Hypothesis

Model of the *c*-subunit oligomer in the membrane domain of F-ATPases

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Abstract A model is described of a dodecameric complex consisting of the integral membrane component subunit *c* of the H^+-transporting F_o domain of *Escherichia coli* F-ATPase. A high-resolution partial structure of monomeric subunit *c* resulting from ^{1}H-NMR studies [1] was used for constructing the model. The validity of the proposed arrangement of protomers in the dodecameric complex was tested by amino acid substitution analysis and chemical, biochemical and genetic data on subunit *c*.

Key words: F-ATPase; Oligomeric transmembrane complex; Proteolipid; Molecular model

1. Introduction

The H^+-transporting F-ATPases from eubacteria, mitochondria and chloroplasts, and their cousins, the H^+-transporting V-ATPases found in the membranes of a variety of vesicular structures and in Archaebacteria, are multisubunit enzymes with a globular catalytic domain attached by a stalk to a integral membrane sector that transports protons. The F-ATPases use energy from a transmembrane proton motive force generated by respiration or photosynthesis to make ATP from ADP and inorganic phosphate, whereas V-ATPases use energy released by ATP hydrolysis to generate a proton motive force across the membrane. The *Escherichia coli* enzyme, a complex of eight different polypeptides, is amongst the simplest known F-ATPases. Its F_1 catalytic domain contains subunits α, β, γ, δ and ε, and subunits *a*, *b* and *c* make the F_o membrane sector. Subunits *a* and *c* are both hydrophobic proteins that are essential for transmembrane proton transport [2]. Subunit *a* may have 5, 6 or 7 transmembrane spans [3–5], and subunit *c* forms a hairpin of antiparallel α-helices across the membrane, the connecting loop making contact with the γ-subunit in F_1 [6]. Each F_o contains one copy of subunit *a*, 10–12 copies of subunit *c*, and two copies of subunit *b*, which provide an important connection between the F_1 and F_o domains [7]. The *b*-subunits have a hydrophobic N-terminal region, probably folded into a single transmembrane α-helix, followed by a highly charged region of about 120 amino acids outside the lipid bilayer [7].

An atomic structure of bovine F_1-ATPase has re-awakened the idea that the enzyme might operate by a rotary mechanism in which the properties of the three catalytic sites of the enzyme are interconverted through a cycle of different binding states, described in a binding change mechanism [8], by the rotation of a central α-helical coiled-coil in the γ-subunit inside the $\alpha_3\beta_3$ assembly. Recent data support a rotary mechanism of this type [9,10]. The formulation of mechanisms of rotary motion generated by the transport of protons through the F_o membrane domain would be helped by an atomic structure, but none is available at present. However, the structure of the 79 amino acid *E. coli* *c*-subunit has been studied by NMR in chloroform/methanol mixtures, resulting in a partial structure containing residues 9–25 and 52–79 [1]. As described below, we have used this structure to build a model of the oligomeric structure of 12 *c*-subunits. The validity of this model has been examined by consideration of other chemical, biochemical and genetic data.

2. Structural model

The molecular model described in this work was constructed at a Silicon Graphics workstation using the macromolecular crystallographic modelling programme 'O' [11]. Transmembrane helices of adjacent protomers were aligned by hand basing on steric restrictions assuming that the contact of adjacent helices is dominated by strong hydrophobic interactions. Helix–helix packing of protomers in the complex was not refined by molecular modelling calculations.

A priori, three circular models can be envisaged which place the 12 *c*-subunits in equivalent positions. In these models, the protomers either interact through their C-terminal α-helices or vice versa, or the C-terminal helix of one protomer interacts with the N-terminal helix of an adjacent protomer. However, the model with interacting C-terminal α-helices is preferred (see Fig. 1) as other arrangements of protomers that have been constructed lack strong hydrophobic contacts and tight packing between adjacent helices.

The inner diameter of the constructed circular model varies between 3.26 nm (bottom) and 2.79 nm (top). The main interactions in the ring are via two hydrophobic clusters of amino acids (residues 52–53, 65–66, 70, 74, 77 and 58–59, 64, 68, 72, 75 in the adjacent protomer). The N-terminal α-helices are peripheral and do not interact with each other, but contacts between adjacent C- and N-terminal helices, via Met^{65} and Ala^{20} and Met^{17}, and Tyr^{73} and Met^{16}, might give additional stability to the complex.

The conserved essential carboxyl-group of Asp^{61} is thought to undergo cycles of protonation and deprotonation during proton transfer through F_o. Proton transport is prevented by covalent modification of its side chain with hydrophobic carbodiimides, such as DCCD. This aspartate is in the middle of the C-terminal helix [12], and in the model is, as would be required by its properties, oriented towards the lipid phase (see Fig. 2A). However, its carboxyl lies in a pocket where

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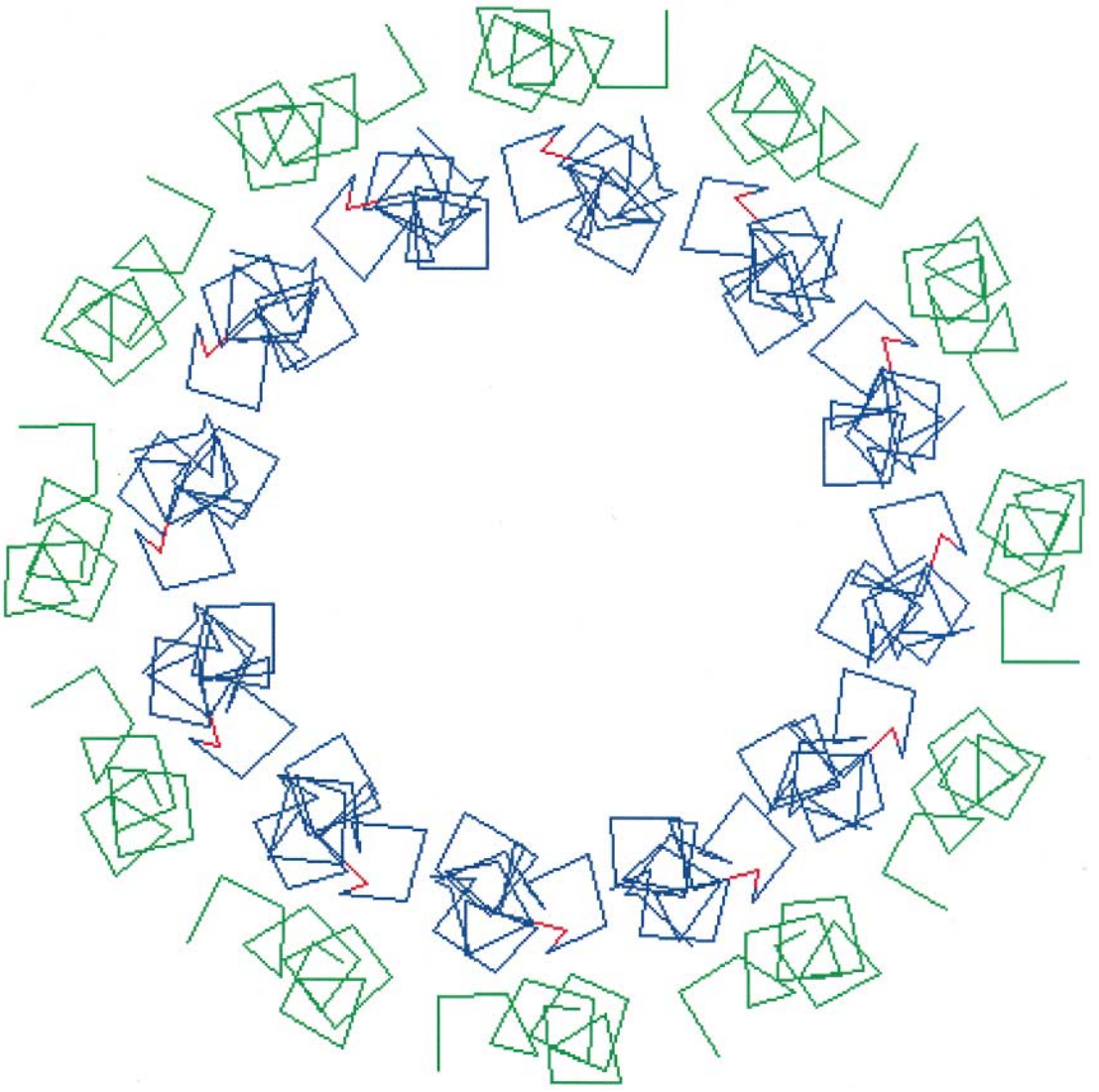

Fig. 1. Peptide backbone model of a dodecameric ring of *c*-subunits of *E. coli* F-ATPase. The model, which is based on the structure of residues 9–25 and 52–79 determined by ^{1}H-NMR [1], is viewed perpendicular to the plane of the membrane from the side to which F_1-ATPase is attached in the intact enzyme. The main interactions between adjacent protomers are via the C-terminal α-helices (blue) with the N-terminal α-helices (green) placed peripherally. The α-carbon atom of residue Asp^{61} is shown in red. Hydrophobic interactions between adjacent C-terminal α-helices involve the side chains of the following amino acids: Phe^{53}–Gly^{58}, Leu^{59}–Gln^{52}, Pro^{64}–Ile^{66}, Met^{65}–Ala^{20} and Met^{17}, Leu^{70}–Val^{68} and Leu^{72}, Tyr^{73}–Met^{16} and Met^{75}–Val^{74}, Ala^{77} and Phe^{76}. Leu^{59} interacts with the peptide backbone of Phe^{53}. The tight packing of the helices in the dodecamer forms a structure exposing a hydrophobic surface to the external membrane phase which is formed by residues Leu^{9}, Tyr^{10}, Met^{11}, Ala^{12}, Ala^{14}, Val^{15}, Gly^{18}, Leu^{19}, Ile^{22}, Gly^{23}, Ile^{26}, Gln^{52}, Val^{56} and the partially buried residues Tyr^{73} and Phe^{76}. Hydrophilic clusters formed by the backbone are exposed to the interior of the dodecameric complex. However, hydrophobic residues are also found at the internal surface of the modelled structure.

it is accessible from the lipid phase without being completely exposed. In F_o, this arrangement may help to avoid the energetically unfavourable exposure of the negative charge resulting from deprotonation to the hydrophobic environment of the membrane. In the double mutant of subunit *c*, $Asp^{61}Gly/Ala^{24}Asp$, the essential carboxyl was transferred from the C- to the N-terminal α-helix, with retention of H^+-transport coupled to ATP hydrolysis [13]. A carboxyl group at position 24 would also be in a pocket and be accessible from the external lipid phase (see Fig. 2B). However, in the model the carboxyl oxygens of Asp^{61} and Asp^{24} are both also accessible from the inside of the dodecameric ring. Therefore, H^+-transport could occur inside the ring up to the conserved carboxyl group of Asp^{61}. Alignment of water molecules at the inside of the ring due to co-ordination by the carbonyl oxygens of Val^{78}, Ala^{77}, Met^{75}, Gly^{71}, Val^{68}, Cys^{67} and Ile^{63} would form the surface along which protons would be transferred. Ions other than hydrogen would be also transported by such a water-coated pore and account for Na^+-transport related to the ATP synthase of *Propionigenium modestum*. The ion specificity of the ATP synthase, however, would be controlled by the structural motif around the DCCD-reactive carboxyl group at position 61 [14].

The exposure of amino acids in *E. coli* subunit *c* in intact F-ATPase and in F_o to the surrounding membrane has been studied by photochemical reaction with the lipophilic, photoreactive agent TID [15]. Residues 4, 8, 10, 11, 15, 19, 54, 57, 65, 73 and 76 were labelled. In agreement with these findings,

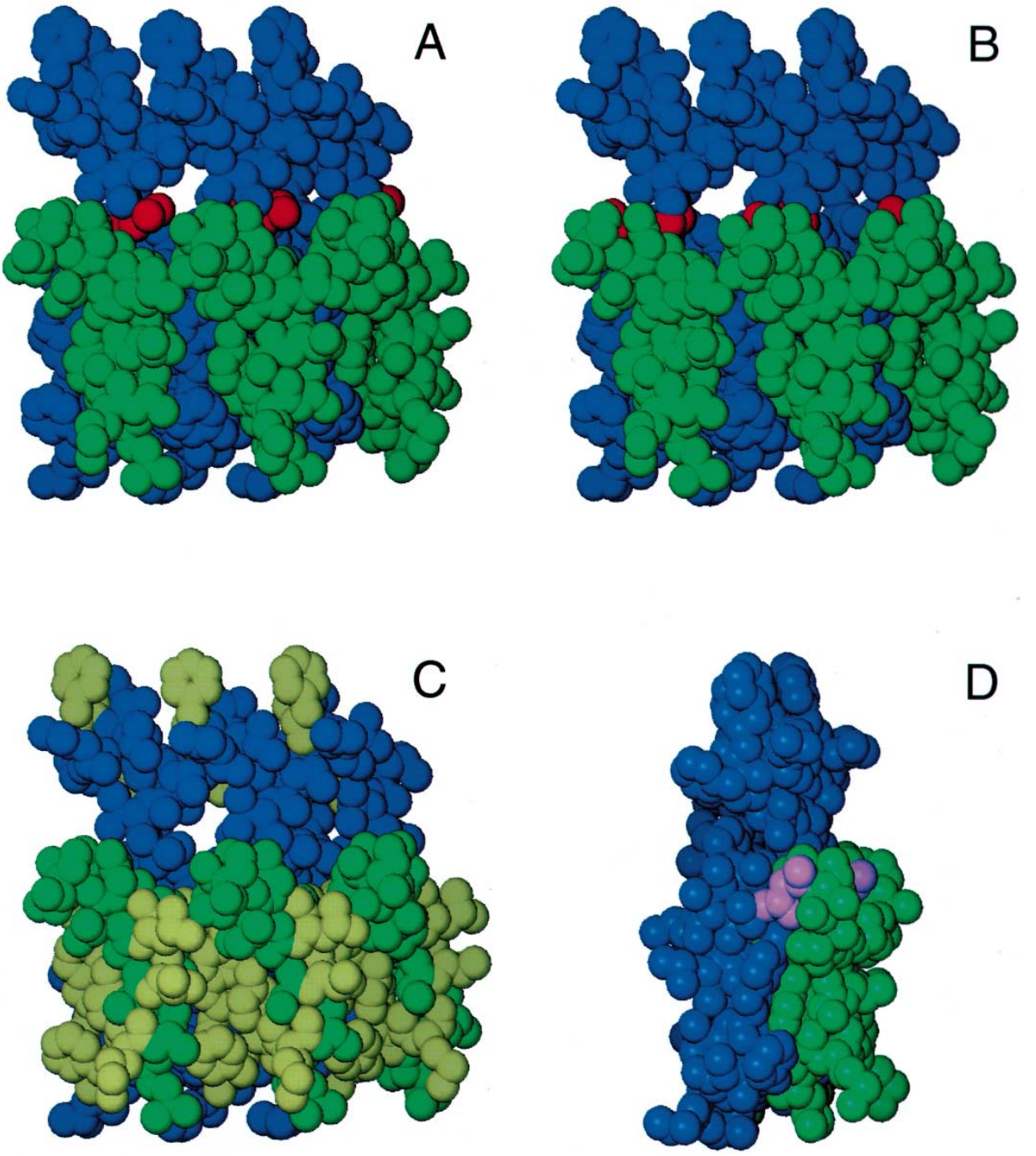

Fig. 2. Van der Waals surface representation of the modelled dodecameric ring of *c*-subunits. Side view of the predicted complex as it would appear from the membrane phase drawn as van der Waals surface plot. For simplicity only three protomers of the modelled dodecamer are shown. A: The C-terminal helices which are exposed to the inside of the modelled ring-shaped complex are coloured blue, the N-terminal helices which are facing the membrane phase are drawn in green. The essential carboxyl group of Asp^{61} which is shown in red, is buried in a pocket formed by adjacent protomers, but still accessible from the lipid phase. B: Van der Waals surface plot of the modelled complex for double mutant A24D/D61G. Equivalent to the situation found for the wild type the carboxyl group essential for proton translocation across F_o (red) is lying in a pocket which is shielded, but still accessible from the membrane phase. C: Surface plot for three adjacent protomers of the modelled *c*-subunit complex. Side chains which are labelled by ^{125}I-TID in intact F_o are shown in yellow. D: Van der Waals surface representation of the modelled *c*-dodecamer after replacing Ala^{25} by threonine as in proton transport deficient mutant Ala^{25}Thr. The hydrophilic threonine side chain which is intercalating between N-terminal (green) and C-terminal (blue) helices of adjacent proteolipid monomers is shown in magenta.

in the model of the *c* dodecamer, residues 10, 11, 15, 19, 73 and 76 are all exposed to the membrane phase, and Met^{57} is exposed to the internal surface of the ring, where it would be accessible to TID reaction (see Fig. 2C). The only disagreements concern residue Met^{65}, which is partially shielded by Ile^{26} in the model, and Phe^{54} (a site of relatively lower reac-

Table 1

	1 15	16 30	31 45	46 60	61 75	76 90	
1	PISUM SATIVUM	----MNPLIAAASVI	AAGLAVGLASIGPGV	GQGTAAGQAVEGIAR	QPEAEDKIRGTLLLS	LAFMEALTIYGLVVA	LALLFANPFV---- 81
2	SPINACIA OLERACEA	----MNPLIAAASVI	AAGLAVGLASIGPGV	GQGTAAGQAVEGIAR	QPEAEGKIRGTLLLS	LAFMEALTIYGLVVA	LALLFANPFV---- 81
3	NICOTIANA TABACUM	----MNPLISAASVI	AAGLAVGLASIGPGV	GQGTAAGQAVEGIAR	QPEAEGKIRGTLLLS	LAFMEALTIYGLVVA	LALLFANPFV---- 81
4	MARCHANTIA POLYMORPHA	----MNPLISAASVI	AAGLAVGLASIGPGI	GQGTAAGQAVEGIAR	QPEAEGKIRGTLLLS	LAFMEALTIYGLVVA	LALLFANPFV---- 81
5	MASTIGCLADUS LAMINOSUS	----MDPLISAASVL	AAALAIGLAAIGPGI	GQGNAAGQAVEGIAR	QPGAEGKIRGTLLLT	LAFMESLTIYGLVIA	LVLLFANPFS---- 81
6	PROPINIGENIUM MODESTUM	MDMVLAKTVVLAASA	VGAGAAMIAGIGPGV	GQGYAAGKAVESVAR	QPEAKGDIISTMVLG	QAIAESTGIYSLVIA	LILLYANPFVGLLG 89
7	ESCHERICHICIA COLI	----MENLNMDLLYM	AAAVMMGLAAIGAAI	GIGILGGKFLEGAAR	QPDLIPLLRTQFFIV	MGLVDAIPMIAVGLG	LYVMFAVA------ 79
8	VIBRIO ALGINOLYTICUS	----METL-LSFSAI	AVGIIVGLASLGTAI	GFALLGGKFLEGAAR	QPEMAPMLQVKMFII	AGLLDAVPMIGIVIA	LLFTFANPFVGQLG 84
9	ENTEROCOCCUS FAECALIS	----------MNYI	AAAIAIMGAAIGAGY	GNGQVISKTIESMAR	QPEMSGQLRTTMFIG	VALVEAVPILGVVIA	LILVFAV------- 71
10	THERMOPHILIC BACTERIUM	---------MSLGVL	AAAIAVGLGALGAGI	GNGLIVSRTIEGIAR	QPELRPVLQTTMFIG	VALVEALPIIGVVFS	FIYLGR-------- 72
11	BACILLUS ACIDOCALDARIUS	---MQLDMVKAIYDI	AAALLLGLAAVGSGV	GDGMVMSKYVEGVAR	QPEARGSIFGSALLG	VALVEAFPVIALAFG	IIILFTKGAF---- 82
12	ZEA MAYS MITOCHONDRION	--------MLEGAKS	IGAGAATIALAGAAV	GIGNVLSSSIHSVAR	NPSLAKQSFGYAILG	FALTEAIASFAPMMA	FLISFVF------- 74
13	PETUNIA	--------MLEGAKS	MGAGAATNASAGAAI	GIGNVLSSSIHSVAR	NPSLAKQLFGYAILG	FALTEANASFAPMMA	FLISFVFQVR---- 77
14	VICIA FABA	--------MLEGAKS	IGAGAATIASAGAAV	GIGNVFSSLIHSVAR	NPSLAKQLFGYAILG	FALTEAIALFALMMA	FLILFVF------- 74
15	YEAST MITOCHONDRION	------MQLVLAAKY	IGAGISTIGLLGAGI	GIAIVFAALINGVSR	NPSIKDTVFPMAILG	FALSEATGLFCLMVS	FLLLFGV------- 76
16	BOS TAURUS	-------DIDTAAKF	IGAGAATVGVAGSGA	GIGTVFGSLIIGYAR	NPSLKQQLFSYAILG	FALSEAMGLFCLMVA	FLILFAM------- 75
17	NEURASPORA CRASSA	YSSEIAQAMVEVSKN	LGMGSAAIGLTGAGI	GIGLVFAALLNGVAR	NPALRGQLFSYAILG	FAFVEAIGLFDLMVA	LMAKFT-------- 81
18	RHODOSPIRILLUM RUBRUM	-------MDAEAAKM	IGAGLAAIGMIGSGI	GVGNIWANLIATVGR	NPAAKSTVELYGWIG	FAVTEAIALFALVVA	LILLFAA------- 75
19	ANABAENA SP.	----MDPLVSAASVL	AAALAVGLAAIGPGI	GQGNAAGQAVEGIAR	QPEAEGKIRGTLLLS	LAFMEALTIYGLVVA	LVLLFANPFA---- 81
20	SYNECHOCOCCUS SP.	----MDPLVASASVL	AAALAIGLASLGPGI	GQGNASGQAVEGIAR	QPEAEGKIRGTLLLT	LAFMESLTIYGLVIA	LVLLFANPFAS--- 82
21	CYANOPHORA PARADOXA	----MDATVSAASVI	AAALAVGLAAIGPGI	GQGTAAGQAVEGIAR	QPEVDGKIRGTLLLS	LAFMEALTIYGLVVA	LALLFANPFV---- 81
22	ANTITHAMNION SP.	----MDSIISAASVI	AAGLAVGLAAIGPGI	GQGSAAANAVEGIAR	QPEVEGKIRGTLLLS	LAFMESLTIYGLVVA	LSLLFANPYTG--- 82
23	GALDIERIA SULPHURARIA	----MDSIISAASVI	AAGLAVGLAAIGPGI	GQGTASAQAVEGIAR	QPEAEGKIRGTLLLS	LAFMEALTIYGLVVA	LSLLFANPFINQ-- 83
24	ODONTELLA SINENSIS	----MDSIISAASVI	AAGLAIGLAAIGPGI	GQGNAAGQAVEGIAR	QPEGENKIRGTLLLS	LAFMEALTIYGLVVA	LALLFANPFNG--- 82
25	PINUS THUNBERGII	----MDPLISAASVI	AAGLSVGLASIGPGV	GQGTAAGQAVEGIAR	QPEAEGKIRGTLLLS	LAFMEALTIYGLVVA	LALLFANPFV---- 81
26	GLYCINE MAX	----MNPIISAASVI	AAGLAVGLASIGPGV	GQGTAAGQAVEGIAR	QPEAEGKIRGTLLLS	LAFMEALTIYGLVVA	LALLFANPFV---- 81
27	PAVLOVA LUTHERII	----MNPIISAASVI	AAGLSVGLAAIGPGI	GQGSAAGQALEGIAR	QPEAEGKIRGTLLLS	LAFMEALTIYGLVVA	LSLLFANPFTAS-- 83
28	EUGLENA GRACILIS	----MNPIICAASVI	GAGLAIGLGAIGPGI	GQGTASGKAIEGLAR	QPEAEGKIRGTLLLS	LAFMEALTIYGLVVA	LAIIFANPFV---- 81
29	HAEMOPHILUS INFLUENZAE	-----METVITATII	GASILLAFAALGTAI	GFAILGGKFLESSAR	QPELASSLQTKMFIV	AGLLDAIAMIAVGIS	LLFIFANPFIGLLN 84
30	BACILLUS MEGATERIUM	----------MGLI	ASAIAIGLAALGAGI	GNGLIVSKTIEGTAR	QPEARGTLTSMMFVG	VALVEALPIIAVVIA	FMVQGK-------- 70
31	BACILLUS SUBTILIS	----------MNLI	AAAIAIGLGALGAGI	GNGLIVSRTVEGIAR	QPEAGKELRTLMFMG	IALVEALPIIAVVIA	FLAFFG-------- 70
32	BACILLUS CALDOTENAX	---------MSLGVL	AAAIAVGLGALGAGI	GNGLIVSRTIEGIAA	QPELRPVLQTTMFIG	VALVEALPIIGVVFS	FIYLGR-------- 72
33	B. STEAROTHERMOPHILUS	---------MSLGVL	AAAIAVGLGALGAGI	ANGLIVSRTIEGIAR	QPELRPVLQTTMFIG	VALVEALPIIGVVFS	FIYLGR-------- 72
34	BACILLUS ALCALOPHILUS	----------MGLL	GAAIVAGLAAVGGAI	AVAIIVKSTIEGVTR	QPELKGTLQTLMFIG	VPLAEAVPIIAIVMG	FLIMGNA------- 71
35	BACILLUS FIRMUS	----------MAFL	GAAIAAGLAAVAGAI	AVAIIVKATIEGTTR	QPELRGTLQTLMFIG	VPLAEAVPIIAIVIS	LLILF--------- 69
36	T. FERROXIDANS	--MDAHTIIVAATAI	AVGIIFGAAGLGSAI	GWGLITSKTIEGITR	QPEMRPQLLVNTFIF	AGLMESFPFIILAFG	FWFLFANPFLG--- 84
37	MYCOBACTERIUM LEPRAE	----MDPMIAQGALI	GGGLIMAGGAIGAGI	GDGMAGNALVSGIAR	QPEAQSRLFTPFFIT	VGLVEAAYFINLAFM	ALFVFATPVK---- 81

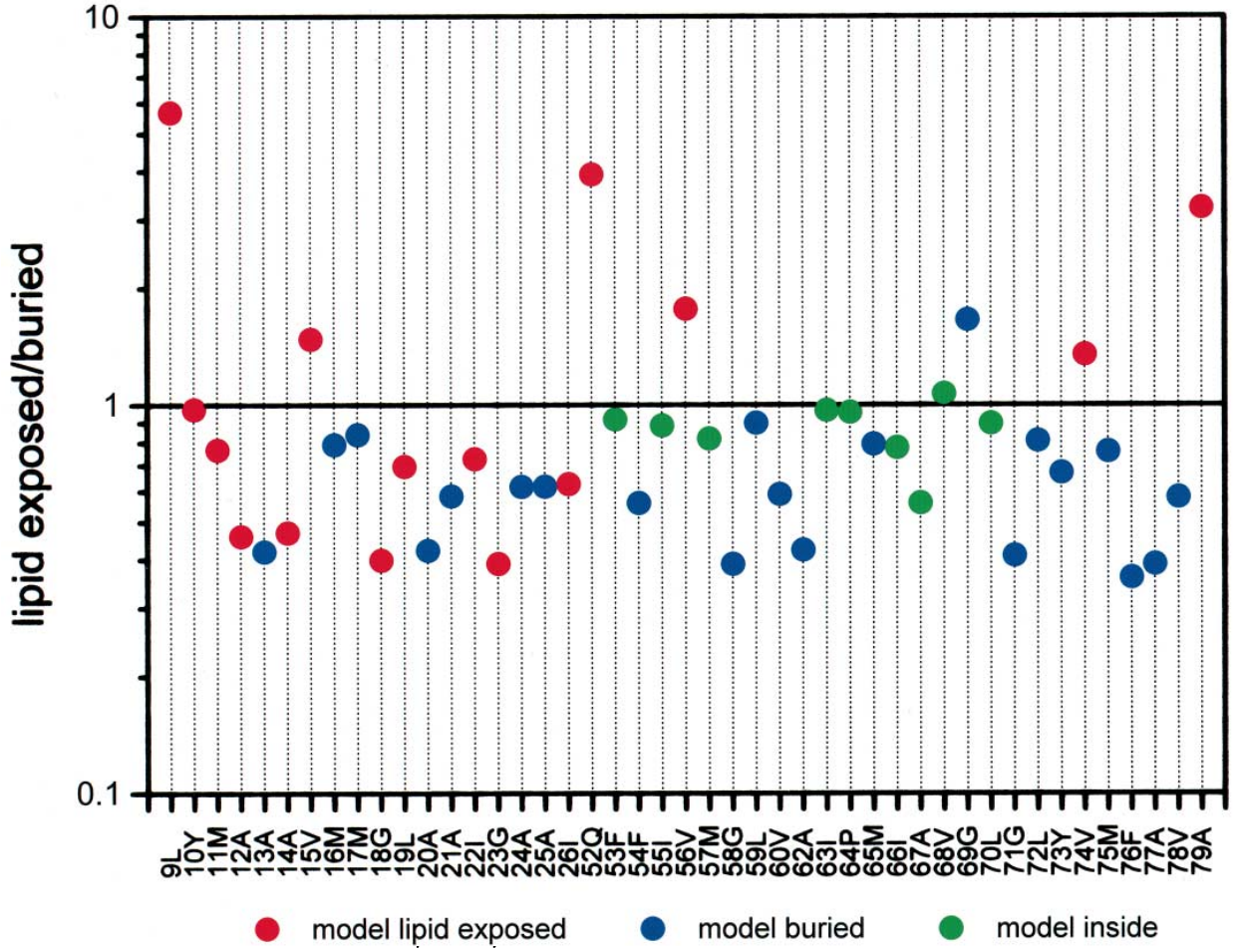

Fig. 3. Predicted orientation of side chains in a transmembrane complex formed by subunit *c*. Amino acid substitution tables for lipid accessible and buried residues were used in combination with an alignment of 37 different *c*-subunits (Table 1) to calculate the probability that a certain side chain is located at the interface between two adjacent monomers or exposed to the lipid phase. Only residues resolved by ^{1}H-NMR [1] were considered in the calculation. The tendency that a certain residue is found in a lipid accessible or a buried environment is expressed by calculating the ratio of the individual probabilities. Lipid exposed residues show ratios >1, buried residues <1. Different colours were used to emphasise the orientation of the residues predicted in the model. Side chains which are placed in the model at the interface between adjacent monomers are coloured blue. Lipid accessible, external residues are coloured red and residues located inside the ring-shaped complex are coloured green.

tion with TID), which could be shielded by the N-terminal helix (assuming that it continues in the same direction beyond Ile^{26} as reported for the resolved residues Ala^{22}–Ile^{26}). A different labelling pattern was found after subunit *c* had been solubilised in SDS. Solubilisation exposes those residues which have been buried or shielded by others in the native structure providing information about the interface of adjacent, interacting subunits in the complex. Residues Met^{16}, Met^{17}, Ile^{22}, Ile^{26}, Gly^{27}, Ile^{28}, Gly^{29}, Ile^{30}, Phe^{53}, Gly^{58}, Gly^{69} and Gly^{71} which are not labelled in functional, membrane integral subunit *c* are marked at a relative intensity of 100, 48, 10, 4, 2, 4, 2, 4, 27, 27, 23 and 14. In the proposed model Met^{16} and Met^{17} are both deeply buried at the interface between adjacent subunits. The side chain of Phe^{53} is partially shielded by Met^{57} and Ile^{63}, and residue Gly^{58}, which is lying in a groove, is covered by Phe^{53}, Ile^{63} and Met^{57}. The glycine in position 69 is also buried in the structure. Only residues Ile^{22}, Ile^{26} and Gly^{71} are exposed towards the external or internal side of the ring-shaped complex. The overall error in assignment resulting from comparison of the labelling data obtained with solubilised EF_o to the alignment of the residues in the model is in the order of 10%. Thus both labelling studies, those of native and those of solubilised subunit *c*, are basically in agreement with the proposed model.

Several non functional mutations have been reported for subunit *c* of *E. coli* in the literature [see [16] for review]. Some might be understood in the framework of the proposed model. Mutants, such as Gly^{23}Asp and Gly^{58}Asp, which fail to assemble subunit *c* in the membrane are expected to change the interacting surface of adjacent protomers. In the proposed oligomeric complex the aspartyl side chain of Gly^{58}Asp mutants would interfere with the aromatic ring system of Phe^{53} of an adjacent *c*-subunit. In Gly^{23}Asp mutants, where subunit *c* is not integrated into the membrane, the proposed *c*-dodecamer would be destabilised by exposure of a potentially charged residue towards the lipid phase. Other mutants still allow assembly of the transmembrane F_o-sector but affect H^+-translocation probably by structural restrictions. The mutation Pro^{64}Leu in subunit *c* eliminates a kink in the C-terminal, transmembrane helix. Residue 64 plays a crucial role in stabilising the proposed dodecamer complex by its direct interaction with Ile^{66} in the adjacent protomer. Changing this in-

teraction might result in rearrangements within the structure — maybe in the alignment of the proposed, internal water cluster — which modify proton transport across the F_o-sector. In another non-functional mutant Ala^{25} was replaced by threonine. Fig. 2D illustrates the modified structure of the mutant which is deduced from the proposed model. A hydrophilic side chain is placed into a buried, hydrophobic environment which is located between N- and C-terminal helices and adjacent N-terminal helices. This replacement should destabilise the *c*-dodecamer and result in a different local folding pattern or a different alignment of adjacent protomers which might affect proton translocation in the transmembrane region.

The alignment of the protomers predicted by the model was tested by substitution analysis. Amino acid substitution tables calculated for residues in membrane proteins [17] were used in this approach to discriminate buried and exposed surfaces in the *c* dodecamer. Substitution tables for external, lipid accessible residues were calculated on the basis of the three-dimensional structure of two homologous bacterial photosynthetic reaction centres [18,19] and an alignment of their sequences with sequences of related proteins [17]. Tables for buried residues were computed from aqueous proteins [20] as the interior structure of aqueous and membrane proteins is similar [21]. Thus, data based on the specific interior fold of the reaction centre and restrictions resulting from bound cofactors were avoided. We used an alignment of 37 homologous sequences of *c* subunits (see Table 1) to compare the proposed orientation of the residues in the model with the alignment predicted from substitution analysis. The assignment resulting from these substitution patterns is shown in Fig. 3. In general, the predicted orientation of the residues is in agreement with the modelled structure. However, significant contradiction is found for residues Ala^{12}, Ala^{14} and Gly^{18}, Gly^{23}, Gly^{69}. While the model suggests a lipid-accessible orientation for Ala^{12}, Ala^{14}, Gly^{18} and Gly^{23}, their substitution pattern puts these residues into a buried environment. On the other hand, Gly^{69} which is a residue that is buried in the modelled complex turns out to be lipid accessible from the data obtained by substitution analysis. The side chains of Tyr^{10}, Met^{11}, Leu^{19} and Phe^{76}, which are all accessible by TID in the native F_o-complex and therefore probably accessible from the membrane phase, are placed in a buried environment by the prediction. Isoleucines in positions 22 and 26, residues which are accessible towards TID only after solubilisation of EF_o in SDS, are also assigned to a buried surface by substitution analysis. In the modelled complex they are located at the external lipid accessible phase. In summary, the disagreement between modelled and predicted assignment is in the order of 20%. Contrary assignments occur mainly for small residues located in a lipid accessible orientation in the model which is placed in a buried environment by the substitution analysis. The contradiction might be resolved by using substitution tables obtained from membrane proteins for buried residues. These tables should be available soon by using the molecular structure of cytochrome oxidase [22,23] as a reference resulting in a more accurate substitution analysis.

3. Conclusion

The proposed model of a transmembrane sub-complex of the membrane sector of F-ATPases is in agreement with most of the data reported in the literature. Constructing the model we assumed a stoichiometry of 12 *c*-subunits per complex based on data reported by [24]. A stoichiometry of 10 protomers per complex as suggested by [25], however, would not alter the proposed model substantially as a slight turn of adjacent C-terminal helices would correct the lower number of subunits per complex and maintain the proposed contacts between adjacent monomers. Contrary orientations compared to labelling studies are proposed for Ala^{12}, Ala^{14}, Ile^{22} and Ile^{26} which are placed in a lipid-accessible orientation in the modelled complex. The insufficient reactivity of TID towards alanine might explain why no label was incorporated at these positions. Weak labelling if Ile^{22} and Ile^{26} was found after solubilisation and suggests a buried orientation of these residues. However, the absence of any label in experiments with unsolubilised F_o-complex might results from the low reactivity of the probe towards isoleucine.

Problems resulting from a different reactivity of residues towards probes are avoided if internal probes are used in labelling studies. Mutagenic introduction of cysteines at specific sites within a protein was applied to resolve the structure and arrangement of membrane-spanning segments in a number of membrane proteins [26,27]. Sulfhydryl reagents of different hydrophobicity and disulfide cross-linking were used in these studies to resolve the position of modified residues. Tryptophan scanning mutagenesis was used to reveal the structure of the transmembrane proton channel MotA and MotB [28,29] by introducing single tryptophan residues at consecutive positions within the transmembrane segments. The bulky, hydrophobic tryptophan is only tolerated in positions facing the lipid phase, but not at buried, interacting surfaces of the transmembrane segments. Both approaches, cysteine and tryptophan mutagenesis, are under investigation at the moment to test the proposed model for the membrane integral proteolipid complex of *E. coli*.

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