BUTYLHYDROXYLAMINE INHIBITS H^+-DRIVEN ATP SYNTHESIS OF THE $TF_1 \cdot F_o$-ATPase INCORPORATED INTO LIPOSOMES

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1. Introduction

Alkylhydroxylamines (R–NHOH) and hydroxylamine (NH_2OH) are known as reagents for activated carboxyl groups and have been used, for example, to identify the carboxylphosphate of the Na^+,K^+-ATPase [1,2]. Though it appears to be generally accepted that no activated phosphates are formed in the H^+-ATPases [3], we were encouraged by the following reasons to look for it once more:

(i) The proposed minimum chemistry of protons, ADP and phosphate

$$ADP + P_i + 2H^+ \rightarrow ATP + H_2O$$

does not appear to be supported by the experience of chemistry [5]. It is more probable that water formation precedes the phosphorylation reaction and this implies the interaction of two functional groups of the ATPase, for example of a carboxyl with a tyrosyl group to a tyrosyllactone [5]. Thus acid–base reactions between protons and phosphate or ADP are excluded;

(ii) Vesicles reconstituted from purified $TF_1 \cdot F_o$-ATPase and phospholipids of the thermophilic bacterium PS 3 were described by Y. Kagawa and N. Sone [6], who were able to synthesize ATP in high yield (40–150 nmol ATP/mg protein) by proton jump experiments.

In this system it is possible to study the action of nucleophilic trapping reagents, like alkylhydroxylamines, directly on the ATP synthase reaction, the forward reaction, instead of on the ATPase reaction.

Abbreviations: DCCD, dicyclohexylcarbodiimide; ANS, 8-anilinonaphthalene-1-sulfonate; 9-AA, 9-aminoacridine

In contrast to the action of hydroxylamine on mitochondria [7], no redox reaction of these reagents with the electron transport complexes may take place. Additionally, they do not need to pass the membrane for their expected reaction with the ATPase molecule and are added together with the basic buffer to the vesicles having an acidic internal environment. Thus, the reactive species of alkylhydroxylamines, the free bases, are present from the beginning of the proton jump experiment. It is reported here that butylhydroxylamine inhibits ATP synthesis of $TF_1 \cdot F_o$ vesicles in proton jump experiments.

2. Materials and methods

Propyl- and butylhydroxylamine were synthesized by reduction of the corresponding 1-nitroalkanes [8] and purified to analytical grade by column chromatography on Sephadex LH 20 with water as the solvent. The DCCD-sensitive ATPase complex $TF_1 \cdot F_o$ and the phospholipids of the thermophilic bacterium PS 3 were prepared as in [6]. ANS- and 9-AA fluorescence were measured in a Perkin-Elmer Fluorescence Spectrometer MPF-4 as in [6]; for details see legends of fig.1 and fig.2. Net ATP synthesis was assayed as in [6]; for details see legend of table 1.

3. Results and discussion

Net ATP synthesis (89 nmol ATP/mg $TF_1 \cdot F_o$) by proton jump experiments in vesicles, reconstituted from purified $TF_1 \cdot F_o$-ATPase and phospholipids of thermophilic bacterium PS 3, was inhibited by hydroxylamine (NH_2OH) and its alkylderivatives of

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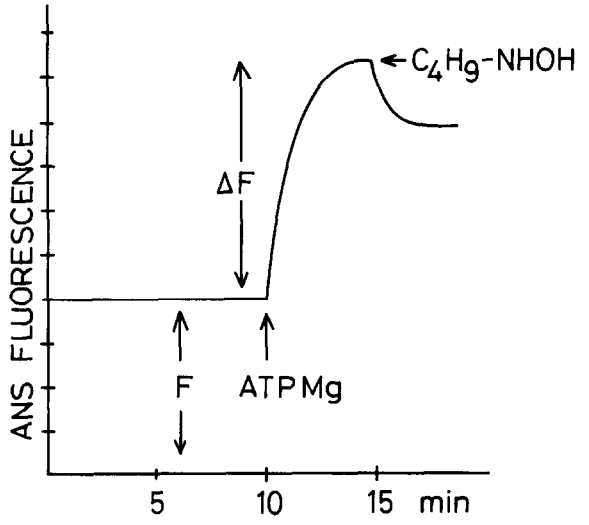

Fig.1. The effect of butylhydroxylamine on ANS-fluorescence enhancement induced by the addition of ATP. Experimental conditions: To 50 μl vesicles (40 μg $TF_1 \cdot F_O$) in 2 ml buffer of 50 mM Tris–sulfate (pH 8.0), 2 mM $MgSO_4$ and 20 μg ANS at 45°C, a mixture of 0.5 μmol ATP and 0.25 μmol $MgSO_4$ were added after 10 min. Addition: 5 μmol butylhydroxylamine. Excitation: 365 nm; emission: 480 nm.

Table 1
Inhibition of ATP synthesis by alkylhydroxylamines

Conditions	cpm	% Inhibition
Without vesicles	60	–
With vesicles	470 (89 nmol ATP/g mg $TF_1 \cdot F_O$)	0
With vesicles + NH_2OH	350	30
With vesicles + C_3H_7–NHOH	180	71
With vesicles + C_4H_9–NHOH	110	88

The reconstituted vesicles (40 μg of $TF_1 \cdot F_O$, 2 mg of PS 3 phospholipids) were first incubated in an acidic medium (pH 5.5, final vol.: 0.25 ml) containing 10 μmol malorate, 1 μmol of ADP and 0.1 μg of valinomycin at 40°C for 10 min. Then, 0.25 ml of an alkaline medium containing 40 μmol glycylglycine (pH 8.5), 7.5 μmol KCl, 0.5 μmol $MgSO_4$, 5 μmol of [^{32}P]phosphate (1.5 × 10^6 cpm), 25 μmol glucose, 10 units of hexokinase and 5 μmol inhibitor were added. Incubation time: 30 s. This test sample (0.5 ml) was mixed with 2 ml of ammonium molybdate solution and 0.1 ml triethylamine as in [6]. Final vol.: 2.6 ml. To count Cerenkov rays, emitted from esterified ^{32}P, 1 ml of the aqueous phase was taken

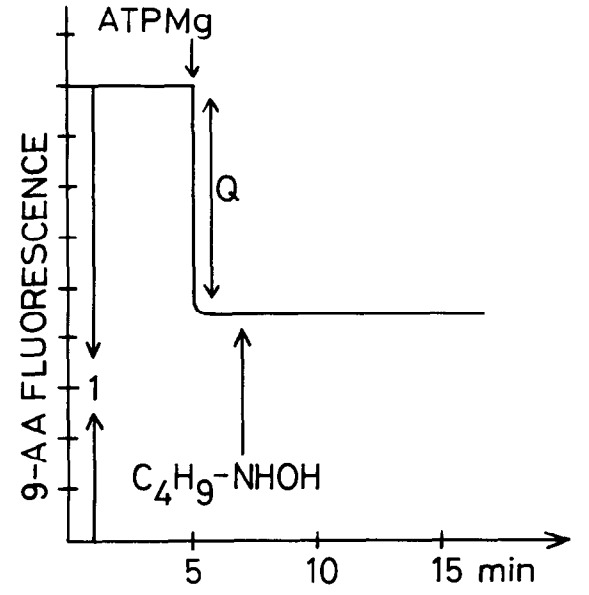

Fig.2. The effect of butylhydroxylamine on 9-AA fluorescence quenching induced by the addition of ATP. Experimental conditions: To 50 μl vesicles (40 μl $TF_1 \cdot F_O$) in 2 ml buffer of 20 mM Tricine–NaOH (pH 7.7), 50 mM $NaNO_3$, 2 mM $MgSO_4$ and 4 μM 9-AA at 30°C, after 5 min 5 μmol ATP and 25 μmol $MgSO_4$ were added. Addition: 5 μmol butylhydroxylamine. Excitation: 365 nm; emission: 451 nm.

lower chain lengths (R–NHOH). A concentration of 1 mM propylhydroxylamine (C_3H_7–NHOH) prevented ATP synthesis by ~25%, whereas hydroxylamine had no effect. With a final concentration of 10 mM, which correspondended to that of orthophosphate, the butyl derivative and hydroxylamine depressed ATP synthesis nearly completely, and only to 70% of the original value respectively (table 1).

It was impossible to distinguish in the proton jump experiments between inhibition and uncoupling of ATP synthesis, because it was not possible to measure the proton gradient, ΔpH, if the basic buffer was added together with butylhydroxylamine. We therefore used the reverse reaction, the ATPase reaction, to build up ΔpH and the membrane potential Δψ, and then to study the action of butylhydroxylamine on ΔpH and Δψ.

ΔpH was determined qualitatively by quenching of 9-AA (9-aminoacridine) fluorescence and Δψ by the enhancement of ANS (8-anilinonaphthalene-1-sulfonate) fluorescence as in [9]. The inhibitory concentration of 10 mM butylhydroxylamine had no effect on ΔpH (fig.1), indicating that the quenching of 9-AA could not be relieved, as by uncouplers. The same concentration suppressed slightly the enhance-

ment of ANS, supporting the interpretation of the experiments with 9-AA that the membrane was not permeable to butylhydroxylamine in the uncharged form. In contrast to this inhibitor, Tris was able to pass the vesicle membrane. It increased $\Delta\psi$, probably by the formation of the corresponding ammonium ion, with concomitant decrease of ΔpH [9]. It was concluded that butylhydroxylamine is an inhibitor and not an uncoupler of ATP synthesis by an artificially imposed $\Delta\mu H^+$ in reconstituted H^+-liposomes. It is reasonable, although still speculative, to assume by analogy to chemistry, that butylhydroxylamine reacts with an activated carboxyl group, which may be phosphorylated or not. If the latter is not phosphorylated, butylhydroxylamine has to compete with the same concentration of orthophosphate for this activated group within 10–20 s, because half of the amount of ATP is synthesized within 5–10 s. It should be possible to identify trace amounts of such activated carboxyl groups using highly labelled radioactive inhibitor. These experiments are in progress.

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