EPR spectroscopy of 5-DOXYL-stearic acid bound to the mitochondrial uncoupling protein reveals its competitive displacement by alkylsulfonates in the channel and allosteric displacement by ATP

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Abstract Competition of fatty acids (FA) and alkylsulfonates with 5-DOXYL-stearic acid (5-SASL) binding to isolated mitochondrial uncoupling protein (UcP) is demonstrated using EPR spectroscopy. A distinct peak of the bound 5-SASL (h_{+II}) decreased with increasing concentration of competitors. Since alkylsulfonates are UcP substrates, it suggests that the FA binding site is located in the anion channel. Moreover, with increasing ATP the h_{+II} peak decreased and was smoothed with the 'micellar' peak into a single wider peak. A pH of 8.5 reversed this effect. It could reflect an allosteric release of 5-SASL from the ATP binding site which mimics the ATP gating mechanism.

Key words: Uncoupling protein; Anion channel; Alkylsulfonate; Fatty acid binding site; 5-DOXYL-stearic acid

1. Introduction

The mechanism of thermogenesis in brown adipose tissue (BAT) [1] involves the regulated short-circuiting of the proton motive force in BAT mitochondria due to the tissue-specific uncoupling protein (UcP). The UcP probably mediates fatty acid cycling across the membrane [2–4] by transporting anionic fatty acids which then flip-flop back via the lipid bilayer in a protonated form. The complete cycle thus results in the H^+ uniport. To prove such a mechanism, one must demonstrate, at least, that the known monovalent amphiphilic substrates of UcP [5,6], such as alkylsulfonates, mutually compete with fatty acids.

Spin-labeled, DOXYL-stearic acids were successfully used to study binding in soluble proteins [7–11]. Previous EPR studies of Ježek and Freisleben [12] indicated binding of 5-DOXYL-stearic acid (5-SASL) to the isolated UcP. This has been demonstrated as the reproducible broadening of the low field peak in its EPR spectrum or as the increase in h_{+II}/h_{+1M} ratio between the peak heights assigned with the immobile and mobile probe portion, respectively. By increasing the UcP concentration while decreasing detergent and using an EPR spectrometer with improved resolution, we have been able to record EPR spectra of 5-SASL bound to UcP with clearly separated peaks of the immobilized and mobile fractions. This allowed us to study the competition with other substrates and ligands of UcP.

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Abbreviations: BAT, brown adipose tissue; NEM, *N*-ethylmaleimide; 5-SASL, 5-DOXYL-stearic acid, where DOXYL is 4,4-dimethyl-3-oxazolinyloxy-residue; N^6-SL-ATP, N^6-(2,2,6,6-tetramethyl-piperidin-4-yl-1-oxyl)-ATP ; TEA, tetraethylammonium; TES, *N*-Tris(hydroxymethyl)methyl)-2-aminoethane-sulfonic acid; UcP, uncoupling protein.

2. Material and methods

5-DOXYL-stearic acid and most other chemicals used were purchased from Sigma Chemie GmbH (D-80240 Deisenhofen, Germany); alkylsulfonates were from Lancaster Synthesis (D-63155 Mühlheim/Main, Germany). N^6-SL-ATP was synthesized according to [13].

BAT mitochondria were isolated from Syrian hamsters by a standard method in a medium containing 5 mg bovine serum albumin per ml. Isolation of UcP has been performed by the step-wise elution method using octylpentaoxyethylene extraction as decribed previously (12). For EPR measurement several fractions were collected and pressure concentrated, first, up to 0.5 mg/ml. The detergent content was subsequently reduced by passage through Bio-Beads SM2 (Bio-Rad) (40 mg beads/mg protein). This procedure was essential for obtaining the 'bound' EPR signal with 5-SASL. The final concentration process was then done using Centricons (cut-off M_r 10,000; Amincon, UK), until 2 mg/ml had been reached.

2.1. EPR spectrospopy

EPR spectroscopy was accomplished in a 50 μl flat chamber with a Bruker ESP 300 E Digital EPR spectrometer operating in the X-band mode. A microwawe power of 6.3 mW and peak-to peak modulation amplitudes of 0.8 Gauss were routinely employed. Typical composite EPR spectra of bound 5-SASL (Fig. 1) contained low field peaks h_{+I} split into two peaks, h_{+1M} and h_{+II}, corresponding to 'mobile' (micellar) and immobilized fractions, respectively [11,14–16]. The apparent intensity of the h_{+II} taken from zero or the ratio h_{+II}/h_{+1M} increases with binding of the probe.

3. Results

3.1. EPR spectra of 5-DOXYL-stearic acid bound to the uncoupling protein

Fig. 1 shows at the top the EPR spectrum of 124 μM 5-DOXYL-stearic acid in the presence of concentrated UcP (30 μM, i.e. approx. 2 mg protein/ml). The spectrum shows a clear separation of two peaks, h_{+1M} and h_{+II}, corresponding to the 'mobile' and immobilized probe fractions, respectively. The immobilized probe also exhibits a distinct h_{-II} peak which allows for accurate determination of the $2A_{zz}$ parameter. This was, on average, 47.8 ± 0.6 Gauss (n = 11), whereas 5-SASL in 2% OctylPOE exhibited a $2A_{zz}$ of 33.4 Gauss (cf. bottom spectrum in Fig. 1). When the same UcP sample was precipitated in the capillary by trichloroacetic acid, the signal of the immobilized probe dissappeared. Moreover, we found that Bio-Beads treatment of UcP was absolutely essential for obtaining the separation of the h_{+1M} and h_{+II} peaks. With untreated

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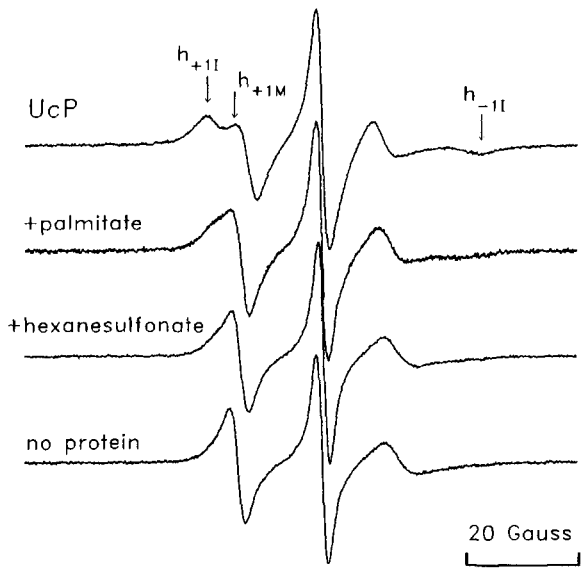

Fig. 1. EPR spectra of 5-DOXYL-stearic acid bound to the uncoupling protein and their changes after addition of palmitic acid and hexanesulfonate. Traces from the top: (1) control; (2) 2 mM palmitic acid; (3) 27 mM hexanesulfonate; (4) 2% octylpentaoxyethylene, no protein. 124 μM 5-SASL and 30 μM (2 mg/ml) uncoupling protein were present in 5 mM TEA-TES, 30 mM TEA_2SO_4, 0.2 mM TEA-EDTA, pH 7.2. Palmitic acid and hexanesulfonate stock solutions contained 2% octylpentaoxyethylene in order to keep a constant viscosity. The arrows indicate peaks of the immobilized (h_{+1I} and h_{-1I}) and mobile probe (h_{+1M}).

samples, h_{+1I} appeared only as a shoulder and $2A_{zz}$ reached barely 42 Gauss (between a shoulder representing h_{+1I} and a tiny apparent h_{-1I} peak).

The main features of the high resolution EPR spectra of bound 5-SASL to UcP are summarized in Table 1. Our data resemble that found for 5-SASL binding to human serum albumin [7,8]. In our case of detergent micelles, the typical free signal is missing and is substituted by a spectrum reflecting partial immobilization. This is superimposed onto the spectrum of the typically immobilized 5-SASL.

3.2. *EPR spectra of 5-DOXYL stearic acid in the presence of palmitic acid and three model alkylsulfonates*

When excess fatty acid, such as 2 mM palmitic acid, was added after 5-DOXYL-stearic acid, the low field peak splitting dissappeared (Fig. 1). From the peak heights of 'immobile' h_{+1I} peaks we calculated percents of released 5-SASL due to competition. This was approx. 20% and 40% of released 5-SASL at 1 and 2 mM palmitic acid, respectively.

The similar prevention of 5-SASL binding was also achieved with three typical UcP substrates, octadecanesulfonate (Figs. 2 and 3), undecanesulfonate (Fig. 3) and hexanesulfonate as well (Fig. 1). The direct transport via UcP of the last two has been confirmed by Ježek and Garlid [3,4,6,17]. The displacement is illustrated in detail in Fig. 2, showing transposition of the h_{+1I} peak with regard to the h_{+1M} peak with increasing octadecanesulfonate concentration. At low (1 mM) concentrations of octadecanesulfonate, an intermediate h_{+1W} peak appears, and the triplet peak exists at low field. The corresponding triplet can be observed at high field, too. Above 2 mM concentration, the original first 'immobilized' peak disappeared and the intermediate peak was gradually shifted towards the 'mobile' peak (Fig. 2). In the high field region, the corresponding h_{-1I} peak disappeared, while the intermediate h_{-1W} peak can be recognized up to 3.2 mM octadecanesulfonate. Above 3.7 mM concentrations, EPR spectra show peaks of the mobile probe fraction only, similar to the EPR spectra in detergent micelles (Fig. 1, Table 1).

In spite of the complicated pattern of the EPR spectra described above, it is possible to plot the approximate dose–responses for displacement of 5-SASL by alkylsulfonates using the heights of the h_{+1I} peaks (Fig. 3). This yields an IC_{50} of 2.6 mM for octadecanesulfonate, 30 mM for undecanesulfonate, and about 35 mM for hexanesulfonate (not shown). Dose–responses were also calculated from the increase in the mobile peak h_{+1M}, yielding an IC_{50} of 3.8 mM for octadecanesulfonate, 24 mM for undecanesulfonate, and 30 mM for hexanesulfonate. When the ratio h_{+1I}/h_{+1M} is used to plot the dose–responses, the half-maximum effects occurred at concentrations of 2.2 mM for octadecanesulfonate, 7.7 mM for undecanesulfonate, and 26 mM for hexanesulfonate. The latter values are in good agreement with the published transport data – relations between the affinities of given alkylsulfonates for UcP are the same [3,6]. Note that all values are higher by of one order of magnitude due to the micellar environment.

3.3. *Effect of purine nucleotides on 5-DOXYL stearic acid binding to the uncoupling protein*

Addition of the native ligand ATP to the complex of 5-SASL with UcP strongly affected the EPR spectra (Fig. 4A). The changes in the EPR spectra with increasing [ATP] were less complicated than those described above for alkylsulfonates, exhibiting only transitions between two fractions (Fig. 4A). With increasing [ATP] the h_{+1I} peak decreased and the 'micellar' peak increased, resulting in smoothing into a single wider peak. From the intensities of the h_{+1I} peaks a dose–response for 5-SASL release by ATP was calculated, yielding an IC_{50} of 0.5 mM when an aqueous ATP solution was added (not shown), or of about 3 mM when the ATP stock solution also contained the detergent (cf. Fig. 4A) in order to ensure a constant viscosity.

Alkaline pH (8.5), which is known to reduce ATP binding [18], reversed this effect of ATP on 5-SASL binding in a such

Table 1
EPR parameters of 5-DOXL-stearic bound to uncoupling protein (5-SASL:UcP of 4:1) after addition of other substrates or ATP

Conditions	Height of the 'immobile' h_{+1I} peak[a]	Ratio of 'immobile/mobile' h_{+1I}/h_{+1M}	$2A_{zz}$ (Gauss)
2% OctylPOE[b], no UcP	9	0.25	33.4
2% OctylPOE, no UcP +2 mM palmitic acid	8.4	0.24	33.0
Control UcP	17	1.42	47.8
UcP + 2 mM palmitic acid	10	0.54	31.9
UcP + 3.7 mM octadecanesulfonate	7	0.14	32.1
UcP + 40 mM hexanesulfonate	7	0.23	32.3
UcP + 1.9 mM ATP	10	0.72	31.7
UcP + ATP, pH 8.5	16	1.29	47.7

[a]Arbitrary units.
[b]Octylpentaoxyethylene.

way that the original 'bound' spectrum re-appeared (Fig. 4B). UcP samples without previous Bio-Bead treatment showed hardly any pH effect. In these samples at pH 8.5, only a slight increase in a shoulder representing the h_{+II} occurred.

To evaluate whether a reciprocal effect exists, i.e. effect of fatty acids on nucleotide binding to UcP, we have employed N^6-SL-ATP. Similarly to 8-SL-ATP [19], N^6-SL-ATP exhibits a signal of the immobilized probe, reflecting binding. A Scatchard plot (not shown) yielded a K_d of 13 μM. This is five times lower than the reported K_d for C8-SL-ATP [19]. When we tested the effect of palmitic acid up to 5 mM on the N^6-SL-ATP binding to UcP, we found no effect at all.

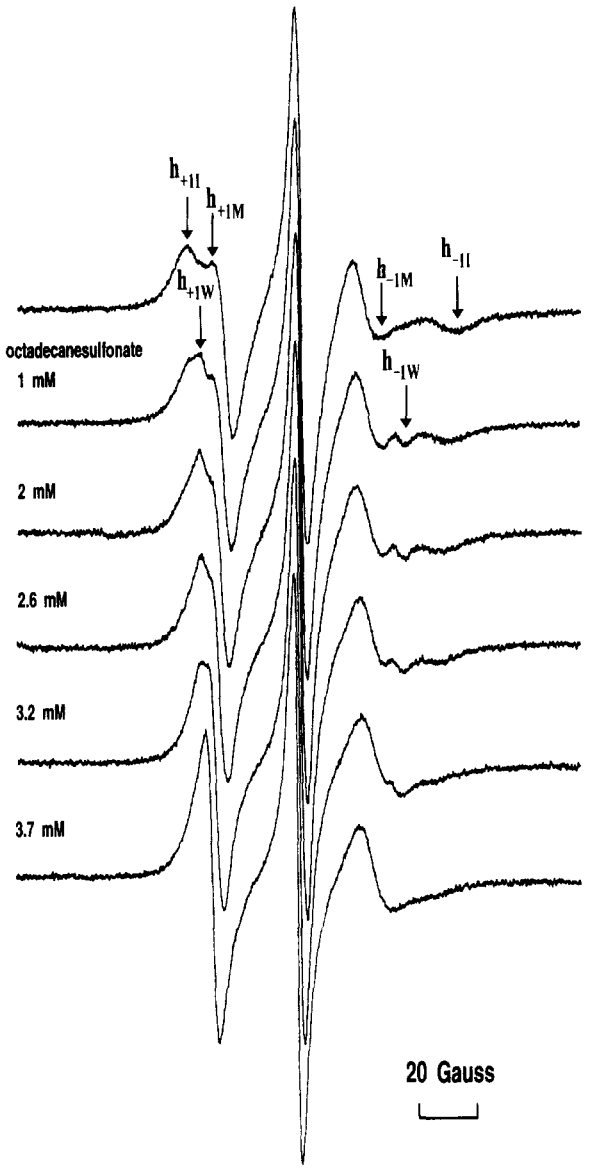

Fig. 2. Effect of octadecanesulfonate on the EPR spectra of 5-SASL bound to the uncoupling protein. Spectra of 124 μM 5-SASL in the presence of 30 μM (2 mg/ml) UcP, 5 mM TEA-TES, 30 mM TEA_2SO_4, 0.2 mM TEA-EDTA, pH 7.2, and increasing concentration of octadecanesulfonate added after 5-SASL were recorded under the same conditions. Traces from the top: (1) control; (2) 1 mM; (3) 2 mM; (4) 2.6 mM; (5) 3.2 mM and (6) 3.7 mM octadecanesulfonate. The arrows indicate peaks of the immobilized (h_{+II} and h_{-II}), weakly immobilized (h_{+1W} and h_{-1W}) and mobile probe (h_{+1M} and h_{-1M}).

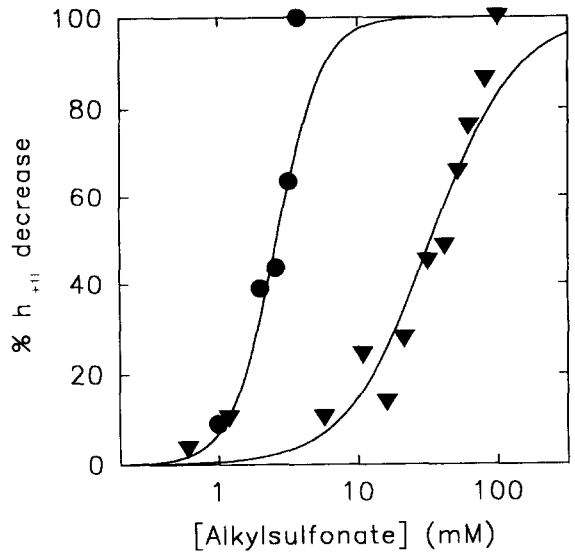

Fig. 3. Dose–responses for displacement of bound 5-DOXYL-stearic acid on UcP by octadecanesulfonate (●) and undecanesulfonate (▼). Dose–responses were plotted using the absolute height of the peak h_{+II} (with regard to zero), when values at a maximum sulfonate dose were taken as a 100% release. Measurements were done as described in Figs. 1 and 2. Theoretical fits were calculated using the Hill equation. The IC_{50} derived from these plots were 2.6 and 30 mM, respectively (Hill coefficients around 2.5 and 1.5).

4. Discussion

Our results confirm the existence of a binding site for fatty acids on the uncoupling protein and show that this binding site is identical, at least with a part of the anion channel (transport pathway) (cf. [3,4,6,17]). The strong evidence for this is provided by the competition of known UcP substrates with 5-SASL. Since fatty acids were reported to inhibit alkylsulfonate uniport [3,4], the competition of alkylsulfonates and fatty acids is mutual. All our data give further support to the recent re-evalution of the transport mechanism of UcP [2–6,17]. These results clearly favor the fatty acid cycling mechanism over the traditionally considered protonophoric mechanism: anionic fatty acid is likely to pass through the UcP while its return in a protonated form via the membrane causes the H^+ uniport flux.

The immobilized component in the EPR spectra of 5-SASL in the presence of UcP must be due to the specific binding of 5-SASL to the fatty acid binding site, because with the same protein (UcP) but without Bio-Beads pretreatment, immobilization of the probe did not occur. This excludes the possibility that the 'bound' spectrum results from an entrapment in non-specific binding sites at the detergent/protein interface. Interestingly, our series of EPR spectra upon titration with alkylsulfonates exhibited clear peak triplets in both low and high field regions. The existence of the intermediate peak corresponding to a weakly immobilized probe most probably reflects a situation in which 5-SASL is partially released from the binding site by alkylsulfonates, and is still semi-attached. Alternatively, formation of less fluid micelles after addition of alkylsulfonates would explain these spectra. Since addition of alkylsulfonates to detergent micelles containing 5-SASL alone does not produce the weakly immobilized component, we can rule out this possibility.

Surprisingly, 5-SASL spectra were also affected by ATP (this work) and GDP (cf. [12]). In the previous report [12], the increased peak half-width was ascribed to an enhancement of 5-SASL binding [12]. However, we now demonstrate that the spectral changes rather indicate a loss of binding. Under conditions when ATP binding is weak, i.e. at alkaline pH, the spectral changes are reversed to the original 'bound' form. Certain DOXYL fatty acids embedded in short chain phospholipids and with the DOXYL group closer to the terminal methyl group have been reported to give similar spectral changes, i.e. larger $2A_{zz}$ values at high pH [9]. Protonation of the carboxyl group here leads to deeper insertion into the membrane, and deprotonation reverses this effect. In our system of a long chain detergent, such an effect should not occur, as proven by experiments in the absence of protein. Hence, we conclude that ATP causes partial displacement of bound 5-SASL from UcP and the probe re-binds at alkaline pH which releases ATP [18].

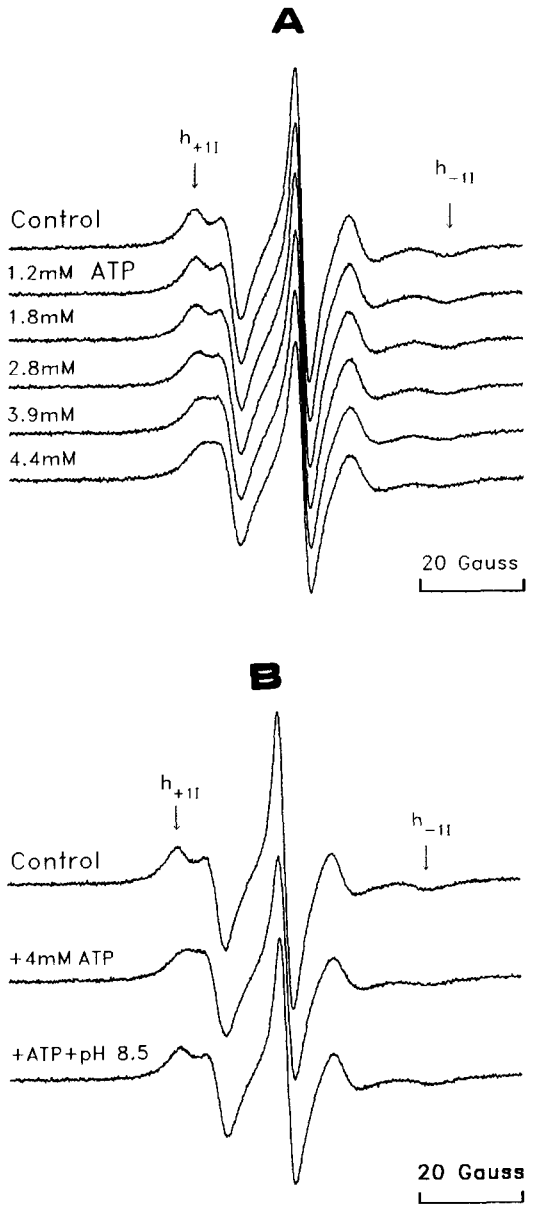

Fig. 4. EPR spectra of 5-DOXYL-stearic acid bound to the uncoupling protein after addition of ATP. (A) Series of subsequent ATP additions; ATP was added after 5-SASL to the UcP sample at concentrations indicated at each trace. The ATP stock solution contained 2% octylpentaoxyethylene. (B) Re-binding of the 5-SASL in the presence of ATP after elevation of pH to 8.5. Traces from the top represent subsequent additions in a typical experiment: (1) control; (2) 4.4 mM ATP added; (3) pH elevated up to 8.5 after ATP addition by using 2 μl of 0.2 M Tris-Cl, pH 8.5.

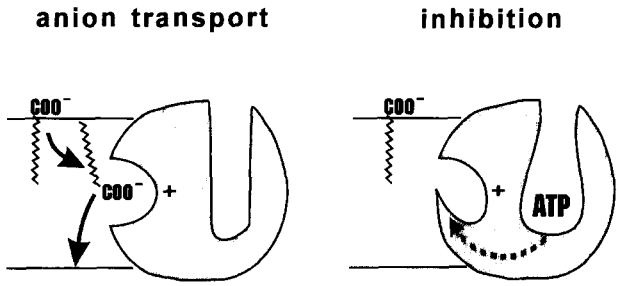

Fig. 5. Model for the molecular mechanism of gating by nucleotides in UcP. The two states, when either transport or nucleotide inhibition takes place, are schematically shown. Under the functional, i.e. transporting, state the internal anion binding site in UcP is exposed and is accessible also for fatty acid anions. Upon reaching the site, anionic fatty acid (or other anions) can move to the opposite side of the membrane. Such a transport mechanism is just possible because the internal binding site lowers the energy barrier represented by the membrane and allows anions to pass (cf. [3,6,22]). Purine nucleotide inhibition is caused by conformational changes which make the anion binding site inacessible for anions. The conformational changes originate in and progress from the water-filled cavity of the ATP binding site [23], where ATP may reach the 'bottom' of the protein.

It has been shown that fatty acids do not influence ATP binding [20] nor inhibition by GDP [3]. We have confirmed this by employing N^6-SL-ATP. Palmitic acid up to 5 mM did not affect its binding. Since the influence between ATP and fatty acids occurs in one direction only, and GDP is a non-competitive inhibitor of anion transport by UcP [3], we can conclude that the displacement of 5-SASL by ATP is allosteric (Fig. 5). This displacement mimics the inherent molecular mechanism of gating. Probably, conformational changes switched by ATP (cf. [21]) prevent exposure of those residues which are essential for binding of anionic substrates. They cause the inaccessibility of the internal anion binding site for anions. The existence of the internal binding site (an 'energy well' [6,22]) has been suggested previously because of the lack of external binding sites for substrates on UcP [6], the increasing affinity of more hydrophobic substrates to UcP [3,6], and the two-energy barrier profile derived from the flux voltage characteristics [22].

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