Cysteine residues are not essential for uncoupling protein function

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The uncoupling protein (UCP) of brown adipose tissue is a regulated proton carrier which allows uncoupling of mitochondrial respiration from ATP synthesis and, therefore, dissipation of metabolic energy as heat. In this article we demonstrate that, when UCP is expressed in *Saccharomyces cerevisiae*, it retains all its functional properties: proton and chloride transport, high-affinity binding of nucleotides and regulation of proton conductance by nucleotides and fatty acids. Site-directed mutagenesis demonstrates that sequential replacement by serine of cysteine residues in the UCP does not affect either its uncoupling activity or its regulation by nucleotides and fatty acids, and therefore establishes that none of the seven cysteine residues present in the wild-type UCP is critical for its activity. These data indicate that transport models involving essential thiol groups can be discounted and that chemical modification data require critical re-evaluation.

INTRODUCTION

Uncoupling protein (UCP) is a 33 kDa protein that is exclusively found in mitochondria from brown adipocytes. UCP acts as a proton short-circuit which allows uncoupling of respiration from ATP synthesis for the purpose of heat production, and its activity is regulated by purine nucleotides (inhibitors) and non-esterified fatty acids (activators) [1]. UCP is related to other mitochondrial transporters such as the adenine nucleotide translocator, the phosphate carrier, the oxoglutarate carrier etc., thus forming a family of homologous proteins [2,3].

Much effort is being put into an attempt to elucidate the molecular mechanism of transport of this carrier family, and chemical modification is providing valuable information in this respect. The use of thiol-specific reagents has allowed the identification of two types of SH groups: modification of type I residues leads to inhibition of transport, while a pore-like state is induced when type II residues are modified [4,5]. In the case of UCP, chemical modification of cysteine residues has already been used by several groups to study its transport mechanism [6–8]: reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) or mersalyl leads to inhibition of proton translocation [7,8], while reaction with maleimides results in a transient inhibition of transport, followed by the formation of a highly conducting channel [6]. Therefore identification of the modified SH groups and examination of their contribution to UCP function would be of interest.

Recombinant expression of proteins followed by site-directed mutagenesis is a very powerful tool with which to investigate structure–function relationships and therefore to obtain an insight into transport mechanisms. UCP offers a unique opportunity for this sort of approach, since it is absent from all eukaryotic cell types except brown adipocytes. In fact, this situation has stimulated the development of expression systems for UCP in various cell types [9,10] including yeasts [11,12]. However, the contribution of site-directed mutagenesis to this field is very modest [13,14] and so far no reports have appeared concerning mutagenesis of UCP.

In this paper we present data on the introduction of UCP into yeast mitochondria by recombinant expression and demonstrate that UCP incorporated into these mitochondria displays the same bioenergetic properties as in brown adipose tissue mitochondria. The consequences of the replacement of cysteine residues in UCP by site-directed mutagenesis are also presented. It emerges that none of the seven cysteines of UCP is essential for its transport activity.

EXPERIMENTAL

Construction of expression vectors for wild-type or mutated UCP

UCP cDNA sequence between its internal *Sac*I site (110 nucleotides before the ATG codon) and the *Pst*I site created during the cloning procedure [15,16] was introduced in a pTZ19 vector (Pharmacia). This plasmid (ZU25) was linearized with *Pst*I, repaired with Klenow enzyme and self-ligated in the presence of an excess of *Eco*RI linkers (Appligene). This led to the ZIbiRI plasmid, from which UCP cDNA could be excised with *Eco*RI. This insert was ligated to the vector pSELECT (Promega), suitable for mutagenesis, and the resulting plasmid was named pSU+. Replacement of each of the seven cysteines present in UCP by serine was carried out by site-directed mutagenesis of a cDNA insert in pSU+ according to the manufacturer's instructions. The UCP cDNA sequence, free of 5′ and 3′ untranslated regions, was obtained by enzymic amplification, with the 5′ primer OL5 (CGA*GAATTC*ATGGTGAGTTCGACAACTTC) and the 3′ primer OL6 (CAA*GAATTC*TATGTGGTGCAGTCCACTGT), complementary to the UCP cDNA sequence. This procedure was designed to introduce two *Eco*RI sites (italic) as close as possible to ATG and TAG codons (underlined). After repair with Klenow enzyme and cutting with *Eco*RI, this amplification product was introduced into the pYeDP-1/8-10 vector [17]. Two different plasmids resulted from this final cloning step: pYeDP-UCP+, in which the UCP coding sequence was expected to give a mRNA coding for UCP, and pYeDP-UCP−, in which the cDNA was in inverse orientation and thus would give the antisense RNA. Mutated UCP cDNA sequences from pSU+-derived plasmids were obtained by enzymic amplification using *Taq* DNA polymerase (Cetus) or Vent DNA polymerase (Bio-

Abbreviations used: UCP, uncoupling protein; YUP, yeast uncoupling pathway; PMSF, phenylmethanesulphonyl fluoride; $DiSC_2(5)$, 3,3′-diethylthiacarbocyanine iodide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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labs) with OL5, and with OL10 (CAA*GAGCTC*TATGTGGTGCAGTCCACTGT) as a 3′ primer. The resulting DNA product was repaired with Klenow enzyme, and cut with *Eco*RI and *Sac*I. This allowed forced cloning in the pYeDP-1/8-10 vector. This procedure was not applicable to the mutant C7/S, since the triplet coding for Cys^{304} is present in OL10; for this mutant another oligonucleotide, PCROL2C (GCATAGGAGCCCAGCATAGGAGCCC), complementary to the 3′ uncoding sequence of UCP cDNA, was used. The resulting DNA was inserted between the *Eco*RI and *Sma*I sites of pYeDP-1/8-10. In each case the cDNA insert coding for UCP in the pYeDP vector was sequenced to check that the cDNA coding sequence had the expected properties.

The diploid yeast (*Saccharomyces cerevisiae*) strain W303 (a/α; *ade2-10; his3-11,15; leu2-3,112; ura3-1; can1-100; trp*−) from Instituto Biomedicas (Madrid, Spain) was used. The pYeDP-UCP expression vectors were introduced into yeast by electroporation and transformants were selected for uracile autotrophy.

Growth of yeast and induction of UCP expression

Clones of transformed yeast were plated on to SD (0.67% yeast nitrogen base, 2% agar, 2% glucose, 0.1% casamino acids, 20 mg/l tryptophan, 40 mg/l adenine) plates and grown in liquid SD in order to maintain the repression of the *Gal–Cyc* promoter and to avoid any selective process due to UCP expression. When induction of UCP expression was required the growth medium did not contain glucose, but galactose (0.4–2%) was present.

For the preparation of mitochondria, a compromise had to be reached between selection pressure for the plasmid in minimal medium and conditions yielding a sufficient amount of good quality mitochondria. This was achieved by the following procedure. A preculture grown in SP (0.67% yeast nitrogen base, 0.1% KH_2PO_4, 0.12% $(NH_4)_2SO_4$, 0.1% glucose, 2% lactic acid, 0.1% casamino acids, 20 mg/l tryptophan, 40 mg/l adenine, pH adjusted to 4.5 with KOH) to 4–5 absorbance units was diluted to a final absorbance of 0.3 unit for overnight growth in 600 ml of YPL (1% yeast extract, 2% bactopeptone, 3% lactic acid, pH adjusted to 4.5 with KOH) at 28 °C with vigorous shaking to ensure good oxygenation. Galactose (0.4% final) was added 6 h before harvesting.

Isolation of yeast mitochondria

Mitochondria were prepared following a procedure derived from the one described by Guérin et al. [18]. Protoplasts were prepared by enzymic digestion with cytohelicase, and mitochondria were isolated by differential centrifugation after homogenization of protoplasts. The resuspension of the final protoplast pellet and all subsequent centrifugations were performed in mitochondrial buffer [0.6 M mannitol, 2 mM EGTA, 1 mM EDTA, 10 mM Tris/maleate, 0.5 mM Na_2HPO_4, 2% BSA, 1 mM phenylmethanesulphonyl fluoride (PMSF) and 1 μg/ml pepstatin, pH 6.8]. The final mitochondrial pellet was resuspended in the same buffer and protein was determined by the Biuret assay using BSA as standard.

Measurement of UCP activity in mitochondria

Mitochondrial respiration was measured at 20 °C in a Hansa-Tech oxygen electrode chamber. The incubation medium contained 0.65 M mannitol, 20 mM Tris/maleate, 0.5 mM EGTA, 2 mM $MgCl_2$, 1 mg/ml BSA and 10 mM K_2HPO_4, pH 6.8. The mitochondrial concentration was 0.15 mg/ml. NADH (3 mM) was used as substrate, because of the ability of *S. cerevisiae* mitochondria to oxidize externally added NADH. The mitochondrial membrane potential was monitored continuously under the same conditions using the potential-sensitive fluorescent probe 3,3′-diethylthiacarbocyanide iodide [$DiSC_2(5)$] in an SLM Aminco SPF-500 spectrofluorimeter with excitation at 643 nm and emission at 680 nm (4 nm bandwidth) [19].

Mitochondrial permeability to protons or chloride ions was determined from the rate of decrease of light scattering during passive swelling [20,21]. Mitochondria (0.5 mg/ml) were suspended either in 0.2 M potassium acetate/5 mM K_2HPO_4 or in 0.2 M KCl/1 mM K_2HPO_4, plus 2 mM $MgCl_2$, 1 mg/ml BSA, 10 μM atractylate, 12.5 μg/ml oligomycin, 0.5 μg/ml antimycin A and 10 mM Tris/maleate, pH 6.8. Swelling was initiated by the addition of 1 μM valinomycin; further additions are described at the appropriate point in the text.

GDP binding to mitochondria was performed essentially as previously described [22]. Mitochondria (0.5 mg/ml) were incubated for 1 min in a buffer containing 0.6 M mannitol, 2 mM EGTA, 5 mM K_2HPO_4, 1 mg/ml BSA, 10 mM Tris/maleate, 0.3 μCi/ml [^{14}C]sucrose and 1 μCi/ml [^{3}H]GDP, with concentrations of GDP ranging from 0.1 to 8 μM. Further additions are described in the text and in Figure legends.

Immunodetection of UCP

Precipitation of yeast proteins was performed as described in [23]. Gel electrophoresis of proteins and the Western blotting procedure have been previously described [24]. Quantification of UCP in mitochondria was performed with an enzyme-linked immunoassay. Briefly, 0.1–4 μg of mitochondrial protein was placed in each well of a Nunc-immunopolysorp plate and incubated overnight at 4 °C. Unspecific sites were saturated with a 1% solution of dried milk in PBS. Rabbit anti-UCP serum diluted 1:160 was added and the mixture was incubated for 1 h at 37 °C. After extensive washing, sheep anti-rabbit IgG (linked to peroxidase) diluted 1:1500 was applied to the wells. Final colour development was performed with *o*-phenyldiamine and plates were read with a Bio-Rad microplate reader with a filter of 490 nm. Calibration of the assay was performed using rat UCP (1–20 ng), purified as previously described [25].

Materials

Restriction and modification enzymes were from Appligene or Biolabs. Taq polymerase used was from Cetus or ATGC; alternatively, Vent DNA polymerase from Biolabs was also used. Isotopes were obtained from Amersham International. Basic components for growth media were from Difco Laboratories. Pepstatin was from Boehringer Mannheim. $DiSC_2(5)$ was from Molecular Probes Inc. Cytohelicase was from IBF Biotechnics. Sheep anti-rabbit IgG and nitrocellulose membranes were from Bio-Rad Laboratories. All other reagents were of the highest purity available.

RESULTS AND DISCUSSION

Conditions for UCP expression in *S. cerevisiae*

In our construct, the rat UCP coding sequence is inserted behind the *Gal–Cyc* promoter [17]; thus the expression of UCP is completely repressed by glucose and strongly induced by galactose (Figure 1, lanes 2 and 3). Little or no variations were observed in the level of expression, obtained after 6 h of induction, when the concentration of galactose was varied from 0.4 to 2% (results not shown). Compared with our previous constructs [26], the removal of 5′ and 3′ non-coding sequences

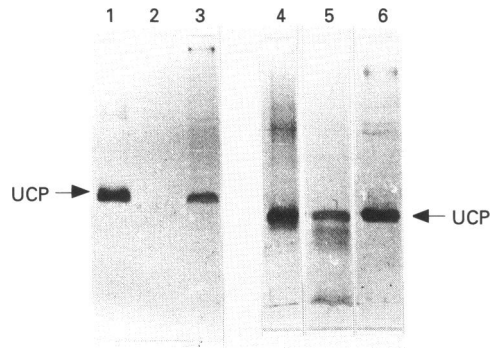

Figure 1 Immunoblot analysis of UCP expression in pYeDP-UCP+ cells and incorporation of the protein into mitochondria

Lanes 1 and 4, 1 μg of purified UCP; lanes 2 and 3, protein from approx. 10^6 yeast cells grown in SD or SG respectively; lanes 5 and 6, 90 μg of protein from mitochondria isolated in the absence of protease inhibitors (lane 5) or with pepstatin, PMSF and EDTA in the preparation buffers (lane 6). Further details are given in the Experimental section.

improved considerably the amount of UCP produced in yeast (results not shown).

Observation of UCP activity requires both a competent expression system and the preparation of good-quality mitochondria. Protocols recommend the use of minimal medium to prevent loss of the pYeDP vector; however, our attempts to prepare mitochondria from yeast grown in this medium invariably led to a low yield of mitochondria with an extremely leaky inner membrane, even in the absence of UCP expression (pYeDP-UCP− strain). We have developed a procedure which includes a first step of growth in lactate minimal medium (SP), which maintains the *Gal–Cyc* promoter in a repressed state, thus ensuring selection for a high copy number of the plasmid without the potentially deleterious UCP expression. The second step is overnight growth in YPL, after inoculation at 0.3 absorbance units, in order to obtain a better quality mitochondrial preparation. Although galactose is a fermentable carbon source, it does not repress respiratory functions to any great extent [27]; thus growth in 2% lactate plus 0.4% galactose did not have an effect on the mitochondrial preparation, as compared with the one obtained without induction (YPL alone) (results not shown). The loss of pYeDP plasmid due to the overnight growth in complete medium and the 6 h induction of UCP expression still allowed the recovery of mitochondria containing a large amount of UCP. Starting from 600 ml of the YPL overnight culture (final absorbance 5 units) we obtained approx. 2.5 g of cells (fresh weight), and the mitochondrial preparation procedure yielded 15–20 mg of mitochondrial protein suspended in 500 μl.

Homogenization of protoplasts leads to the release of lumenal vacuolar proteases, which have been found to be a major source of problems [28]. UCP present in yeast mitochondria is indeed sensitive to proteases during the mitochondrial preparation procedure (Figure 1, lane 5), and thus detection of UCP activity in mitochondria is dependent on the use of PMSF, pepstatin A and EDTA in the preparation buffers to inhibit the main vacuolar proteases. In the presence of these three compounds, only a 33 kDa immunoreactive protein was detected on Western blots probed with anti-UCP serum (Figure 1, lane 6).

Quantification of UCP in yeast mitochondria

The use of an enzyme-linked immunoassay, with purified rat UCP as standard, allowed quantification of UCP incorporated into pYeDP-UCP+ mitochondria, giving a value of 24 ± 2 μg of UCP/mg of mitochondrial protein (two independent determinations from three mitochondrial preparations). This represents around 40% of the value reported in the literature for UCP concentrations in the brown fat of fully cold-adapted rats (50–80 μg/mg) [29,30]. However, it is not possible to establish from this value the amount of functional UCP in the membrane, since it is now well documented that in mice and rats UCP can exist in the membrane in a masked form, which is recognized by antibodies but is inactive (it does not transport ions or bind purine nucleotides [31]). This inactive form can account for up to 90% of the UCP present in brown-fat mitochondria. Thus, in order to quantify the amount of active UCP, its nucleotide binding capacity must be determined.

GDP binding to yeast mitochondria

ATP and ADP are the predominant cytosolic nucleotides, and hence regulators of UCP *in vivo*, but they are rarely used in binding studies with brown-fat mitochondria due to the interference caused by the adenine nucleotide translocator. In these mitochondria, GDP and GTP bind with high affinity only to UCP and are not taken up via the translocator [32], and therefore are widely used for both the analysis and quantification of UCP function. [^{3}H]GDP binding experiments with control yeast mitochondria (pYeDP-UCP−) (Figure 2a) showed that GDP does bind to yeast mitochondria and that this binding increases with time. In the presence of 10 μM atractylate (an inhibitor of the adenine nucleotide translocator), binding is significantly reduced. Scatchard analysis of [^{3}H]GDP binding after a 1 min incubation revealed that, in the presence of atractylate, there exists a binding site with a dissociation constant of 1.3 ± 0.1 μM and a capacity of 0.32 ± 0.01 nmol/mg of mitochondrial protein (Figure 2b).

When binding experiments were performed (in the presence of atractylate) with mitochondria isolated from the pYeDP-UCP+ strain, the Scatchard plot revealed the presence of two binding sites (Figure 2b). Computer fitting of the data again revealed a site with a capacity of 0.31 ± 0.06 nmol/mg, although the calculated dissociation constant now appears higher (4 ± 3 μM). The other site has a higher affinity (0.4 ± 0.2 μM) and a capacity of 0.17 ± 0.10 nmol/mg. Although computer calculation of dissociation constants of two sites with similar affinity should always be treated with caution, it is interesting to note that the calculated dissociation constant for the high-affinity site is in the range of the observed GDP binding affinity determined for UCP from rat brown adipose tissue mitochondria [30,33]. Therefore these experiments demonstrate that 50% of the protein determined immunologically (0.33 nmol of UCP dimer/mg) binds nucleotides and can be ascribed to functional UCP. The remaining protein may represent either masked protein, as in the rat, or a post-translational modification specific to yeast.

The influence of UCP on the proton and chloride ion permeabilities of yeast mitochondria

The presence of UCP should increase membrane permeability to protons and chloride, which should be enhanced by fatty acids and inhibited by nucleotides [1]. The rate of mitochondrial swelling in potassium acetate and KCl in the presence of valinomycin is a measure of the permeability of the inner membrane to protons and chloride respectively [20,21]; thus the nucleotide-sensitive component of the salt-induced swelling is a measure of UCP activity. However, in *S. cerevisiae* mitochondria there is interference with another permeability pathway, termed the yeast uncoupling pathway (YUP), which is activated by ATP [34] and other purine nucleotides (S. Prieto, F. Bouillaud and E. Rial, unpublished work) and is inhibited by millimolar con-

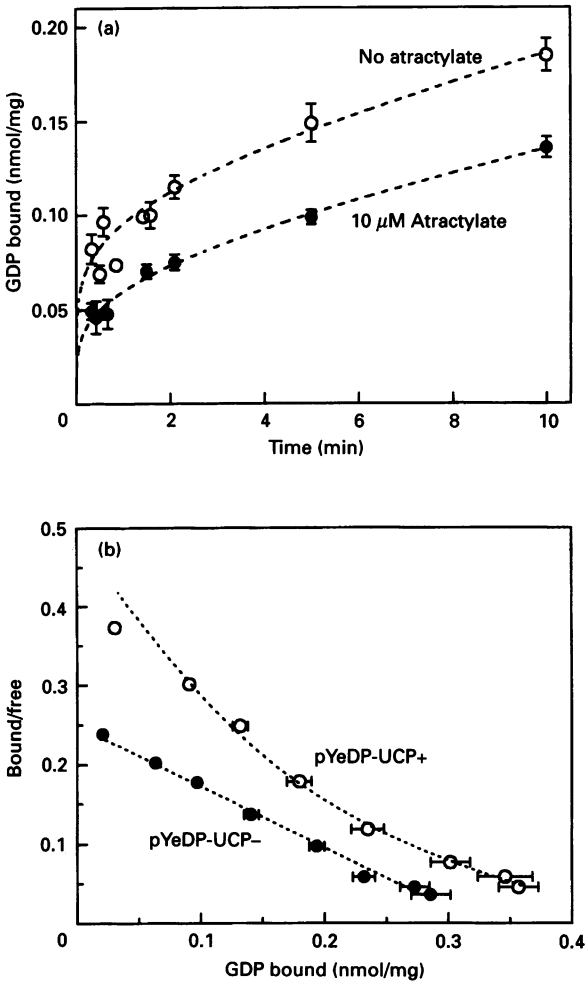

Figure 2 [^{3}H]GDP binding to mitochondria isolated from pYeDP-UCP− and pYeDP-UCP+ yeast

(**a**) Effect of atractylate on the binding of GDP to pYeDP-UCP− mitochondria. Mitochondria were incubated for different periods of time in a buffer containing 1.1 μM [^{3}H]GDP (1 μCi/ml), either in the absence (○) or in the presence (●) of 10 μM atractylate. Data points represent the means ± S.E.M. of two independent experiments performed in duplicate. (**b**) Scatchard plot for the binding of GDP to pYeDP-UCP− (●) and pYeDP-UCP+ (○) mitochondria. Experiments were performed in the presence of 10 μM atractylate and concentrations of [^{3}H]GDP ranging from 0.1 to 8 μM. Horizontal bars represent S.E.M.s for 3–4 independent determinations performed in duplicate.

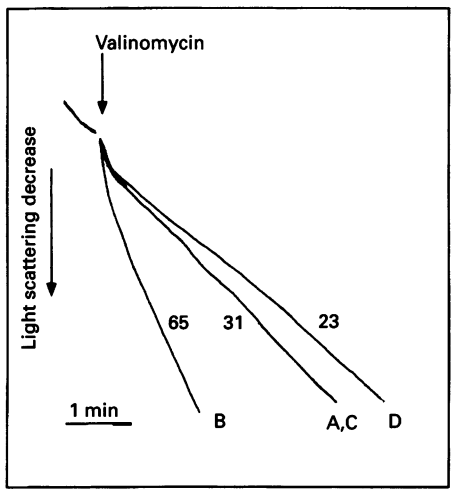

Figure 3 Effect of GDP and phosphate on the swelling of pYeDP-UCP+ mitochondria in potassium acetate plus valinomycin

Mitochondria were incubated in the medium described in the Experimental section with the following additions: A, none; B, 300 μM GDP; C, 10 mM phosphate; D, 300 μM GDP plus 10 mM phosphate. Swelling was initiated by the addition of 1 μM valinomycin as indicated by the arrow. Numbers represent the rates of swelling in arbitrary units.

centrations of phosphate [34]. Figure 3 shows the effect of nucleotides on the rate of swelling in potassium acetate plus valinomycin of pYeDP-UCP+ mitochondria. In the absence of phosphate, addition of GDP increased mitochondrial proton permeability (Figure 3, traces A and B), thus reflecting the activity of YUP. However, when YUP was inhibited (i.e. in the presence of phosphate), the proton permeability of pYeDP-UCP+ mitochondria was lowered by the addition of GDP (Figure 3, traces C and D). The significance of these differences can be judged when the effects of GDP on pYeDP-UCP+ and pYeDP-UCP− mitochondria are measured (Figure 4a): only in mitochondria from UCP-expressing yeasts is there a proton leak that can be inhibited by GDP (Figure 4a).

In order to facilitate the quantification of UCP activity in yeast mitochondria, Figures 4(c)–4(f) present the above data but after subtraction of the 'basal mitochondrial permeability'. This basal permeability is defined as the proton (or chloride; Figures 4c and 4f) permeability measured in pYeDP-UCP− mitochondria without addition of nucleotides. In Figure 4(d) it is shown that the higher proton permeability seen in pYeDP-UCP+ mitochondria can be inhibited by 90% with 300 μM GDP; this should correspond to UCP-induced proton permeability.

The effects of fatty acids on these two types of mitochondria are shown in Figures 4(b) and 4(c). Addition of palmitate at a 4:1 molar ratio to BSA (unbound fatty acid concentration is around 1.3 μM prior to the addition of mitochondria [35]) enhances the proton permeability in pYeDP-UCP+ mitochondria only, and this increased permeability can be inhibited by GDP. UCP is also known to catalyse movement of a variety of anions, including chloride and bromide [1,20,36]. Figures 4(c) and 4(f) demonstrate that the presence of UCP in yeast mitochondria also induces a GDP-sensitive chloride permeability. These swelling experiments have established, therefore, that incorporation of UCP in the inner membrane of yeast mitochondria results in permeability properties consistent with those found in brown fat mitochondria, and this supports the view that molecular properties of UCP are retained in this expression system.

The influence of UCP on mitochondrial respiration

The characteristics of the regulation of UCP function depend on the magnitude of the proton electrochemical potential gradient. Thus, while at low potentials (i.e. swelling experiments) the nucleotide concentrations required to inhibit transport are in the micromolar range, at high membrane potentials (i.e. during respiration) millimolar concentrations are required to attain good respiratory control [37,38]. In order to investigate the behaviour of UCP in yeast mitochondria under respiring conditions, experiments were performed in which the rate of respiration and membrane potential were monitored simultaneously. Phosphate (10 mM) was included in the incubation medium in order to avoid effects on YUP when nucleotides were added [34]. Figure 5 shows the effects of the addition of palmitate and GDP on these two parameters, and the expected differences between pYeDP-

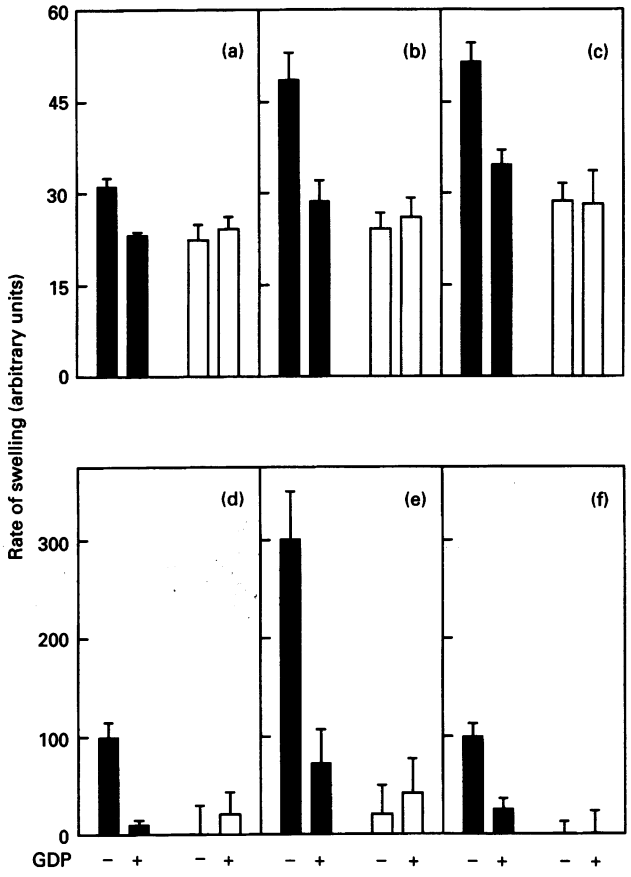

Figure 4 Effect of GDP and palmitate on the permeability properties of pYeDP-UCP+ and pYeDP-UCP− mitochondria

Top panels = rates of swelling in potassium acetate plus valinomycin (**a** and **b**) or in KCl plus valinomycin (**c**) for pYedP-UCP+ mitochondria (■) and pYeDP-UCP− mitochondria (□). The absence (−) or presence (+) of 300 μM GDP is indicated at the bottom of the figure. (**b**) Effect on the proton permeability of 64 μM palmitate in the presence of 16 μM albumin. The bottom panels show the effect of UCP expression on the mitochondrial permeability to protons (**d** and **e**) and chloride (**f**). The rate of swelling ('basal permeability') of pYeDP-UCP− mitochondria was subtracted in order to facilitate comparison of the effects of GDP and palmitate (**e**) on the permeabilities. Error bars represent the S.E.M.s of 7–10 independent determinations for pYeDP-UCP+ and 4–6 determinations for pYeDP-UCP−, each performed at least in duplicate.

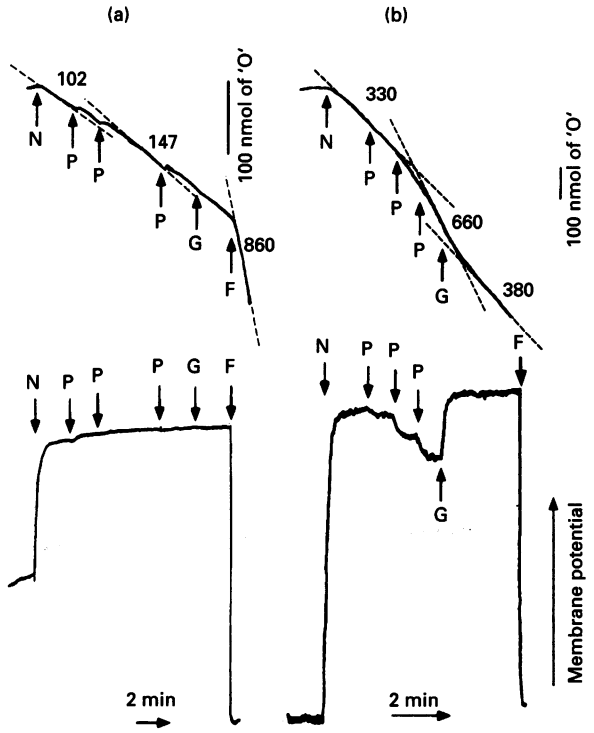

Figure 5 Effect of GDP and palmitate on the mitochondrial membrane potential and respiration of pYeDP-UCP− (a) and pYeDP-UCP+ (b) mitochondria

Additions are as follows: N, 3 mM NADH; P, palmitate (64 μM in **a**, 32 μM in **b**); G, GDP (1 mM); F, FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) (15 μM). For clarity only the values for the rates of respiration at three relevant moments are indicated. Numbers give rates in nmol of 'O'/min per mg of mitochondrial protein. Changes in the fluorescence of the probe $DiSC_2(5)$ were used to monitor variations in mitochondrial membrane potential. No calibration is included, since it is only intended to provide qualitative information on the effects of GDP and palmitate. For further details, see the text.

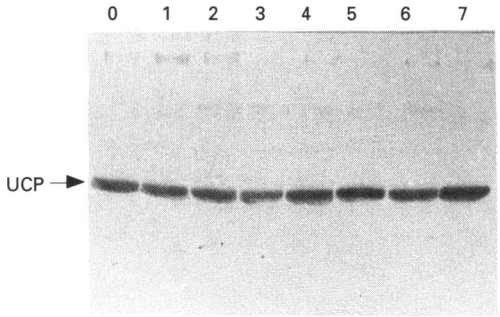

Figure 6 Immunoblot analysis of the expression of mutants of UCP in yeast mitochondria

Lane 0, wild-type UCP; lane 1, C1/S mutant; lane 2, C2/S mutant; lane 3, C3/S mutant; lane 4, C4/S mutant; lane 5, C5/S mutant; lane 6, C6/S mutant; lane 7, C7/S mutant. In all cases, 90 μg of mitochondrial protein was loaded in each lane. See the Experimental section for further details.

UCP+ and pYeDP-UCP− mitochondria are apparent. A protocol was designed so that three successive additions of 32 μM palmitate were made in order to assess the mitochondrial sensitivity to non-esterified fatty acids; 1 mM GDP was then added to reverse the fatty acid effects. Figure 5(b) demonstrates that a first addition of 32 μM palmitate led to a slight decrease in membrane potential. After the third addition of palmitate (i.e. 96 μM final concentration) there was a considerable drop in membrane potential and respiration was stimulated 2-fold. Addition of 1 mM GDP reversed the effects of palmitate. Figure 5(a) shows the lack of effect of palmitate and GDP on pYeDP-UCP− mitochondria.

Site-directed mutagenesis of cysteine residues in UCP

As mentioned in the Introduction, much work is being carried out with the aim of elucidating the mechanism of transport of this carrier family, and chemical modification has pointed to cysteine residues as an important key to the transport mechanism. In the present paper, a first contribution to the study of UCP by site-directed mutagenesis is presented. Seven UCP mutants were obtained by replacement of each cysteine by a serine residue (Cys^{24}, Cys^{188}, Cys^{213}, Cys^{224}, Cys^{253}, Cys^{287} and Cys^{304}, numbered C1/S to C7/S) and were expressed in yeast using the vector

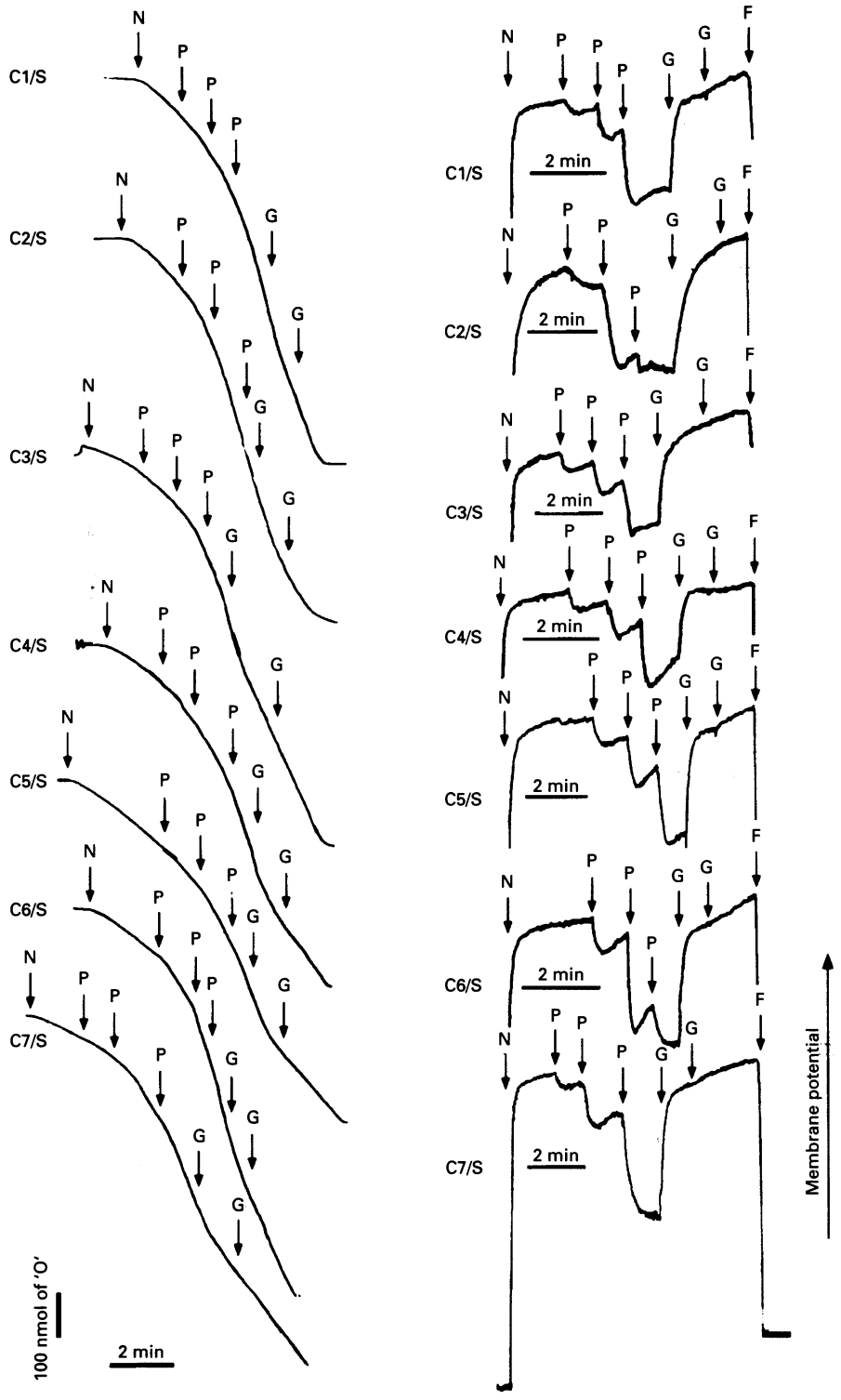

Figure 7 Influence of palmitate and GDP on the respiration and membrane potential of mitochondria isolated from yeast expressing UCP mutants

Additions: N, 3 mM NADH; P, 32 μM palmitate; G, 1 mM GDP; F, 15 μM FCCP. For further details see the legend to Figure 5 and the Experimental section.

described above. Serine was chosen as a substitute of cysteine in order to eliminate the thiol group without altering significantly the polarity or bulkiness of the residue. Preparation of mitochondria from these mutants was performed using the same conditions as for pYeDP-UCP+. The level of incorporation of mutated UCP was similar to that observed for pYeDP-UCP+ (Figure 6). When their properties were examined under respiring conditions, all the mutants showed a qualitatively unchanged response to added fatty acids and GDP (Figure 7), as compared with pYeDP-UCP+ mitochondria (Figure 5). Quantitative dif-

ferences were, however, apparent and they are currently being analysed in detail in our laboratory.

Concluding remarks

The present paper introduces a protocol for the high-level expression of UCP and its mutant forms in *S. cerevisiae* and the study of its function *in situ*. The use of a highly inducible promoter has allowed us to combine the need for good quality mitochondria (grown in complete medium with a non-fermentable substrate) with that of UCP incorporation into the mitochondrial inner membrane at elevated concentrations. It is important to emphasize the need for the study of the protein within mitochondria, since assessment of UCP function requires analysis of its behaviour at both low and high membrane potentials. Once appropriate mitochondrial preparations are available, then studies at the level of the isolated protein or in the reconstituted system can be performed following previously described methodologies [25,39].

During this study, two important obstacles had to be negotiated, and this led to two significant findings: the nucleotide-sensitive YUP [34] and the binding of GDP to two sites, one of which, interestingly, is atractylate-sensitive. YUP induces a proton leak, when nucleotides are present, which does not allow observation of UCP activity unless phosphate is included in the medium. GDP binding to the outer face of the mitochondrial inner membrane of control yeast does not allow quantification of UCP in pYeDP-UCP+ yeast unless a very detailed Scatchard analysis is performed. A previous report on the recombinant expression of UCP in yeast gave a value of 0.4 nmol of GDP binding per mg of mitochondria [11]; no details were given on the protocol used or on the binding parameters in control yeast, but this value may indicate a significant contribution from the two binding sites described in the present paper. Particular attention should be paid in future to the atractylate-sensitive component of GDP binding. Atractylate is considered to be a specific inhibitor of the adenine nucleotide translocator, which in mammals has been reported to be highly specific for ADP and ATP [40]. For yeast mitochondria we have found no report on the assay of the specificity of the various isoforms of the adenine nucleotide translocator. If this atractylate-sensitive GDP binding is due to one of the isoforms of the carrier, this would be a first report on a broader specificity for this carrier in *S. cerevisiae*.

The activity of UCP expressed in yeast shows all the characteristics described for UCP in brown adipose tissue mitochondria: proton and chloride transport, high-affinity GDP binding to the protein, enhancement of UCP proton conductance by fatty acids and inhibition by nucleotides. Combination of immunological titration of UCP and of GDP binding experiments has demonstrated that 50% of the protein incorporated into the yeast mitochondria is inactive, and could represent the masked form of the protein with the same characteristics as those described for UCP in rat mitochondria [31].

Substitution of each of the seven cysteine residues in UCP with serines did not affect either the incorporation of the protein into the mitochondria, or the ability of fatty acids to increase UCP conductance and of GDP to reverse this effect. A more detailed study is currently in progress in order to obtain a quantitative analysis of the ability of these two ligands to regulate UCP activity. However, two important conclusions can be derived from the results presented here. First, the existence of disulphide bonds in UCP is unlikely. Reactions between 5,5′-dithiobis-(2-nitrobenzoic acid) and SDS/urea-denatured UCP titrated only three out of seven cysteine residues, and had led to the proposal of the presence of up to two disulphide bonds [41]. A second hypothesis, which can now also be discounted, concerns the general transport model proposed by Robillard and Könings [42], which had also been considered as a possibility for UCP [8], where a dithiol–disulphide interconversion was an integral part of the transport mechanism. This hypothesis has also been dismissed in the case of the *lac* permease from *Escherichia coli*, where only one out of eight residues has been shown to be important (although not essential) for activity [43].

The work presented here is the first report on the application of site-directed mutagenesis to elucidate the mechanism of transport of the UCP. Furthermore, it establishes the conditions necessary to observe UCP activity in isolated yeast mitochondria and sets a protocol for the production of a large amount of mutated forms of UCP which can be used for biophysical studies in the future.

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