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Coenzyme Q is an obligatory cofactor for uncoupling protein function

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Uncoupling proteins (UCPs) are thought to be intricately controlled uncouplers[1–3] that are responsible for the futile dissipation of mitochondrial chemiosmotic gradients, producing heat rather than ATP. They occur in many animal and plant cells[4–9] and form a subfamily of the mitochondrial carrier family[10]. Physiological uncoupling of oxidative phosphorylation must be strongly regulated to avoid deterioration of the energy supply and cell death, which is caused by toxic uncouplers. However, an H^+ transporting uncoupling function is well established only for UCP1 from brown adipose tissue[2,8,9,11], and the regulation of UCP1 by fatty acids, nucleotides and pH remains controversial[2,12–14]. The failure of UCP1 expressed in *Escherichia coli* inclusion bodies to carry out fatty-acid-dependent H^+ transport activity inclusion bodies[15] made us seek a native UCP cofactor. Here we report the identification of coenzyme Q (ubiquinone) as such a cofactor. On addition of CoQ_{10} to reconstituted UCP1 from inclusion bodies, fatty-acid-dependent H^+ transport reached the same rate as with native UCP1. The H^+ transport was highly sensitive to purine nucleotides, and activated only by oxidized but not reduced CoQ. H^+ transport of native UCP1 correlated with the endogenous CoQ content.

Whereas UCP1 from brown adipose tissue (BAT) can be isolated and is thus functionally well characterized[11], the isolation of more recently discovered UCPs has not been possible because of their low tissue content[8,9]. For this reason and for mutagenesis studies, we expressed recombinant UCPs in yeast and in *E. coli*[15–20]. Our inability to reconstitute H^+ transport activity from UCP1 expressed as inclusion bodies (IB-UCP1) in *E. coli* prompted us to consider several causes. (1) The reconstituted IB-UCP1 may not attain the full native configuration. (2) The reconstituted IB-UCP1 may still contain traces of the anion detergent sarcosyl used to solubilize UCP1, which, as a fatty-acid analogue, may prevent fatty-acid binding. (3) A covalent or (4) non-covalent cofactor present in the native UCP1 may be missing.

To first bring soluble IB-UCP1 into a native state, we thoroughly removed the sarcosyl used for solubilization of inclusion bodies by substitution with digitonin and anion-exchange treatment. We succeeded in restoring the nucleotide-binding capacity of soluble IB-UCP1 (Fig. 1a) with the fluorescent derivative dansyl-GTP, which had been used to test the nucleotide binding of native UCP1 (ref. 21). The decrease of fluorescence caused by competition with excess ATP corresponded to the specific binding of UCP1. We further tested for native protein configuration with the carboxyl reagent, Woodward reagent K, which blocks with high specificity

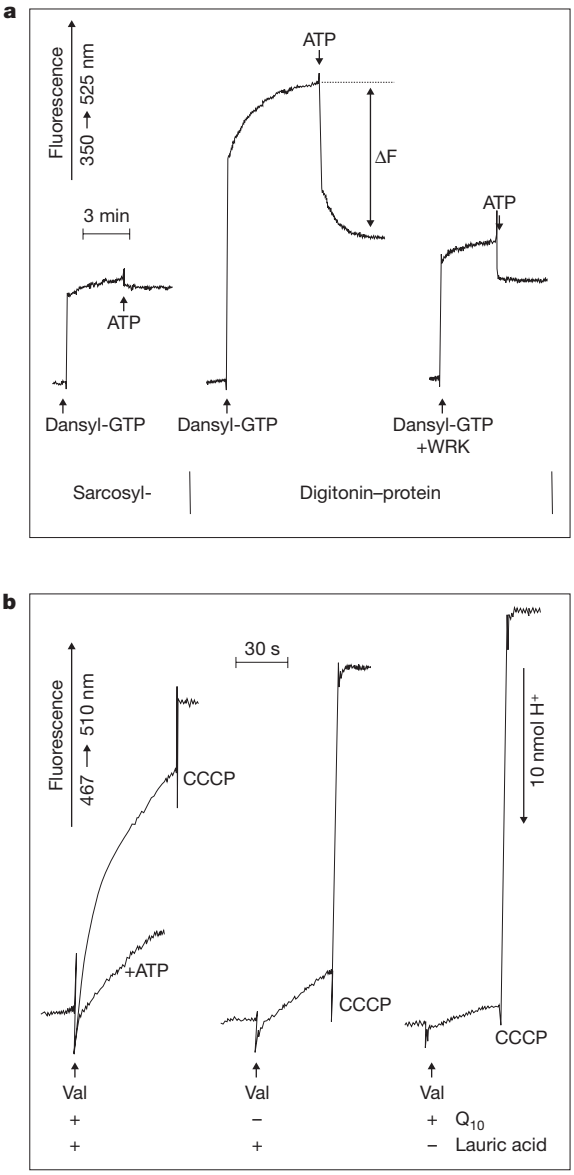

Figure 1 Functional integrity of refolded recombinant IB-UCP1 from *E. coli*. **a**, Fluorescence response of 2′-O-(dimethylamino-naphthaline-1-sulfonyl)-GTP (dansyl-GTP) binding to sarcosyl- and digitonin-treated IB-UCP1. Dansyl-GTP (5 μM) was added to a solution of 45 μg ml^{-1} UCP1 in 10 mM MES/HEPES buffer containing 0.3% digitonin, pH 6.5 at 10 °C. To differentiate the UCP1-linked binding, 0.5 mM ATP was added to displace the fluorescent ligand. To confirm binding to native UCP1, 10 μM Woodward reagent K was added 30 min before dansyl-GTP. **b**, Recording of H^+ influx into vesicles reconstituted with digitonin-treated IB-UCP1. H^+ influx was monitored by the fluorescence of pyranine. Vesicles (50 μl), containing 1.25 μg protein and 0.42 mg phospholipid, were added to a medium containing 0.5 mM HEPES pH 7.3, 1 μM pyranine, 0.5 mM EDTA, 280 mM sucrose in a final volume of 330 μl at 10° C; 125 μM lauric acid and 2 nmol CoQ_{10} were added where indicated. Valinomycin (Val; 2.5 μM) was added to induce K^+ efflux and thus a membrane potential. The uncoupler carbonylcyanide m-chlorophenylhydrazone (CCCP) (1 μM) was added to a determine the vesicle capacity for H^+ uptake.

the carboxyl group E190, necessary for nucleotide binding[18,22]. On incorporation into vesicles, UCP1 was able to transport Cl^- but not H^+. As these results excluded causes (1) and (2) above, it seemed most probable that a mitochondrial cofactor was missing from IB-UCP1. The lack of a covalent component (3) could also be excluded, because after partial removal of the detergent Triton X-100, mitochondrial UCP1 could be brought in a state where it was also unable to transport H^+, although Cl^- transport and inhibition by nucleotides were restored (data not shown). The existence of a noncovalent cofactor remained the most logical explanation.

On the assumption that it was a lipid cofactor, we extracted lyophilized mitochondria using lipid solvents and screened the extracts for their ability to activate H^+ transport. Chloroform extracts were prepared not only from mitochondria of BAT and UCP1-expressing yeast, but also from UCP1-free bovine heart, to determine whether the activating component is confined to UCP1-containing mitochondria. The rates shown in Fig. 2a were evaluated from the recordings of H^+ uptake (Fig. 1b). All extracts, independent of whether mitochondria contained UCP1, stimulated H^+

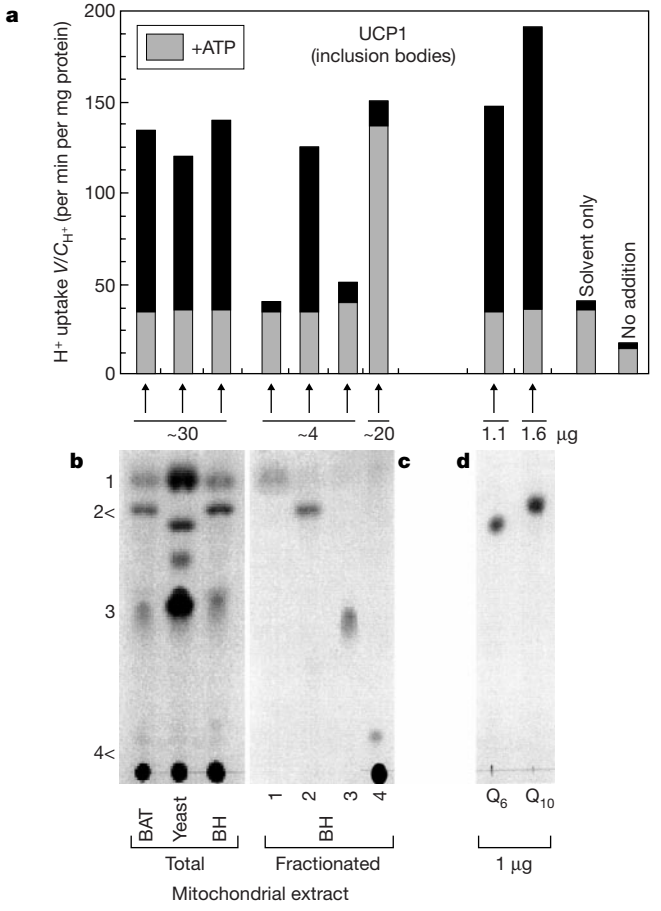

Figure 2 Identification of CoQ as the activating component in mitochondrial lipids of H^+ transport by UCP1. **a**, The activation of H^+ transport in reconstituted digitonin-treated UCP1 proteoliposomes by total lipid extracts from mitochondria (BAT, brown adipose tissue; BH, bovine heart), by fractionated lipids from BH and by CoQ_6 and CoQ_{10}, and in the presence of 125 µM lauric acid. Black bar corresponds to the H^+ transport portion sensitive to 20 µM ATP. Added lipids are analysed in the silica thin-layer chromatograms. H^+ transport rates are evaluated from recordings of H^+ uptake, as shown in Fig. 1. Results are presented as initial H^+ transport rates (V, µmol per min per mg protein) divided by the capacity of the vesicles (C, µmol) determined by addition of CCCP. The transport rates range from 30 to 120 µmol per min per mg protein. **b**, Mitochondrial extracts on silica thin-layer chromatograms using cyclohexane:ethylacetate (30:20) as the mobile phase. Mitochondria from BAT, yeast and BH were lyophilized and extracted with chloroform overnight at 4 °C. After filtration, the extracts were dried under vacuum; the yield is about 20% lipid by mass of mitochondrial protein. **c**, The chloroform extract (1.5 ml) of BH mitochondria was applied to a silica column (volume 20–25 ml) at room temperature using chloroform for equilibration and elution. Fraction 1, triglycerides; 2, activating compounds; 3, fatty acids; 4, phospholipids. **d**, Thin-layer chromatography of CoQ_6 and CoQ_{10} (1 µg) under the same conditions.

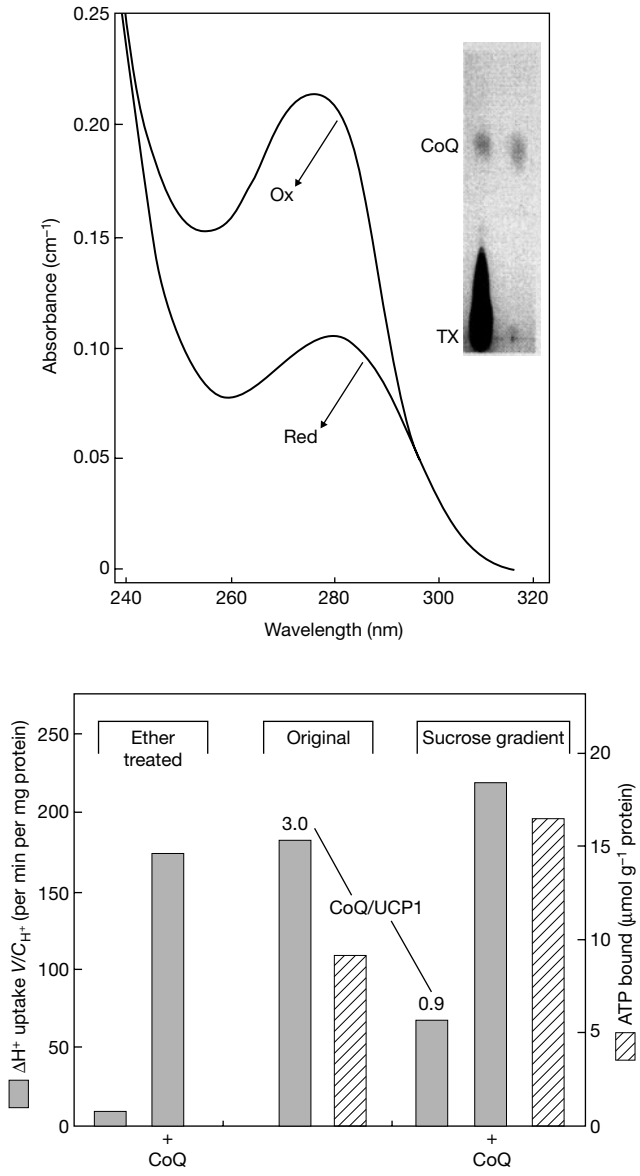

Figure 3 Identification of CoQ in native isolated mitochondrial UCP1. **a**, Absorbance spectrum of CoQ extracted from a native UCP1 preparation. UCP1 (0.5 mg) solubilized from BAT mitochondria by Triton X-100 and purified by hydroxyapatite chromatography was lyophilized and extracted by stirring with 5 ml chloroform overnight at 4 °C. To remove Triton X-100, the extract was applied to a silica column (volume 3 ml, equilibrated with chloroform) (inset). Fractions corresponding to CoQ were pooled and dissolved in 150 µl dichloromethane. CoQ was identified by a decrease in absorbance at 275 nm on addition of $NaBH_4$ (10 mg ml^{-1}). **b**, Endogenous CoQ is a cofactor of mitochondrial UCP1. Treatment with diethylether for removal of all endogenous CoQ by precipitation of UCP1. UCP1 (2 mg in 1.2 ml of medium) was exposed first to 15 ml and then to 5 ml of ether at pH 5 for 20 h. After centrifugation at 15,000*g*, the pellet was solubilized and reconstituted as described for inclusion bodies (see Methods). 'Original' corresponds to mitochondrial UCP1 purified by hydroxyapatite. For partial depletion of CoQ, soluble native UCP1 was purified by sucrose gradient[11]. UCP1 (2.3 mg) was applied to a 12/20% sucrose step gradient and centrifuged for 18 h at 90,000*g*. The peak protein fractions were analysed for CoQ content. Reconstitution of native UCP1 and ATP binding was determined as described[18].

transport when added to the proteoliposomes. This increase was fully inhibited by 20 μM ATP and thus fulfilled our criterion of caused by UCP1.

We initially separated the activating fraction from the bulk lipids by silica thin-layer chromatography. Good separation was achieved with a mobile phase in which the migration of apolar lipids was favoured and phospholipids stayed at the bottom (Fig. 2b). The lipid patterns were similar from mitochondria both with and without UCP1 (compare BAT and bovine heart). We extracted the lipid fractions and screened for activator activity as shown in Fig. 2c. Fraction 4, which contained mostly phospholipids, stimulated only ATP-insensitive H^+ transport. Fractions 3 and 1, which contained free fatty acid and triglycerides did not markedly activate H^+ transport. Fraction 2 produced strong activation, which was largely ATP sensitive. Notably, fraction 2 from yeast mitochondria also contained the activator, although it migrated with apparently lower hydrophobicity.

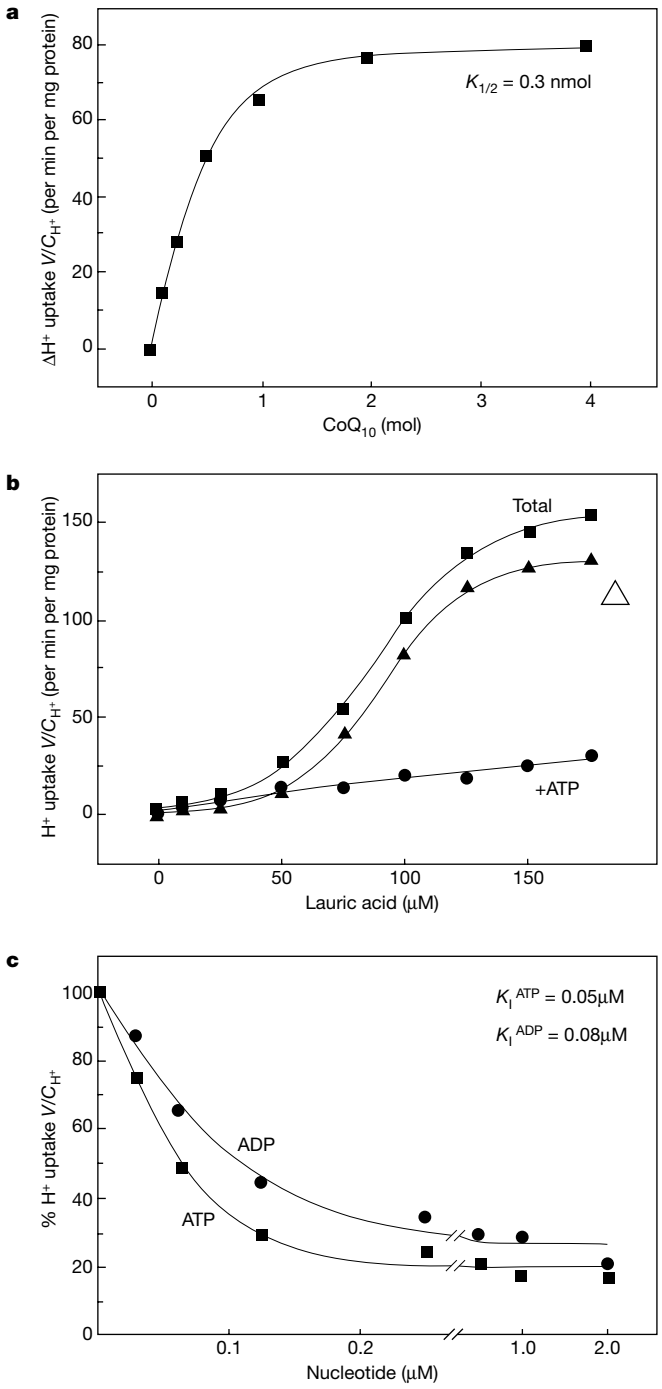

Figure 4 The influence of three regulatory parameters on the H^+ transport activity of recombinant UCP1. **a**, Titration of activity with CoQ_{10} (nmol CoQ per 0.4 mg phospholipid). H^+ transport by reconstituted IB-UCP1 was measured as shown in Fig. 1, with increasing amount of CoQ_{10} at 125 μM lauric acid. The net transport activity shown was obtained by substracting the activity in the presence of 20 μM ATP. **b**, Titration with lauric acid at 2 nmol CoQ_{10}. **c**, Dependence of the inhibition on the concentration of ATP and ADP at 125 μM lauric acid and 2 nmol CoQ_{10}.

We collected a larger quantity of fraction 2 from bovine heart by silica-gel column chromatography; NMR analysis showed that it contained mostly triglycerides. In an ultraviolet spectrum, however, there was a distinct absorption maximum at 274 nm. On reduction with $NaBH_4$, the maximum absorption was reduced and shifted to 290 nm, characteristic of coenzyme Q (CoQ). To identify whether the lipid or CoQ was the activator, we added pure CoQ_{10} to the vesicles and observed a strong stimulation of H^+ transport that could be inhibited by a low concentration of ATP. As shown in the recordings of H^+ uptake (Fig. 1b), CoQ activated H^+ transport only in the presence of fatty acid.

We then determined whether CoQ was only a suitable '*in vitro*' activator for the reconstitution of IB-UCP1, or CoQ was a constituent of the active, isolated mitochondrial UCP1. Mitochondrial UCP1 isolated with Triton X-100 was lyophilized and extracted with chloroform. The extract was fractionated on a silica column so that the bulk of lipids and the excess of Triton X-100 were removed (see insert Fig. 3a), which would block the ultraviolet absorption of CoQ. The eluate equivalent to bovine heart fraction 2 of the mitochondrial extract had a well-defined absorption spectrum corresponding to CoQ (Fig. 3). From the absorbance, we calculated a molar ratio for CoQ:UCP of about 3. As the UCP1 preparation contained a considerable amount of phospholipid (a weight ratio of UCP1 to protein of 5), CoQ may have been distributed between the protein and the phospholipid. We partially removed Triton X-100 and phospholipid by the mild diethylether precipitation of UCP1. H^+ transport of the resolubilized and reconstituted UCP1 was fully dependent on CoQ_{10} addition, as with IB-UCP1 (Fig. 3b).

To prove further the physiological role of CoQ, we determined the dependence of mitochondrial UCP1 on CoQ under conditions in which UCP1 retains its native state throughout the soluble phase. This would eliminate any possible renaturing influence of CoQ on UCP1. We used a sucrose gradient centrifugation to decrease the content of CoQ by removing excess detergent/phospholipid micelles[11]. The amount of UCP1 was determined by ATP binding. The molar ratio CoQ:UCP, as measure by ATP binding, decreased from 3 to 0.9; the H^+ transport activity was reduced to 40%. But by addition of CoQ, the activity reached 120% of the original value (Fig. 3b).

Although other members of the mitochondrial carrier family do not require CoQ for the reconstitution of the recombinant proteins, we considered whether the marginal fatty-acid-stimulated H^+ transport of the ADP/ATP carrier[23,24] is also stimulated by CoQ. In reconstituted vesicles with recombinant ADP/ATP carrier expressed in *E. coli*, no effect of CoQ was observed on H^+ transport, indicating the specificity of UCP in its requirement for CoQ (data not shown).

Titrating the H^+ transport activation with increasing amounts of CoQ determined saturation to be ~2 nMol CoQ (Fig. 4a). Because most CoQ should be absorbed by the phospholipid, the molar ratio of CoQ to phospholipids is critical, and was 1:300 here. The corresponding molar ratio CoQ:UCP is 80:1, indicating a low affinity of UCP1 to CoQ. In mitochondria, the CoQ content corresponds approximately to a density of 1:100 per phospholipid molecule. The dependence on fatty-acid 'concentration' in the presence of saturating amounts of CoQ was nonlinear (Fig. 4b), differing from the linear dependence with reconstituted native UCP1 (ref. 25). Presumably, some fatty acids were sequestered before they became available to the vesicles by the solvent in

Table 1 The dependence of H^+ transport of reconstituted UCP1 on the isoprenoid chain length and on the oxidized and reduced state of CoQ

	UCP1 (*E. coli*)			UCP1 (BAT) (ether treated)		
	CoQ_{ox}	CoQ_{red}	$Q_{ox} + Q_{red}$	CoQ_{ox}	CoQ_{red}	$CoQ_{ox} + CoQ_{red}$
CoQ_{10}	100	7	88	100	6	85
CoQ_6	77	7	70	75	8	68
CoQ_2	25	7	–	30	5	–
CoQ_1	10	5	–	10	4	–
CoQ_0	10	7	–	10	6	–

The percentage of activated H^+ transport rate is shown for reconstituted IB-UCP1 from *E. coli* and for ether-precipitated native UCP1 from BAT, measured as described in Fig. 1 in the presence of 2 nmol CoQ and 125 μM lauric acid. For the competition, 2 nmol CoQ_{ox} + 2 nmol CoQ_{red} were added. Native UCP1 was precipitated with ether, redissolved and treated as described for IB-UCP1. CoQ was reduced by 10 mg ml^{-1} $NaBH_4$ and identified by a decrease in absorbance at 275 nm. The maximum transport rate obtained with CoQ_{10} is equal to 100%. The rates are obtained after subtraction of the activity in the presence of 20 μM ATP.

which CoQ was added. For maximum activity, the concentration of 125 μM lauric acid was the same as with native UCP1. Notably, inhibition of the activated H^+ transport was highly sensitive to purine nucleotide (Fig. 4c). The maximum inhibition was reached already by 0.25 μM ADP or ATP. The inhibition constant, K_I, for ATP (0.05 μM) and for ADP (0.08 μM) was even lower than those determined with reconstituted native UCP1 (ref. 15), but the ratios K_I^{ATP}:K_I^{ADP} were about the same. The 20% nucleotide insensitive H^+ transport activity could be attributed to UCP1 molecules that were inverted in the vesicle with the nucleotide-binding site inaccessible from the outside.

We investigated the structural requirements of CoQ for activation by changing the hydrophobic side chain (Table 1). The activation decreased markedly with shorter isoprenoid chains. Only minor activation remained when one or no isoprenoid unit was present (CoQ_1 and CoQ_0). We considered whether or not the activation depended on the redox state of CoQ. The reduced forms of all CoQ homologues were scarcely able to activate H^+ transport (Table 1), suggesting a regulation of uncoupling *in vivo* by the CoQ_{ox}:CoQ_{red} ratio. There was a possibility that CoQ might undergo a reduction when catalysing H^+ transport, because, owing to the limited capacity of the vesicles, only five H^+ ions per CoQ are transported. In the extracts from rapidly frozen and lyophilized vesicles, however, there was no reduced CoQ, which confirms that CoQ does not act as an oxido-reductant in UCP1.

Our finding that CoQ is an obligatory cofactor for H^+ transport by UCP1 is based on reconstitution of both the native and recombinant protein, where conditions under which UCP1 acts in mitochondria may be only partially realized. However, the specific and obligatory dependence on the presence of CoQ argues for a physiological role of CoQ in the uncoupling of oxidative phosphorylation by UCP1, with several implications. First, it solves the paradox that UCP1 from inclusion bodies of *E. coli* cannot achieve its function under reconstitution conditions in which other mitochondrial carriers, such as the carriers for ketoglutarate[26], citrate[27], phosphate[28] and ADP/ATP (our own unpublished data) become fully active. Second, it suggests a new regulatory dimension as the uncoupling activity of UCP1 depends on the redox state of CoQ; in other words, the availability of CoQ_{ox}. There seems to be no interaction of CoQ_{red} with UCP1, as CoQ_{red} scarcely activates H^+ transport and barely competes with CoQ_{ox}. The elucidation of such a regulation requires further studies on mitochondria. Third, with a ratio of 2.4 molecules of UCP1 to 5 molecules of CoQ per cytochrome a,a_3 complex in brown fat mitochondria[11], activation may also depend on a competitive recruitment of CoQ between the respiratory chain and UCP1. Fourth, unlike fatty acid, CoQ is not an H^+ carrier and its role is expected to be more regulatory. As both are membrane bound, we presume that the molecular role of CoQ resides in cooperation with fatty acid, on the basis of a physical contact of both components at the membrane UCP1 interface. By forming a hydrogen bond between undissociated fatty acid (FAH) and the quinone oxo group, CoQ_{ox} might facilitate the entrance of FAH or of H^+ delivered from FAH into the H^+channel of UCP1. Fifth, our findings are based on the paradigm role of UCP1 in the UCP subfamily. As other UCPs are not available in native form, owing to the incomplete reconstitution of recombinant *E. coli* expressed UCPs[15], widely diverging functions have been proposed[8,9]. It is expected that the activator role of CoQ is universal to all UCPs, whether from animals or plants, as preliminary data show that CoQ is an obligatory activator of UCP2 and UCP3 expressed in *E.coli.* Last, the suggested role of UCP, particularly in plants[7,29], to suppress oxygen radicals generated by CoQ, and the interesting influence of CoQ derivatives on the mitochondrial permeability transition pore[30] now acquire a new perspective with the interaction of UCPs with CoQ. □

Methods

Expression, isolation, renaturation and reconstitution of inclusion bodies

We expressed UCP1 from hamster BAT in *E. coli* cells BL21 (DE3) transformed with the plasmid pET-24a containing the UCP1 gene. Expression was induced by isopropyl β-D-thiogalactoside. Inclusion bodies were isolated as described[15]. For renaturation, 5 mg of inclusion bodies were dissolved in 400 μl of 2% (w/v) sarcosyl in buffer A (50 mM NH_4HCO_3, 2 mM dithioerythritol (DTE) and 2 mM phenylmethanesulphonyl fluoride (PMSF), pH 8.0), followed by centrifugation at 13,000 *g* for 30 min. The supernatant was diluted by buffer A to a final sarcosyl concentration of 0.5% and protein of 3 mg ml^{-1} and further diluted to a final concentration of 0.6 mg ml^{-1} by buffer B (1 mM DTE, 50 μM β-mercaptoethanol, 100 mM potassium phosphate, 0.2% digitonin (water soluble form) and 0.1% sarcosyl, pH 8.0). The soluble protein was concentrated threefold by pressure dialysis at 4 °C and diluted again threefold with buffer B without sarcosyl. This step was repeated three times, and then twice with buffer C (1 mM DTE, 1 mM EDTA and 10 mM potassium phosphate, pH 8.0). The whole process took ~10 h. To remove completely sarcosyl, 1 ml of digitonin-solubilized inclusion bodies (~1.5 mg ml^{-1}) was treated with 21K Dowex mesh 20–50 (800 mg CL^- form and 20 mg OH^- form) by shaking at 4 °C overnight. The yield of renatured protein was 80–90%. The reconstitution of UCP1 in phospholipid vesicles followed essentially a described method[15,18].

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The PSI-H subunit of photosystem I is essential for state transitions in plant photosynthesis

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Photosynthesis in plants involves two photosystems responsible for converting light energy into redox processes. The photosystems, PSI and PSII, operate largely in series, and therefore their excitation must be balanced in order to optimize photosynthetic performance[1]. When plants are exposed to illumination favouring either PSII or PSI they can redistribute excitation towards the light-limited photosystem. Long-term changes in illumination lead to changes in photosystem stoichiometry[2,3]. In contrast, state transition is a dynamic mechanism that enables plants to respond rapidly to changes in illumination. When PSII is favoured (state 2), the redox conditions in the thylakoids change and result in activation of a protein kinase[4–6]. The kinase phosphorylates the main light-harvesting complex (LHCII) and the mobile antenna complex is detached from PSII. It has not been clear if attachment of LHCII to PSI in state 2 is important in state transitions. Here we show that in the absence of a specific PSI subunit, PSI-H, LHCII cannot transfer energy to PSI, and state transitions are impaired.

PSI is a multi-protein complex; in higher plants, it consists of 17 different subunits[7]. The PSI reaction centre complex has a core consisting of 13 different subunits, all the electron transport factors, and about 90 molecules of chlorophyll. Surrounding the core complex are proteins of the light harvesting complex I (LHCI), which bind additional chlorophyll molecules. Four different LHCI proteins appear to be present in a fixed stoichiometry of two copies per PSI reaction centre[7,8]. Whether LHCII normally associates with PSI in state 2 has been a controversial question. While some data have shown the PSI antenna size to increase in state 2 *in vivo*[6,9], there is no available information about the significance of this for state transitions. To investigate the involvement of PSI in state transitions we have generated a series of transgenic *Arabidopsis* plants lacking specific subunits of PSI. Plants lacking PSI-H, -K, -L, or -N (ΔPSI-H, ΔPSI-K, and so on) were relatively unaffected in growth, and showed nearly normal rates of photosynthesis under optimal conditions[10–12] (Table 1). State transitions can be detected as differential changes in fluorescence from PSII when leaves are exposed alternately to light favouring PSII and light favouring PSI (Fig. 1). Plants lacking PSI-H or PSI-L were highly deficient in state transitions as evidenced in the relative fluorescence change, F_r (Fig. 1). Furthermore, non-photochemical fluorescence quenching was identical in PSII- and PSI-light. In contrast, plants lacking PSI-N or PSI-K showed almost normal state transitions. Plants with downregulation of PSI-L had a secondary loss of most of PSI-H (Table 1). As plants lacking PSI-L were slightly less affected in state transitions

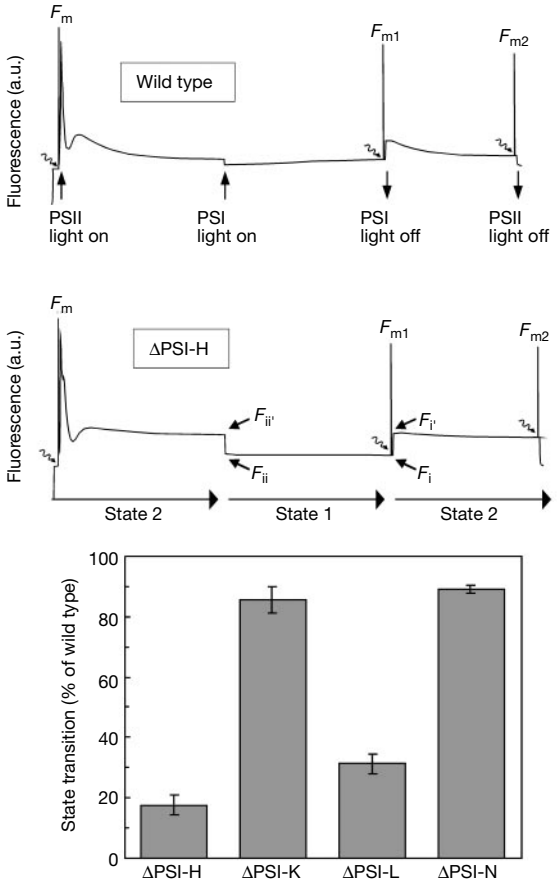

Figure 1 Measurements of transitions between state 1 and state 2. Dark-adapted leaves were exposed to either light favouring PSII (blue) or light favouring PSI (far-red). The initial high PSII fluorescence decreases as photochemical and non-photochemical quenching increase. Fluorescence decreases further when illumination includes PSI light and increases again when illumination is changed to favour PSII. Each bar represents the average (± s.e.) for 5–6 plants. Wavy arrows point to saturating flashes (0.8 s) given 30 s before changing the PSI or PSII light.