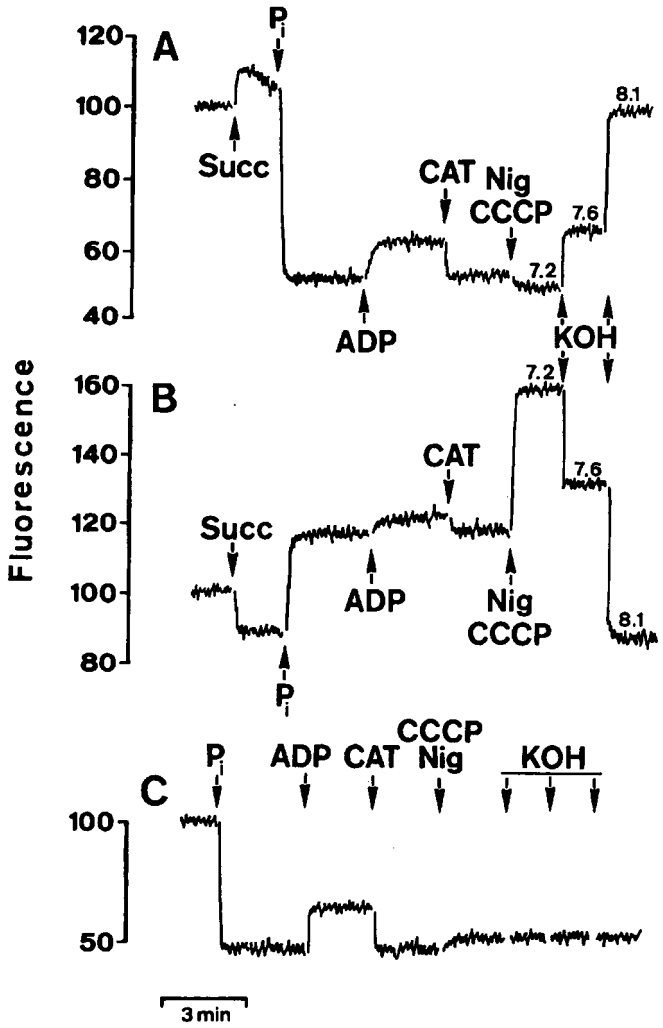

Fig. 5. Changes of carboxy-SNAFL-1 fluorescence in mitochondria.
Mitochondria loaded with carboxy--SNAFL-1 were incubated in the standard medium (pH 7.2) supplemented with 1.6 μM rotenone. CCCP was added to final concentration of 1.6 μM; other additions were as in Fig. 2. Numbers at the traces after addition of nigericin and CCCP indicate pH of the medium. Excitation and emission wavelengths were, respectively, 540 nm and 620 nm for trace A, 490 nm and 540 for trace B, and 535 nm and 580 nm (pH-insensitive wavelength pair) for trace C

indicating that, in this case, fluorescence changes can by no means be explained simply by alteration in the concentration of the two ionic forms of the dye.

These results, taken together, may point to quenching of the fluorescence of both forms by phosphate and ATP, thus leading to underestimation of intramitochondrial pH in trace A and overestimation in trace B of Fig. 5. This assumption is supported by the finding that, when excitation and/or emission wavelengths were set at the isosbestic points, addition of phosphate produced a decrease and addition of ADP (which lowers intramitochondrial ATP/ADP ratio) an increase of the fluorescence, as exemplified in Fig. 5 trace C for one of the wavelength pairs. In all cases, the effect of ADP was abolished by carboxyatractyloside. Addition of ATP to mitochondria pretreated with oligomycin, rotenone and nigericin produced a decrease of carboxy-SNAFL-1 fluorescence (not shown).

Similarly as with DMCF and BCECF, also in carboxy-SNAFL-1-loaded mitochondria A23187 produced a decrease and Mg^{2+} an increase of the fluorescence of both the protonated and deprotonated forms of the probe (Fig. 6). Neither P_i, ADP, ATP nor Mg^{2+} affected emission spectra of free carboxy-SNAFL-1 (obtained by acid hydrolysis of carboxy-SNAFL-1/DA) at pH 7.0 and 8.0 and at excitation wavelengths 490 and 540 nm.

DISCUSSION

Among a number of fluorescent pH indicators used to monitor intracellular H^+ concentration [5 - 8], BCECF has been almost the only one applied so far for mitochondria. The present paper adds two other fluorescein derivatives, namely DMCF and carboxy-SNAFL-1, as probes for intramitochondrial pH measurements. However, it also points to the inter-

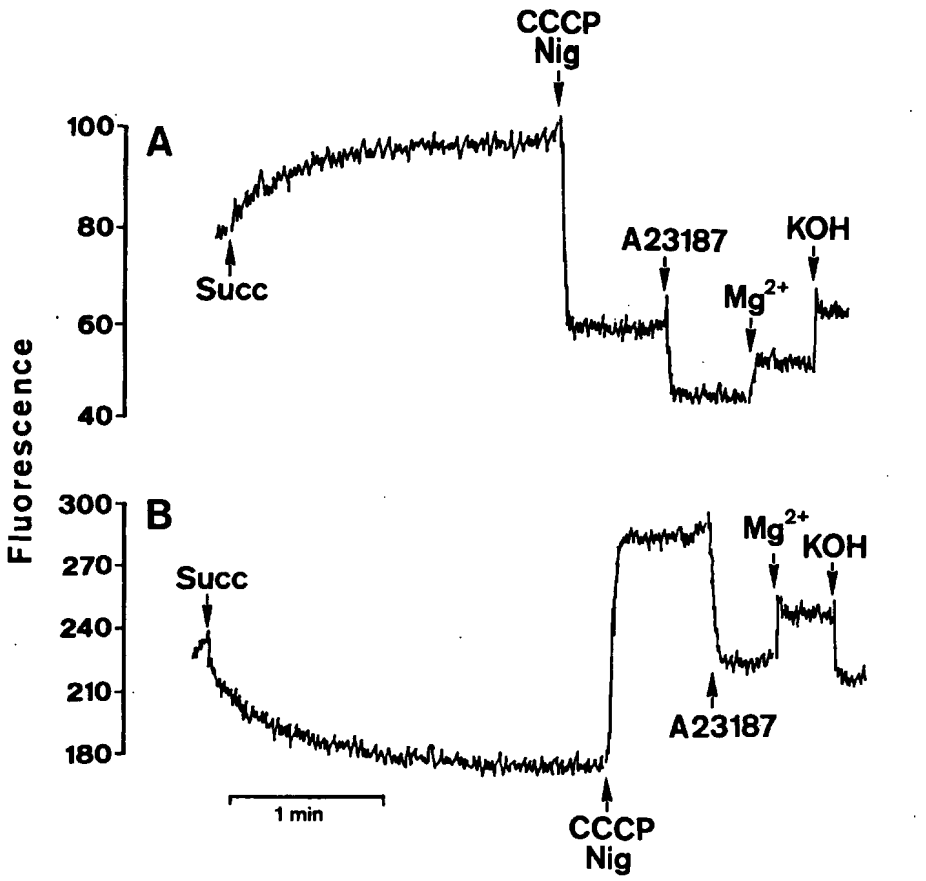

Fig. 6. Effect of A23187 and Mg^{2+} on carboxy-SNAFL-1 fluorescence in mitochondria.
Incubation conditions were as in Fig. 3. Excitation and emission wavelengths were, respectively, 540 and 620 nm in trace A, and 490 and 540 nm in trace B

ference in the fluorescence response of these probes by intramitochondrial orthophosphate and ATP. As an effect, changes of the fluorescence upon transition from State 4 to State 3 do not reflect alterations of ΔpH. As determined from the distribution of a weak penetrant acid, this transition is accompanied by a small decrease of ΔpH, i.e. by acidification of the matrix compartment [1, 21] (see also present work). Meanwhile, upon addition of ADP to respiring coupled mitochondria the fluorescence of DMCF mimics alkalization of the matrix compartment, the fluorescence of BCECF, recorded under fixed excitation wavelength, remains unchanged, and changes of the fluorescence of carboxy-SNAFL-1 may be interpreted as either acidification or alkalization, depending on the excitation/emission wavelength pair used. Thus, the response of the fluorescent probes following addition of ADP to respiring mitochondria does not reflect changes of intramitochondrial pH.

This paradoxical change of fluorescence was not due to changes in light scattering of the mitochondrial suspension, as contraction of mitochondria produced by a rapid increase of medium osmolarity had no such effect.

Both DMCF and carboxy-SNAFL-1 reacted to addition of ATP to fully deenergized mitochondria. Since neither ATP nor ADP affect fluorescence of the probes outside mitochondria, the conclusion could be drawn that their effect on the intramitochondrially entrapped probe is indirect. As the intramitochondrial ATP/ADP ratio considerably differs from State 4 to State 3 [22, 23], our results may suggest that this ratio affects fluorescence of the probes used. This assumption is supported by the fact that the unexpected effect of ADP addition can be prevented or reversed by carboxyatractyloside, blocker of the adenine nucleotide exchanger, but not by oligomycin, inhibitor of ADP phosphorylation. Moreover, carboxyatractyloside, added after oligomycin, has no effect (Fig. 2).

Another support is given by the observation that addition of ATP, but not ADP, to deenergized mitochondria decreases DMCF fluorescence, thus mimicking alkalization of the matrix (Fig. 2). The most likely explanation seems the chelating by ADP and ATP of divalent metal ions, Mg^{2+} and Ca^{2+}. In fact, because of a tenfold difference in their complexing affinity towards Mg^{2+}, higher for ATP than for ADP [24], a change of the ATP/ADP ratio may considerably alter the intramitochondrial concentration of free magnesium ions whose concentration, 0.35 mM [25], is one order of magnitude lower than the concentration of ADP and ATP (free plus complexed).

This assumption was supported by experiments with mitochondria made permeable to Mg^{2+} by the divalent metal cation ionophore A23187 (Figs. 2, 3 and 6). The mechanism by which Mg^{2+} and Ca^{2+} increase the fluorescence of all three examined probes remains unclear. Both cations slightly increased the fluorescence of free DMCF and BCECF outside mitochondria at neutral pH and low ionic strength but were without effect on the fully ionized probes (at alkaline pH) and at high ionic strength. It should be, therefore, supposed that these divalent cations may only interact with the probes entrapped inside mitochondria. It can be speculated, for instance, that they increase the fluorescence quantum yield by facilitating the interaction of the probe with the inner mitochondrial membrane or with soluble matrix proteins. Partial screening of the membrane surface potential or a more specific interaction with membrane phospholipids can be considered.

In conclusion, the present investigation introduces two new fluorescent probes, DMCF and carboxy-SNAFL-1, which can be used to monitor intramitochondrial pH changes. However, it also points to limitations of the applicability of these probes to conditions when no considerable changes of intramitochondrial free Mg^{2+} are expected. This limitation also applies to BCECF used at single excitation wavelength mode.

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