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Mutational analysis of *Arabidopsis thaliana* plant uncoupling mitochondrial protein

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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]).

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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

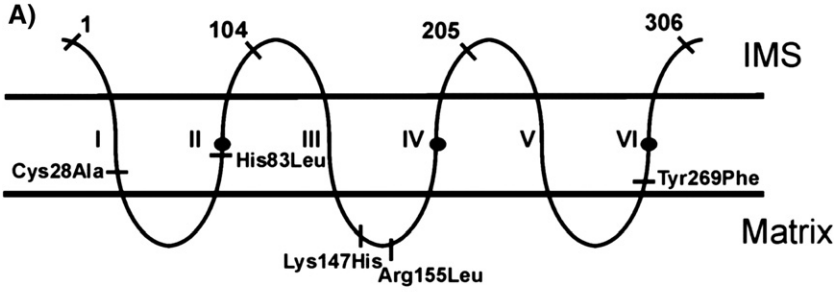
2.1. Materials

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.