Role of Intrahelical Arginine Residues in Functional Properties of Uncoupling Protein (UCP1)†

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ABSTRACT: The functional role of the four intrahelical arginines in uncoupling protein (UCP1) from brown adipose tissue were studied in mutants where they were replaced by noncharged residues. Wild-type and mutant UCP1 were expressed in *Saccharomyces cerevisiae.* As measured in isolated UCP1, nucleotide binding was largely lost in mutants of R83, R182, and R276 occurring in three repeated domains and common to mitochondrial carrier family, whereas mutation of the UCP typical R91 shows normal binding capacity but >20-fold lower binding affinity and a near loss of pH dependency of binding. In reconstituted UCP1, fatty acid dependent H^+ transport is retained in all four mutants, but inhibition by nucleotide changes according to the binding ability of UCP1. Cl^- transport is inhibited only by mutations of arginines in the first domain (R83 and R91). Also in isolated mitochondria H^+ transport and respiration with all four mutants is similar to wt, and inhibition by GDP is found only in R91T. The three "regular" arginines are suggested to influence the nucleotide binding site indirectly via a charge network and the "extra" R91 directly via an ion bond with the previously characterised pH sensor E190. The mutants were also used to assess intrahelical control of UCP1. In the yeast cells expressing UCP1, the aerobic growth could be reduced by fatty acid addition only with the nucleotide insensitive mutants. This demonstrates an intracellular control of UCP1 by nucleotides and fatty acids.

The mitochondrial uncoupling protein (UCP1)[1] from brown adipose tissue (BAT) plays an important role in thermogenesis by allowing dissipation of energy from the H^+ electrochemical gradient across the inner mitochondrial membrane (*1*−*3*). UCP1 is a member of the mitochondrial carrier family (*4*, *5*), and the paradigm of the UCP subfamily (see, for review, ref *6*). Besides transporting H^+, UCP1 is also able to transport small anions, in particular Cl^-, however, at much lower rate. The uncoupling activity by UCP1 is increased by FA and is inhibited by purine nucleoside di- or triphosphates.

The functional characteristics of UCP1 have been investigated in considerable details, particularly using the isolated and reconstituted native UCP1 from BAT (see, for review, ref *3*). One approach has been mutagenesis to elucidate the role of selected residues in nucleotide binding, H^+ and Cl^- transport. For this purpose UCP1 was transfected into *Saccharomyces cerevisiae*, where it is well expressed and inserted into the mitochondria (*7*−*10*). This approach proves to be fruitful for UCP1, particularly in identifying residues involved in the intricate pH control of nucleotide binding, but also to recognize residues having an apparent role in FA dependent H^+ transport and in Cl^- transport (*10*−*13*). In this program most of the residues mutagenised by us had a negative charge.

Intrahelical arginines concentrated in the second helix of the three repeat domains in UCP1 are within a certain variability typical for the mitochondrial carriers (*14*). Their mutational "neutralisation" drastically inhibits functions, for example, the ADP/ATP transport in the yeast AAC2 (*15*). In UCP1, besides the three "regular" arginines an additional arginine R91 is located in the second helix which occurs in all members of the UCP subfamily but also in the ketoglutarate and citrate carrier (see, for review, ref *14*). In previous work, Garlid's group (*16*, *17*) mutated the three "regular" arginines and showed inhibition of nucleotide binding for the R83 and R182 but no influence by the mutation in the third domain. H^+ transport was reported to become insensitive to nucleotides for all three mutations.

Here, we report on the consequences of charge neutralizing all four intrahelical arginines. We screened the effect of these mutations at cellular, mitochondrial, isolated, and reconstituted level of UCP1. In all mutants the nucleotide binding is abolished. However, the H^+ transport remains unaffected, whereas the Cl^- transport is suppressed in two cases. These mutants also serve to demonstrate that the uncoupling by UCP1 within yeast cells is controlled by nucleotide binding and by fatty acids. This vindicates our stand of the physiological importance of those regulatory factors and contradicts recent publications, that they do not play a role in vivo (*18*, *19*).

MATERIALS AND METHODS

Materials. n-Decylpentaoxyethylene ($C_{10}E_5$), Dowex 1-X8 (200−400 mesh), and 2-Br-dodecanoic acid were obtained

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[1] Abbreviations: WT, wild-type; UCP, uncoupling protein; BAT, brown adipose tissue; AAC, ADP/ATP carrier; dansyl-GTP, 2′-*O*-[5-(dimethylamino)-naphthaline-1-sulfonyl]-GTP; FA, fatty acid; BrLA, 2-bromododecanoic (lauric) acid; PMSF, phenylmethanesulfonyl fluoride; MQAE, *N*-(ethoxycarbonylmethy)-6-methoxyquinoliniumbromside; Mops, 4 morpholine propane-sulfonic acid; CCCP, carboxyl cyanide m-chlorphenylhydrazone; OD, optical density.

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from Fluka. [^{14}C]GTP was from Amersham Corp. 2′-*O*-Dansyl-GTP (2′-O-[5-(dimethylamino)-naphthaline-1-sulfonyl]-GTP) was synthesized as described by Huang and Klingenberg (*20*). The fluorescence dyes MQAE [*N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide] and pyranine (8-hydroxpyrene-1,3,6-trisulfonic acid, trisodium salt) were purchased from Molecular Probes.

Methods. UCP1 was expressed in the diploid yeast (*S. cerevisiae*) strain W303 transformed with vector pEMBLyE4 which contains the coding sequences for hamster brown adipose tissue wild-type UCP1 (*10*) or the mutants (R83Q, R91T, R182Q, and R276Q). Gene expression was under the control of gal 10/cyc1 promoter. Yeast cells were grown at 29 °C in selective lactate medium and expression of UCP was induced by adding 0.4% galactose for 6 h before harvesting. Mitochondria were then isolated from protoplasts as previously described (*10*).

Mitochondria. Respiration of UCP1 containing mitochondria was measured with the platinum electrode, and H^+ transport of mitochondria was recorded with a fast response pH electrode (Russel, Scotland) as described previously (*12*, *21*). For measuring respiration, mitochondria (0.1 mg/mL) were suspended in a buffer consisting of 0.65 M sorbitol, 10 mM KCL, 10 mM Tris, and 0.05% bovine serum albumin, pH 6.8. Substrate for respiration was 3 mM NADH. Lauric acid (100 μM) was added to induce uncoupling and GDP (500 μM) for its inhibition. For H^+ transport measurements, isolated mitochondria (1.5 mg/mL) were suspended in 0.6 M mannitol, 100 mM KCl, 2.5 mM KH_2PO_4, and 1 mM MOPS, pH 7.2, to a final volume of 300 μL. Electron transport was blocked by addition of 4 μM antimycin, and the ADP/ATP carrier was inhibited by the addition of 5 μM bongkrekate and 10 μM carboxyatractylate. Lauric acid (50 μM) was added as an activator of UCP1. The H^+ release, initiated by addition of 2 μM valinomycin, was calibrated by addition of 10 mM H_2SO_4 in steps over 20 nmol of H^+. The total H^+ release capacity was recorded after addition of 2 μM CCCP. The H^+ transport inhibition was measured in the presence of 50 μM GDP.

Nucleotide binding to isolated mitochondria was performed with the fluorescence nucleotide derivative dansyl-GTP as described previously (*20*). Fluorescence was recorded on MPF-44A fluorescence spectrophotometer (Perkin-Elmer). To unmask UCP1 from residual endogenous bound ATP, mitochondria at a concentration of 5 mg of protein/mL in incubation buffer containing 250 mM sucrose, 1 mM EDTA, 1 mM PMSF, and 20 mM Hepes, pH 8.0, were shaken with Dowex (120 mg/mg of protein) at room temperature for 1 h (*20*). For the fluorescence measurements, mitochondria were filled into a cuvette (5 × 5 mm) at 1 mg protein/mL incubation buffer, pH 6.8, containing 10 μM carboxyatractylate. The specific fluorescence (ΔF) due to binding at the nucleotide binding site of the UCP1 was obtained by subtracting from the total fluorescence the residual fluorescence measured after addition of 1.5 mM ATP.

Isolated and Reconstituted UCP1. Wild-type and mutant UCP1 were isolated from yeast mitochondria basically as described (*22*, *10*). Mitochondria were solubilized at a protein concentration of 15 mg/mL for 45 min at 0 °C with Triton X-100 at a ratio of 1.2 mg of detergent per mg of protein in the presence of 20 mM Na_2SO_4, 0.2 mM EDTA, 1 mM PMSF, and 20 mM Mops, pH 6.7. The suspension was centrifuged at 10^5 g for 30 min at 4 °C. The supernatant was purified on a hydroxyapatite column (5 mL for 15 mg of mitochondrial protein) and eluated by 20 mM Na_2SO_4, 0.2 mM EDTA, and 5 mM Mops, pH 6.7, at room temperature. The UCP1-containing fraction was collected and concentrated by pressure dialysis to 2 mg/mL, with a yield of about 1.5−2.0% of mitochondrial protein.

The measurement of [^{14}C]GTP binding to isolated protein followed the published procedure using Dowex for removal of free ^{14}C GTP (*9*). UCP1 (200 μg/mL) was added to a buffer containing 15 mM Mops and 1 mM PMSF. To this, [^{14}C]GTP in concentration from 1 to 30 μM with specific activity of 9 dpm/pmol was added. After 30 min incubation at 0 °C, 50 μL of the sample was applied to a column (2 × 60 mm) of 20 mg Dowex 1X 8 (Cl^- form) and washed twice with 100 μL of H_2O. In the eluate, the bound [^{14}C]GTP was determined by scintillation counting. The binding rate of GTP was measured with an automated rapid mixing and separation sampling machine developed in our laboratory (*10*). The reconstitution of isolated UCP1 into phospholipid vesicles as well as measurement of H^+ and Cl^- transport into reconstituted phospholipid was based on the procedures introduced for UCP1 from brown fat adipose tissue (*21*, *23*) and followed the protocol described (*10*).

RESULTS

Nucleotide Binding. For a determination of UCP1 content in mitochondria, the titration with dansyl-GTP was used, as in our previous work on UCP1 mutants expressed in yeast. It proved to be more reliable than [^{14}C]GTP binding, where a high background binding was measured in control mitochondria from yeast cells without the plasmid carrying the UCP1-DNA. According to these measurements in all four mutants, the binding was decreased to the detection limit (data not shown). The question arose whether by these mutations the affinity of UCP1 or the incorporation of protein in mitochondria was affected. An estimation of the UCP1 content by ELISA titration showed indeed a lower but still clearly defined content of UCP1 in the mutant mitochondria. As compared to wt, the content amounted to 30, 35, 30, and 60% in the mitochondria of R83Q, R91T, R182Q, and R276Q, respectively. This was confirmed by the distinctly lower yield of partially purified mutant proteins [about 0.5−0.7% of the total mitochondrial protein as compared to wt (1.5−2.0%)].

The isolated UCP1 enabled a systematic binding study using [^{14}C]GTP. The three mutational charge neutralizations of the common intrahelical arginines R83Q, R182Q, and R276Q abolished binding of GTP at neutral pH (Table 1). At low pH (<5.5), a small binding capacity was observed. For R83Q, as extrapolated in the mass action plot, the B_{max} was 5.5 μmol/g protein, and for R182Q and R276Q, 6.5 and 6.3 at pH 4.5. At pH 5.5, It decreased to 3.3 and 1.3 μmol/g protein, respectively. The binding affinity was reduced more in R83Q than in R182Q and R276Q.

On mutational charge neutralization of the more UCP1 typical arginine (R91) almost normal binding capacity was retained (~12 μmol/g protein) up to pH 7.0, but decreased by about 30% at pH 7.5. The affinity was low throughout the pH range studied as compared to wt. In view of the known pH dependence of the affinity for nucleotides, the

Table 1: GTP Binding to Wild-Type and Mutant UCP[a]

uncoupling protein UCP1	pH	K_D (μM)	B_{max} (μmol/g prot)	$K_{on} \times 10^{-3}$ (M^{-1} s^{-1})
wild-type	4.5	0.28 ± 0.01	13.5 ± 0.6	0.59 ± 0.03
	5.0	0.31 ± 0.01	13.2 ± 0.7	0.47 ± 0.02
	5.5	0.37 ± 0.02	12.6 ± 0.8	0.31 ± 0.02
	6.0	0.39 ± 0.02	12.2 ± 0.9	0.25 ± 0.02
	6.5	1.05 ± 0.06	12.0 ± 0.6	0.20 ± 0.01
	7.5	7.90 ± 0.63	10.2 ± 0.9	0.03 ± 0.004
R83Q	4.5	11.5 ± 0.6	5.5 ± 0.4	
	5.0	12.1 ± 0.8	4.0 ± 0.3	
	5.5	32.9 ± 2.6	1.3 ± 0.1	
	6.0	0	0	0
	6.5	0	0	0
	7.5	0	0	0
R91T	4.5	15 ± 1.2	11.8 ± 0.6	0.11 ± 0.01
	5.0	16 ± 1.1	11.8 ± 0.7	0.08 ± 0.008
	5.5	27 ± 2.1	11.9 ± 0.5	0.05 ± 0.005
	6.0	31 ± 2.5	11.3 ± 0.6	0.04 ± 0.003
	6.5	39 ± 3.1	9.0 ± 0.8	0.02 ± 0.002
	7.5	56 ± 5.1	8.0 ± 0.7	0.01 ± 0.005
R182Q	4.5	2.40 ± 0.09	6.5 ± 0.3	
	5.0	2.60 ± 0.11	5.8 ± 0.5	
	5.5	4.50 ± 0.23	3.3 ± 0.2	
	6.0	0	0	0
	6.5	0	0	0
	7.5	0	0	0
R276Q	4.5	4.50 ± 0.22	6.3 ± 0.4	
	5.0	4.60 ± 0.36	5.0 ± 0.4	
	5.5	8.00 ± 0.56	1.3 ± 0.1	
	6.0	0	0	0
	6.5	0	0	0
	7.5	0	0	0

[a] The K_D values were evaluated from [^{14}C]GTP titration of 200 μg/mL UCP at 0 °C. The B_{max} values were obtained from the mass action plot. The rate constants (k_{on}) were evaluated from time study of [^{14}C]GTP binding to UCP at 15 °C. Results for each UCP1 type were measured with two different protein preparations.

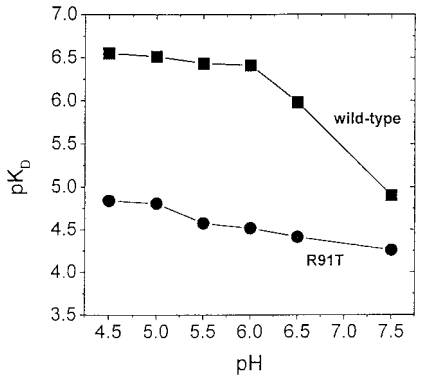

FIGURE 1: pH dependence of dissociation constants of GTP binding to wt and R91TUCP1. The K_D values were evaluated from the [^{14}C]-GTP titration. UCP1 (0.2 mg of protein/mL) was incubated in 15 mM Mops buffer and 1 mM PMSF at 0 °C for 30 min with increasing concentrations (1−30 μM) of [^{14}C]GTP. Binding was determined by the "anion exchange method" (*33*). The samples were passed through a small column (2 × 60 mm) containing 20 mg of wet Dowex 1-X8 (Cl^- form) and the bound nucleotides were determined by scintillation counting of the eluate.

K_D was determined in the pH range from 4.5 to 7.5. Interestingly, in R91T the pH dependency of the affinity (pK_D) for the binding of GTP was nearly flat (Figure 1). The affinity increases only 2-fold whereas in wt UCP1 from pH 6.0 to 7.5 it decreases about 20 times (see also ref *3*).

The rate of nucleotide binding has been used to supplement the information on the affinity. The binding rate of [^{14}C]-GTP to isolated UCP1 from yeast was measured by an automated mixing, sampling, and separation apparatus. The evaluation according to a second-order reaction gives rate constants (k_{on}) listed in Table 1. However, because of a too low affinity and B_{max} of R83Q, R182Q, and R276Q, the binding rates could only be measured in R91T, and were found to be 5−10 times slower than in wt. From pH 6.5 to 7.5, the binding rates k_{on} decreased only 2-fold for the R91T mutant, consistent with the low pH influence on the binding affinity but different from the 7-fold decrease for wt.

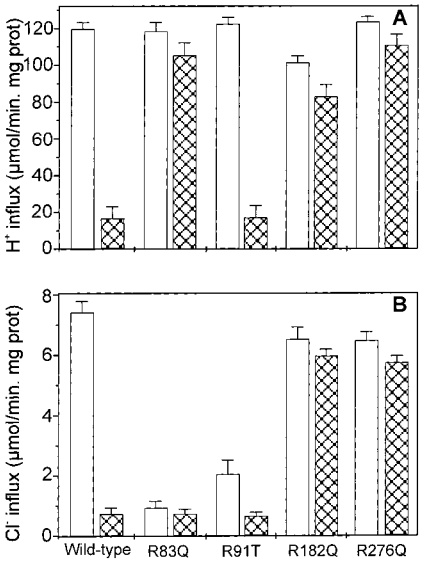

FIGURE 2: H^+ and Cl^- transport into proteoliposomes reconstituted with purified wt and mutant UCP-1. H^+ influx was measured as the change in external pH monitored by pyranine fluorescence at λ_{exc} = 467 nm and λ_{em} = 510 nm. Cl^- influx was monitored by fluorescence of MQAE, loaded into the vesicle, at λ_{exc} = 355 nm and λ_{em} = 460 nm at pH 6.8 and 10 °C. For H^+ transport measurement, a 50 μL portion of vesicles was added to 0.5 mM Hepes buffer, pH 7.5, containing 1 μM pyranine, 0.5 mM EDTA, and 280 mM sucrose to a final volume of 330 μL. Valinomycin of final concentration 2.5 μM was added to generate membrane potential in the presence of 125 μM laurate. H_2SO_4 was added in steps of 20 nmol of H^+ to adjust the pH to 6.8. The uncoupling with CCCP (1 μM) was used to determine the capacity of H^+ conductance across the vesicles. For Cl^- transport, a 50 μL portion of vesicles was suspended in 4 mM sodium phosphate buffer containing 155 mM KCl to a final volume of 385 μL. The Cl^- influx rate was monitored after addition of 2 μM valinomycin. Tributyltin acetate (40 μM) was added to equilibrate internal and external chloride. The Cl^- influx rate was calculated from the fluorescence data as described (*10*). Values from three different reconstituted proteins.

Reconstituted System. On reconstitution of isolated UCP1 into phospholipid vesicles, the H^+ and Cl^- transport capacities were measured. In all these mutant UCP the lauric acid activated H^+ transport rate was almost the same as in wt (Figure 2A). ATP (100 μM) only weakly (20%) inhibits the H^+ transport activity of R83Q, R182Q, and R276Q, whereas with R91T nearly full inhibition (~85%) was achieved. This agreed for all mutants with the results of nucleotide binding. The Cl^- transport was differently affected by these mutations (Figure 2B). Almost no Cl^- transport activity was measured in R83Q, and in R91T only about 25% of the wt activity. This activity was inhibited to the basal level by ATP. In R182Q and R276Q mutants the Cl^- transport activity was slightly inhibited by ATP. In conclusion, charge neutralization of the positive residues R83 and R91 suppresses Cl^- transport but not H^+ transport.

Nucleotide and Fatty Acid Effects on H^+-Permeability of Yeast Mitochondria. To ascertain whether the effects of the mutations were the same in the original mitochondrial environment, uncoupling and H^+ transport by UCP1 were

Table 2: Transport Activities of Wild-type and Mutant UCPs in Mitochondria[a]

	mitochondria				
	H^+ transport (nmol/min mg)			respiration (%)	
UCP1	−	+GDP	Δ	LA activation	GDP inhibition
control (−UCP)	7.5 ± 1.9	7.5 ± 1.5	0	25 ± 3	0
Wt	28.4 ± 2.1	9.2 ± 2.3	19.2	86 ± 8	91 ± 3.5
R83Q	24.0 ± 1.8	24.4 ± 1.3	0	63 ± 14	0
R91T	27.6 ± 1.9	13.8 ± 1.3	13.8	69 ± 9	34 ± 5
R182Q	19.6 ± 1.1	16.0 ± 2.0	3.6	51 ± 7	9 ± 2
R276Q	30.4 ± 2.2	28.4 ± 1.9	2	64 ± 12	0

[a] Transport activities of UCP1 in mitochondria are measured as described in the Materials and Methods. The uncoupling effect on mitochondrial respiration due to UCP1 is presented as percentage of fatty acid-activated respiration and as percentage of GDP-sensitive portion of this activation. ($n = 3-4$ different mitochondria preparations).

studied in isolated mitochondria. The data of the respiratory control and the H^+ transport using our previously introduced procedure (*12*) are summarized in Table 2. The mutants showed almost the same fatty acid dependent H^+ uptake activity as wt; only in R182Q it is decreased. In line with the results on reconstituted UCP1, this H^+ transport was nucleotide insensitive in R83Q, R182Q, and R276Q. In R91T, the inhibition by nucleotide was about 65% as compared to wt (92%), but still significantly higher than in the other intrahelical arginine mutants. The uncoupling as measured by the respiration revealed a similar pattern. Lauric acid activated respiration in all mutants, although less than in wt, particularly in R182Q. GDP abolished the lauric acid induced uncoupling almost fully in wt and to about half in R91T whereas in all three mutants of the "regular" intrahelical arginines the uncoupling was GDP resistant.

The fact that the H^+ transport rates in mitochondria are the same for three mutants, as wt, although the level of expression is only one-third of wt requires comment. In the mitochondrial assay, the turnover of UCP1 is 50−100 times lower than in the reconstituted vesicles and limited by the low capacity for H^+ uptake rather than by the UCP1 content within certain limits. This is true to a some extent also for the uncoupling measurement respiration, where the uncoupled respiration varies less between mutant and wt than UCP1 content.

Intracellular Regulation of UCP1 in Yeast. These arginine mutants of UCP1 provide a unique opportunity to study the intracellular regulation of UCP1 in yeast cells. The fact that UCP1 expressing yeast cells grow well indicates that uncoupling activity by UCP1 must be low. The question arises, why UCP1 is inhibited although, as shown with the isolated mitochondria, it is potentially active in H^+ transport. Two possibilities are obvious: UCP1 is inhibited by nucleotides or it is not activated due to the lack of fatty acids. To differentiate these regulatory influences, the nucleotide insensitive mutants were most welcome. As an indirect indicator of an uncoupling by UCP1, we measured the aerobic growth rate of the yeast cells under 2-Br-lauric acid (BrLA) which is an excellent activator of UCP (*21*) but cannot be metabolized. The control experiments with nontransformed cells gave the UCP1 unrelated uncoupling effect of FA, since also the control cells were affected by 15 μM BrLA as shown in the insert of Figure 3B. Cells expressing wt-UCP1 were not more sensitive to BrLA than untransformed cells (Figure 3A). This indicates the existence of an intracellular inhibition of UCP1 by nucleotides. As a consequence, growth of cells with the mutants of UCP1, R83Q, R182Q, and R276Q, unable to bind nucleotides, should be retarded. Interestingly, growth rate was the same as with wt-UCP1 (Figure 3A). Only on addition of BrLA to the cells, growth was decreased about 2.5-fold (Figure 3A). Differently, yeast expressing R91T had the same growth as wt-UCP. This adds to the evidence that inhibition by nucleotides might be responsible for the suppressed uncoupling activity of UCP1 in vivo (Figure 3B). In conclusion, in yeast, the uncoupling potential of UCP1 depends on the addition of fatty acid since there is no significant difference in the cell growth between wt and all these mutants. Obviously the level of endogenous fatty acids in yeast cells is insufficient to activate UCP1.

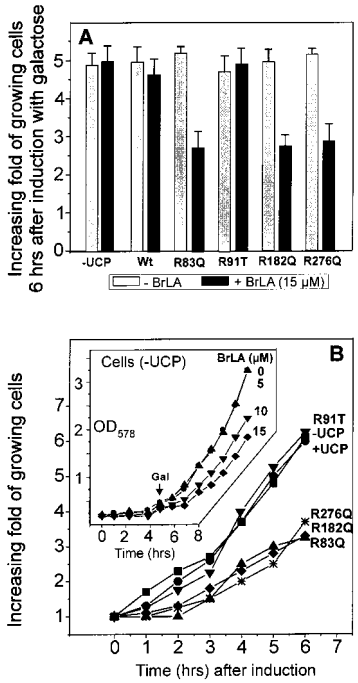

FIGURE 3: Intracellular regulation of UCP1 as monitored by the growth rate of wt and the arginine mutants. (A) Comparison of the growth with and without 1.5 μM 2-bromo-dodecanoic acid (BrLA) expressing wt and mutant UCP1, 6 h after induction with galactose. ($n = 3$). (B) Concentration dependence effect of BrLA on the growing of control yeast cells (without UCP1) (insert figure), and the growth at constant concentration (15 μM BrLA) of wt and of mutant transformed cells. Yeast transformants were grown at 29 °C in a medium containing 0.67% yeast nitrogen base, 0.1% glucose, 0.1% KH_2PO_4, 0.12% $(NH_4)_2SO_4$, 2% lactate, and 0.1% amino acid mixture, pH 6.5, to an OD_{578} of 2−3. The yeast cells were diluted to a final OD_{578} of 0.01 with selective lactate medium (0.67% yeast nitrogen base, 0.05% glucose, 0.1% casamino acid, 2% lactate, 20 mg/L tryptophane, and 40 mg/L adenine, pH 6.0) and grown at 29 °C with vigorous shaking till an OD of 0.5. Galactose at a final concentration of 0.4% was then added to induce the expression of UCP.

DISCUSSION

The present set of mutations of positive residues in UCP1 concentrates on the four intrahelical arginines. The single mutational charge neutralization enlarges our assignments of charged residues to UCP1 functions. They also provide an opportunity to gain some insight into the intracellular regulation of UCP1. The mutational effects were studied on

four levels of integration: cells, mitochondria, isolated UCP1, and reconstituted proteoliposomes and comprise the affinity and pH dependence of nucleotide binding, H^+ and Cl^- transport, uncoupling of respiration. Further, the intracellular regulation of UCP1 were investigated. The mutagenesis of the three repeat arginines occurring in the second helix of each repeat domains was previously reported by Garlid's group (*16*, *17*), but the evaluation confined to nucleotide binding at the isolated protein and to H^+ transport in proteoliposomes.

Nucleotide Binding. By the charge neutralizing mutations of all four intrahelical arginines, nucleotide binding is affected but H^+ transport remains unchanged. This extends the previous experience that in UCP1 nucleotide binding is more sensitive than H^+ transport to manipulation of residues by reagents or by mutations. Several amino acid reagents including arginine reagents affect nucleotide binding but none is known to inhibit H^+ transport (*24*−*27*). The nucleotide binding site had been mapped with the covalent insertion of reactive ATP derivatives (*28*−*30*) and by mutagenesis (*9*, *10*, *13*, *16*, *17*, *31*). Most of the identified residues are located in the third domain. Therefore, among the four intrahelical arginines, R276 is a candidate for the nucleotide binding site. However, the near abolishment of binding also by the mutations in the first (R83Q) and second domain (R182Q) would argue for a broader distribution of the nucleotide interactions or require a different explanation. Our results agree and extend the mutational effects of neutralization of R 83 and R 182 in rat UCP1 reported by Modriansky et al. (*17*). However, they are at variance with the claim of the paradox that charge neutralization of R276 retains nucleotide binding although the inhibition of H^+ transport was lost. This anomaly was not observed in our hands.

At first sight, the suppression of binding by the neutralization of these three arginines would indicate that they represent the positive residues responsible for the interaction with the di-and triphosphate moiety of the nucleotides. In fact, also in the ADP/ATP carrier neutralization of the three homologous arginines impairs the interaction of nucleotides as measured by a strongly decreased transport activity (*15*). The transport of the higher charged ATP^{4-} was much more sensible to introduction of the positive charge defect than that of ADP^{3-}. However, homologously positioned positive residues also occur in other mitochondrial carriers which do not deal with nucleotides, such as the carriers for phosphate (*4*), ketoglutarate (*32*) and citrate (*33*), which could argue against a specific role of these residues in nucleotide binding. They are also thought to interact with helix terminal acid groups of adjacent tilted helices, as deduced from second site mutation in the AAC (*34*). Breaking this ion bond by neutralizing the intrahelical arginine would change the tilt of the helices. In conclusion, it seems that the intrahelical arginines in UCP1 interact with negatively charged ligands within a charge network where a mutational change defect would destabilize the charge balances and impair nucleotide binding.

Interestingly, nucleotide binding in these mutants proteins emerges at pH <5.5, but at a reduced binding capacity. We propose that the charge defect by the mutations causes a conformational instability where part of UCP1 is in a state incompetent for binding. This instability depends on the pH and is possibly linked to the ionization state of E190 (*3*, *35*) which has a p$K = 4.0$. At low pH in the undissociated state of E190 the positive charge defect in the mutants is compensated and the binding state of UCP1 becomes more populated. However, the transition between the binding and nonbinding state is slow and thus even with increased nucleotide concentration only the portion of UCP1 already in the binding state can be loaded. Differently, the mutant R91T can be visualized to be fully in a binding competent state, albeit with a low affinity, because R91 is involved directly in the binding whereas the three "regular" arginines are visualized to be more engaged in maintaining the competent conformation.

The additional intrahelical positive charge R91 is in a different turn of the transmembrane helix 2 as the three repeat arginines. It occurs also in the ketoglutarate and citrate carrier (*32*, *33*). In R91T the binding capacity is nearly normal but the affinity drastically diminished. At the same time, the pH dependence has nearly vanished. The location of R91 at the same turn of helix 2 as the pH sensor E190 (*3*, *36*) in helix 4 suggests that R91 carries the counter charge to E190 in the ion pair which has been postulated to control the entrance to the binding pocket for the phosphate moiety (*3*, *35*−*37*). On protonation of E190, the gate is opened to the binding pocket and R91 becomes free to interact with the nucleotide phosphate moiety. The loss of pH dependence and the drastic decrease of binding affinity in R91T is in line with this role. In accordance, it was shown that on breaking this ion bond in E190Q the binding affinity increases (*10*) since the entrance to the binding pocket is opened and the interaction with the unscreened positive residue is facilitated.

H^+ and Cl^- Transport. All four arginine mutants retain full H^+ transport activity. Only in R91T it can be inhibited by nucleotides. These findings emphasize again that in UCP1 the nucleotide binding site is not an integral part of the H^+ path (see, for review, ref 3). The mutational effects on Cl^- transport are more diverse. Both in R83Q and R91T, Cl^- transport is abolished, pointing to an intimate interaction of the Cl^- path with the second helix. Arginine residues important for anion permeation have also been found in mammalian anion channels, such as for the cystic fibrosis transmembrane conductance regulator (*38*) and the inhibitory glycine receptor (*39*). These mutants also underline the independence of H^+ transport from Cl^- transport, as it was previously observed in the E167Q mutant (*12*). They disagree with the FA anion transport theory of the FA dependent H^+ transport (*40*), where the capability of UCP1 to transport several anions is interpreted to be utilized also for the transport of FA anions. Together with the rapid flip flop of undissociated FA an H^+ transport would be facilitated.

Intracellular Regulation. These mutants also provide an opportunity to gain insight into the controversial field of the in vivo regulation of UCP1. The necessity for a tight control of such a potentially dangerous component as UCP1 is obvious. The activation by FA and the inhibition by nucleotides combined with its control by pH provide an intricate control machinery to cope with the short-term regulation of uncoupling. Notwithstanding, for the in vivo situation the regulation by nucleotides has been negated (*41*) and more recently even the regulation by FA questioned, using mitochondria from UCP1 expressing yeast (*18*) and mitochondria from UCP1 ablated mice (*19*). Surprisingly,

the authors ignore the well-established pH control of nucleotide binding, whereby at pH >7.5 the nucleotide inhibition is relieved (*35*), and thus, FA should be able to induce uncoupling despite the presence of nucleotide.

Here we used the growth curves of UCP1 expressing yeast cells under the assumption that it monitors the intracellular uncoupling. The comparison between wt-UCP1 and the three nucleotide insensitive mutants indicates that within the yeast cell wt-UCP1 is blocked by nucleotides. However, this difference between wt and mutants emerged only when UCP1 was activated by addition of FA, providing evidence for an intracellular regulation by FA, at least in the yeast model. The finding that UCP1 mutants remain inactive without FA addition, although they cannot be blocked by nucleotides, indicates that yeast cells lack endogenous free FA. The free, i.e., not Mg^{2+} bound nucleotide concentration, appears to be sufficient to block even R91T although it has a much lower affinity than wt. Admitting that the parameters governing UCP1 in brown fat adipose cells are somewhat different from those in yeast, the principal control by FA and nucleotide can be expected to be similar. At the prevalent low pH in yeast cells (*42*), the affinity for nucleotide binding dominates but at pH >7.5 wt-UCP1 might be relieved from nucleotide blockage.

Recently Hagen et al. (*43*) reported a stimulation of respiration in UCP1 bearing yeast cells by additional 2Br palmitate, both when wt and R276L-UCP1 were expressed. This would indicate either that UCP1 is not inhibited in the cells by nucleotides or that 2-Br palmitate causes an unspecific uncoupling. The latter interpretation would be accommodative with our results, which also indicate an unspecific effect by 2-Br laurate at higher concentrations (Figure 3A, insert). In conclusion, the data illustrate the importance of FA for the intracellular uncoupling of UCP1 in the yeast cell model and thus argue against the recent claims that FA do not control UCP linked uncoupling (*17*, *18*). They support our stand, that results gained from isolated UCP1 can be extrapolated to the in vivo environment.

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