INHIBITION OF PROTON CONDUCTION BY CHEMICAL MODIFICATION OF THE MEMBRANE MOIETY OF PROTON TRANSLOCATING ATPase

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1. Introduction

Biomembranes that synthesize ATP contain a reversible H^+-translocating ATPase, named $F_0 \cdot F_1$ as a coupling device [1,2]. We have shown that upon reconstitution into phospholipid vesicles the purified enzyme ($TF_0 \cdot F_1$) from a thermophilic bacterium PS3 can catalyze the formation of an electrochemical proton gradient by ATP hydrolysis and carry out ATP synthesis coupled to H^+ conduction [3–5].

The coupling device is composed of an enzyme (TF_1) with ATPase activity and a membrane moiety (TF_0) with H^+ conductivity [6–9]. The preparation of TF_0 was obtained by treating $TF_0 \cdot F_1$ with urea and removal of contaminants on a CM-cellulose column. The purified preparation contains only 2 kinds of subunits [10]. Band 6 protein (13 500 daltons) is able to bind TF_1 and thus named TF_1-binding protein. Band 8 protein (6500 daltons) is a proteolipid, specifically binds *N,N'*-dicyclohexylcarbodiimide (DCCD) and therefore is named DCCD-binding protein like its analogs from other sources [11–15]. Analysis of inhibition by DCCD has shown that an oligomer of DCCD-binding protein is responsible for H^+ conduction [16]. DCCD-binding protein consists of 64 amino acid residues (mainly hydrophobic ones), which include a single tyrosine residue but neither histidine, lysine, tryptophan nor cysteine [16]. Since DCCD is known to inhibit H^+ conduction by binding covalently to a glutamate residue [12,13], chemical modification of other H^+-dissociable residues might give some information on the mechanism of H^+ permeation across the membrane. This communication reports that modification of either tyrosine or arginine residue results in the loss of H^+ conduction.

2. Experimental

2.1. *Preparations*

H^+-translocating ATPase ($TF_0 \cdot F_1$) and soluble ATPase (TF_1) from the thermophilic bacterium PS3 were purified as in [6,7]. H^+-conducting hydrophobic moiety (TF_0) was prepared by treating $TF_0 \cdot F_1$ with 7 M urea as in [10]. The method for reconstitution of TF_0 with partially-purified soy bean phospholipids into vesicles (TF_0-vesicles) and the method for loading TF_0-vesicles with KCl were carried out as in [9].

2.2. *Assay methods*

H^+-conductivity of vesicles was followed using 9-aminoacridine as an indicator of pH inside the vesicles [3], in response to an artificial membrane potential (inside negative) imposed by K^+ diffusion mediated by valinomycin as in [9]. TF_1-binding activity was measured by ATPase activity of the TF_1 that was co-precipitated with the vesicles [10]. Amino acid composition was analysed for the hydrolyzate of protein in 6 N HCl at 110°C for 20 h using Durrum D-500 amino acid analyser.

2.3. *Reagents*

Lactoperoxidase was obtained from Boehringer Mannheim, tetranitromethane, chloramine T and rose bengal from Wako Pure Chemical (Osaka), phenylglyoxal and glyoxal from Tokyo Kasei (Tokyo). The other chemical reagents and inhibitors were obtained as in [10,16]. Tetranitromethane was washed 3 times with water to remove impurities and used as an ethanolic solution.

3. Results and discussion

3.1. *Effect of tyrosine modification on H^+ conduction*

Dye-sensitized photo-oxidation is known to inactivate enzymes by degrading histidine, tryptophan, cystein, methionine and tyrosine residues [17]. Table 1 shows that illumination of TF_0 in the presence of rose bengal caused considerable inhibition of H^+ conductivity after reconstitution with phospholipid vesicles (exp. 1). DCCD-binding protein (band 8 protein, 6500 daltons) has been shown to be responsible for H^+ conduction [10] and this protein contains neither histidine, tryptophan nor cysteine [16]. Thus the inactivation of H^+ translocation is probably caused by degradation of either methionine or tyrosine residue.

Chloramine T which is known to oxidize methionine residues [18] affected neither H^+ conduction nor TF_1-binding (exp. 2). On the other hand some modifiers of tyrosine-inactivated H^+ conduction (exp. 3). However, lactoperoxidase did not inhibit either activity probably because tyrosine residues were not accessible to the enzyme.

3.2. *Nitration of tyrosine residue in DCCD-binding protein*

Treatment of TF_0 vesicles with tetranitromethane resulted in the inhibition of H^+ conduction (fig.1). Half maximum inhibition of H^+ conduction was obtained by 2.5 mM tetranitromethane and almost complete inhibition was obtained at 8 mM.

Since 3-nitrotyrosine is stable in 6 N HCl at 110°C [19], modification of tyrosine residues in DCCD-binding protein can be followed by amino acid

Table 1
Inhibitory effects of tyrosine modifying reagents on H^+ conduction

Reagents	Incubation (min)	Relative activity (%)	
		H^+-conduction	TF_1-binding
Exp 1			
None	0	100	100
Rose bengal (5 μg/ml)	2	42	115
Rose bengal (5 μg/ml)	9	25	113
Rose bengal (5 μg/ml)	20	10	104
Exp. 2			
None	30	100	100
Chloramine T (4 mM)	30	96	108
Chloramine T (10 mM)	30	76	92
Exp. 3			
None	10	100	100
Tetranitromethane (8 mM)	10	7	96
I_2 (0.23 mM in 0.7 mM NaI)	10	28	92
NaI (10 mM) with LPO	10	106	88
NaI (10 mM) with LPO	20	91	125

Exp. 1. TF_0-vesicles (0.2 ml) were photooxidized in 10 mM tricine/NaOH (pH 8.0) in total vol. 0.5 ml at 4°C with stirring under a 500 W tungsten lamp at 10 cm. Exp. 2 TF_0-vesicles (0.2 ml) were incubated in 80 mM Tris/HCl (pH 8.0) in total vol 0.5 ml at 25°C Exp. 3 TF_0 vesicles (0.3 ml) were treated with tetranitromethane as in fig.1, iodine [19] or LPO, lactoperoxidase [20]. After 6 ml of a 0.5% solution of histidine (exp. 1), methionine (exp. 2) or 2-mercaptoethanol (exp. 3) had been added, TF_0-vesicles were collected by centrifugation and assayed for H^+ conduction and TF_1 binding. TF_1-binding assay in exp. 1 was carried out in the dark. Control values of H^+ conduction and TF_1-binding were 2 6 (exp. 1), 0.9 (exp. 2) and 1.1 (exp. 3) μg ion/min·mg TF_0 and 5.3 (exp. 1), 4.3 (exp. 2) and 3.9 (exp. 3) units/mg TF_0, respectively

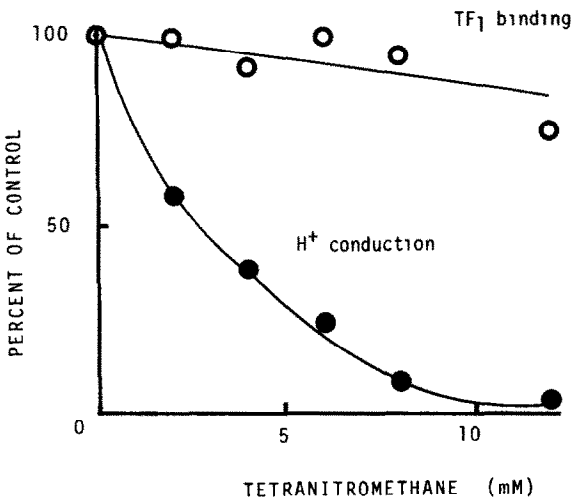

Fig.1 Effect of tetranitromethane concentration on H^+ conductivity and TF_1-binding activity of TF_0 vesicles. TF_0 vesicles (0.2 ml) were incubated with tetranitromethane in 0.2 M Tris/HCl (pH 8.0) in total vol. 0.5 ml for 10 min at 25°C Other conditions were as in exp 3 of table 1.

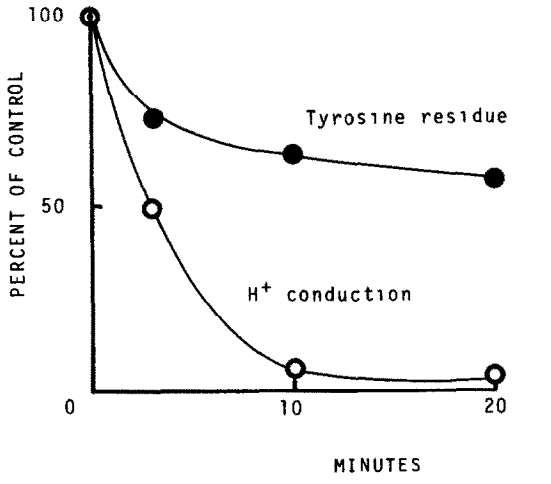

Fig.2. Time course of nitration of tyrosine residue and inhibition of H^+ conduction by tetranitromethane. TF_0 (0.39 mg protein) was mixed with 5 mM tetranitromethane in 0.2 ml of 0.2 M Tris/HCl (pH 8.0) at 25°C. At the indicated time 10 μl 2-mercaptoethanol was added to stop the reaction. An aliquot (0.1 ml) was used to reconstitute vesicles for the assay of H^+ conduction, and the rest (0.3 ml) was used to prepare proteolipid fraction (band 8 protein) as in [16] and its amino acid composition was analysed.

analysis. Figure 2 shows that after 10 min incubation, when 1/3rd of a tyrosine residue is nitrated, almost all H^+ conduction ceased. Since this protein has been determined to contain 1 tyrosine residue/molecule (6500 daltons), nitration of only 1 tyrosine residue/3 proteins resulted in total loss of H^+ conduction. A similar observation has been made with DCCD inhibition of TF_0 [16] and F_0 of *Escherichia coli* [12,21]. This led to the view that the H^+ channel in $(T)F_0$ represents an oligomer of DCCD-binding protein.

3.3. *Effects of arginine modifiers*

Table 2 lists the effects of glyoxal and phenylglyoxal on TF_0 vesicles. Both H^+ conduction and TF_1-binding activities were inhibited. Kinetics of the inhibition by phenylglyoxal are shown in fig.3. The fact that TF_1-binding activity decreases faster than H^+ conduction might indicate that there are two inhibition sites in TF_0: one is probably on band 6 protein which binds TF_1 and another is on band 8 protein which is responsible for H^+ conduction. Band 8 protein extracted from TF_0 treated with 40 mM phenylglyoxal for 60 min at 25°C contained 1.56 mol intact arginine, while 4.01 mol were present prior to the treatment. Evidence has been reported for the presence of arginine residue(s) susceptible to this reagent near the uncoupler-binding site [22].

3.4. *Structure of H^+ channel*

DCCD-binding protein from PS3 is an extremely hydrophobic protein; it contains only 5 glutamic

Table 2
Inhibitory effects of arginine modifying reagents

Reagents	Relative activity (%)	
	H^+-conduction	TF_1-binding
None	100	100
Glyoxal (69 mM)	36	93
Glyoxal (345 mM)	26	67
Phenylglyoxal (79 mM)	12	19

TF_0-vesicles (0.3 ml) were incubated in 80 mM Tris/HCl (pH 8.6) in a total volume of 0.5 ml at 25°C for 30 min. After 6 ml of 0.2% solution of arginine hydrochloride was added, TF_0-vesicles were collected by centrifugation and assayed for H^+ conduction and TF_1-binding Control values of H^+ conduction and TF_1-binding were 0 9 μgion/min·mg TF_0 and 4.3 units/mg TF_0, respectively

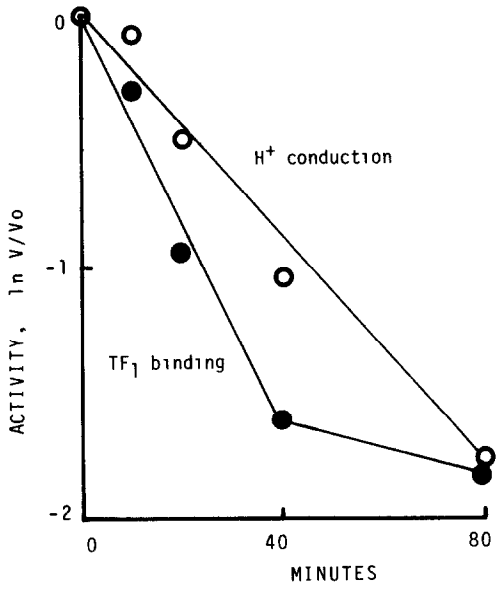

Fig.3. Time course of inactivation by phenylglyoxal of H^+ conduction and TF_1-binding activities. TF_0-vesicles (0.3 ml) were incubated with 59 mM phenylglyoxal in 0.1 M triethanolamine/HCl (pH 8.5) in total vol. 0.5 ml at 25°C. Other conditions were as in table 2.

acids, 1 aspartic acid (some of them may be amides), 4 arginines, and 1 tyrosine [16]. Modifications of such residues, a tyrosine residue by tetranitromethane, arginine residue(s) by phenylglyoxal, and a carboxyl group by carbodiimide [16], results in the blocking of H^+ conduction. Moreover results with tetranitromethane (fig.2) and with DCCD reported [16] indicate that the H^+ channel in TF_0 is made of an oligomer (possibly trimer) of DCCD-binding protein. Thus H^+ conduction through the membrane moiety of H^+-translocating ATPase might proceed through a hydrophilic channel formed by very hydrophobic proteins. Protonation and deprotonation of the polar groups facing the channel should be important for the H^+ movement. Bacteriorhodopsin, a light-driven H^+ pump, is also known to possess a H^+ channel moiety besides a H^+ pumping site containing retinal [23]. A model favourable to the present report has recently been reported [24].

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