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Subunit *c* of F_1F_0 ATP synthase: structure and role in transmembrane energy transduction

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Introduction

In this review, I summarize recent work from my laboratory that addresses the role of subunit *c* in the function of F_1F_0 ATP synthases. H^+ transporting F_1F_0 ATP synthases utilize the energy of a transmembrane electrochemical proton gradient to catalyze ATP synthesis during oxidative phosphorylation. The F_1 sector catalyzes the transphosphorylation reaction, and the F_0 sector functions as the H^+ translocase. When the two sectors are coupled, the enzyme functions as a reversible, H^+ transporting ATPase. The F_1F_0 ATP synthase of *E. coli* is composed of eight subunits, where the stoichiometry of subunits in F_1 is $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ and that in F_0 is $a_1b_2c_{10\pm1}$ [1]. Dicyclohexylcarbodiimide (DCCD) covalently reacts with aspartyl-61 in subunit *c* to block proton translocation and the coupled ATPase reaction in F_1. Reaction with one subunit *c* per F_0 is sufficient to completely abolish activity [2]. For reasons given below, we now believe that subunit *c* plays a key role in not only catalyzing H^+ translocation, but also in harnessing the energy from H^+ translocation via conformational changes that are transmitted to F_1 to promote release of the ATP product. Insights into how the structure of the protein may relate to function are also summarized. The background and many of the conclusions are more thoroughly documented in a recent review [3].

Transmembrane folding of subunit *c* in F_0

Subunit *c* is thought to fold in the F_0 sector of the membrane like a hairpin with two membrane traversing α-helices (see Fig. 1). Residues in both membrane tranversing helices are labeled by hydrophobic agents that are thought to react from the apolar phase of the lipid bilayer [3]. Further, an epitope within the region Lys-34 to Leu-45 is exposed on the F_1 binding side of the membrane [4,5]. This physical evidence is supported by genetic studies. Reaction of DCCD with Asp-61 in transmembrane helix-2 is slowed by mutation of Ala-24 to Ser [6] or Ile-28 to Thr or Val [7] in helix-1. Additional support derives from isolation of suppressor mutations, where the function abolished by mutation of Pro-64 to Leu or Ala in helix-2 was restored by a second substitution of Pro for Ala-20 in helix-1 [8,9]. The nearness of Ala-24 to Asp-61 is also supported by the finding that the essential Asp can be moved from position 61 in helix-2 to position 24 in helix-1 with retention of function [10].

Native-like folding of subunit *c* in chloroform / methanol / H_2O

Dr. Mark Girvin of our laboratory is in the process of determining the three-dimensional structure of isolated subunit *c* in chloroform/methanol/H_2O solvent (Ref. 11; unpublished data). Our interest in this structure is now heightened, having recently proven that native features of structure are preserved in this solvent. The environment around Asp-61 is preserved such that it retains its unique reactivity with DCCD. Further, the Ile-28–Thr mutation continues to slow the rate of reaction of DCCD with Asp-61, which strongly suggests that the protein still folds like a hairpin. The Ile-28–Thr mutation also affects the chemical shift (i.e. environment) of protons on both residues 60 and 61. Our most recent data show NOEs between residues on helix-1 and residues on helix-2, at both ends of the membrane spanning region. This means that the protein must be folding like a hairpin such that the two helices come to within 5 Å of each other.

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Further information on the folding of subunit *c* was derived from an experiment where Asp-61 was specifically labeled with a nitroxide derivative of DCCD (NCCD; *N*-[2,2,6,6-tetramethylpiperidyl-1-oxyl]-*N'*-[cyclohexyl]-carbodiimide). The idea was to define proton resonances close to the nitroxide radical based upon the extent of paramagnetic broadening. The resonance of protons within a radius of 10–11 Å should be broadened beyond detection, whereas resonances of protons in the range of 11–22 Å should be predictably broadened and the intensity only partially reduced. The results are summarized in Fig. 1. Significantly, resonances in both helices were affected by the nitroxide group. For example, the α carbon of Ala-24 must lie within 12 Å of the nitroxide radical. Based upon these distances, we have developed a model for this region of subunit *c* and the helical-helical interactions. The piperidyl ring containing the nitroxide group lies in a pocket between the side chains of Met-57 and Val-60. The side chain of Leu-31 on helix-1 is on the opposite face of the binding pocket. An energy minimized version of the model lacking NCCD shows the β-carboxyl of Asp-61 lying between the side chains of Ala-24 and Ile-28.

Primary role of aspartyl-61 in proton translocation

The hypothesis that proton translocation is mediated by a protonation-deprotonation of Asp-61 is not yet proven. The possibility was initially suggested by studies in which the specific reaction of DCCD with Asp-61 was shown to block F_0-mediated H^+ translocation (reviewed in Refs. 3 and 12). Stronger support for the idea comes from the characterization of randomly isolated mutants in which substitution of Gly or Asn for Asp-61 was shown to totally abolish proton translocation function [3,12]. The lack of function of Asn at this position strongly suggests that the residue must do more than hydrogen bond, i.e., that it must be able to bind and release a proton. A reversible, ATP-driven proton pump requires at least one H^+ (or H_3O^+) binding site, the pK_a of which must change during the proton translocation cycle [3]. Asp-61 is the best candidate for this binding site.

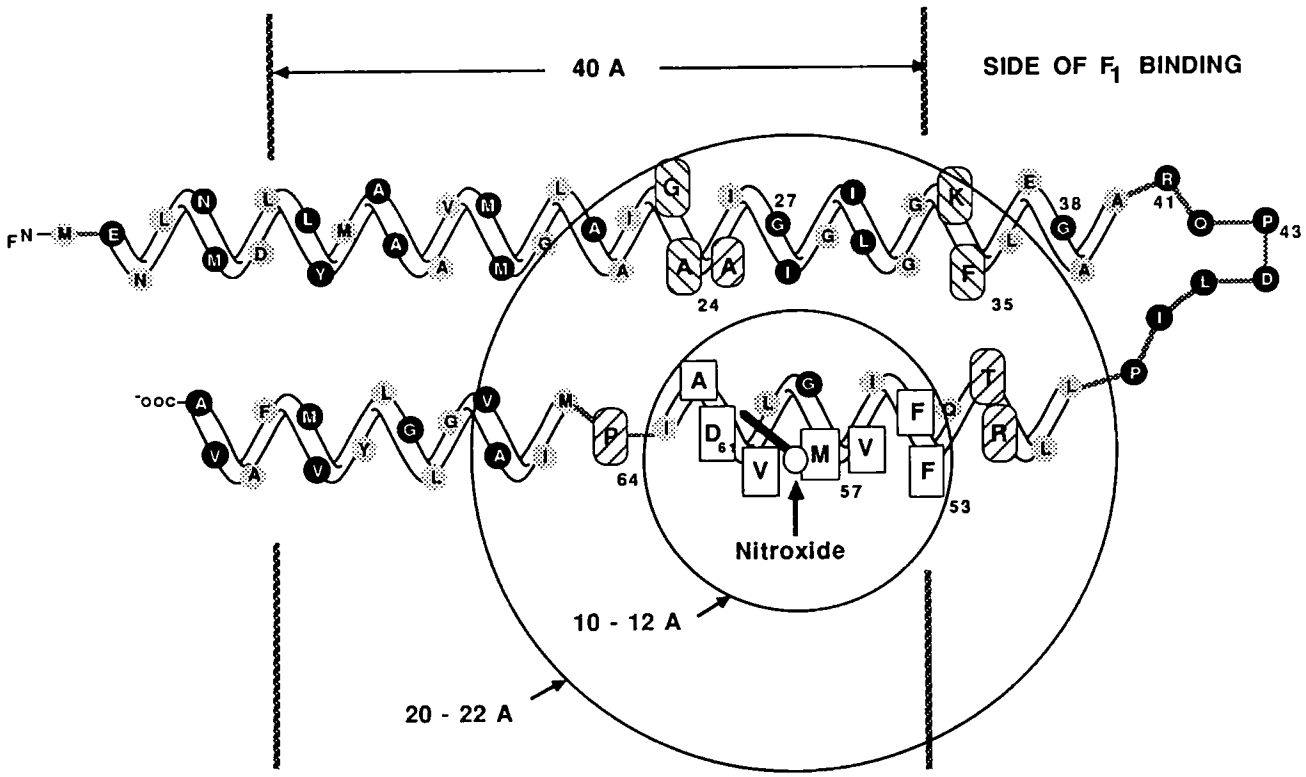

Fig. 1. Proton resonances of subunit *c* broadened by paramagnetic nitroxide of NCCD. The hairpin model for subunit *c* traversing the lipid bilayer is discussed elsewhere [3,10]. The position of the piperidyl ring and N → O radical is indicated by the thick line and open circle drawn between V60 and M57. Residues where the α-CH proton resonance intensity is reduced to zero are highlighted by the open boxes in the inner ring. Residues where the α-CH proton resonance is broadened but is still detectable are indicated by the hatched ovals in the outer circle. Sequence specific assignments have not been made for other residues within these circles, and hence the distances from the nitroxide cannot be estimated.

TABLE I

Coupling properties of polar loop mutants of subunit c

Mutant (Reference)	ATP driven H^+ pumping	F_1 binding (conditions) [a]			H^+ leaky F_1–F_0
		I	II	III	
R41–K [b]	0	+	≪ WT		Yes
R41–H [b]	0	< WT			Yes
Q42–E [15]	0	+	+		Yes
Q42–A, Q42–G [16]	< WT	+	≪ WT		> WT
Q42–V [16]	= WT	+			> WT
P43–S, P43–A [14]	< WT	+	+	≪ WT	No

[a] F_1 binding conditions given in Refs. 14–16.
[b] Fraga, D., Miller, M., Oldenburg, M., Hermolin, J., and Fillingame, R., unpublished data.
[c] WT = wild type.

Coupling function of the polar loop

Mutant analysis has led to the hypothesis that the polar loop of subunit *c* plays a key role in coupling proton translocation to the conformational events in F_1 leading to ATP synthesis. The sequence surrounding Arg-41–Gln-42–Pro-43 is highly conserved among species with no variations reported in either the Arg or Pro residues. Several conclusions emerge from the analysis of 38 substitutions in the region from Glu-37 to Leu-45 [13]. First, only Arg-41 is absolutely required for function. In contrast, the totally conserved Pro-43 can be replaced by Ser or Ala with only minor perturbations of function [14]. All of the other residues are tolerant to at least limited changes, but sufficiently drastic changes in any residue result in loss of function. The ensemble of conserved amino acids in the region may collectively maintain the essential features of structure and explain the tolerance to single amino acid changes. Function is totally abolished in the Arg-41–Lys and Gln-42–Glu polar loop mutants, both of which exhibit an 'uncoupled' phenotype (Table I). Under conditions where F_1 appears to be normally bound to the membrane, ATP hydrolysis is uncoupled from proton translocation. Further, the binding of F_1 no longer blocks the proton channel of F_0, i.e., a passive proton leak occurs through the F_0–F_1 interface. Several mutations in Gln-42 and Pro-43 show near normal function, but still exhibit minor indications of an uncoupled phenotype (Table I). In sum, these phenotypes suggest that the loop region may be involved in the transmission of a conformational change from F_0 to F_1 which is driven by proton translocation and ultimately coupled to ATP product release.

Essential aspartyl can be moved from helix-1 to helix-2

Miller et al. isolated pseudorevertants of the Asp-61–Gly mutant of subunit *c* by selecting for small colonies that grew on a succinate carbon source via oxidative phosphorylation [10]. The pseudorevertants retain the Asp-61–Gly mutation, but substitute Asp for Ala-24. The Asp-24–Gly-61 double mutant grew at 2/3 the rate of wild type on succinate minimal medium and exhibited reduced ATP-driven proton translocation activity (< 20% of wild type). Hence, subunit *c* can function with the essential carboxyl group anchored to either residue 61 on helix-2 or residue 24 on helix-1. We presume that these two residues lie close to each other in the membrane, and that the carboxyl group ends up in approximately the same position. The results also suggest that helix-1 and helix-2 may interact as a unit during proton translocation, and that the structure of this unit is not significantly disrupted in the double mutant. In considering this interaction, it is of interest that the region of Gly-23 to Gly-29 of helix-1 is one of the two most conserved areas of the protein with a consensus sequence of GXGXGXG. It seems likely that the structure provided by this part of the unit is important in either proton translocation itself or in propagating the conformational change that is coupled to H^+ translocation.

Helical-helical interactions between subunit *c* and subunit *a*

The Asp-24–Gly-61 subunit *c* mutant grows more slowly than wild type on a succinate carbon source. We wondered whether the structure surrounding the newly placed Asp-24 was optimal, and hence decided to select third site optimizing mutations that generated larger colonies on succinate agar plates. Eighteen independent, optimizing mutations have now been mapped (Ref. 9; Fraga, D. and Fillingame, R., unpublished data). To our surprise, only 5 of these lie in subunit *c* and the other 13 in subunit *a*. Of the 13 optimizing mutations found in subunit *a*, 10 map to three residues, i.e., Ala-217, Ile-221 and Leu-224. These residues would all lie on the same face of an α-helix, and in at least some models of subunit *a* [3,17], the same transmembrane helix would also include the Arg-210 residue, which is essential for proton translocation [17]. We predict that during the process of proton translocation, a helical-helical interaction is required between the helical unit of subunit *c* that anchors the essential carboxyl group and a transmembrane helix of subunit *a* that includes residues 217–224.

Relationship of subunit stoichiometry to mechanism

As discussed above, Asp-61 of subunit *c* is thought to protonate and deprotonate as each proton is translocated through F_0. At least 3 (perhaps 4) H^+ must be translocated for each ATP synthesized. The translocation of these three protons must be coupled to

conformational changes in F_0 which are ultimately transmitted to F_1 to result in ATP product release. I envision a coupling mechanism in which three subunits *c* are sequentially protonated, but then where the 3 H^+ are released simulataneously in an event coupled to the conformational change [3]. Since subunit *a* is also known to play an essential role in proton translocation [3,17], it may serve in the sequential loading of the three subunits *c*.

At least two, and perhaps all three, $\alpha\beta$ subunit pairs are believed to alternate during catalysis [3]. If three subunits *c* are used per ATP synthesized, and all 3 $\alpha\beta$ pairs alternate in a complete cycle of catalysis, then a total of 9 subunits *c* would be required to provide the 3×3 protons translocated. Nine is very close to the actual stoichiometry of subunit *c* measured in the F_1F_0 complex [1].

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