[2,3-^{3}H]Propylhydroxylamine inhibits ATP synthesis of TF_1F_o liposomes in proton jump experiments

Evidence for an activated carboxyl group in the DCCD-binding protein

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ATP synthesis of $TF_1 \cdot F_o$ liposomes in proton-jump experiments was inhibited by *N*-[2,3-^{3}H]propylhydroxylamine. High radioactivity was shown by SDS–PAGE to be incorporated into the proteolipid. The activation of one of its carboxyl groups, which was apparently trapped by the hydroxylamine derivative, may proceed by the protonation of an undissociated carboxyl group as part of the proton translocation. If ADP was omitted, no trapping reaction occurred. Thus binding of ADP to TF_1 appeared to be necessary for such an activation of a carboxyl group.

Inhibition *ATP synthesis* *Tritiated N-propylhydroxylamine* *H^+-jump* *Modified proteolipid*

1. INTRODUCTION

The chemical mechanism of ATP synthesis by H^+-ATPases may now be studied not only by use of the reversed reaction, the ATPase reaction of the purified, water-soluble F_1-enzymes, but also by the forward reaction, because reconstituted systems of several purified ATP synthases ($TF_1 \cdot F_o$, $BF_1 \cdot F_o$, $CF_1 \cdot F_o$) in liposomes have been described [1–3]. The ATP synthase $TF_1 \cdot F_o$ of the thermophilic bacterium PS3 was the most effective, synthesizing 40–150 nmol ATP/mg protein in proton jump experiments, if incorporated into liposomes of PS3 phospholipids [1]. This excellent preparation of Y. Kagawa and N. Sone allowed us to test the possibility whether phosphate may be activated in this ATP synthase reaction by protons via a carboxyl phosphate. We proposed in [4,5] that water formation should precede the phosphorylation reactions to avoid simple acid–base reaction of protons with phosphate or ADP anions. Therefore we had to assume an activated ester, for example tyrosyl carboxylate, as precursor of the expected carboxyl phosphate.

The classical trapping reagents for activated carboxyl groups, hydroxylamine (NH_2OH) and its *N*-alkyl derivatives (R-NHOH), could be used in this chemical approach because, in contrast to studies with well-coupled mitochondria [6,7], no redox centers were present in the liposomes with the purified ATP synthase $TF_1 \cdot F_o$ and therefore no oxidation of these compounds may occur, for example, of hydroxylamine (NH_2OH) to nitrogen oxide (NO). Furthermore, these reagents were present in their active form, the free base, because they were added in the basic buffer of proton jump experiments, together with phosphate. In an earlier

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Abbreviations: DCCD, dicyclohexylcarbodiimide; ^{3}H-PHA, [2,3-^{3}H]propylhydroxylamine; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis

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paper [5] we were able to show that *N*-alkylhydroxylamines of increasing chain length inhibited ATP synthesis in these experiments. An uncoupling reaction was excluded, because the electrochemical proton gradient ΔpH of $TF_1 \cdot F_o$ liposomes, built up by ATP hydrolysis, did not collapse by the addition of the inhibitory concentration of *N*-butylhydroxylamine (C_4H_9NHOH), the most reactive derivative [5].

We used *N*-propylhydroxylamine (C_3H_7NHOH) in the following experiments, because a seemingly simple synthesis of its tritiated compound with a high specific activity of 600 mCi/mmol was described by Yoda et al. [8], which allowed them to identify the carboxyl phosphate of Na^+/K^+-ATPase [9]. This highly specific activity was assumed by us to be necessary for trapping trace amounts of an activated carboxyl group during a proton jump experiment, because 50% of ATP were synthesized within 5 s [1]. In this paper we report that in proton jump experiments with $TF_1 \cdot F_o$ liposomes in the presence of [2,3-^{3}H]propylhydroxylamine ([^{3}H]PHA) high amounts of radioactivity could be detected by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) predominantly at an M_r of 7500, which corresponded to the relative mobility of the DCCD-binding protein.

2. MATERIALS AND METHODS

[2,3,-^{3}H]Propylhydroxylamine: $^3HCH_2-{}^3HCH-CH_2-NHOH$

(spec. act. 600 mCi/mmol) was synthesized essentially according to Yoda et al. [8] by reduction of the corresponding 1-[2,3-^{3}H]nitropropane (spec. act. 2 Ci/mmol), which was a product of Amersham Buchler (Braunschweig). The purification procedure was simplified by separating the hydrochloride of this hydroxylamine derivative on Sephadex LH 20 with water as the eluant. After evaporation the residue was extracted with isopropanol/ether (10:1, v/v), in which NH_4Cl was insoluble. When this procedure was repeated 4 times, we obtained the hydrochloride as a slightly yellow oil, which was contaminated by minor amounts of propylamine, as detected by ^{1}H-NMR. The ATP synthase $TF_1 \cdot F_o$ and phospholipids of the thermophilic bacterium PS3 as well as the corresponding $TF_1 \cdot F_o$ vesicles were prepared as in [1]. Net ATP synthesis in proton jump experiments was assayed as in [1]. If 10 mM [2,3-^{3}H]propylhydroxylamine was used as inhibitor, 10 mM phosphate were added without [^{32}P]phosphate, for details see fig.1. Each proton jump experiment was taken out of the water bath of 40°C after 30 s and centrifuged at 25°C at 200000 × *g* for 30 min. The supernatant was discarded and the pellet resuspended in a 1:1 mixture of the acidic and basic buffer of the proton jump experiments [1]. Centrifugation and resuspension were repeated 6 times. The pellets were analyzed by 10% SDS–polyacrylamide disc electrophoresis according to Weber and Osborn [10] and in parallel runs ^{14}C-labeled proteins, which were purchased from NEN (FRG), were used as M_r markers (5766–46000). The gels were cut into 1 mm slices and solubilized by 0.5 ml H_2O_2 at 70°C for 24 h. Insta-Gel (Packard) was used for counting the radioactive content.

Table 1

Inhibition of ATP synthesis by 10 mM *N*-propylhydroxylamine

Conditions	cpm	% inhibition
Without vesicles	45	–
With vesicles	390 $\left(\frac{60 \text{ nmol ATP}}{\text{mg } TF_1 \cdot F_o}\right)$	0
With vesicles + C_3H_7NHOH	142	72

The reconstituted vesicles (25 μg $TF_1 \cdot F_o$, 3.75 mg of PS3 phospholipids) were first incubated in an acidic medium (pH 5.5, final vol. 0.25 ml) containing 10 μmol malonate, 1 μmol ADP and 0.1 μg 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 μl [^{32}P]phosphate (3.0×10^6 cpm), 25 μmol glucose, 10 units of hexokinase and 5 μmol inhibitor were added. Incubation time was 30 s. This test sample (0.5 ml) was mixed with 2 ml ammonium molybdate solution and 0.1 ml triethylamine and treated as in [1]. Final volume was 2.6 ml. To count Cerenkov rays, emitted from esterified ^{32}P, 1 ml of the aqueous phase was taken

3. RESULTS

Vesicles reconstituted from PS3 phospholipids and purified ATP synthase $TF_1 \cdot F_o$ according to Kagawa and Sone [1] were capable in our preparation of synthesizing 60 nmol ATP/mg $TF_1 \cdot F_o$ in proton jump experiments (table 1). As described in [5], about 72% inhibition of this H^+-driven ATP synthesis was found in the presence of 10 mM propylhydroxylamine (C_3H_7NHOH), if added together with the basic buffer (pH 8.5) to the incubation of $TF_1 \cdot F_o$ vesicles in acidic buffer (pH 5.5) (table 1). In parallel experiments 10 mM [^{3}H]PHA (spec. act. 600 mCi/mmol) were used instead of the cold substance and 10 mM phosphate without the addition of [^{32}P]phosphate. In expt. 1 (fig.1) the proton jump experiments were performed with twice the amounts of vesicles, radioactive substance and buffers as those with cold propylhydroxylamine (table 1), because we expected only minor incorporation of the trapping reagent into the enzyme. If we analyzed ^{3}H-incorporation by SDS–PAGE we found high amounts of radioactivity at a position, which corresponded to the relative mobility of the DCCD-binding protein (fig.1). Expt.2 was the same as expt.1 with the exception that ADP, the primary acceptor for the phosphoryl group, was omitted and that 20 mM instead of 10 mM [^{3}H]PHA were present. In SDS–PAGE only low radioactivity was detected at an M_r ~5000, which may be ascribed to radioactivity unspecifically bound to phospholipids. In expt.3 we used for the blank our standard conditions of table 1, which implied half the amount of vesicles of expt.1 and 2. These vesicles were incubated in the basic medium only, in which 10 mM [^{3}H]PHA were dissolved. The analysis by SDS–PAGE revealed low radioactivity, at the same position as in expt.2.

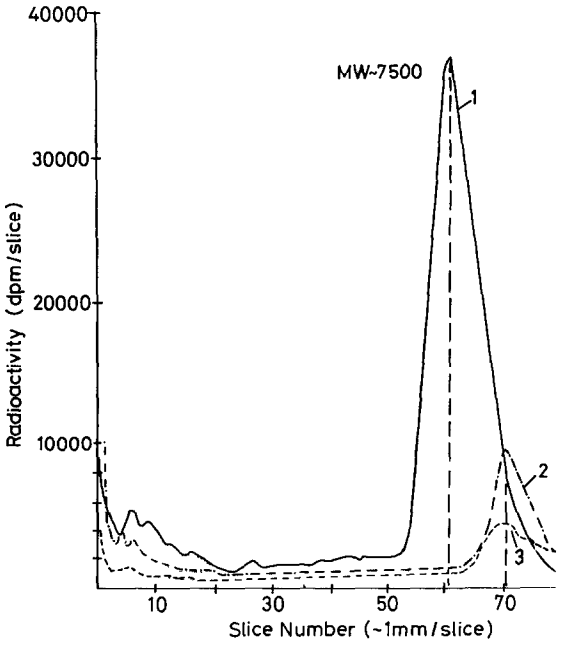

Fig.1. Electrophoretic analysis of $TF_1 \cdot F_o$ vesicles, with or without proton jump in the presence of inhibitory concentrations of [^{3}H]PHA. The experimental details of proton jump experiments are described in the legend of table 1 and in section 2. (Expt.1) 200 μl vesicles (50 μg $TF_1 \cdot F_o$, 7.5 mg PS3 phospholipds): (a) additions, 2-fold as described in table 1 plus 13.2 × 10^9 cpm [^{3}H]PHA (10 mM); (b) omissions, [^{32}P]phosphate. (Expt.2) 200 μl vesicles (50 μg $TF_1 \cdot F_o$, 7.5 mg PS3 phospholipids): (a) additions: 2-fold as described in table 1 plus 26.4 × 10^9 dpm ^{3}H-PHA (20 mM); (b) omissions, [^{32}P]phosphate and ADP. (Expt.3) 100 μl (25 μg $TF_1 \cdot F_o$, 3.75 mg PS3 phospholipids): (a) additions, the vesicles were incubated in the basic medium only as described in table 1, plus 6.6 × 10^9 dpm [^{3}H]PHA; final vol., 0.5 ml; (b) omissions, [^{32}P]phosphate and the acidic medium of table 1.

4. DISCUSSION

We were very surprised to have found by SDS–PAGE, radioactivity mainly incorporated into a low-M_r protein of M_r ~7500, if proton jump experiments with $TF_1 \cdot F_o$ liposomes were performed in the presence of [2,3-^{3}H]propylhydroxylamine.

We had initiated these experiments with the working hypothesis that a carboxyl group of the β-subunit in the $TF_1 \cdot F_o$ enzyme may be involved in the phosphate activating reaction. The β-subunit is considered at present to be the catalytic subunit of the ATPase reaction and modification of one of its carboxyl groups by DCCD apparently inhibited ATP hydrolysis [11].

In contrast to this working hypothesis only very low radioactivity was detected by SDS–PAGE at a position which corresponded to the M_r of the β-subunit, but a very high one was apparently incorporated into the DCCD-binding protein (fig.1, expt.1). This interpretation was supported by the

fact, that $TF_1 \cdot F_o$ liposomes, which were incubated with the same concentration of [^{3}H]PHA, but without proton jump (fig.1, expt.3), showed in SDS–PAGE low amounts of radioactivity at lower M_r (5000). The same pattern of radioactivity was found, if the proton jump experiment of expt.1 was carried out in the absence of ADP (fig.1, expt.2), but with the double concentration of [^{3}H]PHA. These latter results excluded the possibility that the high radioactivity at the M_r of the DCCD-binding protein may be ascribed to unspecific binding. Because [^{3}H]PHA was known and used as specific trapping agent for activated carboxyl groups [8,9], this high radioactivity indicated most likely the existence of such a reactive group in the DCCD-binding protein.

From the present knowledge in this field it appears to be highly unprobable that a carboxyl group of the DCCD-binding protein may be involved in phosphate activation via a carboxyl phosphate. It is, however, an open question where P_i may be bound in the ATP synthase. The finding of Penefsky [12] that ADP inhibited P_i-binding in the soluble mitochondrial F_1-ATPase put in question, whether both, ADP and P_i may be bound on a common reaction coordinate and if the soluble ATPase may be the ATP synthase. Another striking result was that DCCD inhibited ATP synthesis in bacteria, chloroplasts and mitochondria with substoichiometric amounts; in PS3 bacteria only one of three proteolipids was modified by DCCD [13,14]. Because DCCD is a trapping reagent for undissociated carboxyl groups, the two, under these conditions unreactive carboxyl groups were carboxylate anions or were chemically modified.

Much more probable is the interpretation that carboxyl groups were activated to react to hydroxamates ($R_1CO–NR_2–OH$) by the well-known mechanism of proton catalyzed esterification [15]. Thus the activation may be described as protonation of an undissociated carboxyl group ($H\dot{O}=C–OH$) and this oxonium ion may be part of the proton translocation.

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