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Orientation of subunit *c* of the ATP synthase of *Escherichia coli* – a study with peptide-specific antibodies

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Antibodies were raised against a peptide of subunit *c* of the ATP synthase from *Escherichia coli* obtained by cleavage with cyanogen bromide. This peptide comprises the amino acid residues Gly-18 to Met-57 and contains the highly conserved, hydrophilic stretch of subunit *c*. Several conformation-specific populations of antibodies recognized this region both in isolated subunit *c* and in the intact F_0 complex. In antibody binding studies with membrane vesicles of different orientations, recognition occurred only after incubation with everted membrane vesicles, independent of the presence or absence of F_1, although a higher membrane protein concentration was necessary to observe the same antibody binding in the presence of the F_1 part. From these results we conclude that the hydrophilic region of subunit *c* is exposed to the cytoplasmic side of the membrane.

Introduction

The membrane-bound ATP synthase of *Escherichia coli* (F_1F_0; EC 3.6.1.34) generates ATP by utilizing the energy of an electrochemical proton gradient across the membrane. Under anaerobic conditions the enzyme complex can also function in the reverse direction thereby energizing the membrane by ATP hydrolysis [1,2]. The ATP synthase complex consists of two structurally and functionally distinct entities. The water-soluble F_1 complex carries the catalytic and regulatory centres of the enzyme and can be dissociated from the membrane-embedded F_0 complex in the presence of EDTA and a buffer of low ionic strength [3–5]. The membrane-integrated F_0 complex is composed of three different polypeptides of known amino-acid sequence with a stoichiometry proposed to be $1:2:10 \pm 1$ for subunits *a*:*b*:*c*, respectively [6], and constitutes the proton-translocating part of the enzyme [7,8]. From analyses of deletion strains [9] and reconstitution experiments with isolated F_0 subunits [10] it is known that all three subunits are necessary to obtain a F_0 complex functional in proton translocation and F_1 binding.

Subunit and (8,3 kDa) is the most extensively studied subunit of the F_0 complex [7,8,11]. Theoretical calculations based on the primary structure of the protein [7,11] reveal a hairpin-like structure consisting of two hydrophobic stretches separated by a central polar loop. The inhibition of proton translocation through F_0 after binding of DCCD to aspartic acid 61 indicates that this carboxyl group plays an important role in the proton-translocating process [11]. Furthermore, the analyses of mutant strains showed that a single amino-acid substitution in the hydrophilic region resulted in uncoupling of proton translocation from ATP synthesis [12,13], whereas point mutations in the hydrophobic regions produced effects on the assembly of F_0 [14,15] or affected proton translocation [16,17].

Since it has been claimed that subunit *c* plays an important role in proton translocation through F_0 the knowledge about the orientation of subunit *c* within the membrane is a prerequisite for the understanding of the proton-translocating mechanism. Observations supporting a U-shaped conformation of the protein are the following:

(1) subunit *c* has extensive α-helical segments as shown by nuclear magnetic resonance measurements [18];
(2) analyses of DCCD-resistant mutants indicate that Ile-28 is close to Asp-61 [19,20];
(3) the protein regions Leu-4 to Leu-19 and Phe-53 to

Abbreviations: Peptide B6, peptide Gly-18 to Met-57 of subunit *c*; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine.

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Phe-76 are labelled with TID, suggesting that these regions are membrane-integrated [21];
(4) the polypeptide chain exhibits antigenic determinants at both sides of the membrane [22–24]; and
(5) the C-terminal region is located at the periplasmic side of the membrane as known from chemical modification with tetranitromethane and subsequent reduction with sodium dithionite [25].

In this communication we describe binding studies employing antibodies raised against peptide Gly-18 to Met-57 of subunit *c*. Our data show that the hydrophilic loop of subunit *c* is exposed to the cytoplasmic side of the membrane.

Materials and Methods

Materials

Chemicals and chromatography media were purchased from the following companies: MCH-10 HPLC column from Varian (Hamburg); carboxymethyl-cellulose (CM23) from Serva (Heidelberg); alkaline phosphatase-conjugated goat anti-(rabbit IgG), 5-bromo-4-chloroindoxyl phosphate, and Nitro-blue tetrazolium from Sigma (Munich); Ponceau S from Serva (Heidelberg); ABM adjuvant, which contains trehalose dimycolate (0.25 mg/ml) and monophosphoryl lipid A (0.25 mg/ml) in 2% oil and Tween-80, from SEBAK (Aidenbach). All other chemicals were of analytical grade. Flexible poly(vinyl chloride) microtiter plates were obtained from Falcon (Becton Dickinson, Heidelberg). Nitro-cellulose sheets (0.45 μm) were purchased from Schleicher & Schüll (Dassel).

Bacterial growth

E. coli wild-type strain ML308-225 ($i^-z^-y^+a^+$) was grown in minimal medium containing 0.4% glucose [26]. *E. coli* strain CM1470 (F^+ *asnB*32 *thi*-1 *relA*1 *spoT*1 *atp*-706 (*ΔIBEFHA*)), which was kindly donated by Dr. H.U. Schairer (Heidelberg, F.R.G.), was grown in the same medium supplemented with thiamine (0.5 μg/ml) and asparagine (50 μg/ml) [9]. For the preparation of membrane vesicles of both strains cells were harvested in late logarithmic phase. *E. coli* strain KY7485 (kindly donated by Drs. R.H. Fillingame (Madison, U.S.A.) and H. Kanazawa (Okayama, Japan) was grown in a minimal medium [27] containing glucose (1%), thiamine (0.5 μg/ml), arginine (84 μg/ml), and guanine (45 μg/ml) [28]. The growth conditions used were described by Foster et al. [28]. The strain KY7485 carries a λ prophage containing the complete *unc* operon and overproduces the ATP synthase several times after heat induction of the prophage [28].

Preparative procedures

Subunit *c* of the F_0 complex of the ATP synthase was isolated from cells of strain ML308-225 by chloroform/methanol extraction [29,30] and purified to homogeneity by ion-exchange chromatography on carboxymethyl-cellulose [31]. After cleavage of the protein with cyanogen bromide [32] the resulting peptides were separated by HPLC on a Varian MCH-10 column under conditions described by Fimmel et al. [17]. The F_0 complex [33] and subunits *a*, *b* and *c* [10] were purified from F_1-stripped everted membranes of *E. coli* strain KY7485 as described. Everted membrane vesicles of the F_0 deletion strain CM1470 and everted vesicles with and without F_1 of wild-type strain ML308-225 were prepared according to Vogel and Steinhart [34] and tested for intactness by 9-amino-6-chloro-2-methoxyacridine fluorescence quenching [35]. The preparation of membrane vesicles with right-side-out orientation of ML308-225 and CM1470 was carried out as described by Kaback [36]. Right-side-out oriented membrane vesicles were tested for intactness by measurement of [^{3}H]proline uptake [37].

Preparation of antisera

Prior to immunization the HPLC-purified peptide Gly-18 to Met-57 of subunit *c*, which is dissolved in approximately 40% acetonitrile/0.1% phosphoric acid, was freeze-dried. The remaining phosphoric acid was diluted, titrated to pH 6–7, and 1% (w/v) SDS was added. A rabbit was immunized sub- or intracutaneously with approx. 150 μg of the peptide emulsified with 1 ml ABM adjuvant. Booster injections followed in intervals of weeks with the same amount of peptide. Antisera were taken 10 days after immunization by bleeding from the marginal ear vein. The antisera taken after the fifth and sixth immunization were used for the experiments described. Preadsorption of the antisera with everted membrane vesicles of the *unc* deletion strain CM1470 was performed according to Wright and Overath [38] with 10 mg membrane protein per ml antiserum. For a consecutive preadsorption with right-side-out oriented membrane vesicles of CM1470, the following modifications were necessary: 10 mg of membrane protein were incubated with 1 ml anti serum without sonication in order to prevent inversion of the membrane vesicles. After stirring for 2 h at 4°C the vesicles were removed by centrifugation (10 min at 33000 × *g*).

Immunological procedures

The antibody titers of the antisera raised against peptide Gly-18 to Met-57 were determined by ELISA as described [23] with the exception that the incubation with the antisera was continued overnight at 4°C, and the incubation with the alkaline phosphatase-conjugated goat anti-(rabbit IgG) was performed for 4 h at 4°C. With the same modifications the competitive inhibition ELISA was carried out [23]. The incubation times with the substrate varied in the range of 15 to 60

min. Immunoblotting was performed as described by Deckers-Hebestreit and Altendorf [22]. For visualization of transferred proteins the nitrocellulose sheets were stained for 5 min with 0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid. Excess dye was removed by washing the sheets with water. The F_0 subunits were marked for easier identification after immunolabelling. The dye was completely removed by the subsequent quenching of the nitrocellulose sheets with bovine serum albumin. As indicator antibody a goat anti-(rabbit IgG) alkaline phosphatase conjugate was used. The detection of the antibody binding was performed according to Blake et al. [39] with the exception that 50 mM sodium carbonate (pH 9.5) was used as buffer system and that 5-bromo-4-chloroindoxyl phosphate was always freshly dissolved in water. The substrate incubation was carried out for 20 min at room temperature.

Assays

Protein concentration was determined by the method of Dulley and Grieve [40]. SDS-gel electrophoresis (gradient slab gels: 7.5–17.5/%) was carried out as described by Douglas et al. [41]. The protein concentration and purity of the peptide was determined by amino-acid analyses, which were performed on a Biotronik analyzer LC 5000 after hydrolysis of the protein samples for 48 h in 6 M HCl at 105°C.

Results

Binding characteristics of antibodies raised against peptide Gly-18 to Met-57 of subunit c

Polyclonal antibodies were raised in a rabbit against HPLC-purified peptide B6 of subunit *c*, which comprises amino acids Gly-18 to Met-57 (The designation of the CNBr fragments of subunit *c* follows that used by Wachter al. [19].) To this end, subunit *c* was extracted with chloroform/methanol and the peptide was obtained by cyanogen bromide cleavage in 80% formic acid and purified by reversed-phase HPLC with an acetonitrile gradient. Before immunization with the peptide, which occurred in the presence of SDS without a carrier protein, the homogeneity of peptide B6 was ascertained by amino-acid analysis (data not shown). The subunit specificity of the antisera was investigated by immunoblotting and ELISA. The Western blot analysis in Fig. 1B shows that the antisera, in addition to subunit *c*, reacted with several unidentified proteins of the membrane preparation and with copurified proteins in the F_0 preparation. For removal of those unspecific antibodies the antisera were preadsorbed with membrane vesicles of the *unc* deletion strain CM1470 (*ΔuncIBEFHA*) with the consideration that all antibodies with affinity to proteins other than subunit *c* might find their epitopes in the membrane preparations. Indeed, preadsorption of the antisera with everted membrane

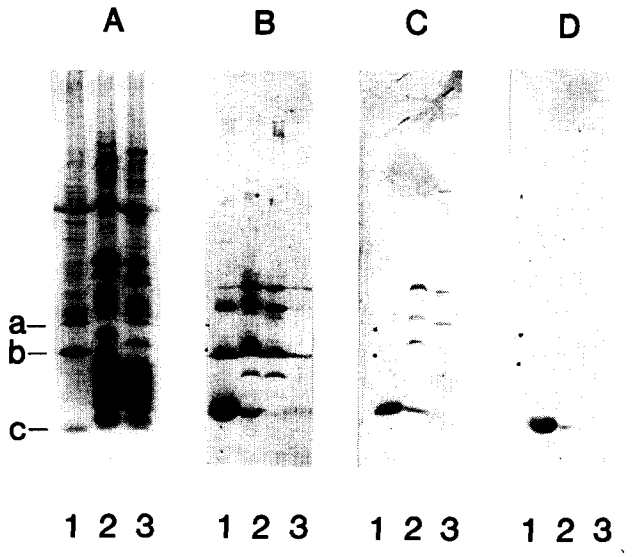

Fig. 1. Recognition of subunit *c* with antibodies raised against peptide Gly-18 to Met-57. Purified F_0 complex (30 μg, lane 1), everted membrane vesicles of *E. coli* strain ML308-225 (*unc*$^+$) (30 μg, lane 2) and CM1470 (*ΔuncIBEFHA*) (30 μg, lane 3) were separated on a SDS-polyacrylamide gel, transferred to nitrocellulose sheets, and incubated with the indicated antisera as described in Materials and Methods. (A) SDS-gel electrophoresis of the protein probes visualized with Serva blue G-250. The distribution of the F_0 subunits in the gel is marked. The nitrocellulose sheets were incubated with anti serum against peptide B6 (B), with the same anti serum preadsorbed with everted membrane vesicles of the *unc* deletion strain CM1470 (C), and with the same antiserum preadsorbed with everted and right-side-out membrane vesicles of CM1470 (D). In each case an antiserum dilution of 1 : 2000 was used.

vesicles removed most of the antibodies that bound unspecifically (Fig. 1C), while the additional preadsorption with right-side-out oriented membrane vesicles of CM1470 resulted in complete loss of unspecific binding (Fig. 1D). It is worthwhile mentioning that in this case a correlation between recognition of antigenic sites of proteins in native membranes and proteins bound to nitrocellulose after SDS-polyacrylamide gel electrophoresis is evident. Due to the preadsorption the amount of antibody reacting with subunit *c* was reduced, which indicated that peptide B6 shares common epitopes with other membrane proteins.

For experiments involving subunit *c* in situ it was important to ascertain that the antibodies raised against peptide B6 also recognized this polypeptide region in subunit *c* isolated under nondenaturing conditions or in the F_0 complex (purification of F_0 subunits under nondenaturing conditions means that these subunits can be reconstituted into phospholipid vesicles to form an F_0 complex active in proton translocation and binding of F_1 [10]). In an ELISA, binding of the antibodies could be observed with both peptide B6, subunit *c*, and the F_0 complex as fixed antigens, whereas the preimmune serum showed no significant binding. However, the titer of half-maximal binding was much lower for subunit *c* and F_0 than it was for peptide B6 (data not shown). The

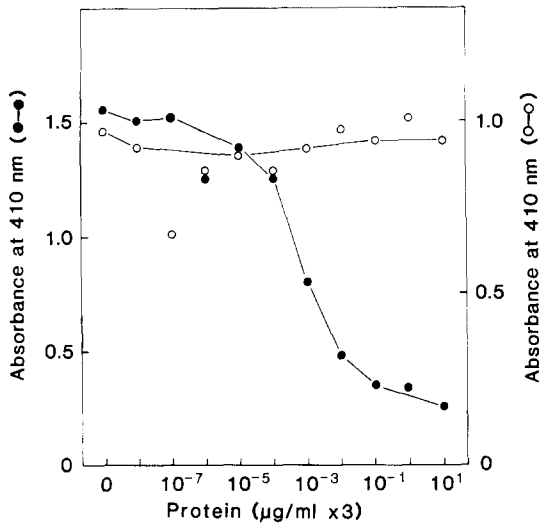

Fig. 2. Conformation specificity of the antibodies raised against peptide Gly-18 to Met-57. Different amounts of the peptide were incubated overnight with the anti-peptide antiserum, which was preadsorbed with everted membrane vesicles of CM1470 and used in a final dilution of 1:100. After preincubation the mixtures were transferred to a microtiter plate coated with 10 µg/ml of F_0 complex (○). After an overnight incubation the mixtures were transfered to a second microtiter plate coated with 3 µg/ml of peptide B6 (●) which was also incubated overnight. Bound antibodies were detected by incubation with an alkaline phosphatase-conjugated goat anti-(rabbit IgG) as indicator antibody and *p*-nitrophenyl phosphate as substrate.

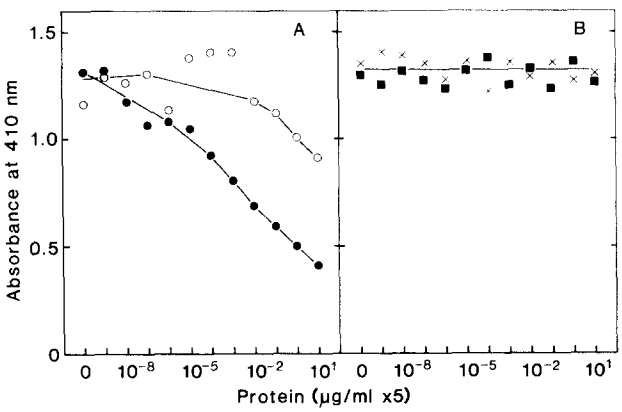

Fig. 3. Competition for anti-peptide antibodies between bound F_0 complex and free F_0 complex or free F_0 subunits. Different amounts of F_0 complex (A, ●), subunit *c* (A, ○), subunit *b* (B, ■) or subunit *a* (B, ×) were incubated overnight with anti-peptide antiserum, which was preadsorbed with everted membrane vesicles of CM1470 and used in a final dilution of 1:100. After preincubation the mixtures were transferred to microtiter plates coated with F_0 complex (10 µg/ml). The microtiter plates were developed as described in the legend to Fig. 2.

reason for this could either be that the antigens were recognized by different populations of antibodies or that the binding affinity of the antibodies depended on the form in which the antigen was applied (isolated vs. protein-incorporated peptide). To distinguish between these alternatives, we performed a competitive inhibition ELISA. Fig. 2 shows the binding of antibodies that were preincubated with different amounts of peptide B6 to the F_0 complex fixed to a microtiter plate. Subsequently, the preincubated antibodies were transfered from the first microtiter plate to a second, which was coated with peptide B6. From this experimental arrangement it can be concluded that no competition for antibodies occurred between free peptide B6 and bound F_0, whereas free and bound peptide B6 showed strong mutual competition. Similar results were obtained when subunit *c* isolated by chloroform/methanol extraction [29,30] was used as competitor for F_0 or peptide B6 (data not shown). In fact, competition for anti-peptide antibodies only occurred between the F_0 complex and subunit *c* provided that the latter was isolated under nondenaturing conditions (see Fig. 3A and Fig. 4). These results show that the antibodies raised against peptide B6 showed a strong conformational specificity towards the amino-acid sequence present in peptide B6, subunit *c* isolated by different procedures, and the F_0 complex.

Specific recognition of subunit c in the F_0 complex

By preadsorption of antisera with membrane vesicles of the *unc* deletion strain CM1470 antibodies were removed that did not interact with subunits of the F_0 complex. However, for studies concerning the orientation of subunit *c* in the membrane it was necessary to ascertain that the antibodies did not crossreact with subunits *a* and *b* either. Therefore, a competitive inhibition ELISA with F_0 as bound antigen was carried out. The results showed that preincubation of the antibodies with different amounts of subunit *a* or subunit *b* dissolved in a cholate-containing buffer (Fig. 3B) or reconstituted into liposomes (data not shown) produced no reduction of the antibody titer. In addition, no

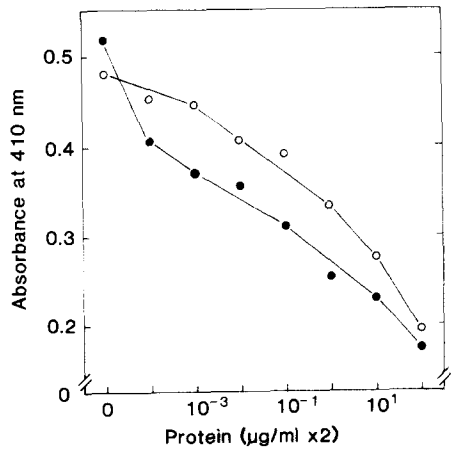

Fig. 4. Competition for anti-peptide antibodies between bound F_0 complex and free proteoliposomes. The conditions used were the same as in Fig. 3 except that the preadsorbed anti-peptide anti serum was preincubated with different amounts of F_0 liposomes (●) or liposomes containing subunit *c* (○).

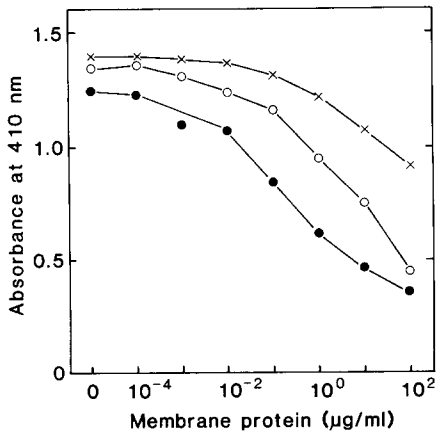

Fig. 5. Competition for anti-peptide antibodies between bound F_0 complex and free everted membrane vesicles. The conditions used were the same as in Fig. 3 except that the anti-peptide antiserum, which was preadsorbed with everted membrane vesicles of CM1470, was preincubated with different amounts of everted membrane vesicles. (×) everted membrane vesicles of CM1470 (*ΔuncIBEFHA*); (○) everted membrane vesicles of ML308-225 (*unc*$^+$); (●) everted membrane vesicles of ML308-225 (*unc*$^+$) depleted of F_1.

binding of the preadsorbed antiserum to subunit *a* and *b* was detected by immunoblotting (Fig. 1).

Preincubation with different amounts of subunit *c* dissolved in cholate-containing buffer caused only a weak competition compared to F_0 (Fig. 3). However, after incorporation of subunit *c* into phospholipid vesicles the binding avidity of the antibodies with regard to subunit *c* increased dramatically (Fig. 4): whereas the protein concentration of free subunit *c* necessary for half-maximal binding of the antibodies was 10^5-fold higher than that of F_0, this factor dropped to approx. 60 after reconstitution of F_0 and subunit *c* into liposomes. Based on (i) this observation, (ii) the absence of crossreactivity with subunits *a* and *b*, and (iii) difficulties in applying phospholipid vesicles as microtiter plate-bound antigen, we decided to carry out the studies on the orientation of subunit *c* with competitive inhibition ELISA using F_0 as fixed antigen.

Accessibility of subunit c in membrane vesicles by anti-peptide antibodies

Two sets of competitive inhibition ELISA were performed to investigate the topography of subunit *c* in the membrane: binding of peptide-specific antibodies to membrane vesicles with right-side-out orientation and to everted membrane vesicles with and without F_1 was tested. For all binding studies membrane vesicles of the mutant strain CM1470, which does not contain any of the F_0 subunits, was used as a control (Figs. 5 and 6). Normally, no competition between bound F_0 and free membrane vesicles of CM1470 for the anti-peptide antibodies occurred; only at very high protein concentrations a slight, obviously unspecific binding to the membranes was observed. In contrast, the antibodies showed strong binding avidities – also at very low membrane protein concentrations – to F_1-depleted everted membrane vesicles of the wild-type strain ML308-225, suggesting that subunit *c* is accessible for the peptide-specific antibodies from the cytoplasmic side of the membrane (Fig. 5). The antibody binding to F_1-stripped wild-type membrane vesicles was about a factor of 10^3 to 10^4 higher than that to membrane vesicles of the *unc* deletion strain. However, an antigen antibody recognition also occurred when the F_1 part remained bound to the F_0 complex (Fig. 5), but a higher membrane protein concentration (approx. 50-fold) was necessary for the same binding. This binding indicates that the F_1 part is not able to cover all antigenic sites of the F_0 complex recognized by the antibodies. Alternatively, this result could be explained by loss of F_1 during the preparation of everted membrane vesicles. However, observations described by our group in a previous paper [22] argue strongly against this possibility: whereas also subunit *c* and subunit *b*-specific antibodies bind to F_1-containing everted membrane vesicles, antibodies raised against subunit *a* do not recognize their antigen in everted membrane vesicles in the presence of the F_1 part.

No significant competition for peptide-specific antibodies could be observed between bound F_0 and different amounts of free membrane vesicles with right-side-out orientation (Fig. 6). Again, a competitive effect occurred at very high membrane protein concentrations as also observed for the control membrane vesicles. The slightly higher binding avidity of the antibodies to the wild-type membrane vesicles compared to the control is possibly due to a small percentage of everted membrane

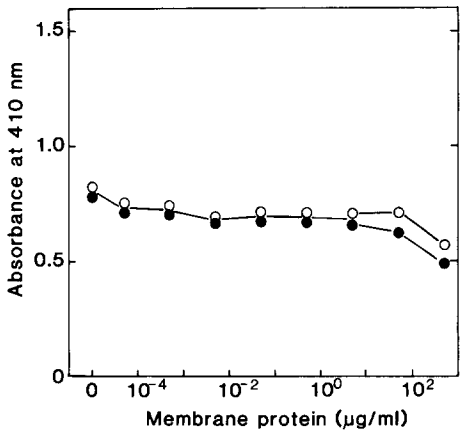

Fig. 6. Competition for anti-peptide antibodies between bound F_0 complex and free membrane vesicles with right-side-out orientation. Microtiter plates were coated with F_0 complex (10 µg/ml). The anti-peptide antiserum, which was preadsorbed with inside-out and right-side-out oriented membrane vesicles of CM1470 and used in a final dilution of 1:40, was preincubated with different amounts of right-side-out membrane vesicles. (○) Right-side-out membrane vesicles of CM1470 (*ΔuncIBEFHA*); (●), right-side-out membrane vesicles of ML308-225 (*unc*$^+$). The microtiter plates were developed as described in legend to Fig. 2.

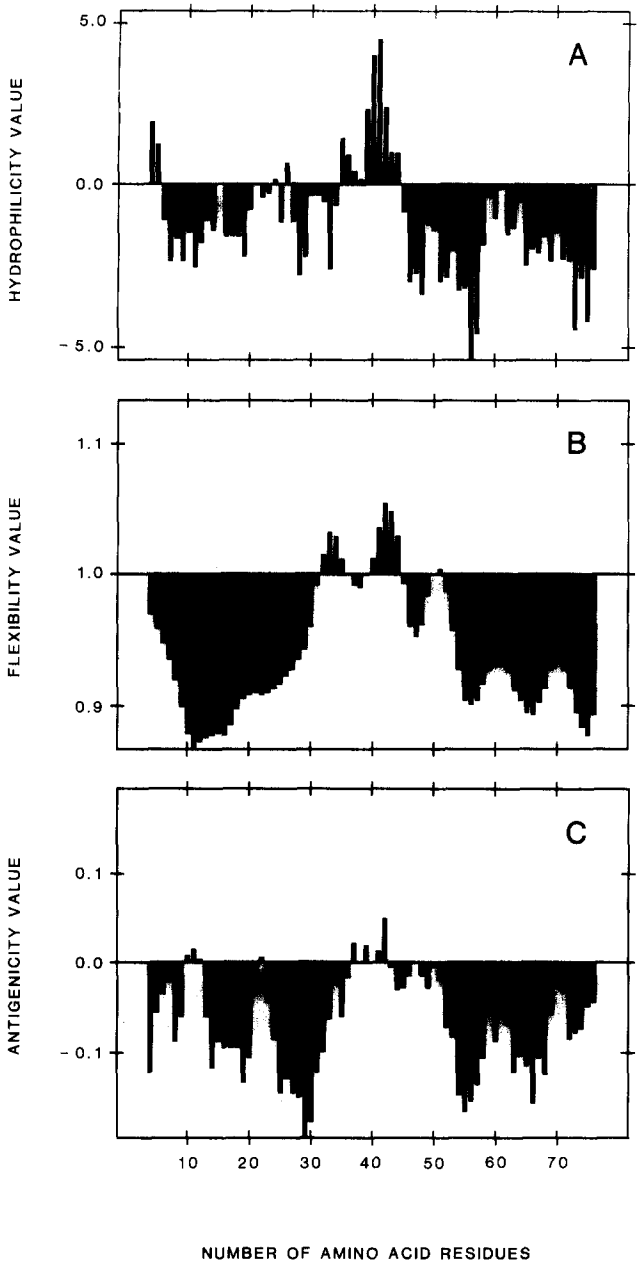

Fig. 7. Prediction of antigenic regions in subunit *c* of the F_0 complex from *E. coli* ATP synthase. (A) Hydrophilicity plot according to Parker et al. [46], (B) flexibility plot with 'rigid' neighbour-analysis according to Karplus and Schulz [47], and (C) antigenicity plot according to the method of Welling et al. [48]. The values for each segment of seven amino-acid residues are plotted at the position of the fourth residue.

vesicles, which is sometimes present in preparations of membrane vesicles with right-side-out orientation.

Discussion

The model favoured for the conformation of subunit *c* is a hairpin-like structure with two hydrophobic membrane-spanning regions connected by a highly conserved hydrophilic region, which is proposed to be located at the cytoplasmic side of the membrane and necessary for interaction with F_1 [7,12,42]. However until now, there has been no direct experimental evidence for such an orientation of subunit *c*. Furthermore, another model for subunit *c* exists, which is based on three membrane-spanning stretches [43]. In this model the conserved hydrophilic region is proposed to be located within the membrane, necessary for the proton-translocating process through F_0. Therefore, antibodies, which do not penetrate biological membranes due to their hydrophilicity and large size [44,45], are an excellent tool to localize the hydrophilic region of subunit *c* with regard to the membrane.

The antibody-binding studies presented here clearly demonstrate that the hydrophilic loop of subunit *c* is exposed to the cytoplasmic side of the membrane, supporting a hairpin-like arrangement for subunit *c* in the membrane (for a model of subunit *c* compare Ref. 25). Furthermore, the recognized polypeptide region is also accessible in the presence of the F_1 part as has been also observed with anti-*c* antibodies [22]. Therefore, it is tempting to speculate that the main antigenic binding site of subunit *c*-specific antibodies [22–24] is very similar to that observed with our anti-peptide antibodies. However, with these antibodies no effect on the function of the F_0 complex could be observed in contrast to subunit-*c*-specific antibodies, which block the proton translocation through F_0 and inhibit the binding of F_1 [42]. Also in the presence of F_1 the anti-*c* antibodies block proton translocation, simultaneously displacing F_1 from F_0 (Deckers-Hebestreit, G., unpublished results). This failure of the anti-peptide antisera may be due to the low antibody titer.

Prediction methods for the localization of sequential antigenic sites in a protein are based on the hydrophilicity [46] or the flexibility [47] of a given polypeptide or depend on the relative occurrence of each amino acid residue in an antigenic site [48]. Calculations for subunit *c* favour the view that the hydrophilic region in the middle of the polypeptide chain is a potential antigenic region (Fig. 7). Although the predictions by different methods show slight deviations, they are all in agreement for region Lys-34 to Leu-46 to contain a sequential antigenic determinant. This region comprises twelve amino acid residues; a region long enough for good antigen-antibody binding, for which an average size of seven amino-acid residues is necessary [49]. However, based on these methods no statement is possible about the presence of discontinuous antigenic determinants in subunit *c*.

The peptide Gly-18 to Met-57 is much longer than the predicted sequential antigenic site of subunit *c*. On the basis of the TID-labelling data [21] it has been concluded that the C-terminal hydrophobic stretch of

the polypeptide chain is integrated in the membrane. No information is available for the location of the glycine-isoleucine-rich region from Gly-18 to Gly-33. However, this region is probably not immunogenic by itself, because a comparison with other known antigenic determinants shows that glycine and especially isoleucine are only rarely present in epitopes [48]. Furthermore, the analysis of a mutant strain with a single mutation in the hydrophilic region revealed that Gln-42 is essential for F_1 binding [12], and, therefore, this amino-acid residue is totally covered by F_1. However, the anti-peptide antibodies were able to bind to F_0 also in the presence of F_1 suggesting that Gln-42 does not participate in antibody binding. In addition, antibody binding studies with F_0 isolated from this mutant strain, in which Gln-42 is substituted by glutamic acid [12], do not show any differences to wild-type F_0 (data not shown). All these data lend support to the notion that the polypeptide region Lys-34 to Arg-41 of subunit *c* which has also been determined as potential antigenic determinant by the predictive measures, is responsible for the binding of the peptide-specific antibodies. Similar results have been obtained by Girvin et al. [50] using an antiserum raised against a synthetic peptide comprising Lys-34 to Ile-46.

Peptide Gly-18 to Met-57 of subunit *c* was isolated under denaturing conditions (in the presence of chloroform/methanol, formic acid, acetonitrile, etc.) and immunized in the presence of SDS without a carrier protein. The characterization of the antibodies obtained showed a high diversity of the immune response and different conformation-specific populations of antibodies, which recognized either peptide B6, subunit *c* or subunit *c* as a component of the F_0 complex. Therefore, at least a partial refolding of the peptide to the 'native' conformation present in subunit *c* and as part of F_0 must take place, although the peptide was isolated under such harsh condition. However, the concentration dependence of inhibition in Figs. 3 and 4 shows that the antibodies involved in the interaction are of medium or low affinity. The refolding of the peptide can possibly be facilitated by a lipid environment due to the hydrophobic regions at the N and the C termini of the peptide (compare Ref. 23). Such an environment can probably be mimicked by the suspension of the peptide in ABM adjuvant prior to immunization or during the presentation of the immunogen by the macrophages. In addition, the diversity of the immune response can partly be explained by the high flexibility of the peptide immunized: due to its size peptide B6 could function as an immunogen by itself, which enhances the flexibility of a molecule in comparison to carrier-fixed haptens. On top of this, the flexibility is increased by the N-terminal region from Gly-18 to Gly-33: this contains six glycine and four alanine residues, all of which have an inherent, high flexibility [47].

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