Transmembrane topology of *Escherichia coli* H^+-ATPase (ATP synthase) subunit *a*

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Received 7 May 1996; revised version received 27 May 1996

Abstract ***Escherichia coli*** **H^+-ATPase subunit *a* is a hydrophobic F_0 subunit. To investigate the topology of the subunit in the membrane, we prepared site-specific polyclonal antibodies against amino-terminal (Ser-3 to Leu-16), middle loop (Lys-167 to Gln-181), and carboxyl-terminal (Thr-259 to His-271) peptide segments. Enzyme-linked immunosorbent assay revealed that these antibodies specifically reacted with subunit *a* of inside-out membrane vesicles, but not with that of right-side-out spheroplasts. Full reactivity appeared when spheroplasts were disrupted with Triton X-100 (0.5%) or by sonication. These results suggest that at least parts of the three peptide segments of subunit *a* face the cytoplasm. Based on these observations, we propose a novel transmembrane topology of subunit *a*.**

Key words: H^+-ATPase; ATP synthase; Subunit *a*; Hydrophobic F_0 subunit

1. Introduction

The H^+-translocating ATPase (F_0F_1) of *Escherichia coli* catalyzes ATP synthesis driven by an electrochemical proton gradient across the cytoplasmic membrane generated by the respiratory chain (for reviews, see [1–4]). The ATPase consists of two portions, F_1 and F_0. The catalytic sector F_1 is present peripherally on the membrane and has five subunits, α, β, γ, δ and ε, while the F_0 sector in the membrane has three subunits, *a*, *b* and *c*, and functions as a proton channel [1–4]. The *a* subunit (271 amino acid residues), coded by the *uncB* gene, is extremely hydrophobic [5,6]. By analyzing a series of nonsense and missense mutants as well as site-directed mutants, it was found that the Arg-210, Glu-219, and His-245 residues are important for H^+ translocation as well as the binding of F_1 [1–4], [7–10]. Furthermore, a region between Gln-252 and Leu-264 contains essential residues for F_0 functions, but seven carboxyl-terminal residues (Ser-265 to His-271) are dispensable [7,8].

To understand the mechanism of H^+ translocation through F_0, determination of the transmembrane topology of subunit *a* is necessary. So far, five transmembrane models of subunit *a*, based on the results of hydropathy analysis [5,6,10], protease digestion [6], chemical cross-linking [11], location of chimeric fusion protein [12,13], and revertant analysis [14], have been proposed. However, there are many discrepancies in the orientations and identification of transmembrane segments among these models. In this study, we prepared three kinds of site-specific polyclonal antibodies against amino- and carboxyl-terminal regions, and a middle loop segment to probe more directly the orientation of the subunit in the membrane. It was found that these antibodies were equally reactive with the *a* subunit of inside-out membranes but not to that of right-side-out vesicle spheroplasts.

2. Materials and methods

2.1. Bacterial strains and preparation of membrane vesicles

Strain DK8 (Δ*uncB-C*, *ilv*::Tn 10) lacking the *unc* operon [15] and the same strain harboring a recombinant plasmid, pBWU13 (carrying the wild-type *unc* operon) [16], were mainly used in this study. The bacterial cells had the highest membrane ATPase activity so far reported, in which about 25% of inner membrane proteins corresponded to F_0F_1-ATPase. Almost all the enzyme was incorporated into inner membranes with the proper orientation as revealed by electron microscopy, ATPase activities, ATP-dependent proton pumping and ATP synthesis [17]. Thus, the bacterial cell is useful for topological studies of F_0F_1. Strains with nonsense mutations in the *a* subunit were also used: KF24, Trp111→end [8]; and KF24A harboring pBB263e, Tyr263→end [7]. Parent strain (KY7230, wild type) was also used [8]. These cells were cultured in a rich medium (L-broth) containing 50 μg/ml ampicillin, harvested by centrifugation, and suspended in 50 mM Tris-Cl buffer (pH 8.0) containing 10 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.2 mM phenylmethylsulfonylfluoride, 5 μg/ml leupeptin and 5 μg/ml pepstatin A [17]. Inside-out membrane vesicles were prepared by passing cells through a French press [18]. Spheroplasts (right side-out vesicles) were prepared by lysozyme treatment [19], and suspended in phosphate-buffered saline comprising 148 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM $NaHPO_4$ (pH 7.2) (PBS) containing 20% (w/v) sucrose and 0.1 mg/ml chloramphenicol.

2.2. Preparation of site-specific antibodies

According to the hydropathy plot of subunit *a* shown in Fig. 1, the following peptides were synthesized and conjugated with keyhole limpet hemocyanin by the addition of glutaraldehyde [20]: peptide aN covering the amino-terminal region, ^{3}SENMTPQDTIGHHL; peptide aC for the carboxyl-terminal region, ^{259}TIVTLSMASGEEH; and peptides aM1 and aM2 for loop regions ^{87}NGSVKDMYHGKSK and ^{167}KMKGIGGFTKELTLQ, respectively. Antibodies were raised by injecting these antigens emulsified with Freund's complete adjuvant into albino rabbits. The antisera (0.5 ml) were diluted 20-fold with PBS containing 0.05% Tween 20 (PBS-Tween), and then incubated at room temperature for 2 h with nitrocellulose sheets (Toyo Roshi Co., Ltd), to which the purified subunit *a* was electrically transferred. The nitrocellulose sheets were washed several times with PBS-Tween, suspended in 2 ml of 0.1 M glycine-HCl (pH 3.0), and then agitated

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Abbreviations: PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; F_0F_1, *Escherichia coli* F_0F_1-ATP synthase

PII S0014-5793(96)00621-7

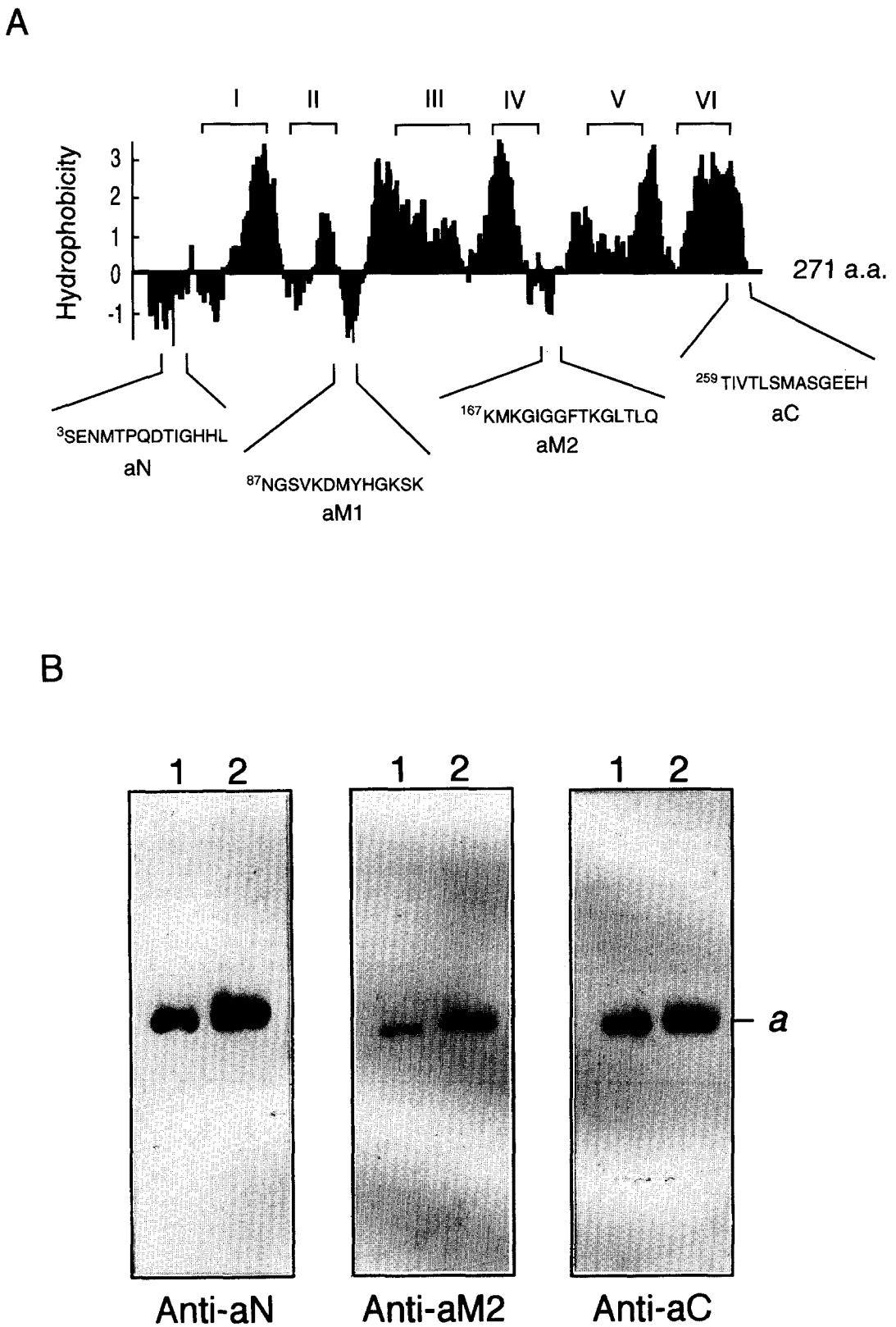

Fig. 1. Design and preparation of site-specific antibodies against subunit *a*. (A) Hydropathy plot of subunit *a* [5,6], and the four different areas used for preparation of the antibodies. (B) Immunoblotting of the purified F_0F_1 (5 μg protein) (lane 1) and inside-out membrane vesicles (100 μg protein) (lane 2) with the purified antibodies at a dilution of 1:500 (for anti-aN and anti-aC) or 1000 (for anti-aM2). The immunological reactivity was visualized by means of the peroxidase reaction with 4-chloro-1-naphthol as a substrate.

vigorously. The solution was immediately neutralized with 1 M Tris, and then bovine IgG (100 μg) was added. The solution was then dialyzed against 10 mM $(NH_4)HCO_3$ for 3 h and used as purified antibodies.

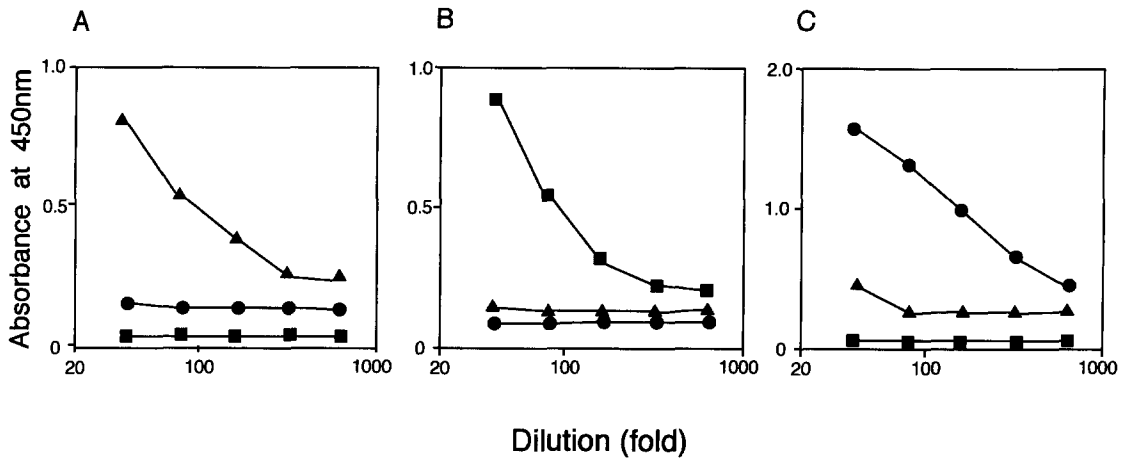

Fig. 2. Specificity of the antibodies. Microtiter plates were covered with a synthetic peptide (0.5 μg) coated with the amino-terminal aN (A), middle M2 (B), or carboxyl-terminal aC (C) region. Then the plates were washed twice with PBS containing 1% bovine serum albumin. The immunoreactivities of the purified antibodies were assayed by ELISA. (▲) Anti-aN; (■) anti-aM2; (●) anti-aC.

2.3. Measurement of antibody binding to membranes

For the enzyme-linked immunosorbent assay (ELISA), the antigen (0.5 μg of synthetic peptide or 0.5 μg of membrane vesicles in 0.1 ml PBS) was incubated in a microtiter plate (type MS-8696F; Sumitomo Bakelite Co., Ltd) for 5 h at 4°C. After washing twice with PBS, the wells were blocked with PBS containing 1% bovine serum albumin for 1 h at 25°C. Then, antibodies (50 ng) diluted with PBS-Tween were added, followed by incubation for 2 h, and washing four times with PBS-Tween. Subsequently, the wells were treated with anti-rabbit IgG conjugated with horseradish peroxidase (Organon Teknika Corp.), washed several times, and then immunoreactivity was assayed using tetramethylbendizine as a substrate (Kirkegaard & Perry Laboratories, Inc.) with an ELISA reader (BioRad).

2.4. Protease treatment

F_1-depleted membrane vesicles (5 μg protein) were incubated in 1 ml of 20 mM Tris-Cl (pH 7.4) containing an equal amount of subtilisin (Sigma) for 1 h at 37°C, and then washed twice (100 000 × *g*, 60 min) with PBS containing phenylmethylsulfonyl fluoride. The degree of digestion of subunit *a* was examined by immunoblotting.

2.5. Other procedures

Published procedures were used for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate [21], immunoblotting [17], purification of F_0F_1 [17], ATP-dependent H^+ transport [17], hydrolysis of ATP [17], and depletion of F_1 from inside-out membrane vesicles [18].

3. Results and discussion

3.1. Preparation of site-directed antibodies against subunit a

Following repeated boosters of synthetic peptides conjugated with hemocyanin, site-specific antibodies against the aN, aC and aM2 regions of subunit *a* could be raised (Fig. 1; henceforth referred to as anti-aN, anti-aC, and anti-aM2 antibodies, respectively). Immunoblotting experiments indicated that these antibodies specifically recognized subunit *a*. The specificity of the antibodies was demonstrated by ELISA assay, since the antibodies reacted with the peptides used as antigens, but not with others (Fig. 2). Furthermore, anti-aN antibodies recognized the truncated subunit *a* with 110 (Trp-111 → end) and 262 (Tyr-263 → end) residues from the amino-terminus [7], while anti-aC antibodies did not react with these fragments (results obtained on Western blotting of mutant

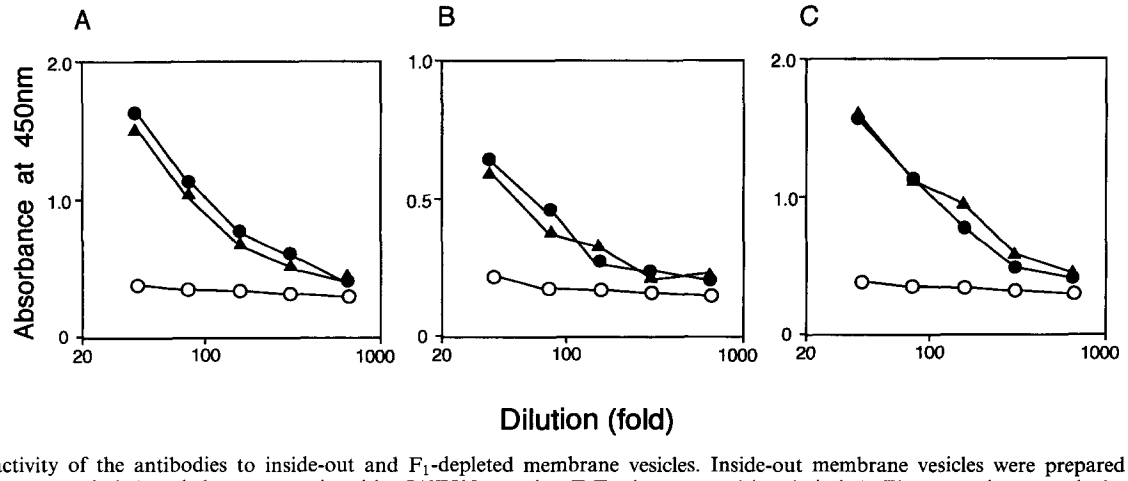

Fig. 3. Reactivity of the antibodies to inside-out and F_1-depleted membrane vesicles. Inside-out membrane vesicles were prepared from DK8 lacking F_0F_1 (open circles) and the same strain with pBWU13 carrying F_0F_1 the operon (closed circles). The F_1 moiety was depleted from the latter membrane vesicles (triangles). Microtiter plates were coated with these vesicles (5 μg protein), and then the immunological reactivities of the purified antibodies were measured by ELISA: (A) anti-aN antibodies; (B) anti-aM2 antibodies; (C) anti-aC antibodies.

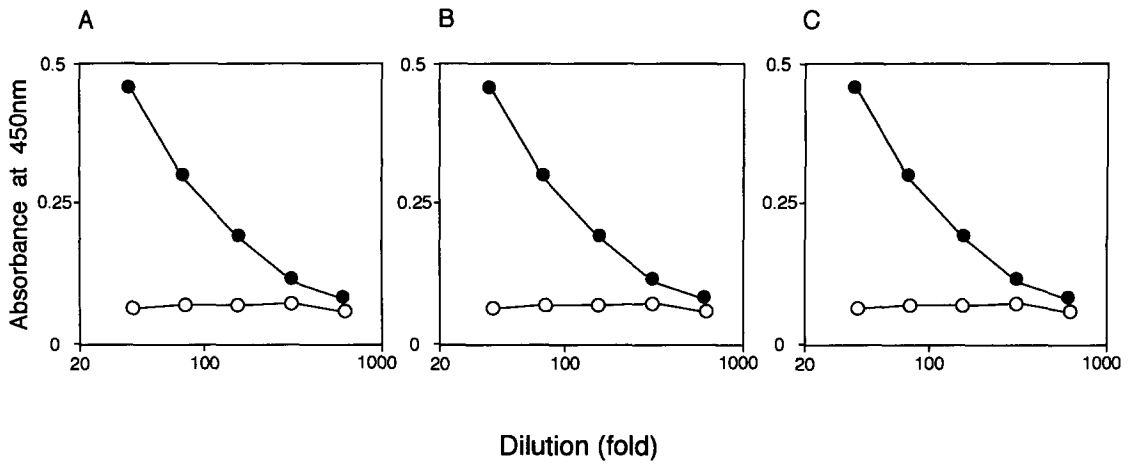

Fig. 4. Reactivity of the antibodies with spheroplasts. Spheroplasts were prepared from DK8/pBWU13, and suspended in PBS in the presence (closed circles) or absence (open circles) of Triton X-100 (0.5%). The microtiter plates were then coated with the vesicles (50 μg protein), and the immunoreactivities with the purified antibodies were measured by ELISA. (A) Anti-aN antibodies; (B) anti-aM2 antibodies; (C) anti-aC antibodies.

membranes; data not shown). Anti-aM2 antibodies did not react with truncated subunit *a* (Trp-111 → end), but reacted with truncated subunit *a* (Tyr-263 → end). Moreover, subtilisin treatment of F_1-depleted inside-out membrane vesicles produced partially digested subunit *a* with an apparent molecular mass of 22 kDa [22]. Anti-aC and anti-aM2 antibodies recognized this fragment, while anti-aN antibodies did not (data not shown). These results clearly indicate that the anti-aN, anti-aC and anti-aM2 antibodies specifically recognize part of the peptide sequences in the subunit. We failed to raise antibodies against the aM1 region, even after several series of attempts.

3.2. Topology of subunit *a* in membrane vesicles

The reactivities of the antibodies with subunit *a* in sealed membrane vesicles should depend on the locations, inside or outside, of the peptide segments. We measured the reactivities of the antibodies with inside-out membrane vesicles and right side-out spheroplasts. All the antibodies reacted with inside-out membrane vesicles (Fig. 3). Inside-out membrane vesicles prepared from a F_0F_1-lacking strain did not show any reactivity with these antibodies.

On the other hand, these antibodies did not react with spheroplasts (Fig. 4). The reactivities apparently increased when spheroplasts were disrupted with Triton X-100 (Fig.

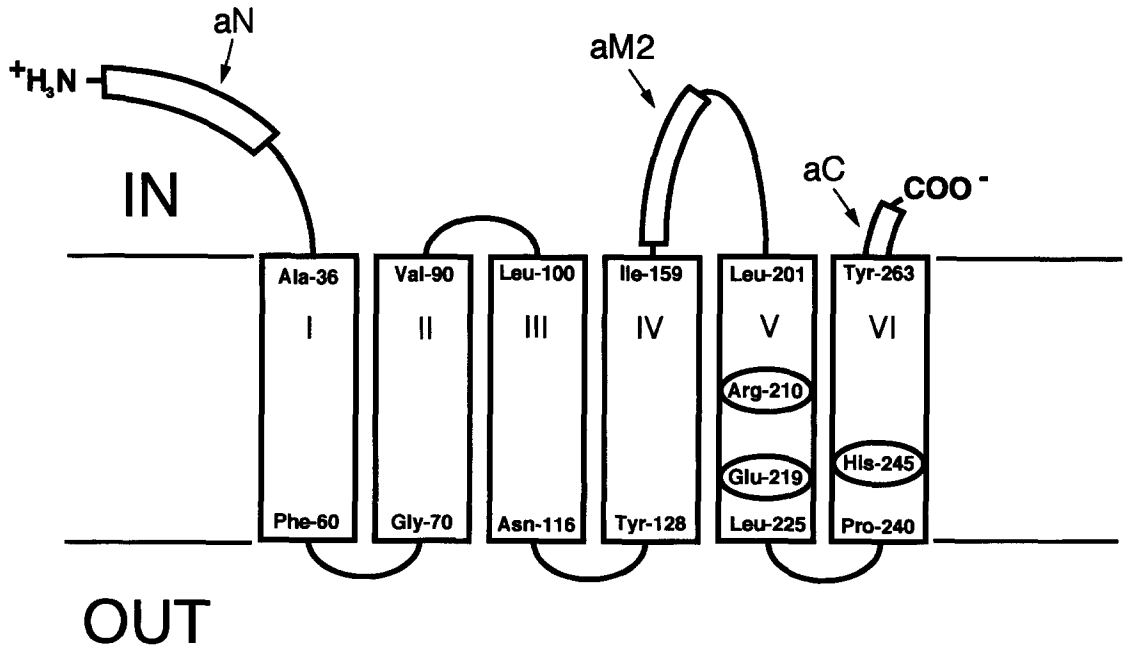

Fig. 5. Proposed secondary structure of subunit *a*. This model proposes six transmembrane segments (I–VI) with amino and carboxyl termini exposed to the cytoplasmic surface (IN). The peptides (aN, aM2 and aC) used for this study are shown schematically. Both the amino- and carboxyl-terminals are located at the cytoplasmic surface.

4). Similarly increased reactivity was observed when spheroplasts (5 mg) were sonicated using a Branson sonifier (30 s at 50% of maximam output) or treated with 1% polyoxyethylene 9-lauryl ether. These results indicate that antigens for these antibodies face the cytoplasm not the periplasm. It is noteworthy that the reactivities of the antibodies used in this experiment did not change on removal of the F_1 moiety by washing of inside-out membrane vesicles with buffer containing EDTA, under these conditions more than 90% of F_1 being released from the membranes (Fig. 3). The exposed F_0 showed passive H^+ translocation inhibited by *N,N'*-dicyclohexylcarbodiimide, and could bind the purified F_1. Essentially the same results were obtained with the wild-type strain KY7230 (not shown). Binding of the antibodies to inside-out membrane vesicles from KY7230 reached about 10% of that from DK8/pBWU13 due to the decreased amount of F_0F_1 in KY7230.

These results suggest that the binding sites of these antibodies are located at the cytoplasmic face of F_0 but different from the F_1 binding domain.

3.3. Transmembrane topology of subunit a based on the results of immunological studies

From the above results combined with a hydropathy plot (Fig. 1), we propose a novel membrane-spanning model for subunit *a*. The subunit contains six transmembrane segments, and the amino and carboxyl terminals as well as the aM2 region face the cytoplasm (Fig. 5). The membrane-spanning segments (I–IV in Fig. 5) were postulated from the hydropathy plot (Fig. 1). This model is apparently similar to those proposed by Hermolin et al. [11], and Walker et al. [5], but has a completely reversed orientation, since the amino- and carboxyl-terminals as well as the aM2 region face the peripheral space in their model. In our model, two of the essential residues (Arg-210 and Glu-219) are located in the 5th transmembrane segment and His-245 in the 6th transmembrane segment, these locations being reasonable for the involvement of these residues in the proton pathway [7]. Cytoplasmic segments (aN, aC and aM2) were accessible to the antibodies, suggesting that the two terminal (amino and carboxyl) regions and the middle one of the *a* subunit are exposed to the surface of the F_0F_1 assembly.

Acknowledgements: This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan, and the Human Frontier Science Program.

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