

# Important amino acid residues of potato plant uncoupling protein (*St*UCP)

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## Abstract

Chemical modifications were used to identify some of the functionally important amino acid residues of the potato plant uncoupling protein (*St*UCP). The proton-dependent swelling of potato mitochondria in K<sup>+</sup>-acetate in the presence of linoleic acid and valinomycin was inhibited by mersalyl ( $K_i = 5 \mu\text{M}$ ) and other hydrophilic SH reagents such as Thiolyte MB, iodoacetate and 5,5'-dithio-bis-(2-nitrobenzoate), but not by hydrophobic N-ethylmaleimide. This pattern of inhibition by SH reagents was similar to that of brown adipose tissue uncoupling protein (UCP1). As with UCP1, the arginine reagent 2,3-butadione, but not N-ethylmaleimide or other hydrophobic SH reagents, prevented the inhibition of *St*UCP-mediated transport by ATP in isolated potato mitochondria or with reconstituted *St*UCP. The results indicate that the most reactive amino acid residues in UCP1 and *St*UCP are similar, with the exception of N-ethylmaleimide-reactive cysteines in the purine nucleotide-binding site.

## Key words

- Plant mitochondria
- Uncoupling protein
- Chemical modification
- Mitochondrial swelling
- Reconstitution

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Research supported by PRONEX,  
FAPESP, CNPq and PADCT/CNPq.  
A.D.T. Costa was the recipient of a  
FAPESP fellowship. Some chemicals  
were purchased through a grant  
(No. 301/98/0568) from the Grant  
Agency of the Czech Republic.

Received April 28, 2000

Accepted September 13, 2000

## Introduction

The functionally well-characterized plant uncoupling mitochondrial protein (PUMP) (1-13) has been cloned from potato (*St*UCP) (14) and *Arabidopsis thaliana* (*At*PUMP) (15) gene libraries. We provided evidence that potato PUMP is a product of the *St*UCP gene (16). Consequently, PUMP has been recognized as a member of the uncoupling protein (UCP) subfamily, homologous with mammalian UCPS such as UCP1 of brown adipose tissue mitochondria (2,17-19), the ubiquitous UCP2 (20), UCP3 of striated muscle (21), and two brain-specific uncoupling proteins, UCP4 (22) and BMCP1 (23). The physiological roles of UCPS in mammals include nonshivering thermogenesis in neonates (UCP1), regulation of weight balance and inflammatory responses such as fever (UCP2), nonshivering thermogenesis in

skeletal muscle (UCP3), and possibly the prevention or regulation of apoptosis in the brain (UCP4, BMCP1). We have hypothesized (2,4-6) that, in plants, *St*UCP may be responsible for a respiratory burst in climacteric fruits and for all physiological events when a sudden cessation of ATP synthesis is required such as during seed formation and senescence. Several climacteric fruits such as tomato (7), banana, mango, apple and others (2) contain *St*UCP. A mild thermogenesis mediated by *St*UCP can accelerate respiration and, hence, metabolic rates (2) and mild *St*UCP-mediated uncoupling leads to a decreased formation of reactive oxygen species (8). Both functions are beneficial for plant growth and development. Thus, fully activated thermogenesis could facilitate plant growth at low temperature, e.g., in roots, tubers (9), or during seed germination. *St*UCP may also play specific roles during

plant senescence and could contribute to processes that maintain the seed dormancy.

All UCPs presumably enable the passage of fatty acid (FA) anions and thus promote FA cycling, leading to H<sup>+</sup> uniport mediated by neutral FA molecules, which results in uncoupling (2,19,24,25). The FA cycling mechanism has been confirmed for UCP1 (26), UCP2 and UCP3 (24) and *StUCP* (6). UCP1 also translocates a wide variety of monovalent, unipolar anions, including short-, medium-, and long-chain alkylsulfonates (27). Hexane and undecanesulfonate transport has also been demonstrated for *StUCP* (5,6). Nevertheless, *StUCP* is not able to translocate small hydrophilic anions such as Cl<sup>-</sup> and pyruvate (5,6) which are good transport substrates for UCP1 (2,19,27,28).

The protein chemistry of *StUCP* has not been studied to the same extent as has UCP1. A 32-kDa *StUCP* has been characterized as a hydrophobic protein which is not retained on hydroxylapatite in the detergent micellar solution (1,6,7). Chemical modifications of reactive amino acid residues, the cleavage pattern produced by proteases, and ligand binding (except for studies with 8-azido-ATP (13)) have not been studied in *StUCP*. In the present study, we examined the effects of several chemical modifiers on *StUCP*-mediated transport as well as *StUCP* inhibition by purine nucleotides. Our results clearly show that the pattern of reactive amino acid residues in *StUCP* is similar to that of UCP1, with the exception that no N-ethylmaleimide (NEM)-reactive cysteines were found in the purine nucleotide-binding site of *StUCP*.

## Material and Methods

### Biological material and chemicals

Potatoes (*Solanum tuberosum*, L. cv. Bintje) were purchased locally. Nucleotides, bovine serum albumin (BSA), valinomycin,

nigericin, iodoacetate, linoleic acid, N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), N-[2-hydroxyethyl]piperazine-N'-[2-ethane] sulfonic acid (HEPES), tetraethylammonium hydroxide (TEA-OH), carbonyl cyanide trifluoromethoxyphenylhydrazine (FCCP), ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), 5,5'-dithio-bis-(2-nitrobenzoate) (DTNB), 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), 2,4,6-trinitrobenzenesulfonic acid (TNBS), pyridoxalphosphate, phenylglyoxal, propranolol, phenylarsine-oxide, eosinmaleimid, 2,3-butadione and phospholipids were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The fluorescent probe SPQ (6-methoxy-N-(3-sulfopropyl) quinolinium) and thiol reagent Thiolyte<sup>®</sup> MB were from Calbiochem (La Jolla, CA, USA). The potassium probe PBFI (potassium-binding benzofuraneisophthalate) was from Molecular Probes (Eugene, OR, USA). All other reagents were commercial products of the purest grade available.

### Isolation of mitochondria and protein determination

Potato mitochondria were isolated as described previously (5,8,9) in medium containing 250 mM sucrose, 10 mM HEPES, pH 7.2, and 0.3 mM EGTA. The protein concentration was 30-40 mg/ml, as determined by the biuret method. A crude fraction was used for swelling studies and for most of the isolations. For some isolations, a Percoll gradient centrifugation was used to remove contamination by plastid proteins, starch and other substances. Qualitatively, transport measurements using the crude fraction gave identical results as those performed with Percoll-purified mitochondria.

### Swelling assay of *StUCP* transport function

Proton-dependent swelling of potato mi-

tochondria (0.2 mg protein/ml) in  $K^+$ -acetate (55 mM  $K^+$ -acetate, 5 mM  $K^+$ -HEPES, 0.2 mM Tris-EDTA, 0.1 mM Tris-EGTA, pH 6.9) initiated by valinomycin in the presence of linoleic acid (16  $\mu$ M) has been used as a standard assay for *St*UCP-mediated transport (5). Since valinomycin allows the uniport uptake of  $K^+$  and neutral acetic acid is able to penetrate the lipid bilayer, an efflux of  $H^+$  is necessary to induce swelling. In our assay, this  $H^+$  efflux was concomitant with linoleic acid cycling which allowed swelling since *St*UCP mediated the uptake of linoleic acid anion, while protonated linoleic acid passed spontaneously through the lipid bilayer by a flip-flop mechanism and released  $H^+$  externally. Hexanesulfonate uniport was assayed as valinomycin-induced swelling in medium containing 51.1 mM  $Na^+$ -hexanesulfonate, 30.8 mM  $K^+$ -HEPES, pH 7.2, 190  $\mu$ M Tris-EDTA and 95  $\mu$ M Tris-EGTA. The side effects caused by the chemical modifiers used, including the induction of mitochondrial swelling without the addition of ionophore and membrane stiffening, were controlled by performing a swelling assay in  $K^+$ -acetate containing nigericin, which does not depend on protein carriers. When a decrease in this rate ( $v_{Nig}[c]$ ) was observed at a given concentration  $[c]$  of modifier, the rates of valinomycin-induced *St*UCP-mediated swelling were corrected by multiplying this decrease by the factor  $v_{Nig}[c = 0]/v_{Nig}[c]$ .

#### Chemical modifications of potato mitochondria

For carrying reactions, mitochondria were resuspended in the sucrose isolation medium (5 mg protein/ml) and aliquots of stock solutions (aqueous or in dimethylsulfoxide) of various reagents were added and incubated for 1 h (unless otherwise indicated) at 0°C. For NEM, DTNB and phenylglyoxal, pH was raised to 8.2 by adding 20 mM Tris-HEPES, pH 8.4, to the stock solution and 2  $\mu$ M propranolol was added.

#### *St*UCP isolation and reconstitution

*St*UCP was isolated from potato mitochondria on hydroxylapatite as described previously (6). The same procedure was used for the isolation of potato mitochondria pretreated with Thiolyte MB or 2,3-butadione. Thirty micrograms of isolated *St*UCP was incorporated into liposomes by detergent removal using Bio-Beads SM2 (BioRad, Hercules, CA, USA) and the vesicles were depleted of the external probe by passage through Sephadex G25-300 spin columns (6). The FA-induced  $H^+$  fluxes initiated by valinomycin were monitored either as the counterflux of  $K^+$ , using PBFI (6,7), or by TES anion quenching of SPQ, as described previously (6,7), on an F-4010 Hitachi fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan). For PBFI monitoring, the vesicles (25  $\mu$ l per assay) contained 75 mM TEA sulfate, 75 mM TEA-TES, pH 7.2, 0.05 mM  $K_2SO_4$  and 300  $\mu$ M PBFI. The external medium contained 75 mM  $K_2SO_4$  and 75 mM TEA-TES, pH 7.2. For SPQ monitoring, the vesicles (25  $\mu$ l per assay) contained 84.4 mM TEA sulfate, 28.85 mM TEA-TES, pH 7.2 ( $[TEA]$  was 9.2 mM) and 0.6 mM Tris-EGTA. In the external medium, 84.4 mM  $K_2SO_4$  replaced TEA sulfate.

## Results

#### Effect of hydrophilic SH reagents on *St*UCP-mediated transport in mitochondria

Proton-dependent swelling of potato mitochondria initiated by valinomycin in  $K^+$ -acetate containing linoleic acid was reversibly inhibited by the organomercurial SH reagent mersalyl with an apparent  $K_i$  of 5  $\mu$ M (Figure 1A, only 10-s preincubations). This type of swelling reflected the ability of *St*UCP to translocate linoleic acid anions (5). The effect of mersalyl can be considered as a specific inhibition, since swelling independent of a protein carrier, i.e., the nigericin-induced swelling in  $K^+$ -acetate, was not affected up to 100  $\mu$ M

mersalyl (Figure 1A). Above 100  $\mu\text{M}$ , and above 40  $\mu\text{M}$  in the presence of linoleic acid, mersalyl induced nonspecific permeability changes which were observed as mitochondrial swelling without the ionophore. Some mitochondrial preparations were more sensitive to mersalyl and this made measurements with them more difficult.

To avoid the interference of nonspecific permeability changes, we used Thiolyte MB, a covalently interacting SH modifier. Mitochondria were preincubated for 1 h with increasing Thiolyte MB doses (Figure 2). The  $\text{IC}_{50}$  for Thiolyte MB was around 500 nmol/mg protein. Carrier-independent swelling was not significantly affected by Thiolyte MB, indicating that the modification of the SH groups in

*S*tUCP inhibits the transport activity of this protein. Carboxymethylation by iodoacetate (which also affects SH groups) also inhibited *S*tUCP transport activity at higher doses ( $\text{IC}_{50}$  of 100  $\mu\text{mol/mg}$  protein), but only with 10-s preincubations (Figure 1B). Ellman's reagent (DTNB) inhibited the activity by 18 and 31% at 1000 and 3000 nmol/mg protein, respectively, after a 2-h incubation, as calculated from the rates corrected for the nonspecific effect (incubations at  $\text{pH} > 8$  lead to preswelling after a few hours). In contrast, NEM and other hydrophobic SH reagents (eosinmaleimide, phenylarsineoxide) were not inhibitory up to 10  $\mu\text{mol/mg}$  protein. Hexanesulfonate uniport via *S*tUCP was partially inhibited by hydrophilic SH reagents, e.g., by 1000 nmol Thiolyte MB/mg protein.

Figure 1 - Inhibition of proton-dependent swelling of potato mitochondria by mersalyl (A) and iodoacetic acid (B) in  $\text{K}^+$ -acetate buffer. The inhibition by mersalyl of *S*tUCP-mediated transport (filled circles) and nigericin-mediated, protein-independent swelling (open squares) are specific and nonspecific effects of mersalyl, respectively. The solid line represents the fit of the data using the Hill equation with a Hill coefficient of 2, yielding an apparent  $K_i$  of 5  $\mu\text{M}$ . B, The iodoacetate dose-response curve, yielding an  $\text{IC}_{50}$  around 100  $\mu\text{mol/mg}$  protein, has already been corrected for the nonspecific effect produced by this compound. The correction and other details of the measurements are described in Material and Methods.

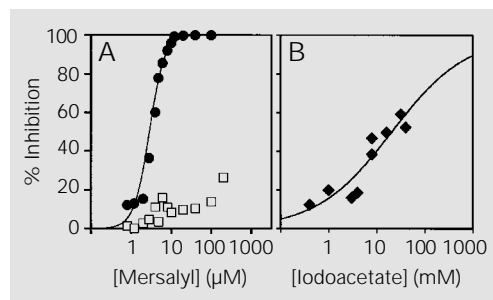
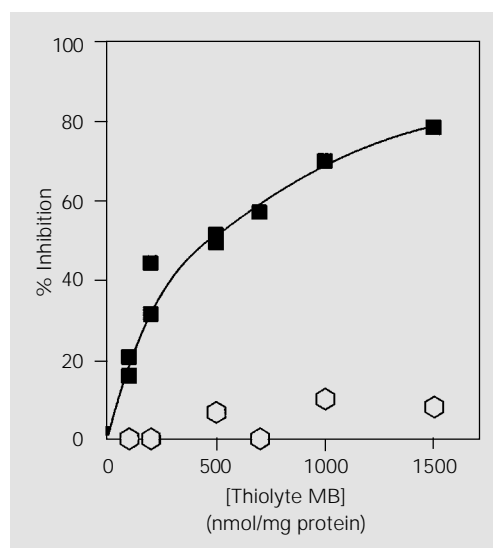


Figure 2 - Inhibition of proton-dependent swelling of potato mitochondria by Thiolyte MB in  $\text{K}^+$ -acetate buffer. The points show the specific effect of Thiolyte MB on *S*tUCP-mediated transport in potato mitochondria (squares) and the negligible, nonspecific effect on nigericin-mediated, protein-independent swelling (diamonds). The solid line represents the best fit curve for the data, when omitting the fourth point. Further details of the measurements are described in Material and Methods.



#### Effect of arginine reagents on ATP inhibition of *S*tUCP-mediated transport

Reagents specific for other amino acid residues did not inhibit transport or prevent the inhibition by ATP at doses up to 10  $\mu\text{mol/mg}$  protein. The reagents tested included DIDS, TNBS and lysine-specific pyridoxalphosphate. Only an arginine-specific reagent, 2,3-butadione, completely prevented the inhibition of linoleic acid transport by 4 mM ATP (Figure 3) at doses above 100 nmol/mg protein (see inset in Figure 3). Thus, a 1-h incubation with 4000 nmol/mg protein 2,3-butadione shifted the ATP dose-response curve so that the extrapolated apparent  $K_i$  was much greater than 10 mM (Figure 3). Surprisingly, phenylglyoxal, a more bulky arginine reagent, had no effect at doses up to 10  $\mu\text{mol/mg}$  protein. NEM, which prevented nucleotide inhibition of UCP, also had no effect on ATP inhibition of *S*tUCP (data not shown).

#### Confirmation of the effects of 2,3-butadione and Thiolyte MB using reconstituted *S*tUCP

The effect of 2,3-butadione on *S*tUCP reconstituted into proteoliposomes after

premodification by 2,3-butadione in mitochondria was identical to that found in potato mitochondria. 2,3-Butadione prevented purine nucleotide inhibition of H<sup>+</sup> efflux, including inhibition by GTP, when the H<sup>+</sup> efflux was monitored with the fluorescent probe PBF1 concomitant with K<sup>+</sup> influx (Figure 4). The inhibitory effect of Thiolyte MB was also confirmed for isolated *St*UCP reconstituted into liposomes for which linoleic acid uniport or concomitant H<sup>+</sup> efflux was detected by TES quenching of the fluorescent probe SPQ (Figure 5). Reconstituted Thiolyte MB-modified *St*UCP showed no transport activity (Figure 5).

## Discussion

The pattern of reactive amino acid residues in *St*UCP was surprisingly similar to that of mammalian UCP1 (29-33; for reviews, see 17-19). This similarity suggests that the structures of *St*UCP and UCP1 are very likely to be closely related, despite only about 40% identity in their sequences (14,15).

The chemical modification of reactive amino acid residues in proteins has been widely used to study protein structure/function relationships. Site-directed mutagenesis has shown that the identification of a residue as essential for a given function is not a straightforward task. In many cases, the effects of modifiers differ from the phenotypes of the corresponding substitution mutants. Interference by the reagent and/or the mutation with the protein function may indicate that i) the residue is essential for that function, i.e., is involved in the required functional interactions (in this case, the substitution mutants have an identical phenotype), ii) the modification of the residue produces steric hindrances which are the actual cause of the altered function (substitution mutations show no such effect), or iii) the residue is important for maintaining a proper conformation of the protein and cannot retain this position after being modified or mutated.

With UCP1, case (i) is valid for its Arg 276, whereas case (ii) has been indicated for its cysteine residues.

When Arg 276 was either substituted in a mutated UCP1 protein (34) or modified by phenylglyoxal and 2,3-butadione (32), purine nucleotide binding and gating were absent. Since the proximal third matrix segment was photolabeled at three different positions with 8-azido-, 2-azido- and 3'-O-(5-fluoro-2,4-dinitrophenyl) adenosine 5'-triphosphate (FNDP-ATP) (35), and since the deletion of residues 261-269 resulted in the lack of nucleotide inhibition (36), it was concluded that the main location of the nucleotide-binding site in UCP1 was located between the fifth and sixth transmembrane segments. This site probably forms a water-filled cavity which penetrates deeply into the membrane close to the opposite surface (35). This cavity in UCP1 is lined with SH residues (C213, C224, C253, C287, C304, and possibly C188). Studies on these residues identified the case (ii) described above, since SH substitution mutants of UCP1 have no disrupted binding or transport (33).

The modification of UCP1 by hydrophobic and hydrophilic SH reagents drastically reduces inhibition by GDP (31). In contrast to UCP1, NEM did not prevent ATP inhibition of transport in *St*UCP. However, trans-

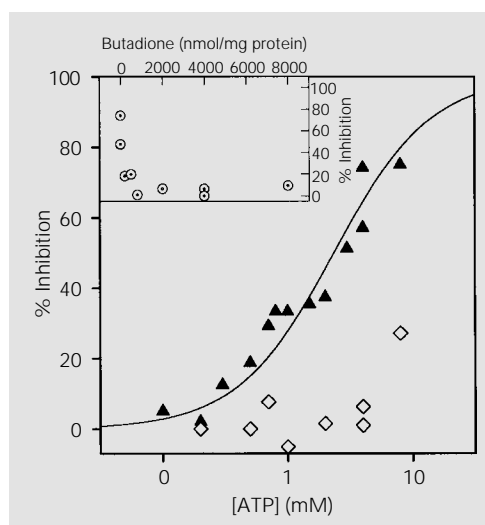


Figure 3 - Prevention of ATP inhibition of *St*UCP-mediated transport following modification of potato mitochondria with 2,3-butadione. The inhibition by ATP of *St*UCP-mediated proton-dependent swelling in K<sup>+</sup> acetate buffer vs log [ATP] is shown for unmodified potato mitochondria (triangles) and mitochondria premodified with 4000 nmol/mg protein 2,3-butadione (diamonds). Inset, Inhibition by 4 mM ATP vs butadione dose in the preincubations. The assay conditions are described in Material and Methods.

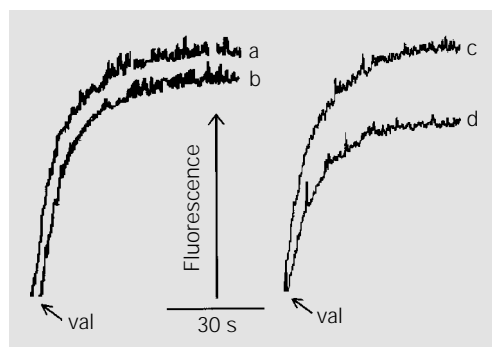


Figure 4 - Lack of inhibition by GTP in proteoliposomes containing *StUCP* from mitochondria modified by 2,3-butadione.  $H^+$  efflux induced by 1.3  $\mu M$  valinomycin (val) in the presence of 53  $\mu M$  linoleic acid was monitored using the fluorescent probe PBFI to measure the concomitant  $K^+$  influx in proteoliposomes containing *StUCP* isolated from mitochondria modified by 2,3-butadione (4000 nmol/mg protein) (traces a,b). The responses of unmodified, reconstituted *StUCP* (control) are also shown (traces c,d). Traces a,c: No further additions; traces b,d: addition of 4 mM Tris-GTP. Vesicles (25  $\mu l$  per assay) contained 75 mM TEA sulfate, 75 mM TEA-TEES, pH 7.2, 0.05 mM  $K_2SO_4$  and 300  $\mu M$  PBFI. The external medium contained 75 mM  $K_2SO_4$  and 75 mM TEA-TEES, pH 7.2.

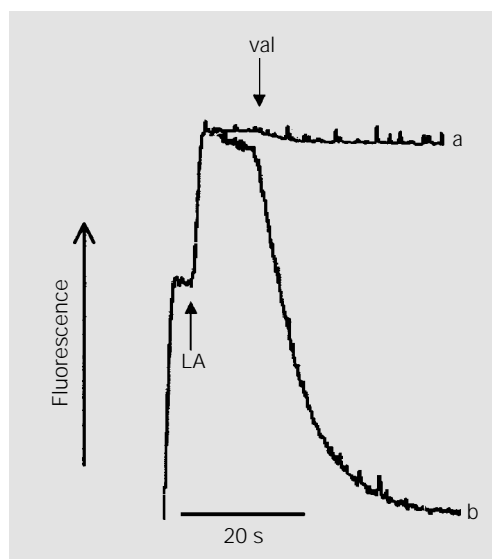


Figure 5 - Lack of  $H^+$  efflux in proteoliposomes containing Thiolyte MB-modified *StUCP*. *StUCP* from mitochondria treated with Thiolyte MB (1000 nmol/mg protein) were isolated and reconstituted into vesicles (trace a). The response of normal reconstituted *StUCP* (control) is shown in trace b.  $H^+$  efflux was monitored by TES quenching of the fluorescent probe SPQ. The addition of 53  $\mu M$  linoleic acid (LA) caused internal acidification of the vesicles, resulting in the flip-flop of neutral fatty acids into the inner lipid leaflet and subsequent dissociation in the internal medium. *StUCP* function was seen as an  $H^+$  efflux (internal alkalization, indicated by the decrease in SPQ fluorescence), initiated by 1.3  $\mu M$  valinomycin (val). This efflux was suppressed in Thiolyte MB-modified samples. Vesicles (25  $\mu l$  per assay) contained 84.4 mM TEA sulfate, 28.85 mM TEA-TEES, pH 7.2, ([TEA] was 9.2 mM) and 0.6 mM Tris-EGTA. In the external medium, 84.4 mM  $K_2SO_4$  replaced TEA sulfate.

port was inhibited by the arginine reagent 2,3-butadione. These findings suggest a probable difference between the purine nucleotide-binding site of UCP1 and *StUCP* and indicate that *StUCP* does not contain modifiable SH groups at or close to the nucleotide-binding site. Alternatively, SH groups may not be important for maintaining the integrity of *StUCP* conformation. These findings agree with the amino acid sequence of potato plant UCP (14,16). Thus, C188 of UCP1 is conserved in UCP2 and UCP3, but is substituted by A197 in *StUCP* (14). Of the two cysteines conserved in the fifth  $\alpha$ -helix of UCP1, 2 and 3, the first, C234, is shifted two residues towards the matrix in *StUCP* such

that its position in the  $\alpha$ -helix is occupied by F231. The second SH (C213 of UCP1) is not conserved in *StUCP* and is substituted by T220. The similarity of the purine nucleotide-binding site in *StUCP* and UCP1 is reflected by the effect of 2,3-butadione, which probably interacts with the conserved arginines in UCPs (and in the mitochondrial carrier gene family as a whole), such as R276 of UCP1 (37), which corresponds to R281 and R278 in *StUCP* and *AtUCP*, respectively (14,15).

Hydrophilic, but not hydrophobic, SH reagents were good inhibitors of UCP1-mediated FA-induced  $H^+$  transport (30). Similarly, in *StUCP* only hydrophilic SH reagents

inhibited *St*UCP-mediated transport of linoleic acid and hexanesulfonate, while hydrophobic SH reagents, arginine, lysine and other modifiers had no effect. Hence, inhibition by hydrophilic SH reagents is common to *St*UCP and UCP1. This inhibitory effect on UCP1 has not yet been fully explained. The SH groups which maintain the integrity of the translocation pathway or, alternatively, participate directly in the translocation mechanism, are probably distinct from those interacting with NEM (in UCP1) and interfere with nucleotide binding after modification

(31). These SH groups are probably located at yet unknown similar positions in the *St*UCP sequence. In addition, the type of interference by SH reagents with the *St*UCP translocation mechanism is likely to be the same as for UCP1. A possible candidate for such a residue is C90, located in the second  $\alpha$ -helix of *St*UCP, which does not have any counterpart in the sequences of UCP1, 2 and 3. Residue C24 of UCP1, absent in *St*UCP, may serve a similar function for C90 in *St*UCP.

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