

Sulfhydryl groups are involved in H^+ translocation via the uncoupling protein of brown adipose tissue mitochondria

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Mersalyl inhibits H^+ transport via the uncoupling protein (UP) in brown adipose tissue (BAT) mitochondria estimated as swelling in potassium acetate (K , 67 μM) or as valinomycin-induced H^+ extrusion in K_2SO_4 (K , 55 μM) and KCl. The swelling in KCl is depressed only slightly. Some other SH-reagents (*p*-hydroxymercuribenzoate, 5,5'-dithiobis(2-nitrobenzoate) and thiolyle DB), but not hydrophobic reagents (*N*-ethylmaleimide and eosin-5-maleimide), exhibit analogous inhibition. Thus an essential SH-group localized at the water-accessible cytosolic surface of UP was found to be involved in H^+ transport via UP but not in Cl^- transport.

Mersalyl; Uncoupling protein; Sulfhydryl reagent; H^+ transport; Sulfhydryl group; (Brown-fat mitochondria)

1. INTRODUCTION

The uncoupling protein of brown adipose tissue mitochondria forms an H^+ transport pathway across the inner membrane. It allows H^+ short-circuiting of respiration-generated protonmotive force and results in heat production in this tissue during acute thermogenesis [1–3]. H^+ transport is inhibited after binding purine nucleoside di- and triphosphates to a site localized at the outer surface of UP [4–8] and activated after specific interaction of UP with free fatty acid [8–10]. All these properties were confirmed also in reconstitution ex-

periments [5–8]. In addition, UP forms a Cl^- transport pathway [7,8], probably different from the H^+ transport pathway [11], which is regulated only by purine nucleotides [7,8,11]. In its natural state UP is a dimer consisting of two identical subunits with M_r 33 000 which bear one nucleotide-binding site [4]. Each subunit contains 306 amino acid residues, the sequence of which was determined directly [12] or derived from cDNA coding the protein [13].

No artificial inhibitor of H^+ or Cl^- transport via UP has yet been found. The suggestion that DCCD specifically inhibits Cl^- transport [14] was questioned (Ježek, P., unpublished and [8,15]), hence any characterization of the transport pathway formed by UP is still lacking.

This work reports for the first time the inhibition of H^+ transport via UP by hydrophilic SH-reagents, which do not affect Cl^- transport via UP. It suggests the participation of SH-group(s) in the H^+ translocation mechanism and thus places the uncoupling protein into the large group of mitochondrial ion-transport carriers possessing SH-groups essential for transport.

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Abbreviations: BAT, brown adipose tissue; BSA, bovine serum albumin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; DTNB, 5,5'-dithiobis(2-nitrobenzoate); EMA, eosin-5-maleimide; NEM, *N*-ethylmaleimide; PMB, *p*-hydroxymercuribenzoate; TBT, tributyltin chloride; UP, uncoupling protein

2. MATERIALS AND METHODS

Valinomycin, oligomycin, rotenone, antimycin A, nigericin, GDP-Na salt, BSA, mersalyl, DTNB, PMB, NEM and dithioerythritol were purchased from Sigma. Thiolyte DB was obtained from Calbiochem and EMA from Molecular Probes. All other chemicals were of analytical grade.

BAT mitochondria from hamsters adapted to the cold (5°C) for at least 3 weeks were prepared according to Hittelman [16]. The protein content was estimated by the method of Lowry et al. [17] using BSA as a standard.

H⁺ transport via UP was estimated either indirectly as valinomycin-induced swelling in 150 mM K-acetate, 5 mM K-Tes, pH 6.9, or directly as valinomycin-induced H⁺ extrusion in 110 mM K₂SO₄ or 150 mM KCl with 2 mM K-Tes (pH 6.9). All media contained 8 μM rotenone, 2.3 μM oligomycin and 1 μM antimycin A. Transport was initiated by the addition of valinomycin (1 μg·(mg protein)⁻¹) usually 20 s after addition of BAT mitochondria to the medium. The accompanying swelling in KCl was followed simultaneously with H⁺ extrusion in the apparatus described elsewhere [11,18]. Initial rates of swelling and H⁺ extrusion were calculated as differences of rates before and after valinomycin addition and were expressed in % *I*₀·min⁻¹ (where *I*₀ is the initial light scattering intensity of the mitochondrial sample) and in nmol H⁺·min⁻¹·(mg protein)⁻¹, respectively [11,18].

The intactness of mitochondria was checked by following ion transport mediated exclusively by added ionophores (1 μg·(mg protein)⁻¹) in the presence of 200 μM GDP, which fully blocks the transport via UP. Thus nigericin-induced swelling in K-acetate, nigericin-induced H⁺ extrusion in KCl or K₂SO₄ and TBT-mediated swelling in the presence of valinomycin and CCCP (5 nmol·(mg protein)⁻¹) were used as control systems. Any decrease of transport rates in these systems was taken as an indication of smoothing of ion gradients or another membrane damage resulting from side effects of the modifiers.

SH-reagents were added either directly to the medium 20 s before valinomycin, or modification procedures were used as in [19] for DTNB and analogously for thiolyte DB and as in [20,21] for NEM and EMA.

3. RESULTS AND DISCUSSION

The impermeant reversible SH-reagent mersalyl inhibits transport via various metabolite carriers in mitochondria [22–24]. It indicates a broad participation of essential SH-groups in the translocation of various substrates. SH-groups attacked by mersalyl are mostly exposed to the water-accessible parts of carrier proteins at the outer surface of the membrane [22–24].

As shown in fig.1, mersalyl also decreases the initial rate of swelling of BAT mitochondria in K-acetate, induced by valinomycin, as well as the initial rate of valinomycin-induced H⁺ extrusion in K₂SO₄. These H⁺ fluxes were shown to be mediated by UP [11,25] as indicated also in fig.1, showing GDP inhibition. The effect of mersalyl can be reversed by the addition of cysteine after (fig.1) or before (fig.2) valinomycin. Thus two independent methods document the reversible inhibition by mersalyl of H⁺ transport via UP.

Because mersalyl was shown to induce K⁺ permeability [26,27] and to penetrate the membrane at higher concentrations [28], one should

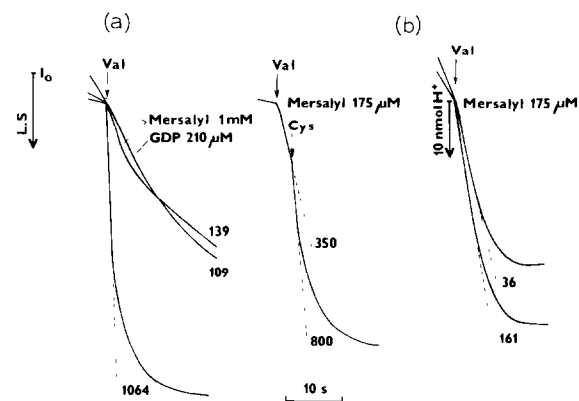


Fig.1. (a) The effect of mersalyl on valinomycin-induced swelling of BAT mitochondria (1 mg protein·ml⁻¹) in 150 mM K-acetate, 5 mM K-Tes (pH 6.9) including oligomycin, rotenone and antimycin A. The inhibition by 210 μM GDP is also shown. The record in the middle shows the reversibility of mersalyl inhibition after addition of cysteine to mM. (b) The effect of 175 μM mersalyl on valinomycin-induced H⁺ extrusion in 110 mM K₂SO₄, 2 mM K-Tes (pH 6.9) and the above-mentioned inhibitors. 1 mg protein·ml⁻¹ of BAT mitochondria was used. Initial rate values are indicated at the curves.

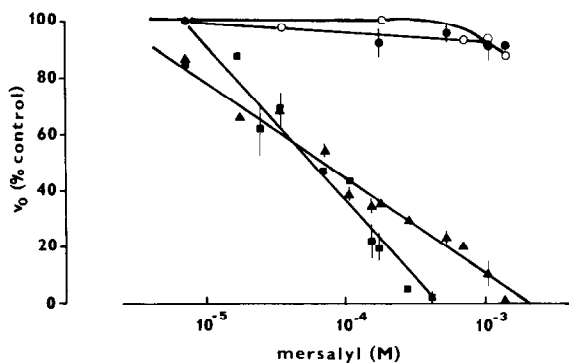


Fig.2. Concentration dependence of mersalyl inhibition. (■) Valinomycin-induced H^+ extrusion in K_2SO_4 ; (▲) valinomycin-induced swelling of BAT mitochondria in K-acetate in the absence of cysteine; (○) in the presence of 3.5 mM cysteine; (●) nigericin-induced swelling of BAT mitochondria in K-acetate. All points represent the mean values of 4–6 experiments (■, ▲) or 2–3 experiments (○, ●). The bars indicate SD. When the bars are omitted, SD was less than the symbol dimension. BAT mitochondria were suspended to 1 mg protein \cdot ml $^{-1}$.

carefully distinguish between the real inhibitory effect and any apparent inhibition caused by smoothing of ion gradients due to any disturbed membrane permeability by mersalyl. As shown in

fig.2, mersalyl up to 1.4 mM does not affect nigericin-induced swelling of BAT mitochondria in K-acetate in the presence of GDP. Therefore, any decrease of the initial rate of swelling in K-acetate indicates the net inhibition of H^+ transport via UP. This is valid also for the case of H^+ extrusion in K_2SO_4 (not shown).

Apparent inhibitory constants (K_i) derived from linearized mersalyl titration on a logarithmic scale (fig.2) are 55 and 67 μ M for H^+ extrusion in K_2SO_4 or swelling in K-acetate, respectively. When the K_i was calculated as $10^{(n_H/a_0)}$ (where n_H is the Hill coefficient and a_0 is the intercept at the ordinate derived from a linearized Hill plot), the results were similar. When Hill plots were drawn for $v_0 + SD$ or $v_0 - SD$ values, the limiting intervals for K_i obtained by the above described calculations were (38–59 μ M) for H^+ extrusion in K_2SO_4 and (56–82 μ M) for swelling in K-acetate. The mean n_H was 1.39 for H^+ extrusion and 0.9 for swelling. The faster approach to zero activity in the case of K_2SO_4 could be due to different reactivity of mersalyl in these different ionic surroundings but still the K_i values of inhibition in K-acetate and K_2SO_4 are quite similar.

When H^+ transport via UP was tested as valinomycin-induced H^+ extrusion in 150 mM KCl simultaneously with swelling, reflecting in this case

Table 1

The effect of mersalyl on H^+ and Cl^- transport via the uncoupling protein and on transport mediated by added ionophores

Conditions		Remaining activities in the presence of	
		210 nmol mersalyl (mg protein) $^{-1}$	420 nmol mersalyl (mg protein) $^{-1}$
UP-mediated transport	(A) valinomycin-induced H^+ extrusion in KCl	50 \pm 8 (6)	32 \pm 19 (9)
	(B) valinomycin-induced swelling in KCl	80 \pm 13 (6)	89 \pm 20 (9)
Transport independent of protein carriers	(C) nigericin-induced H^+ extrusion in KCl + GDP	83 \pm 8 (4)	109 \pm 20 (4)
	(D) TBT-valinomycin-mediated swelling in KCl + GDP + CCCP	80 \pm 8 (4)	81 \pm 1 (2)

Mean values \pm SD (n experiments) are presented. Significant differences were found to be for A vs B at 210 nmol ($P < 0.01$) and at 420 nmol ($P < 0.001$). B vs D and B vs C are not significantly different

Cl^- transport via UP [11,18,29], some difficulties arose due to a more intensive side effect of mersalyl. As shown in table 1, mersalyl at $210 \text{ nmol} \cdot (\text{mg protein})^{-1}$ slightly decreases also the nigericin-induced H^+ extrusion in the presence of GDP and also the swelling mediated by TBT. The decrease in UP-mediated swelling is not significantly different from the decreases in artificial transport systems, while the decrease in initial rates of H^+ extrusion in KCl is indeed significantly different (table 1). However, a further increase of mersalyl concentration to 1 mM leads to real membrane damage and to a swollen state of mitochondria. This confirms the earlier finding that organomercurials induce Cl^- permeability [27] which, in parallel with increased K^+ permeability [26,27], results in swelling without valinomycin. This strong side effect of mersalyl apparent only in KCl could result from the formation of mersalyl- Cl_n^- complexes [30].

All these results suggest that mersalyl specifically inhibits H^+ transport but not Cl^- transport via UP. It further supports the suggestion of two independent transport pathways (H^+ and Cl^- channels) in the functional dimeric unit of UP [11].

In order to characterize the environment of the SH-group(s) found to be essential for H^+ translocation via UP, the inhibitory ability of various SH-reagents was further studied (table 2). Thus PMB exhibits an analogous inhibitory efficiency to mersalyl and covalently modifying SH-reagents DTNB and thiolyte DB also inhibit H^+ transport via UP (table 2). On the other hand, SH-reagents of NEM type, such as NEM and EMA, do not affect transport via UP (table 2). The lack of the effect by these hydrophobic reagents supports the possible localization of the attacked SH-group(s) in the hydrophilic environment. Therefore, this SH-group should be accessible to H^+ from the water and could serve as one of the H^+ binding sites of the H^+ transport pathway.

The sequence of the UP subunit contains 7 Cys, hence 14 SH-groups are present in the naturally occurring dimer [12,13]. According to Aquila's [12] model of UP folding in the membrane obtained on the basis of hydropathy, the only SH-group exposed to the cytosolic water environment should be Cys-304. But at present, it is not possible to identify the critical SH-group with certainty.

The finding of the essential SH-group for H^+

Table 2

Inhibition of H^+ transport via UP by various SH-reagents

SH-reagent	Concentration ($\text{nmol} \cdot (\text{mg protein})^{-1}$)	Method	Remaining activity (% control)
PMB	82	A	57.7 ± 1.7 (3)
DTNB	500	A	69 ± 6 (4)
		B	42 ± 3 (2)
Thiolyte DB	200	A	69 ± 13 (2)
		B	52
EMA	200	B	108 ± 14 (3)
NEM	200	A	110 ± 8 (2)

Mean values \pm SE are presented (*n* experiments). (A) Swelling in K-acetate; (B) H^+ extrusion in KCl. PMB was added directly to the medium 20 s before valinomycin. BAT mitochondria ($4.7 \text{ mg protein} \cdot \text{ml}^{-1}$) were incubated with DTNB in 236 mM sucrose, 16.5 mM Tris-Cl (pH 8.1), 0.9 mM EDTA for 90 min at 20°C under vigorous stirring. For transport measurements aliquots of treated mitochondria were added to media without buffer. Thus 3.5 mM Tris-Cl (pH readjusted to 7) was present. H^+ extrusion was then measured at $2 \text{ mg protein} \cdot \text{ml}^{-1}$. Thiolyte DB was incubated with mitochondria ($5 \text{ mg protein} \cdot \text{ml}^{-1}$) in 250 mM sucrose, 1 mM EDTA (pH 6.9) for 60 min at 20°C . EMA and NEM treatment was analogous (20 min at 0°C) and the reaction was stopped by the addition of dithioerythritol. All measurements were performed as in the case of DTNB. Control samples were incubated with appropriate aliquots of solvents (ethanol or dimethylsulfoxide) under the same conditions. No treatment decreased initial rate of the transport independent of protein carriers more than to 90% of activity in control samples

translocation leads to a consideration of the Robillard and Könings model [31] of ion translocation in UP. It also relates this protein to the large family of mitochondrial translocators possessing essential SH-groups [22–24].

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