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EFFECT OF 2-HYDROXY-5-NITROBENZYL BROMIDE ON PROTON TRANSLOCATION BY THE MITOCHONDRIAL H^+-ATPase

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2-Hydroxy-5-nitrobenzyl bromide, a highly reactive reagent towards tryptophan residues in proteins, is shown to activate the passive proton flux through the inner mitochondrial membrane of bovine heart submitochondrial particles (ETP_H). When added at low concentrations, the reagent increased both the ATPase activity of the particles and the passive proton transport rate through the membrane. The presence of oligomycin reduced the extent of the 2-Hydroxy-5-nitrobenzyl bromide action on the proton conductivity suggesting that it acted primarily on the H^+-ATPase complex. Similar effects were observed on F_1 -depleted particles, whilst no effect was observed on the isolated F_1-ATPase activity.
The results suggest that polypeptides bearing tryptophan residues may be involved in the gating function of proton channels of the mitochondrial membrane and this is particularly evident for the F_oF_1-ATPase complex.

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Abbreviations used: ATPase (F_oF_1-ATPase): adenosine 5'-triphosphatase (E.C. 3.6.1.3.); F_1: soluble mitochondrial ATPase; F_o: membrane sector of the ATPase; ETP_H: electron transfer particles; U-SMP: F_1-depleted submitochondrial particles; HNB: 2-Hydroxy-5-nitrobenzyl bromide; Tris: 2-amino-2-(hydroxymethyl-propane)-1,3-diol; EDTA: ethylenediamine tetraacetic acid.

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The membrane sector (F_o) of the H^+-ATPase (F_oF_1-complex) of coupling membranes functions as a transmembrane proton translocator (1-3). This process is coupled to ATP synthesis or hydrolysis by the catalytic sector (F_1) of the complex.

Recent observations could identify key amino acids in polypeptides of F_o with respect to proton translocation by the assembled F_o complex. These amino acids are apparently located in the a and c subunits of the E. Coli F_o complex (4,5) as well as in homologous subunit counterparts in the bovine mitochondrial F_o F_1 -ATPase (6-9). While no conserved tryptophan residue is present in subunit c (proteolipid), subunit a of E. Coli and its bovine homologous ATPase-6 contain several tryptophan residues, located mainly in the carboxy-terminal region of the polypeptide (6). As the rate of sequence divergence for mammalian mitochondrial genomes is relatively fast (10,11), it is likely that the conserved portions of mitochondrially encoded proteins represent regions of structural and/or functional importance.

Interestingly tryphophan is the most conserved amino acid of the human and bovine mitochondrial genome (12), which might mean that these residues tend to be crucial for the function or structure of the proteins.

In this paper results are presented on the effect of 2-Hydroxy-5-nitrobenzyl bromide (HNB), a highly reactive and selective reagent towards tryptophan residues in proteins

(13,14), on proton conduction through the inner mitochondrial membrane of bovine heart submitochondrial particles and on the hydrolytic activity of mitochondrial H^+-ATPase.

MATERIALS AND METHODS

Electron transfer particles (ETP_H) were prepared from bovine heart mitochondria (15) according to Beyer (16). F_1-depleted submitochondrial particles (urea-particles, U-SMP) were prepared from ETP_H following the method of Racker and Horstman (17). F_1-ATPase was purified from ETP_H according to Beechey et al. (18). Protein was determined by the biuret method (19) in the presence of 1% deoxycholate or according to Lowry et al. (20).

ATPase activity was measured spectrophotometrically at pH 7.5 using an ATP-regenerating system (21). When the activity of particles was assayed rotenone 1 μg was present; 4mM ATP was added to start the reaction. The anaerobic proton release of ETP_H and urea-particles, energized with succinate, was analyzed following the fluorescence changes of the 9-aminoacridine, a commonly utilized probe for the evaluation of transmembrane pH differences in acidic-inside membrane vesicles (22,23). Samples were prepared adding to 1 ml of air-saturated buffer (40 mM glycylglycine, pH 7.8; 0.5 M sucrose; 1 mM EDTA; 6 mM $MgCl_2$; 100 mM KCl) 2 μg valinomycin, 2 μg rotenone, 6 nmol 9-aminoacridine, 2 mg of protein (ETP_H or U-SMP) and 10 μmol succinate. When the anaerobic proton release was analyzed in presence of oligomycin, 1 nmol of inhibitor was added to the assay medium. The assay was carried out at 20°C using a Jasco FP-550 spectrofluorimeter equipped with a thermostating system. The excitation and emission wavelengths used were 400 and 460 nm respectively. Incubation of particles with HNB (from a 0.1M stock solution in acetone, made daily) was performed for 2 min at 20°C in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris/Cl, pH 7, where the reagent is highly selective for tryptophan residues (13).

HNB was purchased from Sigma Chemical Co., St. Louis USA.

RESULTS AND DISCUSSION

Fig. 1 and Table I show that the tryptophan reagent HNB caused activation of the anaerobic relaxation of the proton gradient

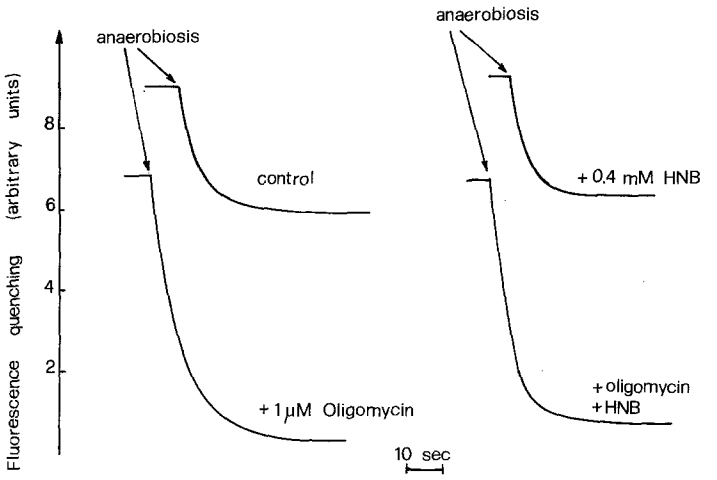

Fig. 1 - Effect of HNB on the rate of anaerobic H^+ release from ETP_H. 2 mg protein were incubated in 1 ml air-saturated 40 mM glycylglycine pH 7.8, 100 mM KCl, 0.5 M Sucrose, 1 mM EDTA, 6 mM $MgCl_2$ containing 2 μg valinomycin, 2 μg rotenone and 6 μM 9-aminoacridine. ETP_H were energized with 10 mM succinate. The curves represent the 9-aminoacridine fluorescence quenching decrease occuring when the anaerobiosis was attained in the reaction mixture.

set up by succinate respiration in "inside-out" submitochondrial particles (ETP_H). The presence of oligomycin, a specific inhibitor of H^+-conduction by the F_o-sector of H^+-ATPase (24), induced a two-fold increase of the fluorescence quenching and a decrease of the anaerobic proton release rate

TABLE I

Effect of HNB on the anaerobic release of respiratory $\Delta\mu_{H+}$ in ETP_H

Addition	rate anaerobic H^+ release $1/t_{\frac{1}{2}}$ (sec^{-1})	% control
Control	0.19	100
0.4 mM HNB	0.33	174
1 μM oligomycin	0.16	84
HNB plus oligomycin	0.21	110

Conditions were the same as those described in the legend of Fig. 1.

TABLE II

Effect of HNB on the ATPase activity of ETP_H and its oligomycin sensitivity

Addition	ATPase activity (μmol/min.mg)	% control
Control	0.42	100
+ 0.4 mM HNB	0.60	143
+ 0.1 μM oligomycin	0.05	12
HNB plus oligomycin	0.05	12

The ATPase activity was determined using an ATP-regenerating system and the oligomycin sensitivity was evaluated adding to the assay medium a molar 10-fold excess of the inhibitor: the amount of enzyme was considered to be 0.12 nmol/mg ETP_H protein, according to Bertina et al. (40).

($1/t_{\frac{1}{2}}$). A two min preincubation with 0.4 mM HNB caused, on the contrary, a 74% stimulation of the proton release rate from ETP_H, whilst the HNB stimulation was lower when oligomycin was present in the medium: the anaerobic H^+release rate increased 31% only.

Table II shows the data concerned with the effect of HNB on the hydrolytic activity of H^+-ATPase in ETP_H. The reagent caused a significant stimulation of the ATPase activity that was still oligomycin sensitive. The above experiments suggest that the tryptophan reagent acted by enhancing primarily the proton conductivity of the H^+-ATPase and, to a lesser extent, the conductivity of other mitochodrial membrane proton channels. Then the stimulatory effects of HNB result mainly from the binding of a hydroxy-nitrobenzyl group (13) to one or more tryptophan residues in polypeptide(s) belonging to the F_oF_1-ATPase complex.

TABLE III

Effect of HNB on the anaerobic release of respiratory $\Delta\mu_{H^+}$ in U-SMP

Addition	rate anaerobic H^+ release	
	$1/t_{\frac{1}{2}}$ (sec-1)	%control
Control	0.46	100
0.4 mM HNB	0.67	146
1 μM oligomycin	0.24	52
HNB plus oligomycin	0.29	63

Conditions were the same as those described in the legend of Fig. 1.

In order to distinguish whether F_o or F_1 polypeptides were modified, we followed the effects of HNB on the ATPase activity of F_1, that contains a single tryptophan in its ε-subunit (26) and on the proton transport activity of F_1-depleted particles. Treatment of isolated F_1-ATPase with 0.4 mM HNB did not result in any appreciable change of the ATP-hydrolytic rate (not shown), indicating that, at the concentration tested, the reagent could not change the catalytic activity of F_1. On the other hand, after removal of F_1 from submitochondrial particles (U-SMP), HNB could still act on F_o polypeptides and in U-SMP similar activation of the protonophoric activity was found (Table III) as in ETP_H. The reagent enhanced the proton efflux rate as indicated by $1/t_{\frac{1}{2}}$ that underwent from 0.46 to 0.67 sec (+46%). Addition of oligomycin to U-SMP decreased the HNB effect as $1/t_{\frac{1}{2}}$ changed from 0.24 to 0.29 (+21%).This clearly indicates the site of HNB effect is the membrane sector F_o of the mitochondrial

ATPase. It remains to be assessed the modification of which F_o polypeptide(s) is responsible for the observed effects.

Tryptophan residues are absent in all types of subunit c (6), however they are present in other subunits which might be involved in H^+-channel of F_o . For instance ATPase-6, polypeptide component of the bovine mitochondrial ATPase, contains three tryptophan residues (12) being homologous to subunit a of E. Coli F_o-ATPase, which is essential for the proton transport (4, 7-9) .Therefore it is likely that tryptophan residue(s) of ATPase-6 may be the main target of HNB in ETP_H.

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