Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1767 (2007) 1412–1417
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbabio

Mutational analysis of *Arabidopsis thaliana* plant uncoupling mitochondrial protein

Regiane Degan Fávaro [a], Jiri Borecký [b], Débora Colombi [a], Aníbal E. Vercesi [b], Ivan G. Maia [a,*]

[a] *Departamento de Genética, Instituto de Biociências, UNESP, Botucatu, SP, Brazil*
[b] *Laboratório de Bioenergética, Núcleo de Medicina Experimental, FCM, UNICAMP, Campinas-SP, Brazil*

Received 28 June 2007; received in revised form 5 September 2007; accepted 20 September 2007
Available online 29 September 2007

Abstract

In this study, point mutations were introduced in plant uncoupling mitochondrial protein AtUCP1, a typical member of the plant uncoupling protein (UCP) gene subfamily, in amino acid residues Lys147, Arg155 and Tyr269, located inside the so-called UCP-signatures, and in two more residues, Cys28 and His83, specific for plant UCPs. The effects of amino acid replacements on AtUCP1 biochemical properties were examined using reconstituted proteoliposomes. Residue Arg155 appears to be crucial for AtUCP1 affinity to linoleic acid (LA) whereas His83 plays an important role in AtUCP1 transport activity. Residues Cys28, Lys147, and also Tyr269 are probably essential for correct protein function, as their substitutions affected either the AtUCP1 affinity to LA and its transport activity, or sensitivity to inhibitors (purine nucleotides). Interestingly, Cys28 substitution reduced ATP inhibitory effect on AtUCP1, while Tyr269Phe mutant exhibited 2.8-fold increase in sensitivity to ATP, in accordance with the reverse mutation Phe267Tyr of mammalian UCP1.

Keywords: Uncoupling protein; *Arabidopsis thaliana*; Site-direct mutagenesis; Proteoliposome; Structure–function relationship

1. Introduction

The uncoupling protein (UCP) is an integral protein of the mitochondrial inner membrane that uncouples oxidative phosphorylation. UCP mediates a fatty acid (FA)-dependent, purine nucleotide (PN)-inhibitable proton leak across the inner membrane [1] that was reported to be activated by superoxide and/or products of lipid peroxidation, such as 4-hydroxy-2-nonenal (HNE) [2,3]. This effect, however, is still controversial since a more recent study provided experimental evidence that HNE can only induce mitochondrial uncoupling via its oxidative product, a hydroxynonenoic acid, in a UCP-independent pathway [4].

The first uncoupling protein (UCP1) was discovered in mice brown adipocytes mitochondria [5] and was shown to be highly tissue specific. UCP1 has a specialized physiological role in generation of metabolic (non-shivering) thermogenesis in hibernating mammals [6], in cold-adaptation of newborn mammals [7,8], and in diet-induced thermogenesis in small rodents [9]. Homologues of UCP1 were subsequently identified in different tissues and the actual known mammalian UCP genes constitute a multigene family composed by five members (UCP1–5) (reviewed in [10]). The physiological roles of UCP2–5 are still under debate; however, given that they are present in mitochondria in much lower amounts than UCP1, their participation in thermogenesis is practically discarded [7,8].

The existence of UCP-like proteins in plants (pUCP), initially referred to as PUMPs (*P*lant *U*ncoupling *M*itochondrial *P*rotein), was firstly demonstrated in potato tuber mitochondria [11]. Thereafter, several genes encoding pUCP have been identified in different plant species (reviewed in [12]). Similar to UCP2–5, pUCPs are present in plant mitochondria in low amounts and are probably not able to promote thermogenesis [13,14]. The physiological roles of pUCPs are most likely related to the control of reactive oxygen species overproduction or to the regulation of mitochondrial energy flow at some stages of plant tissue/organ development (reviewed in [12]).

* Corresponding author. Departamento de Genética, Instituto de Biociências, Universidade Estadual Paulista 'Júlio de Mesquita Filho', 18.618-000, Botucatu, SP, Brazil. Tel.: +55 14 3811 6229; fax: +55 14 3815 3744.
E-mail address: igmaia@ibb.unesp.br (I.G. Maia).

0005-2728/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2007.09.005

In the last years, useful information concerning the mechanism of H^+ transport by mammalian UCP1 has been provided by site-directed mutagenesis experiments (reviewed in [15]). Important amino acid (aa) residues involved in this process have been identified, in particular a C-terminal cysteine (Cys304) that modulates UCP1 proton-translocating activity [16]. Moreover, a critical role for a histidine pair, present in the second matrix loop, in the FFA-activated proton transport of UCP1 was demonstrated [17]. Although not presented in PUMPs, the introduction of such a pair at equivalent positions of a UCP from maize (ZmPUMP; Lys155His/Ala157His) increased 1.55-fold its affinity to linoleic acid (LA) [18]. In contrast, when a patch of 8 amino acid residues (including the His pair) of UCP1 was replaced by the homologous region of UCP2, no alteration in UCP1 proton transport was observed [19].

Mutations compromising PN binding have been also described, namely those affecting three conserved intrahelical arginines (Arg83, Arg182, Arg276) [20,21] (Fig. 1A). In this context, a UCP1 mutant carrying a deletion of residues Phe267, Lys268, and Gly269 was described as being insensitive to nucleotide inhibition [22]. In addition, the intrahelical Glu190 was pointed out as important for the pH regulation of PN binding in UCP1 [23].

In contrast to numerous studies elucidating the structure–function relationships of mammalian UCP1, only one report regarding these aspects within pUCPs has been provided to date [18]. In this respect, UCP1 appears to be the last evolutionary event within uncoupling protein gene family with highly specialized function and therefore cannot be considered as the representative member of this family. For this reason, studies employing other UCPs/pUCPs are important to advance our understanding of the mechanistic and structural properties of uncoupling proteins in general.

In this study, five different aa residues (Cys28, His83, Lys147, Arg155, and Tyr269; Fig. 1) of AtUCP1 (originally called At PUMP1) potentially important for protein activity were selected and mutagenized. Among them, Cys28 and His83 are exclusively found in pUCPs and were chosen to study possible pUCP-specific structure–function relationships. Lys147 occupies the position of the first residue (His147) of an essential His pair present in mammalian UCP1 [17] and was mutated to determine the significance of a positive charge at this location on LA-mediated uncoupling protein activity. The mutation Tyr269Phe was selected as reversal to a Phe267Tyr mutation studied in UCP1 and associated with a decrease of PN inhibition efficiency [22]. Arg155 corresponds to Arg152 in UCP1, a residue known to be crucial for its activation by FA [24], and was investigated to probe common structure–activity relationships among members of the UCP/pUCP family. The biochemical activities and kinetic parameters of these pUCP mutants were examined using recombinant proteins reconstituted in proteoliposomes to avoid possible interferences of other mitochondrial carriers.

2. Materials and methods

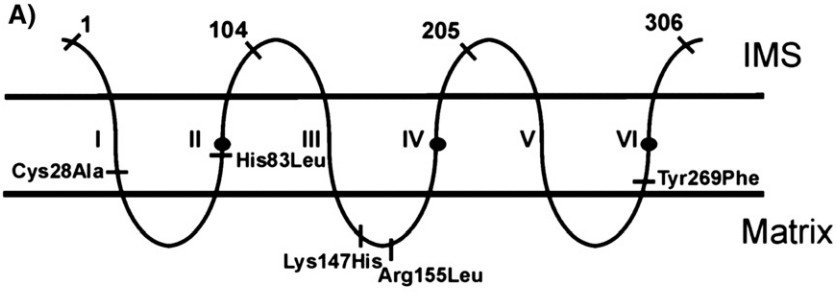
2.1. Materials

Bio-Beads SM2 were from Bio-Rad. The fluorescence probe SPQ [6-methoxy-*N*-(3sulfopropyl) quiminolinium] was from Molecular Probes. Lecithin was a special gift of Dr. Iolanda Cuccovia (Department of Biochemistry, University of São Paulo, Brazil). Nucleotides, LA and other chemicals were from Sigma. HNE was from Cayman Chemical (Ann Arbor, MI).

2.2. Mutagenesis and expression of AtUCP1 mutants

A plasmid pET3d with an inserted *AtUCP1* cDNA under the control of the T7 promoter [25] was used to introduce the site-directed mutations. Mutagenesis was conducted using a QuickChange Site-Directed Mutagenesis Kit (Stratagene). Oligonucleotides were designed to alter *AtUCP1* codons for Cys28 (TGC) to Ala (GCC), His83 (CAT) to Leu (CTT), Lys147 (AAA) to His (CAC), Arg155 (CGG) to Leu (CTG) and Tyr269 (TAC) to Phe (TTC). All recovered mutants were verified by DNA sequencing in an ABI-PRISM 3100 automatic sequencer (Perkin-Elmer).

Escherichia coli BL21(DE3) pLysS cells were transformed with the plasmids containing the wt *AtUCP1* or corresponding mutants. Expression of the



B)

	28	83	147	155	269
AtPUMP1	...EV**C**TIPL...	PGL**H**RQC...	QAEG**K**LAAG...	**R**YSGALN...	PMAF**Y**KGF
StUCP	...EA**C**TLPL...	PGL**H**RQC...	QAEG**K**LPAG...	**R**YSGALN...	PLAF**Y**KGF
haUCP1	...DI**T**TFPL...	AGI**Q**RQI...	QAQS**H**L**H**GI...	RYTGTYN...	PTAF**F**KGF
hUCP3	...DL**V**TFPL...	AGL**Q**RQM...	QASI**H**LGPS...	**K**YSGTMD...	PTAF**Y**KGF

Fig. 1. (A) Schematic transmembrane folding of AtUCP1 showing the positions of the amino acid (aa) residues mutated in this study (Cys28Ala, His83Leu, Lys147His, Arg155Leu and Tyr269Phe). Numbers (1 to 306) represent the positions of the aa residues starting from the initial methionine. The transmembrane domains are labeled by Roman numerals (I–VI). Solid circles represent three conserved intrahelical arginines. IMS — intermembrane space. (B) Selected aligned segments of the aa sequences of AtUCP1 (AtPUMP1; At3g54110), StUCP (CAA72107), hamster UCP1 (P04575) and human UCP3 (P55916) encompassing the mutated residues in AtUCP1 (in bold). Accession numbers were taken from GenBank or Tair database.

genes was induced with 1 mM IPTG (isopropyl-β-D- thiogalactopyranoside) as described previously [25]. The presence of recombinant proteins was analyzed by SDS-PAGE (0.1% SDS, 12% polyacrylamide) stained with Coomassie Blue.

2.3. Reconstitution

Wild type (wt) and mutant AtUCP1 proteins were isolated as described previously [25] and reconstituted (100 μg each) in proteoliposomes consisting of 39 mg lecithin, 1.66 mg cardiolipin, and 0.66 mg phosphatidic acid at lipid/protein molar ratio of 410:1. The proteoliposomes internal medium (IM) contained 28.8 mM tetraethylamonium N-tris [hydroxymethyl]methyl-2-aminoethanesulfonate (TEA-TES; $[TES^-]_{free}$=9.2 mM), pH 7.2, 84.4 mM TEA_2SO_4 and 0.6 mM TEA-EGTA, 2 mM SPQ probe and 84.4 μM K_2SO_4 to set the potassium diffusion potential across the liposomal membrane to ~180 mV [25].

H^+ flux assays were based on the quenching of SPQ fluorescence by TES^- [26]. Vesicles (30 μl; 0.24 mg lipids, ~25 μg protein) were added to 1.5 ml of external medium (EM; 28.8 mM TEA-TES [9.2 mM TES^-], pH 7.2, 84.4 mM K_2SO_4, and 0.6 mM TEA-EGTA). Fatty acids were added after 20 s and the H^+ efflux was initiated by 1.3 μM valinomycin 20 s later. In selected experiments, 10, 30, 60 or 100 μM HNE was added before vesicle addition. The fluorescence was calibrated to $[H^+]$ by the addition of 6 μmol of KOH aliquots in the presence of 1.5 μM nigericin to proteoliposomes suspended in IM. Fluorescent data were converted into "H^+ traces", that correspond to H^+ efflux, by fitting with modified Stern–Volmer equation, $[H^+]=(1/K_q).(F_0-F)/(F-L)$, where F is the experimental and F_0 the unquenched fluorescence. Parameters K_q (quenching constant) and L (background, mostly light scattering) were obtained by linear regression of F vs. $(F_0-F)/[KOH]$ plot. Rates of H^+ efflux were derived from "H^+ traces", multiplied by the internal proteoliposome volume (V, estimated from volume distribution of the SPQ; [1]), and corrected for protein content (9.9 mg lipids corresponding to 1 mg of protein in case of protein-free liposomes) to yield final rates in μmol H^+ min^{-1} (mg prot)$^{-1}$. When expressed per μmol of AtUCP1 dimer, rates represented the minimum values for the turnover number, since not all of the protein was inserted into the vesicles. The kinetic constants were calculated as the mean±SD of the values determined by Lineweaver–Burk and/or Hill plot analyses from three independent experiments.

3. Results

Sequencing confirmed the correctness of clones with introduced point mutations. All mutant proteins were successfully produced in *E. coli* BL21(DE3) and originated polypeptides with an apparent molecular mass of 32 kDa that were present in the inclusion body fraction (data not shown).

3.1. H^+ efflux in proteoliposomes containing AtUCP1 mutants

The proton fluxes mediated by AtUCP1 mutants in reconstituted system were assayed by K^+ diffusion potential (created by $[K^+]$ gradient plus valinomycin) in the presence of LA and the internal $[H^+]$ changes were monitored as SPQ fluorescence [25]. The measurements were performed with initial membrane potential set to ~180 mV. An important property of the system, the basal H^+ permeability of lipid bilayer, was assayed in protein-free vesicles (liposomes). After the addition of LA, an internal acidification of the liposomes was observed, indicating the redistribution of the LA molecules in both leaflets of the lipid bilayer. The addition of 1.3 μM valinomycin caused no significant H^+ movement (data not shown), demonstrating a low H^+ permeability of lipid membrane itself. In contrast, proteoliposomes containing wt and AtUCP1 mutants exhibited a significant H^+ efflux in the presence of valinomycin. Fig. 2A illustrates H^+ fluxes in proteoliposomes containing Cys28Ala mutant in the absence and at three different concentrations of LA. The LA-concentration dependence of H^+ efflux represents protein-mediated H^+ transfer. Interestingly, when LA was replaced by HNE, no significant H^+ efflux was observed in the presence of valinomycin in proteoliposomes containing wt AtUCP1 (data not shown).

3.2. Kinetic of LA transport by AtUCP1 mutants

The kinetic of H^+ efflux mediated by the AtUCP1 mutants was evaluated by varying the total LA concentration. Low protein-independent basal H^+ fluxes simulated in protein-free liposomes, under identical LA and valinomycin concentrations and membrane potential showed linear, non-saturable profile (Fig. 3). Data, corrected for the different (lower) volume of proteoliposomes and different protein concentrations were used for estimation of the basal H^+ flux in proteoliposomes. Evaluation of the kinetics of LA-induced H^+ efflux mediated by the mutants under study produced a concentration-dependent, saturable response that exceeded the H^+ fluxes in liposomes (Fig. 3A). The corresponding Eadie–Hofstee plot (Fig. 3B) yielded an apparent K_m for these mutants 1.3- to 2.6-fold higher than for the wt (Table 1). In comparison to wt AtUCP1, the apparent LA-affinity was strongly reduced for mutants Cys 28Ala (2.6-fold), Lys147His (1.8-fold), and Tyr269Phe (2.4-fold) while the affinity of His83Leu and Arg155Leu mutants to

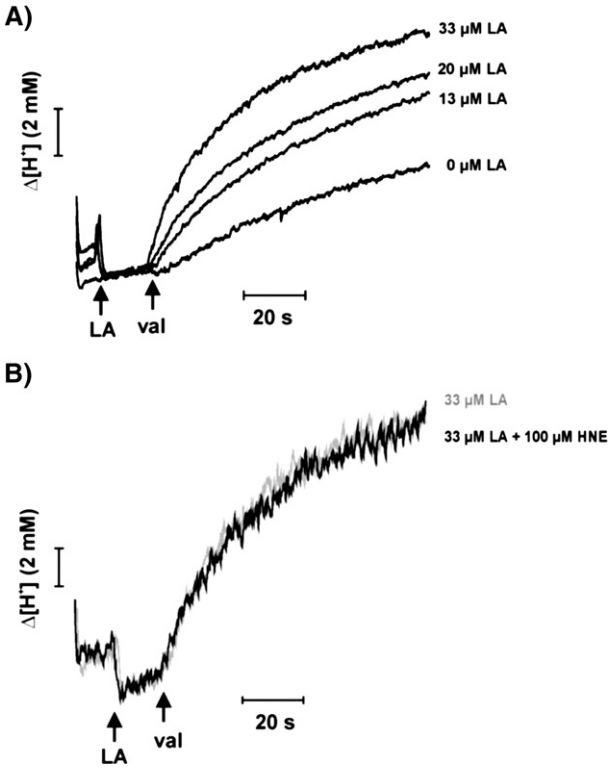


Fig. 2. Representative protein-mediated H^+ efflux in reconstituted proteoliposomes. The measurements were conducted in the presence of 1.3 μM valinomycin (Val) at pH 7.2 at room temperature. (A) Traces show H^+ efflux mediated by the Cys28Ala mutant in the absence or induced by LA at indicated concentrations. (B) Traces show H^+ efflux mediated by wt AtUCP1 in the presence of LA and in the absence (gray trace) or presence (black trace) of HNE.

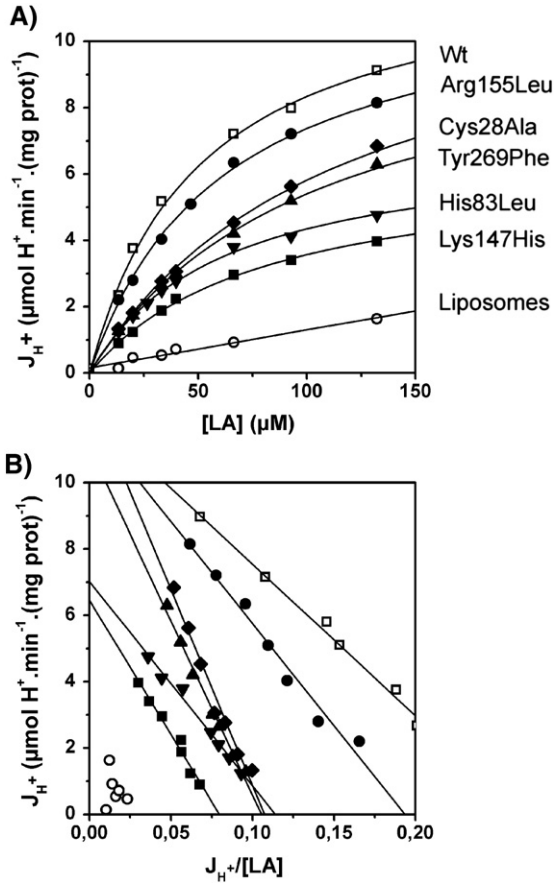


Fig. 3. (A) Kinetics of LA-induced H^+ fluxes in protein-free liposomes (○) and proteoliposomes reconstituted with wt AtUCP1 (□) and mutants Cys28Ala (♦); His83Leu (▼); Lys147His (■); Arg155Leu (●) and Tyr269Phe (▲). Measurements were conducted in the presence of 1.3 μM valinomycin at pH 7.2 at room temperature. The fluxes were calculated per mg of protein (or mg lipids corresponding to 1 mg of protein in case of protein-free liposomes) to yield final rates in μmol H^+ min^{-1} (mg prot)$^{-1}$. Rates of H^+ transport (J) were measured using different concentrations of LA. (B) Eadie–Hofstee plot for kinetics of LA-induced H^+ fluxes in protein-free liposomes and proteoliposomes reconstituted with wt AtUCP1 and mutants (the symbols are according to Panel A). The derived kinetic parameters are listed in Table 1, where the basal flux J_B was subtracted.

LA decreased 1.3-fold and 1.4-fold, respectively. The derived V_{max} values were lower for mutants His83Leu and Lys147His (~50% reduction) with respect to the wt protein (Table 1) while no or a slight change in V_{max} was observed with Cys28Ala, Arg155Leu, and Tyr269Phe mutants. The maximum relative efficiency (V_{max}/K_m ratio) of AtUCP1 mutants reached 0.18 μmol H^+ min^{-1} (mg prot)$^{-1}$ μM^{-1} (Arg155Leu) that represents only 62% of the value found for wt AtUCP1 [0.29 μmol H^+ min^{-1} (mg prot)$^{-1}$ μM^{-1}].

In order to investigate the responses of the studied mutants to HNE, we first determined the kinetics of LA-induced H^+ efflux mediated by wt AtUCP1 in the presence of 10, 30, 60, and 100 μM of HNE. Interestingly, the values of K_m and V_{max} did not alter within the whole range of tested HNE concentrations (Fig. 2B), yielding the single values of 43.8±2.3 μM and 14.5±2.0 μmol H^+ min^{-1} (mg prot)$^{-1}$, respectively.

Table 1
Kinetic parameters for H^+ efflux and ATP inhibition for wild type (WT) and AtUCP1 mutants[a]

	V_{max} (μmol H^+ min^{-1} (mg prot)$^{-1}$)	K_m (μM)	Relative efficiency (μmol H^+ min^{-1} (mg prot)$^{-1}$ μM^{-1})	$K_{i,ATP}$ (mM)
WT	13.2±1.7	46.1±3.5	0.29	0.83±0.04
Cys28Ala	13.0±0.4	118.0±7.9	0.11	3.31±0.18
His83Leu	6.9±0.2	58.4±4.4	0.12	0.93±0.05
Lys147His	6.6±0.2	85.0±5.9	0.08	1.35±0.09
Arg155Leu	11.3±0.9	64.4±4.0	0.18	0.91±0.04
Tyr269Phe	11.4±0.4	109.5±6.5	0.10	0.30±0.02

[a] The values represent the mean±SD from three independent experiments.

3.3. Inhibition of H^+ efflux by PN in proteoliposomes containing AtUCP1 mutants

The inhibitory effects of PN on LA-mediated H^+ efflux in proteoliposomes containing the AtUCP1 mutants were evaluated in the presence of ATP or GDP. The protein-independent, non-specific inhibitory effects of ATP on basal H^+ fluxes were simulated in protein-free liposomes. Under identical LA and valinomycin concentrations and membrane potential, 3 mM ATP had no effect on basal H^+ fluxes (data not shown). The concentration dependence curve for externally added ATP (Fig. 4) at pH 7.2 yielded an apparent K_i of 0.83 mM for wt AtUCP1 as found previously [25]. The dynamic K_i values for ATP obtained for the studied mutants by Lineweaver–Burk analysis are listed in Table 1 and the corresponding concentration dependence curves represented in Fig. 4. The His83Leu and Arg155Leu mutants exhibited ATP inhibition characteristics similar to wt AtUCP1. In contrast, the sensitivity to inhibition by ATP of Tyr269Phe mutant was increased 2.8-fold, whereas Cys28Ala and Lys147His mutants were 4.0-fold and 1.6-fold less sensitive, respectively. The K_i values obtained with GDP were practically the same as found for ATP (data not shown).

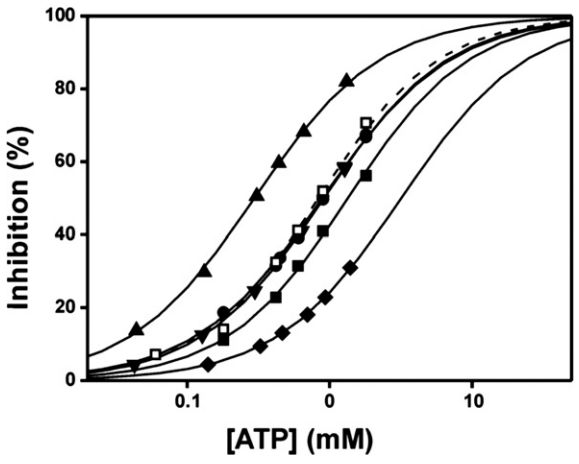


Fig. 4. Inhibition of LA-induced H^+ efflux in proteoliposomes containing AtUCP1 mutants. The Hill plots (solid and dashed lines) represent the concentration dependence of the inhibition of AtUCP1 (only the molecules with the outwardly oriented ATP binding sites were considered [25]). K_i measurements for ATP were performed at pH 7.2 at room temperature with Hill coefficients in the range of 0.95–1.05. Wild type AtUCP1 (□) and mutants Cys28Ala (♦); His83Leu (▼); Lys147His (■); Arg155Leu (●) and Tyr269Phe (▲).

4. Discussion

In this study, point mutations were introduced in AtUCP1 aa residues Lys147, Arg155 and Tyr269, located in the so-called UCP-signatures [27], and in two more residues (Cys28 and His83) found exclusively in pUCP sequences (Fig. 1A and B) [28]. Similar to wt AtUCP1, its mutants mediated H^+ transport across the liposomal membrane that was activated by LA and sensitive to inhibition by PN. Interestingly, AtUCP1 and its mutants were insensitive to activation by up to 100 μM HNE that corroborate the recent findings obtained with mammalian UCP1 [4].

4.1. Arg155Leu mutant

The Arg152 residue is widely conserved in UCPs unless UCP3 (Fig. 1B). The charge neutralization of this residue in mammalian UCP1 led to a ~50% reduction of FA-affinity as well as of its activity [24]. The replacement of the analogous residue in AtUCP1 (Arg155) led to a similar reduction of LA-affinity (by approximately 40%), however, with a slight alteration of V_{max} value, suggesting that a positive charge at this position is crucial for LA-affinity but not for activity of AtUCP1. In line with a mutagenesis study in UCP1 [24], the Arg155Leu mutation did not affect the K_i value for ATP and GDP.

4.2. Lys147His mutant

Previous site-directed mutagenesis experiments in UCP1 revealed that the His pair, His145 and His147, is essential for UCP1-mediated H^+ transport [17,24]. Contrasting with the postulated essentiality of these residues for proton transport, all other UCPs contain only one (UCP3) or no His residues of this pair in its primary aa sequence, although being able to mediate FA-induced uncoupling [29]. Typically, pUCPs have a strong base (Lys) and hydrophobic (Ala or Pro) amino acid residues in the positions corresponding to His pair in UCP1. The presence of Lys residue appears to be crucial for pUCP transport activity, since when a His residue replaced Lys147 present at an equivalent position in AtUCP1, a ~1.8 fold reduction in LA-affinity and ~50% reduction of V_{max} value was observed with an alteration in ATP or GDP inhibition. Therefore, the attenuation of the strong positive charge of Lys147 to His was sufficient to lower 3.6-fold the mutant relative efficiency. On the other hand, a double ZmPUMP mutant containing a complete His pair presented only 1.55-fold higher affinity to LA than the wt protein and no change in its activity [18]. These results are consistent with a key role of Lys147 (a strong base) in pUCP-mediated FA-induced uncoupling being able to replace the two histidines (weak bases) present in mammalian UCP1. Despite these evidences, one cannot exclude the possibility that this residue is intolerant to any amino acid substitution as already suggested [19].

4.3. Tyr269Phe mutant

Residue Phe267 has been previously suggested as being fundamental for PN binding to heterologous UCP1 expressed in yeast. Although its single mutation did not promote qualitative changes in activity of UCP1 in isolated mitochondria, corresponding flow cytometry analyses suggested that this mutation increased the uncoupling of yeast mitochondria *in situ* [22]. Contrary to UCP1, all other UCPs have Tyr residues in the homologous position. This fact led us to further investigate the suggested importance of this Tyr/Phe residue for PN inhibition. The quantitative kinetic characterization of the revertant Tyr 269Phe mutant revealed that its sensitivity to ATP increased almost 3-fold over wt AtUCP1 (Table 1). This finding indicates that the mutation effect is reversible and hence actually confirmed for the first time the very importance of this Tyr/Phe residue for inhibition mechanism of UCPs by PN. Furthermore, about 6-fold lower affinity of AtUCP1 ($K_{i,ATP}$=0.82 mM; [25]), UCP2, and UCP3 ($K_{i,ATP}$=0.76 and 0.65 mM, respectively; [30]) for PN in comparison to mammalian UCP1 ($K_{i,ATP}$= 125 μM; [30]) could be also related to this residue. In addition, the results from mutational analyses of this residue in UCP1 and AtUCP1 corroborate the hypothesis suggesting UCP1 as the evolutionary newest member of the UCP subfamily [29]. UCP1 seems to be the only member of uncoupling protein family involved in thermogenesis [31]. Thus the tight regulation of UCP1 activity should be critical for proper function of this tissue.

4.4. Cys28Ala and His83Leu mutants

The Cys28 and His83 residues, located in the first and second transmembrane domains (Fig. 1A), respectively, were identified as candidates due to their unique conservation in plant UCPs. Residue His83 is exclusively present in pUCPs and is placed in the vicinity of an invariant Arg residue (Arg83 in UCP1) important for PN binding [21] (Fig. 1A). The replacement of His83 (weak base) by a hydrophobic residue (Leu) in AtUCP1 decreased the LA-affinity (by approximately 27%) and the V_{max} (by approximately 52%) but had no effect on ATP inhibition, indicating that the provided positive charge is of significance for optimal proton transport but not for its regulation by purine nucleotides.

The Cys28 residue was found to be probably one of the residues that are crucial for the proper function of pUCPs. The replacement of Cys28 residue for Ala promoted a large decrease of AtUCP1 affinity for both LA (by 61%) and ATP (by 75%). Thus, this residue should be involved in the proper folding of the pUCPs playing a structural rather than functional role. However, no intramolecular S–S bridge within any UCP monomer molecule has been reported. It is also known that the functional unit of uncoupling proteins is a dimer but no involvement of any S–S bridge in monomer interaction was proved so far.

This study brings the first deeper site-directed mutational analysis of a pUCP aimed to reveal the structure–function relationships that might be involved in the mechanism of its uncoupling activity. The AtUCP1 residues His83, Lys147, and Arg155 appear to be associated preferentially with LA-activated proton transport mediated by pUCP whereas Tyr269 residue is important for inhibition of its activity by PN. The plant UCP-specific residue Cys28 should be one of the residues that are essential for proper functioning of pUCPs at all.

Acknowledgments

We are grateful to Dr. P. Arruda for the laboratory facilities. This work is funded by FAPESP (Processo 06/59912-5). R.D.F., J.B. and D.C. are recipients of fellowships from FAPESP.

References

[1] K.D. Garlid, D.E. Orosz, M. Modrianský, S. Vassanelli, P. Ježek, On the mechanism of fatty acid-induced proton transport by mitochondrial uncoupling protein, J. Biol. Chem. 271 (1996) 2615–2620.
[2] M.J. Considine, M. Goodman, K.S. Ecthay, M. Laloi, J. Whelan, M.D. Brand, L.J. Sweetlove, Superoxide stimulates a proton leak in potato mitochondrial that is related to the activity of uncoupling protein, J. Biol. Chem. 278 (2003) 22298–22302.
[3] A.M.O. Smith, R. George Ratcliffe, L.J. Sweetlove, Activation and function of mitochondrial uncoupling protein in plants, J. Biol. Chem. 279 (2004) 51944–51952.
[4] I.G. Shabalina, N. Petrovic, T.V. Kramarova, J. Hoeks, B. Cannon, J. Nedergaard, UCP1 and defense against oxidative stress, 4-hydroxy-2-nonenal effects on brown fat mitochondrial are uncoupling protein 1-independent, J. Biol. Chem. 281 (2006) 13882–13893.
[5] D. Ricquier, J.C. Kader, Mitochondrial protein alteration in active brown fat: a sodium dodecyl sulfate-polyacrylamide gel electrophoretic study, Biochem. Biophys. Res. Commun. 73 (1976) 577–583.
[6] D.G. Nicholls, E. Rial, A history of the first uncoupling protein, UCP1, J. Bioenerg. Biomembranes 31 (1999) 399–418.
[7] G. Argyropoulos, M.-E. Harper, Invited review: uncoupling proteins and thermoregulation, J. Appl. Physiol. 92 (2002) 2187–2198.
[8] J. Mozo, Y. Emre, F. Bouillaud, D. Ricquier, F. Criscuolo, Thermoregulation: what role for UCPs in mammals and birds? Biosci. Rep. 25 (2005) 227–249.
[9] C. Erlanson-Albertsson, The role of uncoupling proteins in the regulation of metabolism, Acta Physiol. Scand. 178 (2003) 405–412.
[10] S. Krauss, C.Y. Zhang, B.B. Lowell, The mitochondrial uncoupling protein homologues, Nat. Rev. 6 (2005) 248–261.
[11] A.E. Vercesi, I.S. Martins, M.A.P. Silva, H.M.F. Leite, I.M. Cuccovia, H. Chaimovich, PUMPing plants, Nature 375 (1995) 24.
[12] A.E. Vercesi, J. Borecký, I.G. Maia, P. Arruda, I.M. Cuccovia, H. Chaimovich, Plant uncoupling mitochondrial proteins, Annu. Rev. Plant Biol. 57 (2006) 383–404.
[13] F.F. Calegario, R.G. Cosso, M.M. Fagian, F.V. Almeida, W.F. Jardim, P. Ježek, P. Arruda, A.E. Vercesi, Stimulation of potato tuber respiration by cold stress is associated with an increased capacity of both plant uncoupling mitochondrial protein (PUMP) and alternative oxidase, J. Bioenerg. Biomembranes 35 (2003) 211–220.
[14] C. Hourton-Cabassa, A.R. Matos, A. Zachowski, F. Moreau, The plant uncoupling protein homologues: a new family of energy-dissipating proteins in plant mitochondria, Plant Physiol. Biochem. 42 (2004) 283–290.
[15] M. Klingenberg, K.S. Echtay, Uncoupling proteins: the issues from a biochemist point of view, Biochim. Biophys. Acta 1504 (2001) 128–143.
[16] M.M. Gonzalez-Barroso, C. Fleury, I. Arechaga, P. Zaragoza, C. Levi-Meyrueis, S. Raimbault, D. Ricquier, F. Bouillaud, E. Rial, Activation of the uncoupling protein by fatty acids is modulated by mutations in the C-terminal region of the protein, Eur. J. Biochem. 239 (1996) 445–450.
[17] M. Bienengraeber, K.S. Echtay, M. Klingenberg, H^+ transport by uncoupling protein (UCP-1) is dependent on a histidine pair, absent in UCP-2 and UCP-3, Biochemistry 37 (1998) 3–8.
[18] R.D. Fávaro, J. Borecký, D. Colombi, I.G. Maia, ZmPUMP encodes a fully functional monocot plant uncoupling mitochondrial protein whose affinity to fatty acid is increased with the introduction of a His pair at the second matrix loop, Biochem. Biophys. Res. Commun. 344 (2006) 194–199.
[19] J. Jiménez-Jiménez, R. Zardoya, A. Ledesma, M.G. de Lacoba, P. Zaragoza, M.M. González-Barroso, E. Rial, Evolutionarily distinct residues in the uncoupling protein UCP1 are essential for its characteristic basal proton conductance, J. Mol. Biol. 359 (2006) 1010–1022.
[20] K.S. Echtay, M. Bienengraeber, M. Klingenberg, Role of intrahelical arginine residues in functional properties of uncoupling protein (UCP1), Biochemistry 40 (2001) 5243–5248.
[21] M. Modrianský, D.L. Murdza-Inglis, H.V. Patel, K.B. Freeman, K.D. Garlid, Identification by site-directed mutagenesis of three arginines in uncoupling protein that are essential for nucleotide binding and inhibition, J. Biol. Chem. 272 (1997) 24759–24762.
[22] F. Bouillaud, I. Arechaga, P.X. Petit, S. Raimbault, C. Levi-Meyrueis, L. Casteilla, M. Laurent, E. Rial, D. Ricquier, A sequence related to a DNA recognition element is essential for the inhibition by nucleotides of proton transport through the mitochondrial uncoupling protein, EMBO J. 13 (1994) 1990–1997.
[23] K.S. Echtay, M. Bienengraeber, M. Klingenberg, Mutagenesis of the uncoupling protein of brown adipose tissue. Neutralization of E190 largely abolished pH control of nucleotide binding, Biochemistry 36 (1997) 8253–8260.
[24] E. Urbánková, P. Hanák, E. Škobisová, M. Růžička, P. Ježek, Substitutional mutations in the uncoupling protein-specific sequence of mitochondrial uncoupling protein UCP1 lead to the reduction of fatty acid-induced H^+ uniport, Int. J. Biochem. Cell Biol. 35 (2003) 212–220.
[25] J. Borecký, I.G. Maia, A.D.T. Costa, P. Ježek, H. Chaimovich, P.B.M. Andrade, A.E. Vercesi, P. Arruda, Functional reconstitution of *Arabidopsis thaliana* plant uncoupling mitochondrial protein (*At*PUMP1) expressed in *Escherichia coli*, FEBS Lett. 505 (2001) 240–244.
[26] D.E. Orosz, K.D. Garlid, A sensitive new fluorescence assay for measuring proton transport across liposomal membranes, Anal. Biochem. 210 (1993) 7–15.
[27] P. Ježek, E. Urbánková, Specific sequence motifs of mitochondrial uncoupling proteins, IUBMB Life 49 (2000) 63–70.
[28] P. Hanák, P. Ježek, Mitochondrial uncoupling proteins and phylogenesis-UCP4 as the ancestral uncoupling, FEBS Lett. 495 (2001) 137–141.
[29] J. Borecký, I.G. Maia, P. Arruda, Mitochondrial uncoupling proteins in mammals and plants, Biosci. Rep. 21 (2001) 201–212.
[30] M. Jabůrek, M. Vařecha, R.E. Gimeno, M. Dembski, P. Ježek, M. Zhang, P. Burn, L.A. Tartaglia, K.D. Garlid, Transport function and regulation of mitochondrial uncoupling proteins 2 and 3, J. Biol. Chem. 274 (1999) 26003–26007.
[31] J. Borecký, A.E. Vercesi, Plant uncoupling mitochondrial protein and alternative oxidase: energy metabolism and stress, Biosci. Rep. 25 (2005) 271–286.