EFFECTS OF N,N'-BIS-(4-TRIFLUOROMETHYLPHENYL)-UREA ON ISOLATED PLANT MITOCHONDRIA AND THYLAKOID MEMBRANES

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Key Word Index - *Solanum tuberosum*; Solanaceae; *Spinacia oleracea*; Chenopodiaceae; phenylurea; plant mitochondria; thylakoids; uncoupling activity.

Abstract

The N,N'-bis-(4-triftuoromethylphenyl)-urea is demonstrated to be a powerful uncoupler of ATP formation in plant mitochondria and thylakoid membranes. In mitochondria, the full uncoupling effect was obtained at a concentration of 3 µM uncoupling agent in the medium, which corresponded to an inner mitochondrial concentration of 7.2 nmol uncoupling agent mg^{-1} protein. The full uncoupling concentration was displaced from 3 to 0.6 µM when bovine serum albumin was omitted from the reaction medium. Up to 10 µM, the studied compound acted as a strictly selective uncoupler. At concentrations higher than 10 µM, a selective inhibition of electron transfer occurred at the level of the external NADH dehydrogenase of the inner mitochondrial membrane; the I_{50} value was 25 µM. In thylakoid membranes, the full uncoupling effect was obtained for a concentration of 0.6 µM uncoupling agent in the medium, which corresponded to a concentration of 25 nmol uncoupling agent mg^{-1} chlorophyll. The addition of bovine serum albumin (0.1%) to the reaction medium reversed this effect. For concentrations greater than 2 µM, inhibition of PSII electron transfer was observed. Full inhibition was obtained at 15 µM uncoupling agent and corresponded to 650 nmol uncoupling agent mg^{-1} chlorophyll. In marked contrast with classical uncouplers such as CCCP (m-chlorophenylhydrazone) or

substituted phenols, the N,N'-bis-(4-triftuoromethylphenyl)-urea cannot be classified among the protonophoric class of uncouplers, due to its inability to exchange protons at pHs between 2 and 9. Its uncoupling action must therefore be attributed either to conformational changes of a transmembrane protein present both in mitochondria and in thylakoid membranes, or to a modification of the arrangement of the phospholipid membrane bilayers.

Introduction

Many substituted phenylureas are well known as PSII inhibitors, acting efficiently on light-dependent electron transfer in chloroplasts [1]. In some technical preparations of diuron [N-(3,4-dichlorophenyl)-N'-dimethyl- urea] an impurity is present which acts as a potent uncoupler [2]. Its structure was established as being that of N,N'-bis-(3,4-dichlorophenyl)-urea. The physiological interest of this molecule led us to synthesize many derivatives of the same chemical family, including N,N'- bis-(4-trifluoromethylphenyl)-urea which was found to be a very toxic molecule towards isolated plant mitochondria and thylakoid membranes.

Results

Synthesis and physical constants of N,N'-bis-(4-trijluoromethylphenyl)-urea

Aniline is known to form symmetrical phenylureas in combination with phenylisocyanate [2-4]. Therefore, N,N'-bis-(4-triftuoromethylphenyl)-urea ($USpCF_3$) was synthesized by using an aniline derivative with the appropriate substitution pattern and a phenylisocyanate with the same substitution pattern on the phenyl ring (see Experimental). The $USpCF_3$ was crystallized several times and its structure carefully characterized: UV λEtOH max (log ε): 264 (4.7); ^{1}H NMR (200 MHz, DMSO-d_6, TMS); δ7.63 (8H, dd, H-2 and H-3), 9.2 (2H, s, N-H); ^{13}C NMR (50MHz, DMSO-d_6); δ152.2 (s, C=O), 143.2 (s, C-1), 122.5 (q, CF_3), 127.2 (s, C-4), 118.2 (s, C-2), 126.0 (d, C-3).

The partition coefficient (P) of $USpCF_3$ was determined by a conventional octanol-water measurement (-log P=3.8), and its solubility in distilled water was found to be close to 5 µM. However, the presence of bovine serum albumin (BSA) in the mitochondrial suspension medium greatly increased the solubility of $USpCF_3$, showing that urea is able to bind to this protein. The solubility increase is also observed when BSA is replaced by

mitochondria or chloroplasts.

Uncoupling activity of $USpCF_3$

Mitochondrial oxygen consumption for electron transfer from succinate to O_2, in the presence of ADP, in a medium containing 0.1% BSA (state III = 262 nmol O_2 min^{-1} mg^{-1} mitochondrial protein) is shown in Fig. 1A. When all the ADP had been utilized, the oxygen consumption (state IV) fell to 37 nmol O_2 min^{-1} mg^{-1} protein and the addition of $USpCF_3$ produced a sharp increase in the rate of electron transfer. Both CCCP (5 µM) and ADP (200 µM), if added at this stage, had no effect. These findings demonstrate that the compound under study, at 3 µM, is able to fully uncouple electron transfer. The same uncoupling effect was observed when succinate was replaced by α-ketoglutarate, exogenous NADH or pyruvate. The spectrophotometric measurement of mitochondrial swelling [5], confirms the existence of this uncoupling activity (Fig. 2). A rapid passive swelling in NH_4CI or NH_4NO_3 isoosmotic medium was obtained upon addition of $USpCF_3$, indicating the entry of H^+ into the mitochondrial matrix space. The lowest concentration which causes complete uncoupling is 3µM.

Half (D_{50}) and full (D_{100}) uncoupling effects were obtained for concentrations in the medium of 5.9 nmol $USpCF_3$ mg^{-1} protein and 13.6 nmol $USpCF_3$ mg^{-1} protein, respectively. When the mitochondrial protein concentration in the medium changed from 0.2 to 1.2 mg protein ml^{-1} , the concentrations in the medium which induced D_{50}, (as expressed by the total amount of $USpCF_3$ introduced in the reaction vessel), remained at a level of 5.8 ± 0.4 nmol mg^{-1} protein.

Cautiously broken class A chloroplasts gave rise to true class C chloroplasts which were unable to perform the Mehler reaction. After addition of methylviologen, they carried out the light-dependent transfer of electrons from water to methylviologen, the re-oxidation of which led to the formation of hydrogen peroxide. This reaction led to an oxygen consumption which was greatly increased by the addition of 0.6 µM $USpCF_3$ (Fig. 3A). This increase remained light-dependent, and a further addition of NH_4CI (5 mM), used as reference uncoupler, did not change the rate of oxygen consumption. $USpCF_3$, therefore, totally uncouples the photophosphorylation system. Under our conditions (23 µg chlorophyll), this full uncoupling effect corresponds to a concentration in $USpCF_3$ of 26 nmol mg^{-1} chlorophyll in the medium. D_{50} was obtained with 13 nmol $USpCF_3$ mg^{-1} chlorophyll in the medium. The same result was obtained when PSI operates alone (ascorbate+DPIP as electron and proton donor; methylviologen, as electron acceptor; in

the presence of a PSII inhibitor). This uncoupling effect probably explains the full inhibition of O_2 evolution in class A chloroplasts which was obtained at 0.6 µM $USpCF_3$ (Fig. 3C), the inhibitory effect of $USpCF_3$ on the photodependent electron transfer being unable to explain such an inhibition of O_2 evolution (Fig. 3B, see below).

In order to see if the uncoupling effect of $USpCF_3$ on phosphorylation processes can be explained by the existence of H^+ transmembrane transport (as is the case of the classical protonophoric uncouplers such as CCCP [6], pentachlorophenol [7] or platanetin [8]), the ability of $USpCF_3$ to change from a neutral to an ionized form was studied. In order to do this, UV spectral shifts dependent on pH changes (between 9 to 2) were looked for. No significant spectral variation of the product was observed when changing the pH of the medium (Fig. 4A). This indicates that $USpCF_3$ remains in a stable neutral form, whatever the pH. The same result was obtained by the classical method of pK_a measurement (Fig. 4B). Under the same experimental conditions, the reference protonophore uncoupler CCCP presented a pK_a value of 5.3 with both methods (Fig. 4).

The uncoupling effect of $USpCF_3$ in mitochondria was greatly increased when BSA was omitted from reaction medium. Under this condition, D_{100} decreased from 3 to 0.6 µM (Fig. 1B). A further addition of 0.1% BSA at this stage re-established oxidative phosphorylation. This indicates the reversibility of the uncoupling activity induced by $USpCF_3$ (Fig. lC). Similar results were also obtained with class A chloroplasts and thylakoïd membranes (results not shown).

The high UV specific absorbance of $USpCF_3$ [λEtOH max (log ε):): 264 (4.7)] allowed us to measure its partition rate between the medium and the mitochondria when the mitochondrial concentration increased (Fig. 5A). In a reaction medium devoid of BSA, $USpCF_3$ at 5 µM attained its solubility limit. In the presence of BSA and mitochondria, the solubility of $USpCF_3$ can easily reach 20 µM and an equilibrium was established between the medium and the mitochondria, dependent on the concentration of the mitochondrial suspension. For an amount of mitochondrial protein between 1 and 1.4 mg ml^{-1}, 0.4 µM $USpCF_3$ remains in the medium containing BSA 0.1%.

When the concentration of $USpCF_3$ in the medium increased from 1 to 20 µM, the percentage of product bound to a quantity of mitochondria corresponding to 0.22 mg protein ml^{-1} remained almost constant and close to 53% of the added product (Fig. 5B) suggesting that, in this range of concentration, no saturation takes place inside the mitochondria. Under these conditions, it is possible to calculate the amount of product bound to mitochondria, in order to obtain either a 50 or a 100% uncoupling effect. These

values are close to 3.1 (= 5.9 x 0.53) nmol $USpCF_3$ mg^{-1} protein for D_{50} and 7.2 (= 13.6 x 0.53) nmol $USpCF_3$ mg^{-1} protein for D_{100}.

Inhibitory activity of $USpCF_3$

Mitochondrial electron transfer from the respiratory substrate to O_2 was studied in an uncoupled state (presence of 5 µM CCCP), using either malate, succinate or exogenous NADH as substrates. If an inhibitor acts on the common part of the electron pathway, (between the quinone pool and O_2), it lowers the rate of oxygen consumption in the same manner, whatever the substrate oxidized. In contrast, when an inhibitor acts preferentially on one of the flavoprotein dehydrogenases (before the quinone pool), the decrease in oxygen consumption differs from one substrate to another. The I_{50} values obtained were 100 and 130 µM for malate oxidized at pH 6.5 and 7.5 respectively, 50 µM for succinate and 25 µM for exogenous NADH (with 0.28 mg protein m^{-1} in the medium). The oxidation of this latter substrate was fully inhibited at a concentration of 200 µM $USpCF_3$, which corresponds to 715 nmol $USpCF_3$ mg^{-1} protein. All these results show that $USpCF_3$ is a moderate inhibitor of electron transfer in mitochondria. The greatest effect was shown when exogenous NADH is the substrate, suggesting a semi-selective effect on the external NADH dehydrogenase of the inner membrane. Under these conditions, I_{50} was obtained for a concentration in the medium of 89.3 nmol $USpCF_3$ mg^{-1} protein. This value is 15-fold higher than the value obtained for D_{50}.

In thylakoid membranes, $USpCF_3$, at concentrations greater than 5 µM, inhibits electron transfer through PSII. The I_{50} value was obtained at 6 µM (Fig. 3B) which corresponds to a concentration of 260 nmol $USpCF_3$ mg^{-1} chlorophyll in the medium. This value is 25-fold higher than D_{50}. 100% inhibition is obtained at 650 nmol $USpCF_3$ mg^{-1} chlorophyll (=65 nmol $USpCF_3$ mg^{-1} thylakoidal protein).

Discussion

Our results show that N,N'-bis-(4-trifluoromethyl-phenyl)-urea ($USpCF_3$) is one of the most powerful uncouplers of ATP formation known at the present time, acting both on mitochondria and on thylakoid membranes. The result of this action is a proton transfer through the biological membranes, leading to a collapse of the proton gradient. This gradient is required for the biosynthesis of chloroplastic and mitochondrial ATP. The efficiency of $USpCF_3$ is comparable to the protonophore reference uncoupler CCCP [6]

and better than pentachlorophenol [7]. The effectiveness of $USpCF_3$ can be compared to that of platanetin, a natural lipophilic flavonoid extracted from plane-tree buds [8]. To obtain a full uncoupling effect, the amount of product associated with mitochondria must reach 7.2 nmol mg^{-1} protein. The great lipophilicity of the molecule under study (- log P = 3.8) suggests that the major part of $USpCF_3$ linked to the mitochondria must be associated with the lipophilic components of the membrane. We can roughly estimate that the internal concentration of the product inside the mitochondrial membrane should be higher than 5 mM for full uncoupling to take place. In fact, if we consider that the lipidic part of the mitochondria represents approximately 30% of the dry weight [9], and that all the bound $USpCF_3$ is associated with this lipophilic fraction, the $USpCF_3$ lipidic concentration can be estimated to be close to 6 nmol mg^{-1} lipid. This represents 5.4 mM if we consider the lipid density as being close to 0.9. All the other well-known uncouplers, which are all very lipophilic compounds, probably also accumulate in great concentrations inside the biological membranes. As a consequence, it seems highly unlikely that the uncoupling effect can be obtained through high affinity binding to membrane components [10, 11]. In comparison, it should be emphasized that the specific binding of PSII inhibitors (such as phenmedipham) to the thylakoidal D_1 protein is close to 2 nmol mg^{-1} chlorophyll, which corresponds to 0.2 nmol mg^{-1} protein [12].

One particularly interesting point which emerged from this study is the role played by BSA on the uncoupling effects of the studied compound. It seems likely that it binds to BSA, maybe through hydrogen bonds between the-NH-and-SH or the S-S sites of the protein [13, 14].

During the course of our experiments, the presence of small molecules coming from the altered mitochondria (even after a Percoll gradient purification) decrease the coupling rate (state III-state IV ratio) by an increase in the H^+ membrane porosity. In the reaction medium, BSA traps these small molecules. The role played by BSA in our experiments has therefore to be attributed, in part, to this binding of small molecules, and, in addition, to the binding of the studied product, as previously shown with other lipophilic compounds [15, 16]. Thus, BSA greatly decreases the concentration of $USpCF_3$ inside the mem- brane of the organelles. It is suggested that this latter effect is associated with the presence of a -NH- group of the urea, as it is associated with the presence of a hydroxyl group in the case of phenols [6].

The most noteworthy characteristic of $USpCF_3$ is that this powerful uncoupler remains non-ionizable at each of the pH values studied. That means that this compound is unable

to transport H^+ through biological membranes, and that it cannot act in the same way as CCCP [17], phenolic compounds [6] or platanetin [8]. Its mode of uncoupling action may be to bring about either a con- formational change in the transmembrane proteins, leading to H+ permeability [18, 19] or a change in the phospholipidic bilayer organization and/or activity [2]. This latter point is presently under study using artificial bilayer vesicles.

Experimental

Synthesis

N,N'-Bis-(4-trifluoromethylphenyl)-urea($USpCF_3$) was obtained by reaction of 4-trifluoromethylphenylisocyanate (0.024 M; 4.48 g) with 4-trifluoromethyl-aniline(0.024 M; 3.86 g), in dry Et_2O, at room temp., for 6 hr, in the presence of triethylamine. White crystals of $USpCF_3$ were filtered under vacuum. After recrystallization from EtOAc, 3.13 g $USpCF_3$ were obtained.

Control of purity

TLC ($CHCl_3$- EtOAc, 7:3) and reversed phase HPTLC (MeOH-H_2O, 17:3) respectively on commercial silica gel plates F_{254} and $RP18F_{254}$ (Merck) were used to check the purity of the $USpCF_3$.

pK_a measurements

Due to the low water solubility of $USpCF_3$, an ethanolic soln (EtOH-H_2O, 3:1) was used for pK_a determination experiments. The pK_as were firstly determined by spectrophotometric methods. The UV spectrophotometric shifts of bands II and I were analysed to determine the pH value for which the equilibrium between the ionized and the neutral form was 50%. The pK_as were also determined using a titrimetric method. Solns (50 ml) containing l mM of the product were automatically titrated with 0.01 M HCI. The pK_a corresponds to the half-neutralization value of the studied product. In this ethanolic soln, CCCP was not completely ionized, so 0.5 ml NaOH (0.1 M) was added to the soln.

Log P measurement.

Experimental values were obtained as previously described [21].

Preparation of chloroplasts

Spinach leaves (*Spinacia oleracea* L. var. Monstrueux de Viroflay) were homogenized in ice-cold extraction medium (0.3 M mannitol, 25 mM sodium pyrophosphate, EDTA 2 mM, BSA 1g 1^{-1}, pH 7.9). The homogenate was filtered and intact class A chloroplasts were prepared by centrifugation [22] and·further purified on a Percoll density gradient. Class C chloroplasts (thylakoids) were prepared, in dist. H_2O, by gentle osmotic shock of intact chloroplasts, just prior to the experiments.

Preparation of mitochondria.

Mitochondria from potato tubers (*Solanum tuberosum* L.) was prepared by methods previously described [23].

O_2 exchange measurements

O_2 exchange was followed polarographically at 25°, using a Clark-type electrode system. For plant mitochondria, the reaction medium contained 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM Pi buffer, and in some cases, 0.1% BSA. All incubations were carried out at pH 7.2. For measuring photosynthetic O_2 exchange, the reaction medium contained 0.3 mannitol, 50 mM MOPS (morpholinopropane sulphonic acid), 2 mM EDTA, pH 7.6. The release of O_2 by class A chloroplasts was measured after the addition of 0.5 mM $NaHCO_3$ and 0.1 mM Na_2HPO_4. In thylakoid membranes (23 µg chlorophyll ml^{-1} medium), electron transfer was measured in the presence of the electron acceptor methylviologen (paraquat, 0.1 mM). NH_4Cl (5 mM) was used as a reference uncoupler. KCN (0.1 mM) was added to inhibit any possible catalase activity.

Uncoupling test for thylakoids.

Measurements of the uncoupling effect were made on light-driven electron flow through PSII +PSI. The uncoupling effect gave rise to an increase in the O_2 consumption rate. A 100% uncoupling effect was obtained when the addition of NH_4Cl (5 mM) did not induce further increases O_2 consumption.

Uncoupling test for mitochondria.

A suspension of intact mitochondria was energized. After a state III-state IV transition, the uncoupling effect of the product added at this stage corresponds to an increase in the oxidation rate. A 100% uncoupling effect was obtained when the rate of O_2 was not

further stimulated by the addition of CCCP (5 µM) or ADP (200 µM). Mitochondrial swelling. Mitochondrial swelling was measured, as previously described [5], by the apparent absorbance decrease at 540 nm. The reaction medium contained 100 mM NH_4NO_3 or NH_4Cl, 15 mM Tris-HCI, 0.1% BSA, pH 7.2. A rapid passive swelling was induced by uncouplers.

Protein and chlorophyll measurements.
Protein contents were determined according to ref. [24] and chlorophyll concentrations were measured as described in ref. [25].

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Fig. 1. Polarographic traces showing the full uncoupling activity of $USpCF_3$ in potato tuber mitochondria. (A) In the presence of BSA (0.1%) in the reaction medium; (B) in the absence of BSA; (C) reversibility of the uncoupling effect. M: Mitochondria (220 µg protein $m1^{-1}$ medium), S: succinate 6 mM +ATP 0.3 mM; ADP: 200 µM; KCN: 30 µM; CCCP: 5 µM. Numbers on traces refer to nmol O_2 consumed min_{-1} mg^{-1} protein.

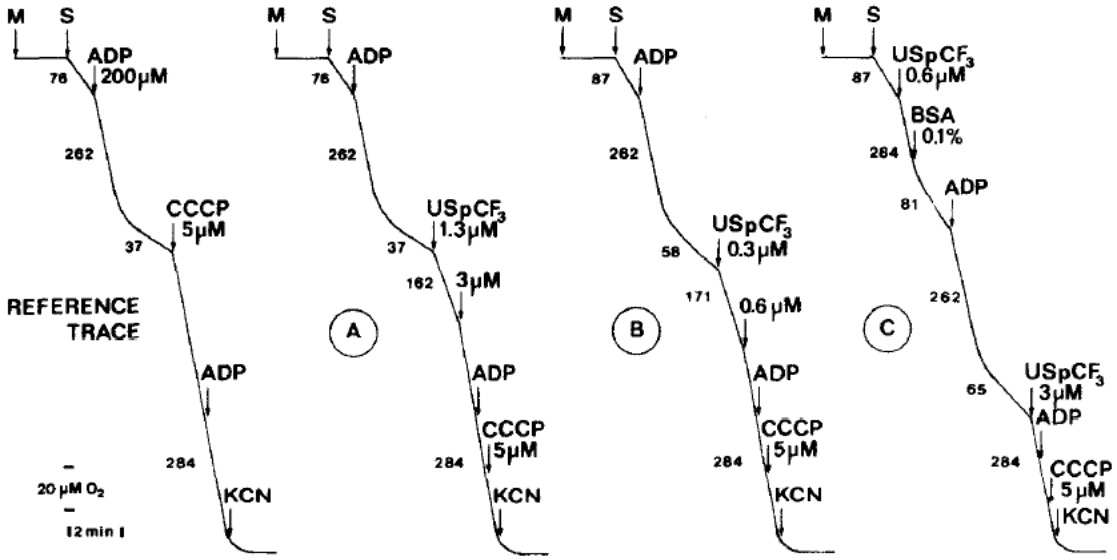

Fig. 2. Swelling of isolated mitochondria in the presence of $USpCF_3$ in NH_4Cl or NH_4NO_3 isoosmotic medium. The decrease in absorbance (△A) of the mitochondrial suspension (0.24 mg protein ml^{-1} medium; BSA 0.1 %) induced by $USpCF_3$ was measured at 540 nm, CCCP was used as a reference uncoupler. U: uncoupler.

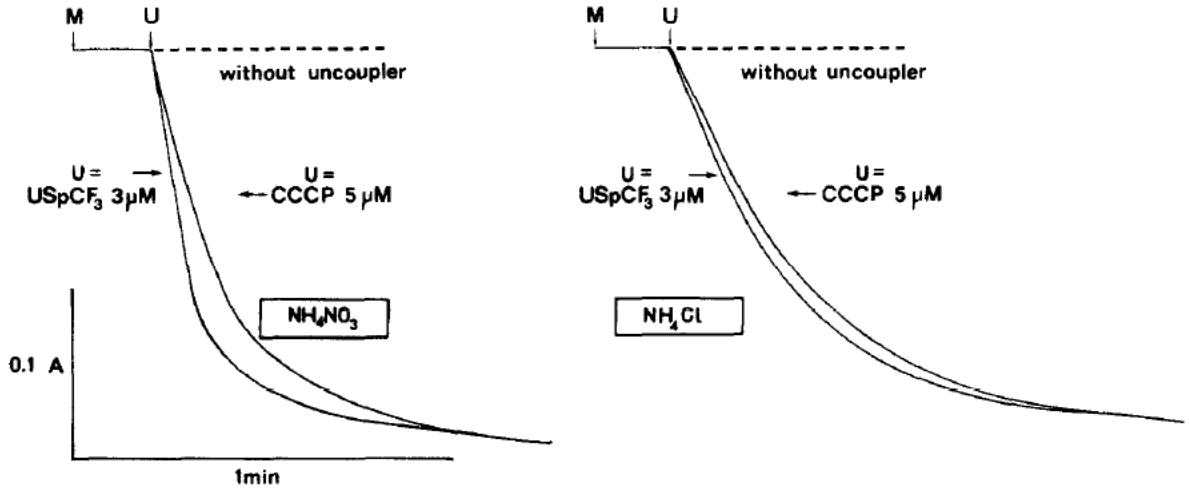

Fig. 3. Uncoupling activity (A) and PSII inhibition (B) of $USpCF_3$ in isolated spinach thylakoids membranes and effect in class A chloroplasts (C). T: Thylakoids (23 µg chlorophyll $m1^{-1}$ medium); A: class A chloroplasts (23 µg chlorophyll m^{-1} medium); KCN: 100 µM; MV: methylviologen: 0.1 mM; NH_4Cl: 5 mM; L: light; D: dark; Asc: ascorbate 0.25 mM; DPIP: 0.1 mM; C: $NaHCO_3$, 0.5 mM and P: $Na_2 HPO_4$, 0.1 mM. Numbers on traces refer to µmol O_2 consumed or emitted hr^{-1} mg^{-1} chlorophyll.

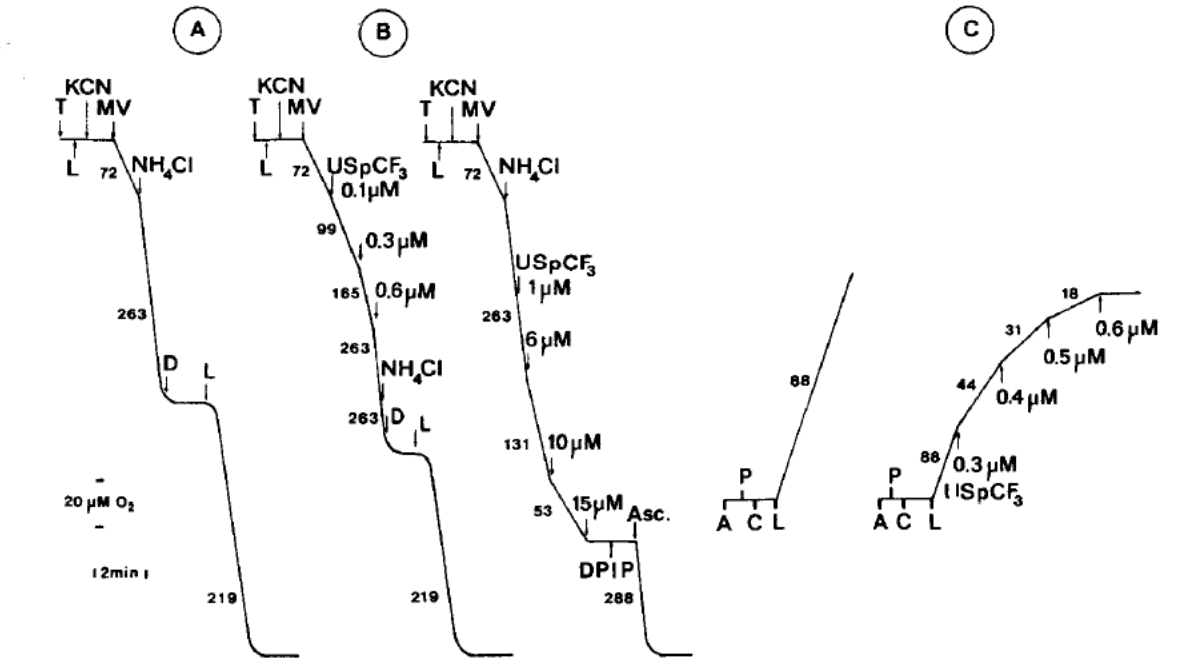

Fig. 4. pK determination for USpCF$_3$ and CCCP. (A) By spectral shift measurements. Spectral changes were measured at 264 nm for USpCF$_3$ (λEtOH max for the molecular form) and at 380 nm for CCCP (λEtOH max for the ionized form); (B) by titrimetry. Curves were obtained by addition of HCl (0.01 M) in CCCP and USpCF$_3$ soln (1 mM, in EtOH-H_2O, 3:1). Reference trace without product: dotted lines.

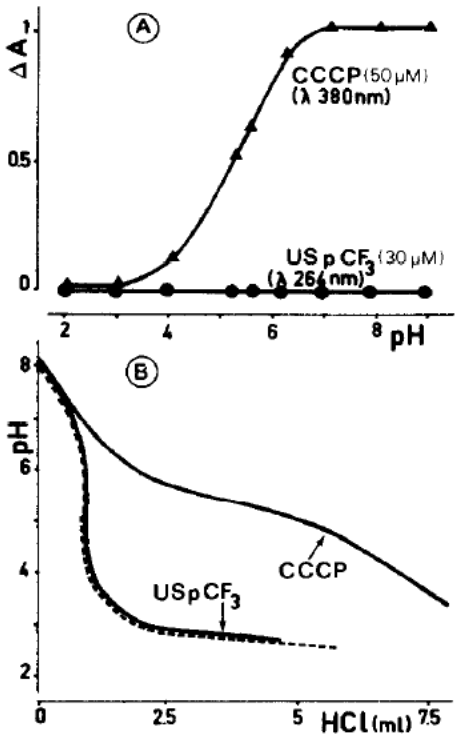

Fig. 5. (A) Partition of $USpCF_3$ between mitochondria and medium. (A) When mitochondrial concentration increases in 1 ml medium containing 5 nmol $USpCF_3$ and (B) when $USpCF_3$ concentration increases in 1 ml medium containing 220 µg mitochondrial protein. All experiments were performed in the presence of BSA 0.1%.