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The flavoprotein Cyc2p, a mitochondrial cytochrome *c* assembly factor, is a NAD(P)H-dependent haem reductase

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Summary

Cytochrome *c* assembly requires sulphydryls at the C*XX*CH haem binding site on the apoprotein and also chemical reduction of the haem co-factor. In yeast mitochondria, the cytochrome haem lyases (CCHL, CC_1HL) and Cyc2p catalyse covalent haem attachment to apocytochromes *c* and c_1. An *in vivo* indication that Cyc2p controls a reductive step in the haem attachment reaction is the finding that the requirement for its function can be bypassed by exogenous reductants. Although redox titrations of Cyc2p flavin ($E_m = -290$ mV) indicate that reduction of a disulphide at the C*XX*CH site of apocytochrome *c* ($E_m = -265$ mV) is a thermodynamically favourable reaction, Cyc2p does not act as an apocytochrome *c* or c_1 C*XX*CH disulphide reductase *in vitro*. In contrast, Cyc2p is able to catalyse the NAD(P)H-dependent reduction of hemin, an indication that the protein's role may be to control the redox state of the iron in the haem attachment reaction to apocytochromes *c*. Using two-hybrid analysis, we show that Cyc2p interacts with CCHL and also with apocytochromes *c* and c_1. We postulate that Cyc2p, possibly in a complex with CCHL, reduces the haem iron prior to haem attachment to the apoforms of cytochrome *c* and c_1.

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Introduction

The *c*-type cytochromes, generically referred to as cytochromes *c*, are ubiquitous metalloproteins carrying iron-protoporphyrin IX as a covalently attached co-factor (Ferguson *et al*., 2008; Hamel *et al*., 2009; Kranz *et al*., 2009; Sanders *et al*., 2010). Because haem is a redox-active molecule, cytochromes *c* typically function as soluble or membrane-bound electron carriers in membrane systems involved in energy transduction (i.e. bacterial membrane, thylakoid membrane, mitochondrial inner membrane) (Thöny-Meyer, 1997; Bonnard *et al*., 2010). The site for haem attachment on apocytochrome *c* is a C*XX*CH motif where each cysteine reacts with a carbon atom in a haem vinyl group to form a thioether linkage. *In organello* and *in vitro* reconstitution experiments with the apoprotein and haem co-factor have established the requirement for redox chemistry in the assembly of *c*-type cytochromes. Both ferro-haem and the cysteine sulphydryls at the C*XX*CH motif are needed for haem ligation to apocytochrome *c* (Basile *et al*., 1980; Nicholson and Neupert, 1989; Nicholson *et al*., 1989; Tong and Margoliash, 1998). While it appears obvious that C*XX*CH sulphydryls need to be maintained reduced if haem is to be attached, the requirement for ferro versus ferrihaem is less intuitive and appears inherent to the chemistry of thioether bond formation (Barker *et al*., 1993). *In vivo*, cytochrome *c* assembly occurs on the *p*-side of the energy-transducing membrane [i.e. bacterial periplasm, thylakoid lumen, mitochondrial intermembrane space (IMS)] and extensive studies in bacteria, plastids and mitochondria revealed that this is a catalysed process under the control of distinct assembly pathways, the so-called systems I, II and III (Ferguson *et al*., 2008; Hamel *et al*., 2009; Kranz *et al*., 2009; Sanders *et al*., 2010). Common to systems I and II is the involvement of multicomponent transmembrane pathways for the delivery and ligation of haem (Goldman *et al*., 1998; Schulz *et al*., 1998; 1999; 2000; Ren and Thöny-Meyer, 2001; Ahuja and Thony-Meyer, 2003; 2005; Dreyfuss *et al*., 2003; Hamel *et al*., 2003; Feissner *et al*., 2006; Ahuja *et al*., 2009; Frawley and Kranz, 2009; Richard-Fogal *et al*., 2009; Richard-Fogal and Kranz, 2010) and the delivery of reducing equivalents to the site of holocytochrome *c* assembly (Beckett *et al*., 2000; Deshmukh *et al*., 2000; Bardischewsky and Friedrich, 2001; Lennartz *et al*.,

2001; Erlendsson and Hederstedt, 2002; Erlendsson *et al.*, 2003; Gabilly *et al.*, 2010; 2011). In bacteria and plastids, the provision of reducing equivalents relies on a membrane thiol-disulphide oxido-reductase and a thioredoxin-like protein that act sequentially to reduce an intramolecular disulphide in the apocytochrome *c* C*XX*CH prior to the haem attachment reaction (Bonnard *et al.*, 2010; Sanders *et al.*, 2010).

In systems I and II, candidate proteins involved in the maintenance of haem in the reduced form have been postulated but the demonstration of such a biochemical activity is still lacking (Frawley and Kranz, 2009; Richard-Fogal *et al.*, 2009; San Francisco *et al.*, 2011).

System III, the maturation pathway under study in this work, was discovered in fungi through the genetic analysis of mutants deficient in membrane-bound cytochrome c_1 and soluble cytochrome *c*, the two *c*-type cytochromes residing in the mitochondrial IMS (Bonnard *et al.*, 2010). In contrast to systems I and II, exhaustive genetic screens failed to uncover system III components involved in the transport of haem (from its site of synthesis in the mitochondrial matrix to the IMS) or delivery of reductants for maintenance of the C*XX*CH sulphydryls (Sherman, 1990). The only defining components of system III are the so-called haem lyases (CCHL and related CC_1HL), a unique class of IMS proteins with a still poorly understood biochemical activity in the assembly process (Dumont *et al.*, 1987; Nargang *et al.*, 1988; Drygas *et al.*, 1989; Zollner *et al.*, 1992; Cervera *et al.*, 1998). In yeast, CC_1HL exhibits a strict specificity towards apocytochrome c_1 while CCHL acts on apocytochrome *c* but also displays intrinsic weak activity towards its non-cognate substrate, apocytochrome c_1 (Matner and Sherman, 1982; Dumont *et al.*, 1987; Stuart *et al.*, 1990; Bernard *et al.*, 2003; Zollner *et al.*, 1992) (Fig. 6). Mutations in either the enzyme CCHL or the substrate cytochrome c_1 modulate the activity of CCHL towards apocytochrome c_1 (Bernard *et al.*, 2005; Corvest *et al.*, 2010). Recent genetic studies show that the identity of the second intervening residue at the C*XX*CH site appears to be a key determinant for interaction of apocytochrome c_1 with CCHL and/or CC_1HL (Corvest *et al.*, 2010).

The CCHL-dependent assembly of cytochrome *c* and c_1 requires Cyc2p, a mitochondrial inner membrane flavoprotein with its redox domain exposed to the IMS, which was known from former studies on cytochrome *c* maturation and re-discovered through a multicopy suppressor screen (Dumont *et al.*, 1993; Pearce *et al.*, 1998; Bernard *et al.*, 2003; 2005). Cyc2p is dedicated to the CCHL pathway and is not required for the activity of CC_1HL (Corvest *et al.*, 2010). Because cytochrome c_1 can be assembled by CCHL or by CC_1HL, the CCHL-dependent assembly of cytochrome c_1 requires Cyc2p but the CC_1HL-dependent assembly of cytochrome c_1 does not (Corvest *et al.*, 2010).

The exact role of Cyc2p in the assembly pathway had not been characterized but the finding that a recombinant form of the molecule exhibits a NAD(P)H-dependent reductase activity suggested a redox-linked function (Bernard *et al.*, 2005). The fact that Cyc2p does not carry a conserved cysteine or cysteine pairs typically present in NAD(P)H-dependent flavoproteins exhibiting disulphide reductase activity (Argyrou and Blanchard, 2004; Bernard *et al.*, 2005) suggests that a disulphide-bonded C*XX*CH is probably not the relevant target of its activity.

In this paper we explore the function of Cyc2p in the cytochrome *c* maturation pathway and postulate that the molecule, possibly in a complex with CCHL, reduces the haem iron prior to haem attachment to the apoforms of cytochrome *c* and c_1 by the lyase. Our model is based on the findings that: (i) the reducing activity of Cyc2p can be bypassed *in vivo* by application of reductants, (ii) a recombinant form of the molecule is able to catalyse the NAD(P)H reduction of hemin in an *in vitro* assay and (iii) Cyc2p interactions with CCHL and with apocytochrome c/c_1 can be detected in yeast two-hybrid.

Results

Reductants bypass the in vivo *requirement for Cyc2p in cytochrome* c *assembly*

The finding that recombinant Cyc2p exhibits a NAD(P)H-ferricyanide oxido-reductase activity suggests that the molecule controls a reductive step in the assembly process (Bernard *et al.*, 2005). To provide evidence for the physiological relevance of such an activity, we tested whether the requirement for Cyc2p *in vivo* could be bypassed by the application of reductants. As shown in Fig. 1A, the slight respiratory growth defect in the *cyc2*-null mutant was rescued by application of TCEP, a strong reducing agent. Analysis of holocytochrome *c* content using both haem staining and immunoblotting in the rescued mutant shows that the restoration of the respiratory growth correlates with enhanced accumulation of holocytochrome *c* (Fig. 1B). Providing exogenous reductants to the *cyc2*-null strain does not result in the increased level of CCHL that is needed for the conversion of apocytochrome *c* to its holoform (Fig. 1B). The cytochrome *c*-deficient phenotype arising from the loss of CCHL is not suppressed by supplying exogenous reductants and remains tight, even upon extended incubation (Fig. 1A) (Bernard *et al.*, 2003). This suggests that the TCEP-dependent rescue of cytochrome *c* deficiency that we observed is specific for the absence of Cyc2p. To solidify the evidence that Cyc2p participates in a reducing pathway for cytochrome *c* assembly, we examined whether reductants could rescue the respiratory growth defect of the *cc*$_1$*hl*-null mutant, a phenotype that can be suppressed by overexpression of *CYC2* (Bernard *et al.*,

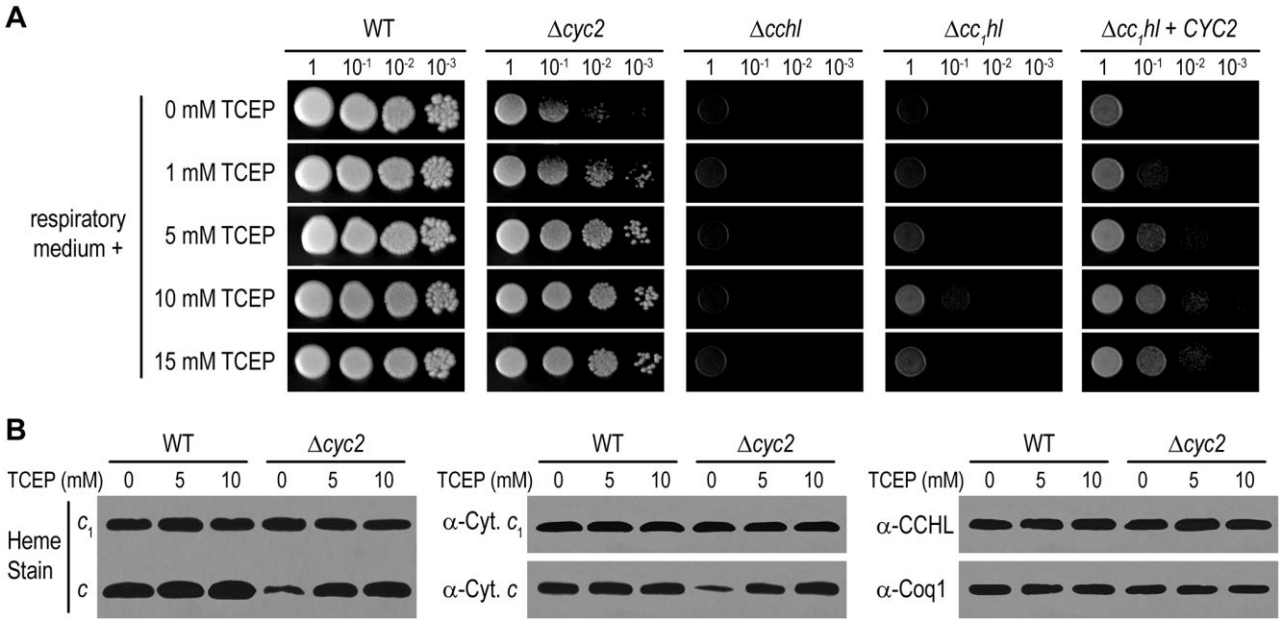

Fig. 1. The requirement for Cyc2p can be rescued by exogenous reductants *in vivo*.
A. TCEP-dependent respiratory rescue. Tenfold serial dilutions of WT (W303-1A), *Δcyc2* (YPH1), *Δcchl* (SMY4), *Δcc*$_1$*hl* (SMY1) and *Δcc*$_1$*hl* + *CYC2* (overexpressing *CYC2*) strains were grown on ethanol/glycerol as respiratory substrate supplemented or not with Tris[2-carboxyethyl] phosphine (TCEP) as indicated, and incubated at 28°C for 3 days.
B. TCEP-dependent restoration of holocytochrome *c* assembly in the *Δcyc2* mutant. Eighty-five micrograms of mitochondrial proteins purified from cells grown in ethanol/glycerol medium supplemented or not with TCEP as indicated were separated through LDS-PAGE for haem staining or SDS-PAGE for immunoblotting as described in *Experimental procedures*. Note the restoration of holocytochrome *c* assembly in the *Δcyc2* mutant by the supply of exogenous reducing agent. Coq1 immunodetection served as a loading control.

2003). Addition of reductants could stimulate, albeit weakly, the respiratory growth of the *cc*$_1$*hl*-null mutant (Fig. 1A). Interestingly, the overexpression of *CYC2* in combination with TCEP treatment significantly enhanced the respiratory growth of the *cc*$_1$*hl*-null (Fig. 1A). Reduced DTT could suppress the respiratory defect of *cyc2*-null and *cc*$_1$*hl*-null strains but less efficiently than TCEP (not shown). No rescue was observed with oxidized DTT, an indication that the chemical rescue is mediated via reductants *in vivo*. These results support the hypothesis that Cyc2p is involved in supplying reducing equivalents during the cytochrome *c* maturation process.

Determination of midpoint redox potentials of Cyc2p flavin and apocytochrome c *haem binding site*

Based on our understanding of the biochemical requirements for cytochrome *c* maturation, we reasoned that either apocytochrome *c*/*c*$_1$ or haem could be the physiological target for Cyc2p-catalysed reduction. A preliminary indication that Cyc2p acts in an electron transfer reaction to reduce a disulphide in apocytochrome *c*/*c*$_1$ C*XX*CH or the haem iron atom can be inferred from comparing the redox potentials of the flavin in Cyc2p, which is the only redox centre in the molecule, versus those of apocytochrome *c*/*c*$_1$ and haem.

The redox potential of Cyc2p was determined experimentally by monitoring the relative amounts of oxidized and reduced flavin in redox equilibrium with known ambient redox potentials, established using the physiological $NADP^+$/NADPH couple (the flavin prosthetic group is fully oxidized in the as-isolated protein and addition of $NADP^+$ alone produced no detectable changes in the flavin spectrum). As can be seen from visible-region spectra obtained during the course of a titration (see the inset in Fig. 2A), no features are present at any E_h value that can be attributed to the presence of either a protonated neutral semiquinone or an unprotonated semiquinone anion (Massey and Hemmerich, 1980). Hence, the data were analysed on the assumption that only two redox states of the flavin are present at significant concentrations (i.e. the fully oxidized and fully reduced flavin) at redox equilibrium at all redox values. The data for this titration gave an excellent fit to the Nernst Equation for a two-electron redox couple with a midpoint potential, $E_m = -280$ mV at pH 7.0 (Fig. 2A). An independent replicate titration gave an excellent fit to the two-electron Nernst Equation with $E_m = -300$ mV (not shown). This second titration produced spectra similar to those shown in Fig. 2A, indicating again that the flavin semiquinone, if present at all, is present at levels below the *c*. 10% detection limit of our measurements. Thus, we conclude that

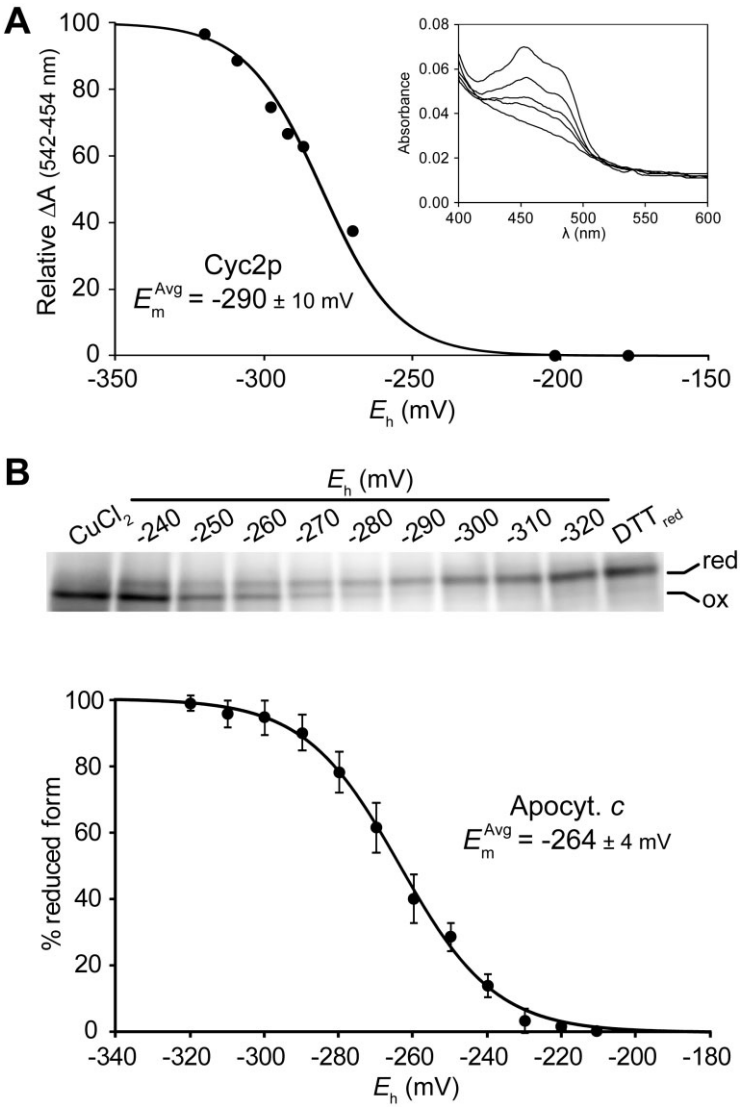

Fig. 2. Redox titrations of Cyc2p flavin cofactor and apocytochrome *c* C*XX*CH.
A. Redox titration of the flavin co-factor of Cyc2p. The titration was carried out as described in *Experimental procedures*. The extent of flavin reduction was monitored by the magnitude of the absorbance decrease at 542 nm minus 454 nm. The solid line represents the best fit of the data to the two-electron Nernst Equation with an E_m value of –280 mV (the E_m = –290 mV value shown on the figure is the average determined from this titration and a second independent titration for which the data are not shown). The inset shows spectra recorded at –270, –282, –292, –298 and –309 mV (from top to bottom).
B. Redox titration of the CXXCH motif in apocytochrome *c*. (Top) Separation of oxidized and reduced forms of apocytochrome *c* under non-reducing SDS-PAGE. Oxidized (non-modified, ox) and reduced (AMS modified, red) forms of apocytochrome *c* were separated on a 16% non-reducing SDS gel, visualized by silver staining and quantified using Image J software (National Institutes of Health). As a control for fully oxidized or fully reduced forms, apocytochrome *c* was incubated in the presence of either 50 μM $CuCl_2$ or 5 mM DTT_{red} respectively. (Bottom) Oxidation-reduction titration of apocytochrome *c*. Oxidized (unmodified) and reduced (AMS modified) forms of apocytochrome *c* for different ambient potentials were quantified by using Image J software. Percentages of reduced form of apocytochromes *c* were plotted as a function of ambient potential (E_h). Lines represent fits of the data to a two-electron Nernst curve. Error bars correspond to the standard deviation calculated from three independent experiments. The E_m value shown on the figure is the average determined from three independent titrations.

the E_m value for the oxidized/reduced flavin couple in Cyc2p is –290 ± 10 mV (the uncertainty shown, which is not unusual for these types of measurements, is simply the average deviation of the two measurements). It should be pointed out that the reduced form of Cyc2p, produced by reduction with NADPH, was not stable in the presence of oxygen, necessitating that these redox titrations be carried out under strictly anaerobic conditions.

Measurement of the redox potential of the cysteine pair at the haem binding site of apocytochrome *c* was carried out using DTT_{red}/DTT_{ox} buffers to poise samples at defined ambient potential (E_h) values, prior to their treatment with the thiol modifier AMS (Motohashi and Hisabori, 2006). AMS increases the molecular weight of a protein by 0.5 kDa per thiol modified, enabling the resolution of the doubly modified, reduced form from the unmodified oxidized form on non-reducing SDS-PAGE gels (Fig. 2B). The titration gave an excellent fit for a single two-electron redox component according to the Nernst equation, an expected finding as thiol/disulphide interconversion is a two-electron reaction. The midpoint potential of apocytochrome *c* C*XX*CH was –264 ± 4 mV, a value that is less negative than the one measured for the flavin cofactor in Cyc2p.

Cyc2p is not active as an apocytochrome c/c_1 *CXXCH disulphide reductase*

Based on the comparison of the midpoint potentials for Cyc2p and apocytochrome *c*, an electron transfer from the flavin cofactor to a disulphide in the apocytochrome *c* haem binding site is a thermodynamically favourable reaction. In order to determine if Cyc2p displays a disulphide reductase activity, we developed an *in vitro* assay and tested whether the soluble domain of Cyc2p that contains the flavin cofactor can catalyse reduction of the disulphide present in oxidized apocytochrome. Recombi-

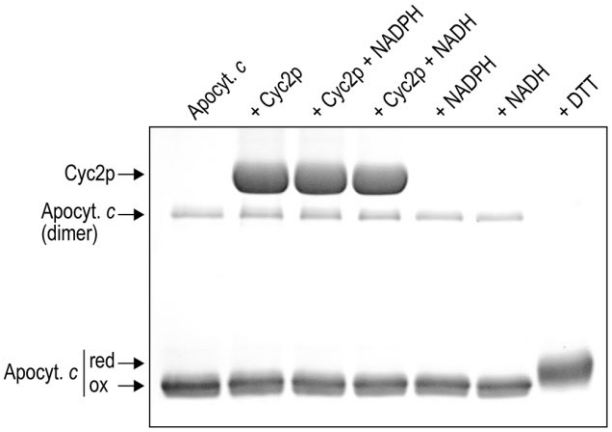

Fig. 3. Cyc2p is not active as an apocytochrome *c* C*XX*CH disulphide reductase. Reduction of apocytochrome *c* by Cyc2p was tested using thiol labelling with AMS as described in *Experimental procedures*. Fully oxidized apocytochrome *c* (1 μM) was incubated anaerobically for 1 h in the presence of Cyc2p (0.5 μM) with or without NAD(P)H (500 μM) as indicated. Incubation of apocytochrome *c* with DTT served as a positive control. Note that in the presence of Cyc2p and co-factor NAD(P)H, no reduced form of apocytochrome *c* can be observed. Similar results were obtained with recombinant soluble apocytochrome c_1 (data not shown).

nant apocytochrome *c* with an intramolecular disulphide at the C*XX*CH motif was incubated in the presence of soluble Cyc2p and an excess of NAD(P)H (NADPH had previously been demonstrated to serve as an electron donor to Cyc2p in Bernard *et al*., 2005). Conversion of the apocytochrome *c* disulphide to the dithiol form was monitored using AMS (a covalent modifier specific for free sulphydryl groups). Even after a 2 h incubation, no conversion of oxidized to reduced apocytochrome *c* was observed (Fig. 3). As a control, we showed that the disulphide in apocytochrome *c* could be chemically reduced by addition of DTT (Fig. 3). We also found that the soluble domain of apocytochrome c_1 carrying a disulphide bond at its haem binding site could not be converted to its reduced form by Cyc2p (not shown). In addition, we failed to detect any disulphide reductase activity using an insulin reduction assay, which is a simple assay to test a generic disulphide reductase activity (Holmgren, 1979; Lennartz *et al*., 2001; Motohashi and Hisabori, 2006) (not shown). We concluded that under the conditions tested, Cyc2p does not act as an apocytochrome *c*/c_1 C*XX*CH disulphide reductase.

Cyc2p catalyses the NAD(P)H-dependent reduction of hemin

To test whether Cyc2p is able to convert ferri to ferrohaem, we used an *in vitro* approach described in *Experimental procedures* that is based on the spectroscopic properties of hemin, the chloride form of iron-protoporphyrin IX. As shown in Fig. 4, a recombinant form of soluble Cyc2p is able to reduce hemin, more efficiently with NADPH as the electron donor than with NADH. In the absence of NAD(P)H to regenerate the reduced form of the enzyme (Cyc2p), only 10% of hemin can be reduced, a reaction that is probably catalysed by the fraction of reduced Cyc2p in the purified protein sample. No reduction of hemin was observed when either NADH or NADPH was added to the reaction mixture in the absence of Cyc2p. Therefore, we concluded that Cyc2p carries a NAD(P)H-dependent haem reductase activity.

Cyc2p interacts with CCHL and apoforms of cytochrome c *and* c_1

To investigate interactions between Cyc2p, the haem lyases and apocytochrome *c* and c_1 substrates, we chose to use the 'split-ubiquitin' system, a molecular tool in yeast that is based on the reconstitution of the ubiquitin molecule C- and N-terminal halves (termed C_{ub} and N_{ub} respectively) as a sensor for membrane protein interaction (Snider *et al*., 2010). In this system, the bait proteins (CCHL, CC_1HL, Cyc2p) are fused to the C-terminal domain of ubiquitin followed by the LexA-VP16 transcription factor (C_{ub}-LexA-VP16). The prey proteins (apocytochrome *c*, soluble domain of apocytochrome c_1, CCHL, CC_1HL) are fused to the N-terminal domain of ubiquitin ($N_{ub}G$). If the bait and prey proteins interact, the functional ubiquitin is reconstituted and recognized by ubiquitin-specific proteases that release the transcription factor, which activates the expression of a reporter system. $N_{ub}G$ is a mutant form of $N_{ub}WT$ that has very low intrinsic affinity for C_{ub} and therefore can associate with C_{ub} only if both ubiquitin fragments are linked to proteins that interact. To insure membrane localization of the bait proteins, we chose to express CCHL, CC_1HL and the soluble domain of Cyc2p as C-terminal fusions to the membrane anchor of the ER protein Ost4. To verify that the bait proteins were expressed and membrane-tethered, the bait constructs were tested by coexpressing the $N_{ub}WT$ protein. $N_{ub}WT$ has a high affinity for the C_{ub} and will constitutively reconstitute the ubiquitin with the fusion bait proteins. Hence, $N_{ub}WT$-dependent expression of the reporter gene (*LacZ*) indicate successful expression and membrane localization of the bait proteins. All the bait constructs expressed proteins at the membrane based on the fact that they activate the *LacZ* reporter only when $N_{ub}WT$ but not $N_{ub}G$ was coexpressed (Fig. 5). Based on the β-galactosidase activity, interactions were detected between Cyc2p and apocytochromes *c*, c_1, CCHL but not with CC_1HL. Moreover, we also provide evidence for physical interaction between the haem lyases, CCHL and CC_1HL, and their cognate apocytochromes *c* and c_1 substrates. Interaction between CC_1HL and cytochrome *c* is a surprising finding, as genetic studies indicate that CC_1HL displays a strict specificity towards apocytochrome

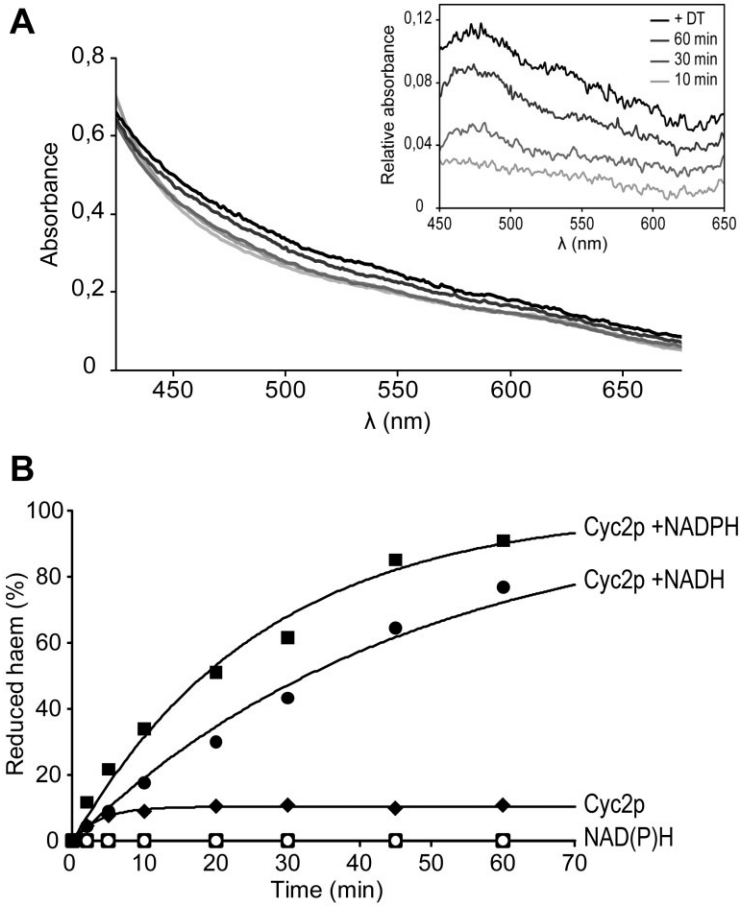

Fig. 4. Cyc2p catalyses the NAD(P)H-dependent reduction of hemin.
A. Hemin reduction spectra. Spectra of hemin by Cyc2p in the presence of NADPH were recorded at t = 0, 10, 30 and 60 min after addition of the enzyme (from bottom, light grey line, to top, black line). Catalytic conditions are described under *Experimental procedures*. The inset shows difference spectra calculated from spectra at 10, 30 and 60 min after ignition of the reaction minus initial spectra (t = 0 min).
B. Haem reductase activity of Cyc2p. Hemin reduction by soluble Cyc2p was measured by the difference in absorbance at 475 nm and at 620 nm as detailed under *Experimental procedures*. Reduction of 20 μM hemin by 10 μM Cyc2p was determined as a function of the time in the presence of 500 μM NADPH () or 500 μM NADH (●). The assay with Cyc2p only (◆) and the nonenzymatic reaction with NADPH only (□) or with NADH only (○) served as controls.

c_1 (Bernard *et al.*, 2003). However, because CCHL is required for the import of its cognate substrate apocytochrome *c* (Dumont *et al.*, 1988; 1990; 1991; Nargang *et al.*, 1988; Mayer *et al.*, 1995) and subsequent conversion to holoform, it is likely that CC_1HL never interacts with apocytochrome *c in vivo*. The interaction between CC_1HL and cytochrome *c* might be explained by the fact that only a limited sequence (including the haem binding site) is required for lyase-apocytochrome recognition. The observation that a 25-residue peptide carrying the C*XX*CH motif

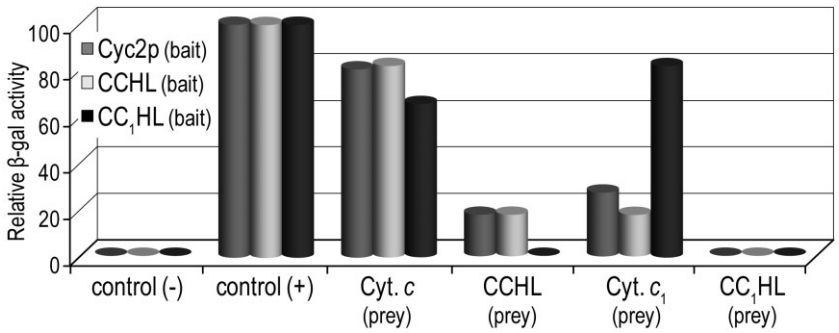

Fig. 5. Yeast two-hydrid-based interaction of Cyc2p with CCHL and apoforms of cytochrome *c* and c_1. Protein interactions between Cyc2p, cytochromes *c* and c_1, CCHL and CC_1HL were monitored by the Split-Ubiquitin system. Bait constructs are fusions of a protein of interest with the C-terminal domain of ubiquitin (C_{ub}) followed by a transcription factor (lexA-VP16). Prey constructs are fusion of a protein of interest with the N-terminal domain of ubiquitin ($N_{ub}G$) carrying a mutation preventing constitutive interaction with C_{ub}. Protein interactions were visualized by the activation of the reporter gene *lacZ*, and the β-galactosidase accumulation were assayed. As a negative control, a prey construct expression $N_{ub}G$ only were used. Expression of wild-type N-terminal domain of ubiquitin ($N_{ub}WT$) as a prey which activates constitutively C_{ub} served as a positive control, and was used to normalize β-galactosidase activities. The standard deviations calculated on three independent assays were in all experiments inferior to 20% of β-galactosidase activity values (not shown).

can be acted upon by the lyase in an *in vitro* reaction supports this view (Veloso *et al*., 1984).

Discussion

In this paper we have further characterized the biochemical activity of flavoprotein Cyc2p, a candidate redox enzyme involved in the assembly of mitochondrial *c*-type cytochromes (Dumont *et al*., 1993; Bernard *et al*., 2003; 2005). We show that: (i) the *in vivo* requirement for Cyc2p can be replaced by supplying exogenous reductants; (ii) even though midpoint potential determinations for the Cyc2p flavin and the cysteine pair in the haem binding site of apocytochrome *c* provide no thermodynamic arguments against a disulphide reductase activity for Cyc2p, in fact Cyc2p does not act as an apocytochrome c/c_1 disulphide reductase; (iii) in contrast, we have demonstrated that Cyc2p exhibits a pyridine nucleotide-dependent haem reductase activity *in vitro*; and (iv) furthermore, using a yeast 2-hybrid assay, we have provided evidence for a physical interaction between Cyc2p and CCHL and also between Cyc2p and apoforms of cytochrome *c* and c_1.

Cyc2p, a pyridine nucleotide-dependent haem reductase dedicated to CCHL

The proposal that the function of Cyc2p is to transfer reducing equivalents was originally based on the finding that a soluble form of the molecule containing the flavin cofactor is able to catalyse the NAD(P)H-dependent reduction of ferricyanide (Bernard *et al*., 2005). Because a disulphide at the C*XX*CH motif and the haem cofactor need to be reduced prior to the haem ligation, we had postulated that Cyc2p could act as an apocytochrome c/c_1 disulphide and/or haem reductase (Bernard *et al*., 2005). The midpoint potential for Cyc2p (–290 mV, Fig. 2), apocytochrome *c* (–264 mV, Fig. 2), apocytochrome c_1 (–266 mV; Corvest *et al*., 2010) and hemin (–230 mV; Conant and Tongberg, 1930) indicate that an electron transfer reaction from the flavin to a disulphide in apocytochrome c/c_1 or ferrihaem is thermodynamically possible. To discriminate between these different thermodynamically feasible targets for Cyc2p activity, we have developed *in vitro* assays to test whether apocytochrome c/c_1 and/or haem can serve as the actual targets for reduction by Cyc2p. While it appears that Cyc2p cannot reduce a disulphide at the haem binding site of apocytochrome *c* or c_1 (Fig. 3), the protein is able to carry the pyridine nucleotide-dependent reduction of hemin (Fig. 4). A specific mechanism for haem reduction was already suspected based on the finding that pyridine and flavin nucleotides are needed for the reconstitution of holocytochrome *c* assembly using mitochondrial extracts or isolated mitochondria (Basile *et al*., 1980; Nicholson and Neupert, 1989). However, since this first report, the molecular identity of the components controlling haem reduction has remained unknown. Our findings are thus very significant in this context. The reduction of hemin catalysed by Cyc2p suggests a possible role for flavin semiquinone in the reaction mechanism, as ferrihaem can only accept one electron at a time. While no flavin semiquinone, a typical intermediate in one-electron chemistry, was detected under the conditions used for our equilibrium titrations experiments, it is possible that semiquinone states could function under turnover conditions. It is also possible that Cyc2p can reduce two haems simultaneously, with one electron from the fully reduced flavin going to each ferric haem. More detailed kinetic and mechanistic studies will be required to explore these different possibilities.

The fact that the effects produced by a loss of Cyc2p can be overcome by the addition of reducing agents such as TCEP or DTT (Fig. 1) supports the hypothesis that the role of Cyc2p in *c*-type cytochrome assembly is to catalyse the supply of reducing equivalents. Interestingly, in a *cc*$_1$*hl*-null context, where cytochrome c_1 is assembled through CCHL and Cyc2p (Fig. 6), provision of exogenous reductants can partially restore respiratory growth (Fig. 1). This TCEP-dependent rescue is further enhanced by the overexpression of *CYC2*, an indication that Cyc2p reducing power might be limiting in conditions where CCHL is the only haem lyase. TCEP is known as a membrane impermeant disulphide reducing agent, while DTT, in contrast, can readily permeates biological membranes (Burns *et al*., 1991; Hsu *et al*., 2005). It is possible that TCEP reduces disulphide bonds in proteins at the plasma membrane, allowing a route for its entry inside the cell and the mitochondrial IMS. Recent studies have revealed that thiol/disulphide exchange in cell-surface proteins impacts the intracellular uptake of exogenous molecules (Aubry *et al*., 2009). The reductant-dependent bypass of Cyc2p function could be attributed to the direct *in vivo* reduction of haem by TCEP or DTT as both reagents are active in reducing hemin *in vitro* (data not shown; Tong and Margoliash, 1998). However, it is also plausible that TCEP acts indirectly and transduces reducing power from outside the cell to the mitochondrial IMS.

We cannot exclude the possibility that Cyc2p acts also as a disulphide reductase but such an activity could not be demonstrated in our *in vitro* assays. Disulphide reductase chemistry is certainly compatible with the presence of a flavin in Cyc2p, as flavoproteins are able to perform the two-electron transfer reaction involved in the reduction of a disulphide to a dithiol. However, it seems unlikely that the protein would be active in reducing both a disulphide in the apocytochrome substrates and an iron atom in the haem molecule. Moreover, Cyc2p appears to lack an additional nonflavin redox centre (such as a conserved

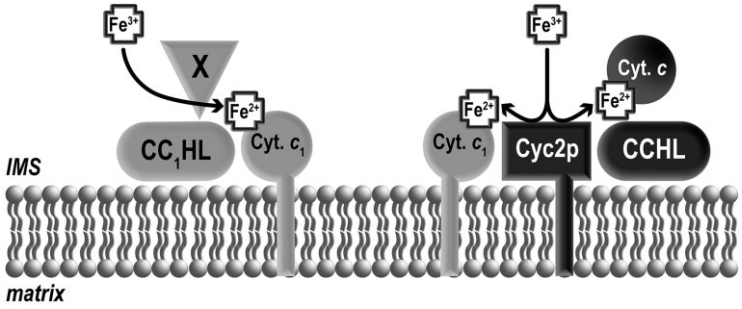

Fig. 6. Cytochrome *c* assembly in fungal mitochondria. In yeast mitochondria, the cytochrome *c* haem lyase (CCHL) displays specificity towards both cytochromes *c* and c_1. The flavoprotein Cyc2p promotes the NAD(P)H-dependent reduction of ferrihaem (Fe^{3+}) to ferrohaem (Fe^{2+}) prior to its covalent attachment to the apoforms of cytochromes *c* and c_1, as demonstrated in an *in vitro* hemin reduction assay (Fig. 4). It is likely that Cyc2p interactions with CCHL and apocytochromes *c* and c_1, shown in a yeast two-hybrid approach (Fig. 5), is critical to ensure the stereospecific haem attachment by the lyase. We have established genetically that Cyc2p is not required for the activity of the cytochrome c_1 haem lyase (CC_1HL), which is strictly specific to cytochrome c_1 (Corvest *et al.*, 2010). Moreover, the absence of direct interaction between Cyc2p and CC_1HL (Fig. 5) strengthens the view that Cyc2p is a haem reductase dedicated to the CCHL pathway. From the observation that NADH and FMN are required for the reconstitution of holocytochrome c_1 assembly in isolated mitochondria (Basile *et al.*, 1980; Nicholson *et al.*, 1989), we postulate that a distinct haem reductase (X), possibly a pyridine nucleotide-dependent flavoprotein (with a FMN cofactor), is devoted to the CC_1HL pathway.

cysteine or cysteine pairs) that is typically present in NAD(P)H-dependent flavoproteins (such as thioredoxin reductase), which exhibit disulphide reductase activity (Argyrou and Blanchard, 2004; Bernard *et al.*, 2005). Interestingly, an apocytochrome *c* C*XX*CH disulphide reductase was recently discovered in the thylakoid lumen, a compartment that is topologically analogous to the mitochondrial IMS (Lennartz *et al.*, 2001; Motohashi and Hisabori, 2006; Gabilly *et al.*, 2010). This suggests that the redox state of the C*XX*CH sulphydryls is probably under the control of dedicated components in the mitochondrial IMS. The identity of such components is currently unknown but a disulphide bond forming pathway has already been described in this compartment (Sideris and Tokatlidis, 2010; Depuydt *et al.*, 2011; Riemer *et al.*, 2011). In plant mitochondria, which assemble *c*-type cytochromes via system I, CcmH a thiol-disulphide oxidoreductase was postulated to reduce a disulphide at the haem binding site of apocytochrome *c* and c_1 (Meyer *et al.*, 2005). However, a direct biochemical demonstration of such an activity is still missing. The redox function of this assembly factor remains obscure but a simple role as a disulphide reductase appears unlikely (Kranz *et al.*, 2009; Bonnard *et al.*, 2010; Sanders *et al.*, 2010).

Two-hybrid experiments indicate that Cyc2p interacts with CCHL but also with both apoforms of cytochrome *c* and c_1 (Fig. 5). This result can be taken as an indication that Cyc2p might also exert some chaperoning activity on its apocytochrome *c* targets. In system I, CcmG was shown to display an apocytochrome *c* chaperone function in addition to its reducing activity (Turkarslan *et al.*, 2008). It is conceivable that the haem reduction and attachment reactions are coupled and that Cyc2p interaction with CCHL and apoforms of cytochromes *c* and c_1 is critical to ensure the stereospecific haem attachment by the lyase. Our study is the first biochemical characterization of a system III cytochrome *c* assembly factor. Earlier attempts to demonstrate the biochemical activity of the CCHL were unsuccessful as purification of the enzyme under an active form could not be achieved.

A CC_1HL-dependent haem reductase?

Previous genetic studies have established that Cyc2p is only required for the activity of CCHL and does not control the CC_1HL-catalysed haem attachment reaction to apocytochrome c_1 (Corvest *et al.*, 2010). Compatible with the view that Cyc2p is a haem reductase dedicated to the CCHL pathway, interactions via yeast two-hybrid could only be detected between Cyc2p and CCHL and not with CC_1HL (Fig. 5). The operation of a distinct haem reduction mechanism for the CC_1HL-dependent assembly of cytochrome c_1 is inferred from the observation that NADH and FMN are required for the reconstitution of holocytochrome c_1 assembly in isolated mitochondria (Nicholson *et al.*, 1989). Similarly to what was observed in the case of the reconstitution of holocytochrome *c* assembly, both redox molecules are required for the chemical reduction of haem. The observation that holocytochrome c_1 assembly is dependent upon FMN and NADH while holocytochrome *c* assembly requires FAD and NADPH can be taken as an indication the two haem lyases have different redox co-factors requirement for the haem reduction reaction. Based on our finding that NADPH-dependent flavoprotein Cyc2p is dedicated to the CCHL pathway, we favour, by analogy, a model where a yet-to-be identified NADH-dependent FMN-containing haem reductase is required for the CC_1HL pathway (Fig. 6). The identity of the

Table 1. Genotypes and sources of yeast strains.

Strain	Genotype	Source
W303-1A	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	R. Rothstein[a]
W303-1B	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	R. Rothstein[a]
YPH1	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyc2::hph*	Bernard *et al.* (2003)
SMY1	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyt2::his5*$^{+}$	Bernard *et al.* (2003)
SMY4	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 cyc3::loxP-kan-loxP*	Bernard *et al.* (2003)
AP4	*MATa ade2 his3 leu2 trp1 ura3 lexA:ADE2 lexA:HIS3 lexA:lacZ*	Obrdlik *et al.* (2004)
AP5	*MATα ade2 his3 leu2 trp1*	Obrdlik *et al.* (2004)

a. Department of Human Genetics, Columbia University.

CC_1HL-dependent reductase is currently unknown as genetic screens for yeast mutants deficient in holocytochrome c_1 did not unveil additional loci beside *CYT1* and *CYT2* that correspond to the apocytochrome c_1 and CC_1HL encoding genes respectively (Lang and Kaudewitz, 1982).

Diversity of haem reduction mechanisms in cytochrome c *maturation*

Investigations in bacterial systems I and II have unveiled that the redox state of the haem iron is controlled via seemingly different mechanisms. In system I, haem is relayed by the chaperone CcmE and postulated to first undergo oxidation and subsequent chemical reduction before haem attachment (Richard-Fogal *et al.*, 2009; Richard-Fogal and Kranz, 2010). Haem reduction was proposed to require the quinol oxidase activity of CcmF, a membrane protein that also catalyses the ligation of haem (Richard-Fogal *et al.*, 2009; San Francisco *et al.*, 2011). In system II, haem is not believed to be reduced by an active mechanism but protected from oxidation, presumably from its site of synthesis (on ferrochelatase) to its site of assembly onto apocytochrome *c*. The current view is that haem is maintained reduced through interaction with histidine ligands within CcsBA, a transmembrane haem channel carrying the haem lyase activity (Frawley and Kranz, 2009). Hence, it is conceivable that haem is delivered as reduced to cytochrome *c* assembly components in some instances. This complexity in terms of requirement for haem reduction is not clearly understood but it is possible that this reflects mechanistic differences in the haem attachment reaction. It has now become apparent that the enzymology of haem attachment is far more complex than initially anticipated.

Experimental procedures

Yeast strains, plasmids and growth conditions

All yeast strains used in this study are listed in Table 1. Constructions of pFL44L-CYC2 and pFL61-$HCCS_{Hs}$ have been described in Bernard *et al.* (2003). Yeast cells were transformed by the lithium acetate procedure (Schiestl and Gietz, 1989) or the one-step technique (Chen *et al.*, 1992). *Saccharomyces cerevisiae* strains were grown at 28°C in medium containing glucose or galactose as fermentable substrate or ethanol/glycerol as respiratory substrate (Dujardin *et al.*, 1980; Hamel *et al.*, 1998; Saint-Georges *et al.*, 2002).

Mitochondrial protein preparation and analysis

Mitochondria were purified as previously described (Bernard *et al.*, 2005) from yeast strains grown in ethanol/glycerol medium supplemented or not with 1, 5, 10 or 15 mM Tris[2-carboxyethyl] phosphine (TCEP, Pierce) as a reducing agent. The protein concentration was measured using the Bradford reagent (Bio-Rad). Detection of mitochondrial holocytochromes *c* and c_1 (haem staining) and immunoblotting were performed as detailed in Bernard *et al.* (2003; 2005).

Overexpression and purification of Cyc2p and apocytochrome c

The sequences encoding the soluble form (amino acids 53 to 366) of Cyc2p and the isoform 1 of cytochrome *c* were amplified by PCR from yeast genomic DNA, and cloned into the NheI/XhoI sites of the hexahistidinyl tag plasmid pET-24b(+) (Novagen). The cysteine C_{109} of cytochrome *c* was replaced by a serine by site-directed mutagenesis. Proteins were overexpressed in *Escherichia coli* BL21(DE3) strain in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside for 5 h at 30°C. Purifications of His-tagged proteins were performed in native conditions. Cells were harvested, resuspended in a phosphate buffer [0.1 M sodium phosphate, 0.1 M NaCl, 0.01% (v/v) Tween 20, pH 8.0] containing 20 mM imidazole, and treated on ice with lysozyme for 1 h. After sonication, the lysate was clarified by centrifugation at 10 000 *g* for 20 min at 4°C. The supernatant was then applied to the Ni-NTA resin (Qiagen). The resin was washed with the phosphate buffer containing 50 mM imidazole, and the proteins eluted with 250 mM imidazole. Samples were then dialysed to remove imidazole, and concentrated by using a centriprep filter unit (Millipore). Concentration of purified proteins was measured by recording the absorbance at 280 nm, and purity was assessed on SDS gel.

Measurement of midpoint redox potentials of Cyc2p and apocytochrome c

An anaerobic reaction mixture (2.5 ml) with 51 nmol of Cyc2p in 100 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl was used to titrate the flavin prosthetic group of Cyc2p. The buffer solution was made anaerobic by bubbling with water-saturated argon for 30 min prior to the addition of Cyc2p, which is oxidized in its as-isolated form. A gentle stream of water-saturated argon was continuously directed at the surface of the reaction mixture, to which an oxygen-removing system, consisting of 20 units of glucose oxidase (Sigma), 500 units of catalase (Sigma) and 2.5 μmol of glucose, had been added to insure that the system remained anaerobic during the course of the titration, which was carried out at ambient temperature. Aliquots of an anaerobic solution containing defined mixtures of $NADP^+$ plus NADPH (the total amount of the two pyridine nucleotides was kept constant at 255 nmol per addition) were added to adjust the ambient redox potential, E_h (calculated from the $NADP^+$/NADPH ratio and the known $E_m = -320$ mV value for the $NADP^+$/NADPH couple at pH 7.0). After redox equilibrium was reached (indicated by the lack of any further changes in the absorbance spectrum of the protein), the spectrum of Cyc2p was measured over the range from 300 nm to 700 nm (neither glucose oxidase nor catalase make any detectable contribution to the absorbance-measured changes at the low concentrations used to insure anaerobicity). The baseline zero point was adjusted to be constant at 700 nm and the absorbance change at 524 nm minus 454 nm used as a measure of the redox state of the flavin. No significant absorbance changes were detected indicative of the presence of a significant amount of flavin semiquinone, allowing the data to be analysed on the basis of the Nernst Equation for a single two-electron couple using KaleidaGraph software (Synergy software) to fit the data.

Redox titrations of cysteinyl disulphide/dithiol couple at the haem binding site of apocytochrome *c* were performed using thiol labelling with 4-acetamido-4′-maleimidylstilbene-2,2′-disulphonic acid (AMS, Invitrogen) (Motohashi and Hisabori, 2006) as detailed in Corvest *et al.* (2010). Redox equilibration was achieved at different ambient potential (E_h) in redox buffers consisting of defined mixtures of oxidized and reduced DTT (DTT_{red}/DTT_{ox}). Data were plotted and fitted to the Nernst equation for a single redox couple by iteration, using the solver of Excel (Microsoft Corporation). The mid-point potential (E_m) was defined as a variable and its value was taken after the target cell, containing the average quadratic variation between the theoretical curve and the experimental data, converged to its minimum value. Best fits were obtained for an *n*-value of 2 (i.e. for a two-electron redox reaction). All E_m values reported correspond to the average of three independent titrations.

Apocytochromes c *disulphide reductase assay*

Reduction of apocytochromes *c* and c_1 by the soluble form of Cyc2p was tested using thiol labelling with AMS (Invitrogen) (Gabilly *et al.*, 2010). Apocytochromes *c* or c_1 (1 μM) were incubated in anaerobic conditions at 25°C for 1 h or 2 h in a buffer containing 0.1 M sodium phosphate (pH 7.0), 0.1 M NaCl, 500 μM NAD(P)H, 0.5 μM Cyc2p. After incubation, samples were treated with 10% trichloroacetic acid (TCA) and allowed to stand on ice for 30 min. Protein precipitates were washed with 1% TCA and then with ice-cold acetone. Samples were resuspended in buffer containing 50 mM sodium phosphate (pH 7.0), 2.5% SDS, 10 mM AMS and incubated at 37°C for 1 h under argon atmosphere. Reduced (AMS modified) and oxidized (unlabeled) forms of apocytochromes *c* were separated by non-reducing SDS-PAGE, and stained with Coomassie Brillant Blue G250. As a positive control, apocytochromes *c* were treated with 5 mM DTT. Reductions of apocytochromes *c* with NAD(P)H only or with Cyc2p without NAD(P)H served as negative controls.

Measurement of haem reduction

The recombinant soluble domain of Cyc2p was assayed for haem reductase activity by difference spectroscopy (Nicholson and Neupert, 1989). The reaction mixture was prepared in a stirred and thermostatted (25°C) cuvette filled to a final volume of 1 ml containing 0.1 M sodium phosphate (pH 7.0), 0.1 M NaCl, 20 μM hemin, 500 μM NAD(P)H. The reaction was initiated by adding 10 μM recombinant Cyc2p. At different times after addition of the enzyme, the spectrum was recorded. The difference in absorbance between the maximum at 475 nm and the minimum at 620 nm ($\Delta A_{475-620}$) was proportional to the concentration of reduced haem (Nicholson and Neupert, 1989). After 1 h reaction, an excess of sodium dithionite was added into the cuvette and the spectrum recorded to obtain the $\Delta A_{475-620}$ of the fully reduced sample from which the proportion of haem that was reduced during the reaction could be calculated. The non-enzymatic reduction of hemin by NAD(P)H only and the reduction of haem by 10 μM recombinant Cyc2p without NAD(P)H served as negative controls.

Proteins interactions by the split-ubiquitin system

The sequences corresponding to cytochrome *c*, CCHL, CC_1HL, and the soluble domains of cytochrome c_1 and Cyc2p were amplified by PCR from yeast genomic DNA, and cloned into SfiI sites of pDHB1 (Dualsystems Biotech) or into B1/B2 sites of $pN_{ub}G$-X (Obrdlik *et al.*, 2004; Rayapuram *et al.*, 2007). Proteins of interest in the bait vector pDHB1 were fused at their N-termini to a small membrane anchor (the yeast ER protein Ost4) and at their C-termini to reporter cassette composed of the C-terminal half of ubiquitin (C_{ub}) and a transcription factor (lexA-VP16). Prey constructs in $pN_{ub}G$-X express N-terminal fusions of proteins of interest with the N-terminal half of ubiquitin ($N_{ub}G$) containing the point mutation $I_{13}G$ that prevents its spontaneous association with C_{ub} (Stagljar *et al.*, 1998). Bait and prey constructs were transformed in the haploid yeast strains AP4 and AP5 respectively (Obrdlik *et al.*, 2004). Bait and prey expressing haploid strains were crossed, and the expression of the reporter gene *lacZ* was monitored as a result of protein interaction. Expression of *lacZ* was measured by β-galactosidase assay (Obrdlik *et al.*, 2004).

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