Biochem. J. (2011) **437**, 381–387 (Printed in Great Britain) doi:10.1042/BJ20110883

ACCELERATED PUBLICATION

The circularly permuted yellow fluorescent protein cpYFP that has been used as a superoxide probe is highly responsive to pH but not superoxide in mitochondria: implications for the existence of superoxide 'flashes'

Markus SCHWARZLÄNDER*[1], David C. LOGAN†, Mark D. FRICKER* and Lee J. SWEETLOVE*[1]

*Department of Plant Science, University of Oxford, South Parks Road, Oxford OX1 3RB, U.K., and †Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5E2

The properties of a cpYFP [circularly permuted YFP (yellow fluorescent protein)] reported to act as a superoxide sensor have been re-examined in *Arabidopsis* mitochondria. We have found that the probe has high pH sensitivity and that dynamics in the cpYFP signal disappeared when the matrix pH was clamped by nigericin. In contrast, genetic and pharmacological manipulation of matrix superoxide had no detectable effect on the cpYFP signal. These findings question the existence of superoxide flashes in mitochondria.

Key words: circularly permuted yellow fluorescent protein (cpYFP), mitochondrion, pH sensitivity, superoxide.

INTRODUCTION

The development of genetically encoded probes that allow quantification of ROS (reactive oxygen species) at a cell-specific and subcellular resolution in living tissues, and in real-time, have facilitated a more sophisticated understanding of the role of ROS in signalling and development [1,2]. Measuring superoxide dynamics in living intact cells, i.e. *in vivo*, is of particular interest as in most systems superoxide is the precursor for most other ROS and RNS (reactive nitrogen species) such as hydrogen peroxide, the hydroxyl radical or peroxynitrite. In mitochondria, where superoxide is generated by the ETC (electron transport chain), superoxide links bioenergetic function and radical dynamics [3]. The superoxide production rate is strongly determined by the functional state of the ETC and it can rise dramatically under stress [4]. Mitochondrial superoxide dynamics are therefore key for the understanding of the interplay between cellular energetic state, redox signalling and stress responses. However, most of our understanding about mitochondrial superoxide has been gathered under *in vitro* conditions, i.e. from isolated organelles. A genetically encoded superoxide sensor therefore has the potential to provide physiologically meaningful insights into mitochondrial superoxide dynamics in a cellular context. A superoxide sensor based on a cpYFP [circularly permuted YFP (yellow fluorescent protein)] has been reported and provided evidence for 'flashes' of superoxide production in single mitochondria of cardiomyocytes [5,6]. This raised important questions about the dynamic nature of mitochondrial free radical processes *in vivo*.

However, doubts have been raised about the specificity of cpYFP as a reporter of superoxide in the mitochondrial matrix [7]. In the present study we have characterized the dynamic fluorescence properties of cpYFP targeted to the matrix of plant mitochondria. Properties of the probe were validated in living plant cells and in isolated mitochondria, and the sensitivity of the probe to mitochondrial bioenergetics, superoxide production and pH were established.

MATERIALS AND METHODS

Plant lines and culture conditions

The mitochondrial-targeted cpYFP construct was engineered by combining the cpYFP coding region in-frame and downstream of the mitochondrial-targeting sequence from *Nicotiana plumbaginifolia* β-ATPase [8] under control of the CaMV (cauliflower mosaic virus) 35S promoter in the binary vector pH2GW7 [9], using Gateway technology (Invitrogen). Hairpin RNAi (RNA interference) constructs were composed of specific and unique fragments of the MnSOD [manganese SOD (superoxide dismutase); At3g10920] sequence, in sense and antisense orientation, in the pOpOff2 vector providing dexamethasone-inducible transcript knockdown [10]. Agrobacterium-mediated transformation of *Arabidopsis thaliana* (accession Columbia, Col-0) was performed by 'floral dip' [11]. Transformants were selected by hygromycin (pH2GW7) or kanamycin (pOpOff2) resistance. Knockdown efficiency of the MnSOD transcript was assessed by semi-quantitative RT (reverse transcriptase)–PCR. Homozygous T_3 lines were selected by segregation of antibiotic resistance. For *in vivo* microscopy, seedlings were cultivated for 7–10 days on vertical half-strength Murashige and Skoog medium agar plates under long-day conditions. For mitochondrial isolation, seedlings were cultured in a hydroponic pot system for 14 days as described previously [12]. For induction of RNAi, 10 μM dexamethasone was added to the medium. Immunoblotting of total seedling extract was performed using a polyclonal MnSOD-specific antiserum as described previously [13,14].

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CLSM, confocal laser-scanning microscopy; cpYFP, circularly permuted yellow fluorescent protein; ETC, electron transport chain; EYFP, enhanced yellow fluorescent protein; GFP, green fluorescent protein; MnSOD, manganese superoxide dismutase; MnTMPyP, manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin; pmf, protonmotive force; RNAi, RNA interference; ROS, reactive oxygen species, SOD, superoxide dismutase YFP, yellow fluorescent protein.

[1] Correspondence may be addressed to either of these authors (email markus.schwarzlander@plants.ox.ac.uk or lee.sweetlove@plants.ox.ac.uk).

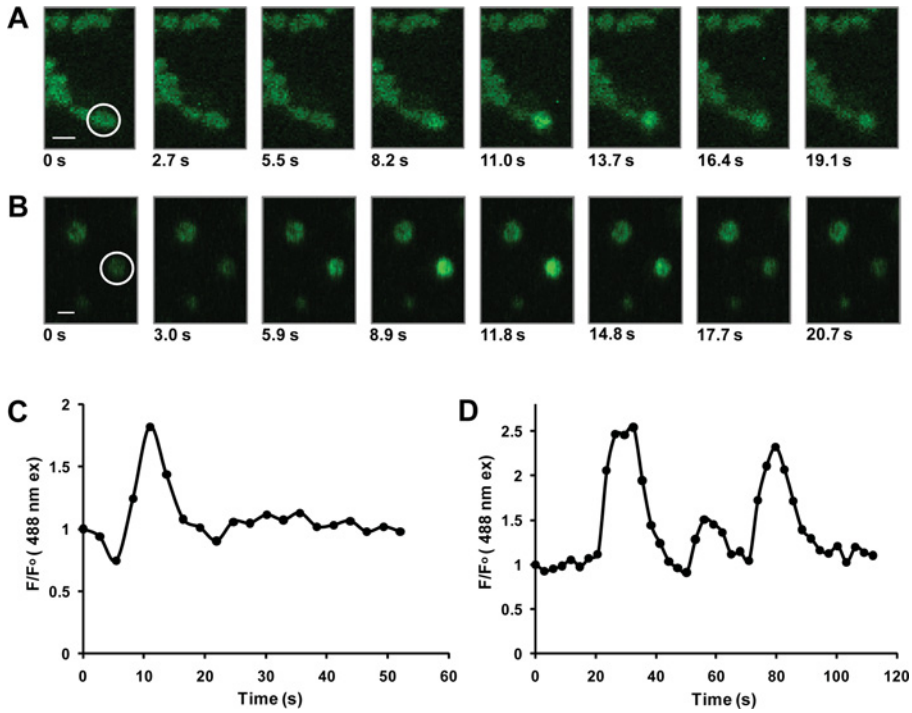

Figure 1 CpYFP flashes in mitochondria of living root epidermis cells and in isolated mitochondria

(**A**) A single mitochondrion (white circle) undergoing a transient increase in cpYFP signal in a living root epidermis cell and (**B**) a transient cpYFP intensity increase in a respiring isolated mitochondrion (white circle) in 'basic incubation medium' supplemented with 10 mM succinate, 0.25 mM ATP and 20 μM rotenone. Scale bar = 1 μm. Normalized intensity trace (**C**) for a single mitochondrion *in vivo* and (**D**) for an isolated mitochondrion. Typical events are shown.

Mitochondrial isolation and assays

Mitochondria were isolated from 14-day-old *Arabidopsis* seedling cultures as described previously [12], except the pH of the wash buffers was adjusted with Tris base instead of KOH. Coupling was measured in a Clark-type oxygen electrode 'Oxygraph' (Hansatech Instruments) as described previously [15] in 'basic incubation medium' [0.3 M sucrose, 5 mM KH_2PO_4, 10 mM Tes, 10 mM NaCl, 2 mM $MgSO_4$ and 0.1 % fatty-acid-free BSA (pH 7.2 adjusted with KOH)].

Microscopy

Living roots were imaged in half-strength Murashige and Skoog medium. Staining with 200 nM MitoTracker Orange CMTMRos (Invitrogen) was performed as described previously [16]. Isolated mitochondria were immobilized on to round glass cover slides by centrifugation (2000 ***g*** for 5 min at 4 °C) in 0.3 M sucrose and 10 mM Tes (pH 7.2 adjusted with Tris base). Immediately before imaging, mitochondria were energized by the addition of medium containing the required respiratory substrate. Microscopy was performed using a Zeiss LSM510 META confocal microscope (Carl Zeiss MicroImaging) equipped with laser lines for 488, 405 and 543 nm excitation. Imaging was carried out with a ×63 lens [Zeiss Plan-Apochromat ×63, NA (numerical aperture) = 1.4, oil-immersion] in multi-track mode with line switching. cpYFP was excited at 488 and 405 nm, and MitoTracker Orange was excited at 543 nm. cpYFP or MitoTracker fluorescence were measured at 505–530 nm and 565–615 nm respectively. Laser output was kept under 2 % of maximal power equalling <2.5 μW for any wavelength as measured in the defocused beam at the backplane of the objective. Pixel size was 70 nm and pixel dwell time was 1.3 μs. Time series were collected with time intervals between 2 and 5 s. Microscopy data were background-subtracted and analysed using custom software as described previously [16].

Fluorimetry

Mitochondria were suspended in 'basic incubation medium' in 96-well plates. The total assay volume was 200 μl. Fluorescence was measured at 25 °C in a Beckman Coulter DTX 880 Multimode Detector equipped with 485 and 405 nm excitation filters, and a 500–530 nm BP emission filter. Data were background-subtracted and processed in Microsoft Excel.

RESULTS

cpYFP flashes in active plant mitochondria

To study the dynamic behaviour of cpYFP in plant mitochondria, we created stable *Arabidopsis* transformants expressing cpYFP in the mitochondrial matrix. Mitochondrial localization of the cpYFP signal with excitation at either 488 nm or 405 nm was confirmed by co-localization with MitoTracker Orange (Supplementary Figure S1 at http://www.BiochemJ.org/bj/437/bj4370381add.htm) and by fluorescence of isolated purified mitochondria (Figure 1B). Probe behaviour was monitored in living root epidermal cells by CLSM (confocal laser-scanning microscopy). The cpYFP signal was relatively homogeneous between all mitochondria of a single cell, whereas the intensity between cells was somewhat variable. At the level of individual mitochondria, infrequent, spontaneous and transient increases in cpYFP fluorescence were observed with 488 nm excitation (Figure 1A). The cpYFP signal in a single mitochondrion increased

transiently in intensity before returning to baseline fluorescence within seconds (Figures 1A and 1C, and Supplementary Movie S1 at http://www.BiochemJ.org/bj/437/bj4370381add.htm). Fluorescence with 405 nm excitation remained unchanged during the event (results not shown). The duration of each event was typically 10–20 s, and the degree of signal increase was 1.3–2.5-fold over baseline. Hence this phenomenon showed similar characteristics in time and amplitude as cpYFP 'flashes' in mitochondria of mammalian cells described by Wang et al. [5]. We therefore adopt the term 'flashes' for the events we describe in the present paper in plant mitochondria.

Another similarity between our findings and those of Wang et al. [5] was the infrequent occurrence of flashes. On average approximately one flash could be observed when monitoring mitochondria in the cortical cytoplasm of a living root epidermal cell for 10 min. Low flash frequency, coupled with the motility of mitochondria in plant cells (moving into and out of the field of observation in three-dimensions), prevented continuous observation of single mitochondria and quantification of flash frequency. We therefore optimized a cell-free system using isolated mitochondria that allowed a more systematic assessment of flashes. Isolated mitochondria were immobilized on to a coverslip and monitored by confocal microscopy. Respiratory control ratios were >2.5 for succinate as the substrate in all experiments. Isolated respiring mitochondria also displayed cpYFP flashes with 488 but not 405 nm excitation (Figures 1B and 1D, and Supplementary Figure S2 and Supplementary Movie S2 at http://www.BiochemJ.org/bj/437/bj4370381add.htm). Spontaneous changes in cpYFP signal intensity were more frequent in this system, making flashes the norm rather than the exception at approximately 0.8 flashes/mitochondrion per min. Similar behaviour was observed with other respiratory substrates (malate/pyruvate and NADH). No flashes occurred in non-respiring mitochondria (state 1) or upon uncoupling (results not shown), suggesting that the presence of a pmf (protonmotive force) was a prerequisite for flashes to occur. Isolated mitochondria therefore provided an appropriate model system to evaluate further the reason for dynamic cpYFP behaviour in the mitochondrial matrix.

cpYFP responds dynamically to changes in mitochondrial respiratory state

cpYFP flashes in the mitochondrial matrix have been linked to respiratory activity and functional ETC [5]. To understand the average probe behaviour in different bioenergetic states, we measured the population response of mitochondrial suspensions in a 96-well plate reader. Different respiratory states were established by substrate or inhibitor feeding. The cpYFP signal responded to those changes in a highly sensitive manner with 485 nm excitation (Figure 2). A supply of respiratory substrate led to a strong increase in probe signal. Substrates with different ETC entry points (succinate, complex II; pyruvate/malate, complexes I and II; and ferrocyanide, complex IV) triggered similar cpYFP responses, although ferrocyanide triggered a less pronounced signal increase. For consistency, succinate was used as a substrate in all subsequent measurements. The absolute signal varied between 3- and 6-fold depending on mitochondrial preparation and storage time of mitochondrial suspensions before use (probably correlating with degree of coupling), but the relative probe response was consistent between individual experiments. The switch from state 2 to state 3 caused by the addition of ADP resulted in a decrease in cpYFP fluorescence, followed by recovery when entering state 4 upon ADP depletion (Figure 2). Uncoupling by CCCP (carbonyl cyanide

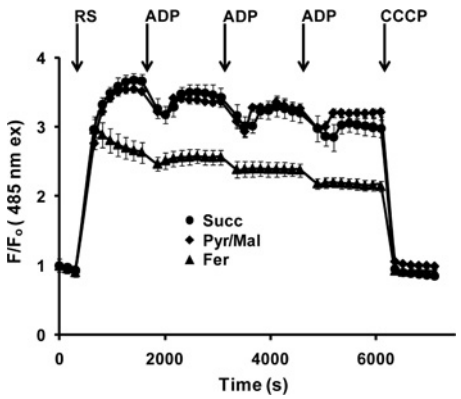

Figure 2 The response of cpYFP fluorescence to different respiratory substrates and states in isolated mitochondria

Freshly isolated *Arabidopsis* mitochondria (~20 μg of protein) expressing cpYFP in their matrix were suspended in 'basic incubation medium' and energized by respiratory substrates (RS). Succ (●), 10 mM succinate, 0.25 mM ATP and 20 μM rotenone; Pyr/Mal (◆), 10 mM pyruvate, 10 mM malate, 0.3 mM NAD and 0.1 mM thiamine pyrophosphate; Fer (▲), 20 mM $K_4[Fe(CN)_6]$. ADP (250 μM) was added as indicated. For uncoupling, CCCP (5 μM) was added. Values are means ± S.D., $n = 5$. The experiment was repeated independently five times with similar results.

m-chlorophenylhydrazone) led to a decrease in signal close to the value of state 1. In contrast, fluorescence at 405 nm excitation remained largely stable over the course of the measurements (Supplementary Figure S3 at http://www.BiochemJ.org/bj/437/bj4370381add.htm). Expressing the data as a ratio of the 485/405 signal was used in a ratiometric approach, but was not required in the plate reader system. The overall pattern of cpYFP signal in different respiratory states appeared to correlate with the magnitude of pmf, and therefore suggests that cpYFP is responsive to a parameter linked to, or downstream of, pmf in plant mitochondria.

Manipulation of matrix superoxide levels does not have an impact on cpYFP dynamics

cpYFP has been used previously as a superoxide sensor based on a 4-fold increase in fluorescence in response to artificially generated superoxide *in vitro* [5]. The correlation of cpYFP signal and pmf in isolated plant mitochondria is consistent with the expected changes in superoxide, as mitochondrial superoxide production increases as a function of pmf [3]. However, other parameters linked to pmf could also be responsible for the altered cpYFP fluorescence intensity. To further evaluate superoxide sensitivity of cpYFP in plant mitochondria, the effect of different complex III inhibitors on the cpYFP signal was measured. Antimycin A inhibits complex III by binding to the 'N' site, strongly stimulating superoxide release, whereas myxothiazol binds to the 'P' site and does not increase superoxide production [4]. We found that both inhibitors caused a decrease in the cpYFP signal (Figure 3A). This suggests that an inhibition of electron transport at complex III, rather than changes in superoxide levels, causes changes in cpYFP intensity. In addition, menadione, a redox-active quinone that mediates superoxide release in mitochondria [17], had no effect on the cpYFP signal (Supplementary Figure S4 at http://www.BiochemJ.org/bj/437/bj4370381add.htm).

The superoxide sensitivity of cpYFP in plant mitochondria was further assessed by treatment of mitochondria under state 2 respiration with three different membrane-permeant superoxide scavengers. Treatments with tiron within the concentration range used by Wang et al. [5] had no effect on the cpYFP signal

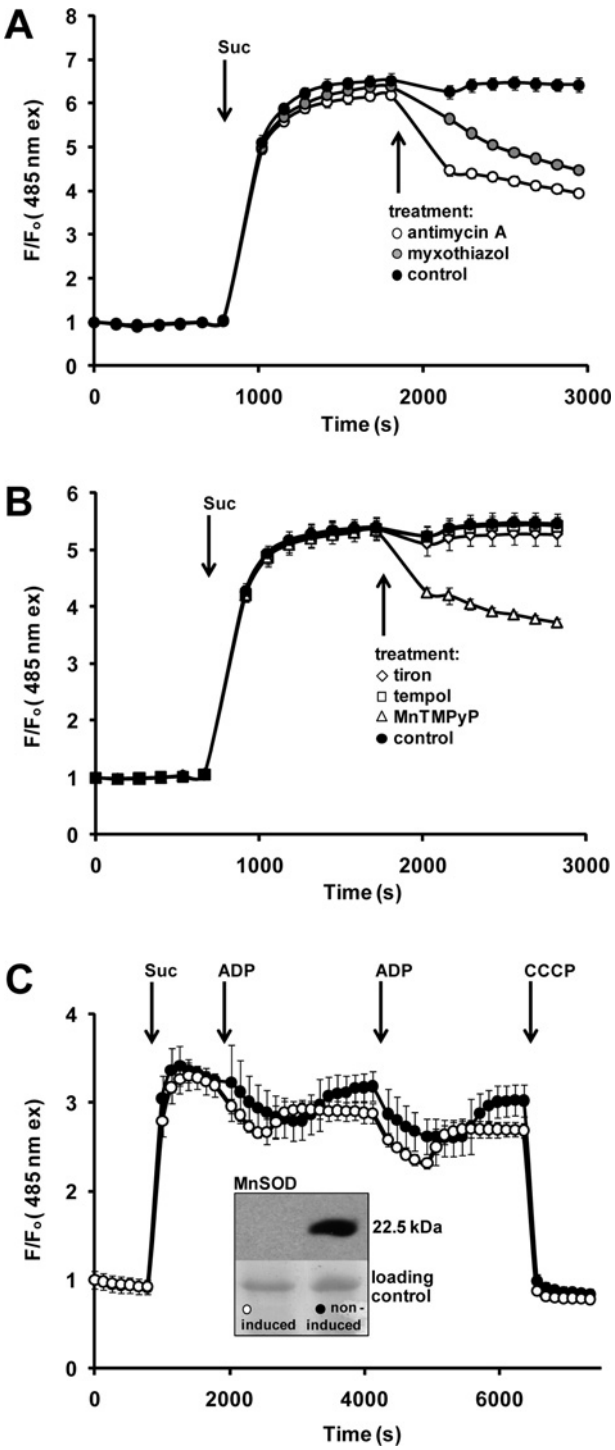

Figure 3 CpYFP response to antimycin A, superoxide scavengers and lack of MnSOD in active isolated mitochondria

Mitochondria (20 μg) were suspended in 'basic incubation medium' and energized (Suc; 10 mM succinate, 0.25 mM ATP and 20 μM rotenone). (**A**) Treatment with the complex III inhibitors antimycin A (1 μM, ○), myxothiazol (1 μM, ●) and solvent control (ethanol, ●). (**B**) Treatment with superoxide scavengers tiron (500 μM, ◇), tempol (100 μM, □), MnTMPyP (20 μM, △) and solvent control (water, ●). (**C**) Response of cpYFP in isolated mitochondria lacking MnSOD (○) to different respiratory states (see Figure 2) compared with control (●). Seedlings of a dexamethasone-inducible MnSOD knockdown line were induced with 10 μM dexamethasone and solvent control (DMSO). Immunoblotting of seedling extract showed the absence of MnSOD protein compared with control from day 4 after induction (inset). Mitochondria were isolated after 5 days. Values are means ± S.D., $n = 4$. The experiments were repeated three times with similar results.

(Figure 3B). In addition tempol, an SOD mimetic that has been shown to alleviate ROS-induced mitochondrial abnormality in plants, when applied at similar concentrations, did not cause any effect [18]. By contrast, MnTMPyP [manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin], another SOD mimetic, led to a significant decrease in fluorescence. However a proportional decrease was also observed at 405 nm excitation upon MnTMPyP treatment (Supplementary Figures S5A and S5B at http://www.BiochemJ.org/bj/437/bj4370381add.htm), and MnTMPyP also decreased baseline cpYFP fluorescence, decreasing the signal beyond its state 1 value (Supplementary Figure S5C). These observations suggest quenching of cpYFP fluorescence by MnTMPyP, which may be a result of the intense brown colour of the compound, rather than an effect due to superoxide removal.

Finally, the response of cpYFP in isolated mitochondria lacking matrix MnSOD was tested. For that purpose cpYFP was expressed in the mitochondrial matrix of a line containing an inducible RNAi construct for suppression of mitochondrial SOD expression. RNAi-mediated suppression of MnSOD was confirmed by immunoblot analysis. MnSOD protein was undetectable from day 4 after induction (Figure 3C, inset). Respiring mitochondria isolated 5 days after induction showed no significant increase in cpYFP signal compared with control (Figure 3C).

In conclusion, there was no significant experimental correlation between mitochondrial ROS and cpYFP fluorescence in isolated *Arabidopsis* mitochondria.

A high pK_a makes cpYFP highly pH-sensitive in the mitochondrial matrix

As no direct evidence for any significant sensitivity of cpYFP to superoxide in plant mitochondria could be found, other parameters linked to pmf were assessed for their ability to modulate cpYFP fluorescence. Strong pH-dependence of cpYFP was reported previously by Wang et al. [5] and cpYFP fluorescence intensity in isolated *Arabidopsis* mitochondria was observed to increase with increasing pH of the medium (Supplementary Figure S6 at http://www.BiochemJ.org/bj/437/bj4370381add.htm). Re-evaluation of the pH properties of cpYFP in mitochondrial lysates revealed a pK_a of approximately 8.6 with a 485 nm excitation (Figure 4A). In addition, the dynamic range of the probe was broad between pH 7 and 10. A >20-fold increase in fluorescence was observed upon alkalization. Addition of 5 μM CCCP did not lead to any changes in lysates (results not shown), confirming that cpYFP was well equilibrated with the medium. In contrast with excitation with 485 nm, the probe showed only minor pH sensitivity with 405 nm excitation (Figure 4B). This allows ratiometric expression of pH sensitivity, independently of probe concentration and without a significant effect on pK_a or the dynamic range (Figure 4C). The strong pH-dependence of cpYFP raises the possibility that pH changes rather than superoxide may account for the observed probe dynamics in mitochondria.

cpYFP dynamics in respiring plant mitochondria represent pH changes

To assess whether pH changes are sufficient to account for the observed probe dynamics in respiring mitochondria, ΔpH was separated from pmf (and thereby superoxide production) using nigericin. pH was clamped by nigericin-mediated K^+/H^+ antiport, whereas the voltage component of pmf was maintained. Addition of nigericin in the presence of K^+ ions not only decreased the cpYFP signal to the same degree as the uncoupler

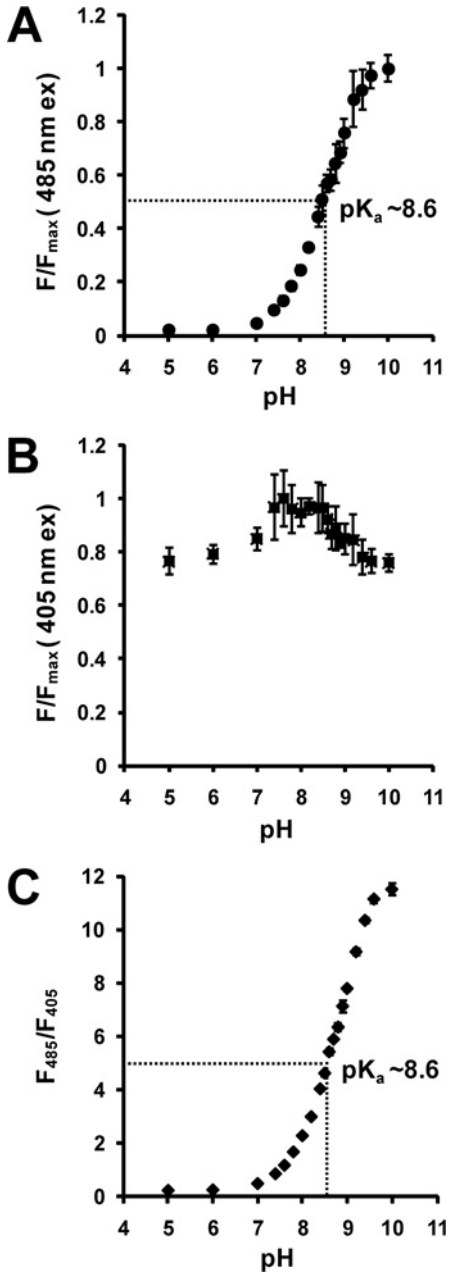

Figure 4 pH-dependence of mitochondrial cpYFP fluorescence

Fluorescence of lysed mitochondria expressing cpYFP in response to excitation at (**A**) 485 nm, (**B**) 405 nm and (**C**) the ratio of 485 nm to 405 nm fluorescence. Mitochondria were lysed by a freeze–thaw cycle. Mitochondrial protein lysate (10 μg) was suspended in 0.3 M sucrose and 10 mM Tes/Tris. The pH was adjusted by titration of Tes with Tris base. pK_a is indicated by dotted lines. Values are means $\pm$ S.D., $n = 3$.

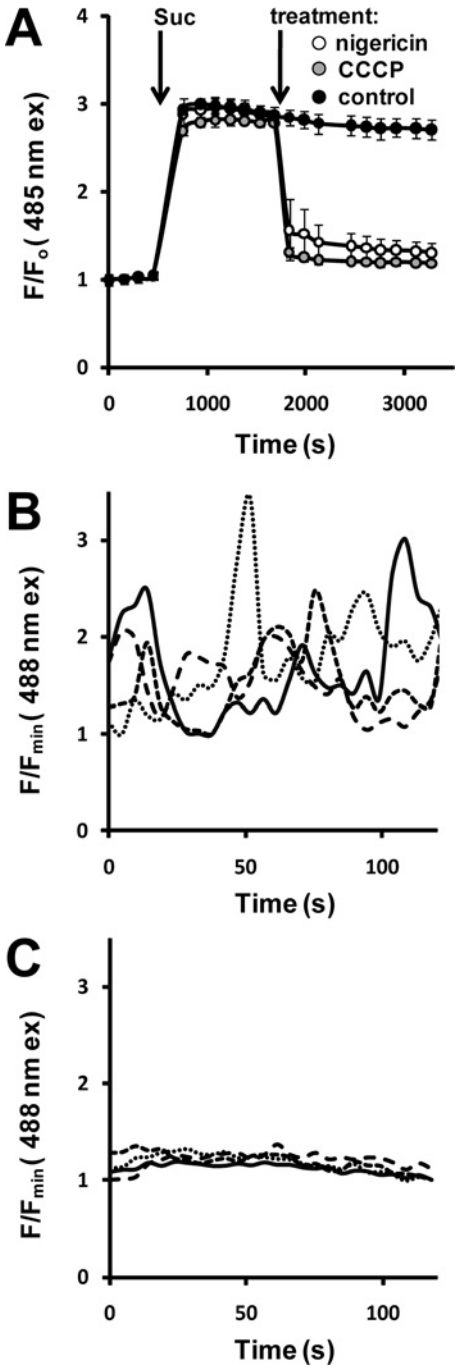

Figure 5 The effect of nigericin on cpYFP fluorescence dynamics in isolated mitochondria

(**A**) Mitochondria (20 μg) were suspended in a medium containing 0.2 M sucrose, 0.1 M KCl and 10 mM Tes (pH 7.2 adjusted with Tris base), energized (Suc; 10 mM succinate and 20 μM rotenone) and treated with nigericin (5 μM, ○), CCCP (5 μM, ◉) and solvent control (ethanol, ●). Values are means $\pm$ S.D., $n = 3$. The experiment was repeated independently three times with similar results. (**B** and **C**) The dynamics of cpYFP signal intensity (488 nm excitation) was measured by CLSM in individual respiring mitochondria under the same conditions as described in (**A**). (**B**) Solvent control (ethanol) and (**C**) 5 μM nigericin. Normalized intensity traces for four representative individual mitochondria are shown. The experiment was repeated three independent times with similar results.

CCCP (Figure 5A), but it also abolished the increase in cpYFP signal upon energization from state 1 to state 2 (Supplementary Figure 5C). Clamping matrix pH was therefore sufficient to stop mitochondrial cpYFP dynamics in mitochondrial suspensions, independently of the presence of a pmf and associated superoxide release. Nigericin also completely abolished cpYFP flashes in single isolated mitochondria monitored by CLSM (Figures 5B and 5C, and Supplementary Movies S3 and S4 at http://www.BiochemJ.org/bj/437/bj4370381add.htm). Flashes were retained when the identical treatments were applied in K^+-free medium (results not shown), demonstrating the specificity of pH clamping in this system. Overall, changes in matrix pH provided a sufficient explanation for the observed cpYFP signal dynamics in mitochondrial suspensions, as well as in single mitochondria.

DISCUSSION

On the basis of the observation of cpYFP flashes in single plant mitochondria, we aimed to elucidate the basis of the probe response. Similar flashing events have been observed in single mitochondria of cardiomyocytes using cpYFP and interpreted as response superoxide bursts in the matrix based on superoxide sensitivity of the sensor in an *in vitro* assay [5]. Using isolated mitochondria as a minimal model system allowed us to validate cpYFP properties *in situ*, using a reductionist approach. Isolated plant mitochondria lack most respiratory metabolites and co-factors allowing tight control of potential probe interactors [19]. The results of the present study confirm previous observations that the mitochondrial cpYFP signal is linked to mitochondrial electron transport and metabolic status [5,6]. Wang et al. [5] showed that flash frequency was strongly inhibited when electron transport was dysfunctional or inhibited in cardiomyocytes. In our hands absolute signal intensity strongly depended on respiratory state, decreased upon ETC inhibition at complex III and flashes were only observed in energized mitochondria. However, we were unable to demonstrate a significant response to matrix superoxide; instead the dynamic behaviour of cpYFP was fully accounted for by its pronounced pH sensitivity.

pH sensitivity of cpYFP was reported by Wang et al. [5], but without any determination of pK_a and the dynamic range. pH sensitivity is common in GFP (green fluorescent protein)-derived probes; however, it can often be ignored when the pK_a of the probe is sufficiently distant from the assay pH. Wang et al. [5] ruled out transient changes in matrix pH being responsible for cpYFP flashes, based on the absence of flashes using a mitochondrial-targeted EYFP (enhanced YFP) as a pH sensor. However, the pK_a of EYFP is approximately 7.1 [20], meaning that it will not be particularly responsive to increases in pH from the physiological value of 7.8–7.9 that has been determined in respiring animal and plant mitochondria *in vivo* [20,21]. Comparably high matrix pH is expected in isolated mitochondria under state 2 and 4 respiration, and a medium pH of 7.2. On the basis of the cpYFP signal, a switch from state 1 to 2 increases matrix pH by approximately 1 unit, i.e. to approximately 8.2, under the conditions used in the present study (see Supplementary Figure S6). The high pK_a of cpYFP at approximately pH 8.6 means that cpYFP will be much more sensitive than EYFP to pH changes in the mitochondrial matrix. For example, given a resting pH of 7.9, a pH increase of 0.4 unit results in a ~2-fold increase in cpYFP fluorescence. This is in contrast with EYFP that would only show a very subtle fluorescence change (~1.1-fold) upon the same pH increase [20]. Moreover, the observed probe responses to changes in respiratory states of isolated mitochondria at buffer pH 7.2 appears, at least qualitatively, in line with the expected pH changes occurring in the matrix. cpYFP is therefore a highly responsive pH sensor in the physiological context of the mitochondrial matrix.

Wang et al. [5] provided good evidence that cpYFP is sensitive to superoxide *in vitro*. It therefore remains possible that cpYFP can report mitochondrial superoxide *in vivo*. However, for reasons stated above, our view is that changes in cpYFP fluorescence in the mitochondrial matrix in plants are more likely to be related to changes in matrix pH *in vivo*. A mechanism for the proposed superoxide sensitivity of cpYFP would help to clarify the situation; indeed Wang et al. [5] have suggested the involvement of two cysteine residues. However, the reactivity of thiols with superoxide is low, which makes such a mechanism unlikely [22]. In contrast, pH-dependence of cpYFP fluorescence provides a straightforward explanation for the observed probe responses: the chromophore can be protonated, inducing a reversible fluorescence increase [23].

The superoxide sensitivity of cpYFP has been scrutinized in a review by Muller [7]. A major criticism included the decreased flash rate upon antimycin A treatment, which generally induces superoxide release into the matrix. Our treatments of isolated plant mitochondria with antimycin A also gave a decrease in cpYFP intensity rather than the inverse, as expected from elevated superoxide production. cpYFP fluorescence was low when respiration was not active, either due to a lack of substrate or inhibition of complex III, and no cpYFP flashes occurred in the absence of pmf. Although plant and mammalian mitochondria differ in many aspects, changes in pH could in principle resolve the apparent contradictions that have been pointed out by Muller [7]. Recently Pouvreau [24] also detected flashes in mitochondria of mouse skeletal muscle cells using a ratiometric pericam sensor that contains the cpYFP sequence. MitoSOX, a superoxide-sensitive chemical dye, was then used as an *in vivo* control for superoxide sensitivity of the sensor. An increase in MitoSOX intensity for a mitochondrion undergoing a flash was suggested. Therefore it is possible that cpYFP flashes in different systems represent different physiological phenomena. However, as the MitoSOX signal only increases slightly during a flash, even though MitoSOX acts as an irreversible and cumulative superoxide stain (with its specificity *in vivo* having been questioned [25]), the possibility of photo-oxidative ROS production during a transient increase in cpYFP fluorescence should be considered as an alternative explanation.

We suggest that cpYFP is a promiscuous sensor that can, in principle, respond to different parameters via changes in signal intensity. Its responsiveness to superoxide and thiol agents *in vitro* has been documented along with its pH sensitivity, and additional effectors cannot be ruled out. However, its pH response is substantial particularly at mitochondrial pH values, and cpYFP dynamics in mitochondria can be abolished when pH is clamped. These findings cast doubt on the existence of superoxide flashes in mitochondria. Moreover they emphasize the vital need to understand how ROS and redox probes work mechanistically [26,27]. Without this understanding the relative contributions of individual parameters to the response of cpYFP under physiological conditions have to be considered carefully in order to draw accurate conclusions.

AUTHOR CONTRIBUTION

Markus Schwarzländer designed and performed the experiments and wrote the paper. Lee Sweetlove, David Logan and Mark Fricker supervised the research and co-wrote the paper.

ACKNOWLEDGEMENTS

We thank Dr Wang Wang (University of Washington, Seattle, WA, U.S.A.) and Professor Heping Cheng (Peking University, Bejing, China) for kindly providing the cpYFP construct, Dr Karl Morten (University of Oxford, Oxford, U.K.) for the gift of myxothiazol, Dr Daniel Kliebenstein (University of California, Davis, CA, U.S.A.) for the MnSOD antiserum, and Dr Ian Moore (University of Oxford, Oxford, U.K.) for the pOpOff2 vector. Stimulating discussions with Professor George Ratcliffe (University of Oxford, Oxford, U.K.) and Professor Andreas Meyer (University of Bonn, Bonn, Germany) have strengthened this work.

FUNDING

M.S. was funded by New College Oxford through a Weston Junior Research Fellowship.

REFERENCES

1 Niethammer, P., Grabher, C., Look, A. T. and Mitchison, T. J. (2009) A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. Nature **459**, 996–999

2 Meyer, A. J. and Dick, T. P. (2010) Fluorescent protein-based redox probes. Antioxid. Redox Signaling **13**, 621–650

3 Murphy, M. P. (2009) How mitochondria produce reactive oxygen species. Biochem. J. **417**, 1–13
4 Moller, I. M. (2001) Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. Annu. Rev. Plant Physiol. Plant Mol. Biol. **52**, 561–591
5 Wang, W., Fang, H., Groom, L., Cheng, A., Zhang, W., Liu, J., Wang, X., Li, K., Han, P., Zheng, M. et al. (2008) Superoxide flashes in single mitochondria. Cell **134**, 279–290
6 Fang, H., Chen, M., Ding, Y., Shang, W., Xu, J., Zhang, X., Zhang, W., Li, K., Xiao, Y., Gao, F. et al. (2011) Imaging superoxide flash and metabolism-coupled mitochondrial permeability transition in living animals. Cell Res., doi:10.1038/cr.2011.81
7 Muller, F. L. (2009) A critical evaluation of cpYFP as a probe for superoxide. Free Radical Biol. Med. **47**, 1779–1780
8 Logan, D. C. and Leaver, C. J. (2000) Mitochondria-targeted GFP highlights the heterogeneity of mitochondrial shape, size and movement within living plant cells. J. Exp. Bot. **51**, 865–871
9 Karimi, M., Inze, D. and Depicker, A. (2002) GATEWAY vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci. **7**, 193–195
10 Craft, J., Samalova, M., Baroux, C., Townley, H., Martinez, A., Jepson, I., Tsiantis, M. and Moore, I. (2005) New pOp/LhG4 vectors for stringent glucocorticoid-dependent transgene expression in *Arabidopsis*. Plant J. **41**, 899–918
11 Clough, S. J. and Bent, A. F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16**, 735–743
12 Sweetlove, L. J., Taylor, N. L. and Leaver, C. J. (2007) Isolation of intact, functional mitochondria from the model plant *Arabidopsis thaliana*. Methods Mol. Biol. **372**, 125–136
13 Morgan, M. J., Lehmann, M., Schwarzlander, M., Baxter, C. J., Sienkiewicz-Porzucek, A., Williams, T. C., Schauer, N., Fernie, A. R., Fricker, M. D., Ratcliffe, R. G. et al. (2008) Decrease in manganese superoxide dismutase leads to reduced root growth and affects tricarboxylic acid cycle flux and mitochondrial redox homeostasis. Plant Physiol. **147**, 101–114
14 Kliebenstein, D. J., Monde, R. A. and Last, R. L. (1998) Superoxide dismutase in *Arabidopsis*: an eclectic enzyme family with disparate regulation and protein localization. Plant Physiol. **118**, 637–650
15 Sweetlove, L. J., Heazlewood, J. L., Herald, V., Holtzapffel, R., Day, D. A., Leaver, C. J. and Millar, A. H. (2002) The impact of oxidative stress on *Arabidopsis* mitochondria. Plant J. **32**, 891–904
16 Schwarzlander, M., Fricker, M. D., Muller, C., Marty, L., Brach, T., Novak, J., Sweetlove, L. J., Hell, R. and Meyer, A. J. (2008) Confocal imaging of glutathione redox potential in living plant cells. J. Microsc. **231**, 299–316
17 Baxter, C. J., Redestig, H., Schauer, N., Repsilber, D., Patil, K. R., Nielsen, J., Selbig, J., Liu, J., Fernie, A. R. and Sweetlove, L. J. (2007) The metabolic response of heterotrophic *Arabidopsis* cells to oxidative stress. Plant Physiol. **143**, 312–325
18 Scott, I. and Logan, D. C. (2008) Mitochondrial morphology transition is an early indicator of subsequent cell death in *Arabidopsis*. New Phytol. **177**, 90–101
19 Douce, R. (1985) Mitochondria in Higher Plants, Academic Press, Orlando
20 Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G. and Tsien, R. Y. (1998) Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. Proc. Natl. Acad. Sci. U.S.A. **95**, 6803–6808
21 Nobel, P. S. (1991) Physicochemical and Environmental Plant Physiology, Academic Press, San Diego
22 Moller, I. M., Jensen, P. E. and Hansson, A. (2007) Oxidative modifications to cellular components in plants. Annu. Rev. Plant Biol. **58**, 459–481
23 Kneen, M., Farinas, J., Li, Y. and Verkman, A. S. (1998) Green fluorescent protein as a noninvasive intracellular pH indicator. Biophys. J. **74**, 1591–1599
24 Pouvreau, S. (2010) Superoxide flashes in mouse skeletal muscle are produced by discrete arrays of active mitochondria operating coherently. PLoS ONE **5**, e13035
25 Zielonka, J. and Kalyanaraman, B. (2010) Hydroethidine- and MitoSOX-derived red fluorescence is not a reliable indicator of intracellular superoxide formation: another inconvenient truth. Free Radical Biol. Med. **48**, 983–1001
26 Cocheme, H. M., Quin, C., McQuaker, S. J., Cabreiro, F., Logan, A., Prime, T. A., Abakumova, I., Patel, J. V., Fearnley, I. M., James, A. M. et al. (2011) Measurement of H_2O_2 within living *Drosophila* during aging using a ratiometric mass spectrometry probe targeted to the mitochondrial matrix. Cell Metab. **13**, 340–350
27 Meyer, A. J., Brach, T., Marty, L., Kreye, S., Rouhier, N., Jacquot, J. P. and Hell, R. (2007) Redox-sensitive GFP in *Arabidopsis thaliana* is a quantitative biosensor for the redox potential of the cellular glutathione redox buffer. Plant J. **52**, 973–986

Received 18 May 2011/27 May 2011; accepted 1 June 2011
Published as BJ Immediate Publication 1 June 2011, doi:10.1042/BJ20110883

Biochem. J. (2011) **437**, 381–387 (Printed in Great Britain) doi:10.1042/BJ20110883

multimedia online data

ACCELERATED PUBLICATION
SUPPLEMENTARY ONLINE DATA

The circularly permuted yellow fluorescent protein cpYFP, that has been used as a superoxide probe, is highly responsive to pH but not superoxide in mitochondria: implications for the existence of superoxide 'flashes'

Markus SCHWARZLÄNDER*[1], David C. LOGAN†, Mark D. FRICKER* and Lee J. SWEETLOVE*[1]

*Department of Plant Science, University of Oxford, South Parks Road, Oxford OX1 3RB, U.K., and †Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5E2

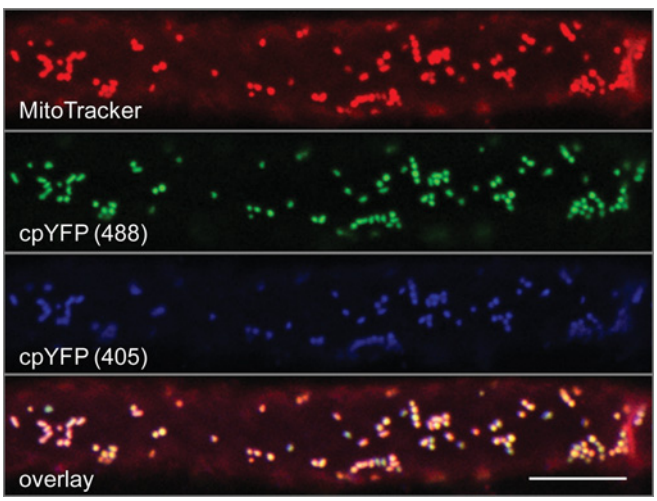

Figure S1 Localization of cpYFP expression

A living root of an *Arabidopsis* cpYFP-expressing line was stained with 200 nM MitoTracker Orange CMTMRos and an epidermal cell was imaged by CLSM. MitoTracker signal, red; cpYFP signal at 488 nm, green; cpYFP signal at 405 nm, blue. Scale bar = 10 μm.

[1] Correspondence may be addressed to either of these authors (email markus.schwarzlander@plants.ox.ac.uk or lee.sweetlove@plants.ox.ac.uk).

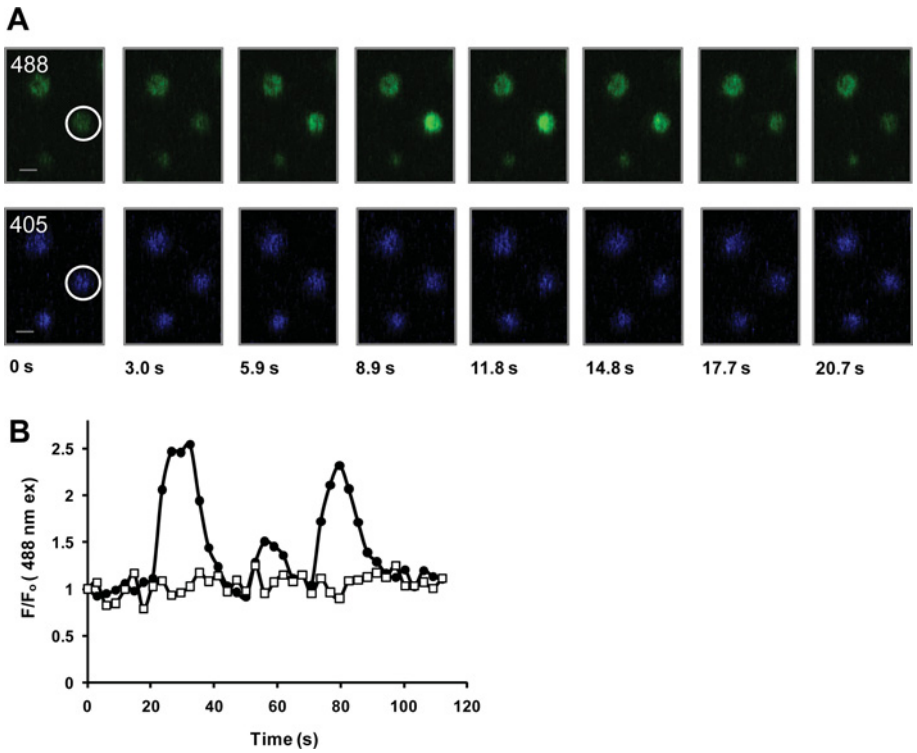

Figure S2 cpYFP fluorescence at 405 nm excitation during flashes at 488 nm excitation in single isolated mitochondria

(**A**)cpYFP fluorescence at 488 nm (green) and 405 nm (blue) excitation in a respiring isolated mitochondrion (white circle) during a flash in 'basic incubation medium' supplemented with 10 mM succinate, 0.25 mM ATP and 20 μM rotenone. Scale bar = 1 μm. (**B**) Normalized intensity trace for a single isolated mitochondrion at 488 (●) and 405 nm (□) excitation.

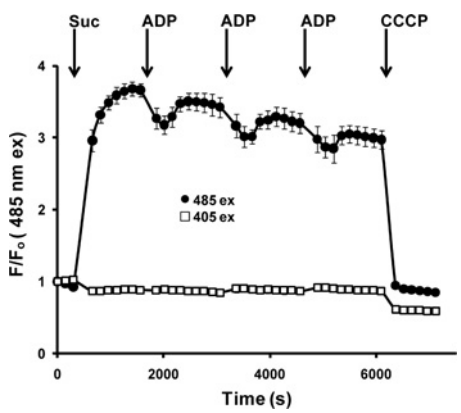

Figure S3 The response of cpYFP fluorescence at 485 and 405 nm excitation to different respiratory states in isolated mitochondria

Mitochondria respiring succinate as shown and described in Figure 1 of the main text. Fluorescence at excitation of 485 nm (●) and 405 nm (□). A signal decrease at 405 nm excitation upon CCCP addition can be accounted for by the absorption of CCCP that is strong at 405 but minimal at 488 nm [1]. Values are means ± S.D., $n = 5$. The experiment was repeated independently five times with similar results.

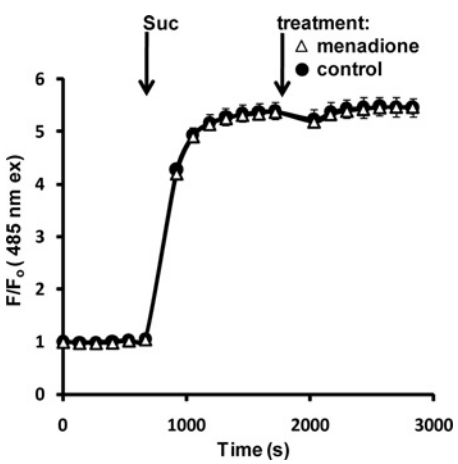

Figure S4 CpYFP response to menadione in active isolated mitochondria

Mitochondria (20 μg) were suspended in 'basic incubation medium' and energized (10 mM succinate, 0.25 mM ATP and 20 μM rotenone). Menadione (30 μM, △) and solvent control (●) were added. Values are means ± S.D., $n = 4$. The experiment was repeated three times with similar results.

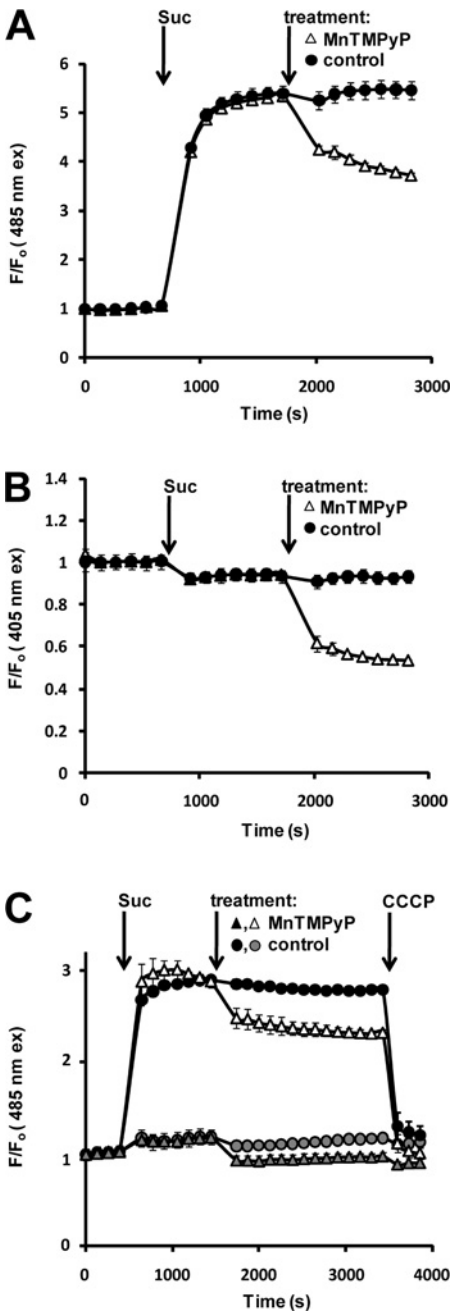

Figure S5 Effects of MnTMPyP on cpYFP fluorescence

Mitochondria (20 μg) were suspended in 'basic incubation medium' and energized (Suc; 10 mM succinate, 0.25 mM ATP and 20 μM rotenone). MnTMPyP (20 μM, Δ) and solvent control (●) were added. CpYFP signal at 485 nm (**A**) and 405 nm (**B**) excitation. In (**C**) mitochondria were energized in the presence (grey symbols) and absence (black and white symbols) of 5 μM nigericin in the medium. MnTMPyP (20 μM, white and grey triangles) and solvent control (black and grey circles) were added where indicated. CCCP (5 μM) was added for uncoupling at the end of the assay. Values are means $\pm$ S.D.. The experiments were repeated three times with similar results.

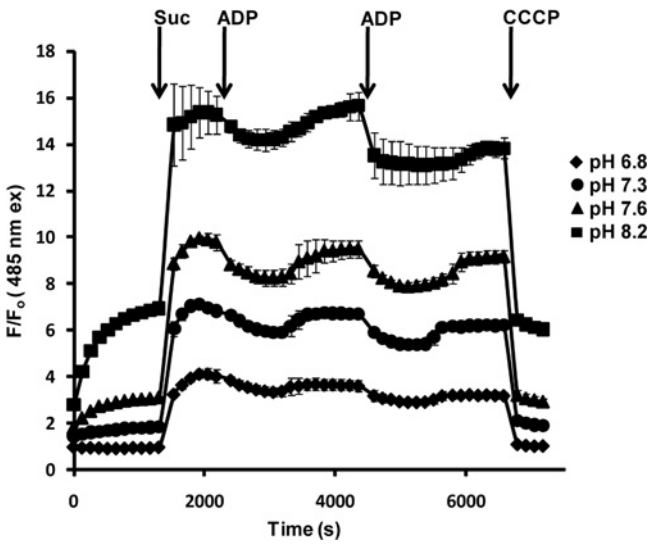

Figure S6 Effect of medium pH on cpYFP fluorescence in isolated mitochondria in different respiratory states

Mitochondria (20 μg) were suspended in 'basic incubation medium' that was adjusted (with KOH) to pH values of 6.8 (◆), 7.3 (●), 7.6 (▲) and 8.2 (■). After equilibration, mitochondria were energized (Suc, 10 mM succinate, 0.25 mM ATP and 20 μM rotenone) and respiratory states were varied as described in Figure 2 of the main text at the end of the assay. Values are means $\pm$ S.D., $n = 3$.

REFERENCE

1 Kaback, H. R., Reeves, J. P., Short, S. A. and Lombardi, F. J. (1974) Mechanisms of active transport in isolated bacterial membrane vesicles. 18. The mechanism of action of carbonylcyanide *m*-chlorophenylhydrazone. Arch. Biochem. Biophys. **160**, 215–222

Received 18 May 2011/27 May 2011; accepted 1 June 2011
Published as BJ Immediate Publication 1 June 2011, doi:10.1042/BJ20110883