Genetic Fusions of Subunit c in the \( F_0 \) Sector of \( \text{H}^+\)-transporting ATP Synthase

**FUNCTIONAL DIMERS AND TRIMERS AND DETERMINATION OF STOICHIOMETRY BY CROSS-LINKING ANALYSIS***

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Phil C. Jones‡ and Robert H. Fillingame§

From the Department of Biomolecular Chemistry, University of Wisconsin Medical School, Madison, Wisconsin 53706

The multicopy \( c \) subunit of the \( \text{H}^+\)-transporting ATP synthase of *Escherichia coli* folds through the transmembrane \( F_0 \) sector as a hairpin of two hydrophobic \( \alpha \)-helices with the proton-translocating aspartyl-61 side chain centered in the second transmembrane helix. The number of subunits \( c \) in the \( F_0 \) complex, which is thought to determine the \( \text{H}^+\)-pumping/ATP stoichiometry, was previously not determined with exactness but thought to range from 9–12. The studies described here indicate that the exact number is 12. Based upon the precedent of the subunit \( c \) in vacuolar-type ATPases, which are composed of four transmembrane helices and seem to have evolved by gene duplication of an \( F_0 \)-type progenitor gene, we constructed genetically fused dimers and trimers of *E. coli* subunit \( c \). Both the dimeric and trimeric forms proved to be functional. These results indicate that the total number of subunit \( c \) in \( F_0 \) should be a multiple of 2 and 3. Based upon a previous study in which the oligomeric organization of \( c \) subunits in \( F_0 \) was determined by cross-linking of Cys-substituted subunits (Jones, P. C., Jiang, W., and Fillingame, R. H. (1998) *J. Biol. Chem.* 273, 17178–17185), we introduced Cys into the first and last transmembrane helices of subunit \( c \) monomers, dimers, and trimers and attempted to generate cross-linked products by oxidation with Cu(II)-(1,10-phenanthroline)₂. Double Cys substitutions at two sets of positions gave rise to extensive cross-linked multimers. Multimers of the monomer that extended up to the position of \( c_{12} \) were correlated and calibrated with distinct cross-linked species of the appropriate doubly Cys-substituted dimers (i.e. \( c_{22}, c_{66}, \ldots, c_{12} \)) and doubly Cys-substituted trimers (i.e. \( c_{32}, c_{62}, c_{12} \)). The results show that there are 12 copies of subunit \( c \) per \( F_0 \) in *E. coli*, the exact number having both mechanistic and structural significance.

\( \text{H}^+\)-transporting \( \text{F}_1\text{F}_0 \) ATP synthases utilize the energy of a transmembrane electrochemical \( \text{H}^+ \) gradient to catalyze formation of ATP. Closely related enzymes are found in the plasma membrane of eubacteria, the inner membrane of mitochondria, and the thylakoid membrane of chloroplasts (1). The enzyme is composed of distinct extramembranous and transmembrane sectors, termed \( F_1 \) and \( F_0 \) respectively. Proton movement through \( F_0 \) is reversibly coupled to ATP synthesis or hydrolysis in catalytic sites of \( F_1 \). Each sector of the enzyme is composed of multiple subunits with the simplest composition being \( a_\beta_\gamma \) for \( F_1 \) and \( a_\beta g_9 \) for \( F_0 \). Based upon a previous genetic and chemical studies of \( F_0 \), the exact stoichiometry of subunit \( c \) in \( F_0 \) was determined by cross-linking of Cys-substituted proteins, which indicated a ring-like arrangement of alternate \( c \) subunits (8–10). The subunit \( c \) oligomeric ring is proposed to rotate with respect to a static \( a_\beta_\gamma \) portion of bovine \( F_1 \) and \( a_\beta g_9 \) for \( F_0 \) in the case of the *Escherichia coli* enzyme (2). Homologous subunits are found in mitochondria and chloroplasts. An atomic resolution X-ray structure of the \( a_\beta_\gamma \) portion of bovine \( F_1 \) shows the three \( a \) and \( \beta \) subunits interacting around a centrally located \( \gamma \) subunit, with the \( \gamma \) subunit interacting symmetrically with the 3 \( \beta \) catalytic subunits (3). Subunit \( \gamma \) was subsequently shown to rotate with respect to the 3 \( \beta \) subunits during catalysis (4–6). Rotation of \( \gamma \) is thought to change the binding affinities in alternating catalytic sites to promote tight substrate binding and product release during catalysis (7). During ATP synthesis, the rotation of \( \gamma \) must be driven by proton translocation through \( F_0 \).

The structure of \( F_0 \) remains to be determined. Electron and atomic force microscopic studies suggest that the \( a \) and \( b \) subunits pack at the periphery of a ring of multiple subunits \( c \) (8–10). The subunit \( c \) oligomeric ring is proposed to rotate with respect to a static \( a_\beta_\gamma \) subcomplex to drive rotation of subunit \( \gamma \) in \( F_1 \) (4, 11–13). Proton binding and release at a conserved Asp/Glu side chain in the center of the membrane (Asp-61 in the case of *E. coli* subunit \( c \)) is proposed to drive rotation of the \( c \) oligomer. The NMR structure of monomeric subunit \( c \) shows that it folds as a hairpin of two transmembrane \( \alpha \)-helices with interacting residues in the two helices located at positions predicted from genetic and chemical studies of \( F_0 \) in situ (14). The NMR model is supported by a cross-linking analysis of Cys-substituted proteins, which indicated a ring-like arrangement of at least 10 and quite probably 12 subunits \( c \) (15). The subunits interact with the front face of one subunit packed against the back face of the next, with the Asp-61 carboxylate lying at the center of the four helices of adjacent subunits. The exact stoichiometry of subunit \( c \) in the \( F_0 \) complex remains uncertain, although 12 copies are favored (16), for reasons briefly reviewed here. Purification of \( \text{F}_1\text{F}_0 \) following *in vivo* labeling suggested a possible range of 9–12 subunits per \( F_0 \) (17, 18). This range of stoichiometry is supported by inhibition experiments where modification of a single subunit \( c \) per \( F_0 \) was sufficient to block all activity (19, 20). The stoichiometry of subunits in \( F_0 \) should directly relate to the stoichiometry of \( \text{H}^+ \) transported per ATP synthesized, if the hypothesis of \( c \)-oligomer rotation is correct (4, 11–13). The \( \text{H}^+/\text{ATP} \) ratio in chloroplasts is now suggested to be 4 (21, 22), which, with three alternating catalytic sites in \( F_1 \), is consistent with 12 subunits \( c \) per \( F_0 \). A homologue of subunit \( c \) is found in a related family...
of vacuolar or V$_{1}$V$_{0}$ ATPases. The V-type subunit c is approximately twice the size of that in F$_{1}$F$_{0}$, exhibiting four apparent transmembrane helices, and appears to have evolved by duplication from an F$_{1}$-like progenitor gene (23, 24). Chemical analysis of a purified V$_{1}$V$_{0}$ ATPase indicates six copies of the V-type subunit c per enzyme (25, 26) and if correct, would imply 12 copies of subunit c in the F$_{1}$F$_{0}$ enzymes. In this study we have made genetic fusions of E. coli subunit c based upon the precedent of the homologous V-type subunit. The subunit c dimers (c$_{2}$) and trimers (c$_{3}$) proved to be functional, which indicates that the stoichiometry of c monomers in F$_{0}$ must be a multiple of both 2 and 3. Following genetic substitution of Cys, oligomers of c$_{2}$ dimers and c$_{3}$ trimers were generated by cross-linking. The maximal size of the cross-linked product corresponded to a multimer of 12 c subunits. The experiments provide compelling support for arrangement of subunit c in F$_{0}$ as a c$_{12}$ oligomer.

**EXPERIMENTAL PROCEDURES**

**Construction of the Subunit c Dimer (c$_{2}$) and Trimer (c$_{3}$)**—The plasmids used in this study are derivatives of plasmid pNOC, which contains the uncE subunit gene uncBEFHE genes (bases 870–3216$^{1}$ coding subunits a, c, b, and d) except for the hC21S substitution in subunit b (15). All three of the F$_{0}$ subunits coded by plasmid pNOC lack Cys. A polymerase chain reaction (PCR$^{2}$) strategy was used to construct the subunit c dimer (c$_{2}$). An oligonucleotide primer, 5’-CCCGGCTAACCCTTCTGGAACGATGGATATCTCTGCT-3’, which at the 3’ end corresponds to the first 19 bases of the sense strand of the uncE gene (bold italic type) and additional sequence (regular type) that encodes the amino acid sequence ANGSLNDGISA. The four bases at the 5’ end of this primer (bold italic type) correspond to the last four bases of the uncE gene. The 11-residue sequence ANGSLNDGISA corresponds to the amino acid sequence linking helices 2 and 3 of the larger and related subunit c of the bovine V$_{1}$V$_{0}$ ATPase (23). PCR was carried out with this primer and an antisense primer (primer 2) hybridizing 3’ to the uncE gene (bases 2174–2189) using plasmid pNOC as the template DNA. The product of the PCR (product A) should encode an 11-amino acid extension lying in-frame with the start of the uncE gene coding sequence.

In another amplification using plasmid pNOC DNA as template, a sense primer (primer 3) hybridizing 5’ to uncE (bases 1844–1860) was used with an antisense primer, 5’-ACCACCTGTTICAGAAAACGCPTAGCCCGCACAAGGCAACATCTAC-3’, designed to incorporate the last 18 bases of uncE at the 3’ end (bold italic type). This primer contains sequence that is complementary to the primer used to generate product A (sequence underlined in both primers). The product of the PCR (product B) should incorporate the additional sequence at the end of uncE (underlined), corresponding to the amino acid sequence ANGSLNDGISA. Primers A and B were used to construct a sequence encoding c$_{2}$ by a PCR strategy. The reaction was set up so that during the first three cycles of a 30-cycle regimen in total, products A and B acted as both primer and template. During these first three cycles, the overlapping complementary DNA sequence at opposing termini in products A and B were predicted to produce a template consisting of two uncE genes linked by sequence encoding the 11-amino acid stretch of ANGSLNDGISA. After the three initial PCR cycles, primers 2 and 3 were added to amplify this product. The final product was then digested with Avai restriction enzyme. A single Avai site is present in the pNOC plasmid, at bases 1976–1981 in the uncE gene in codons for amino acid residues 31 and 32. The product of the final PCR described above will contain two such Avai sites. The Avai fragment cut from the PCR product was ligated into the Avai site of plasmid pNOC to generate the genetic fusion of two subunits c and the plasmid pPJc2. This was confirmed by restriction mapping and sequencing. The above strategy was also carried out whereby a random sequence, designed to be flexible, was used to join the two subunits c. The amino acid sequence used was ASASNGASA and the plasmid encoding the fused c$_{2}$ gene was designated pPJc2R, where R designates random.

A subunit c trimer (c$_{3}$) or tetramer (c$_{4}$) was generated by subcloning the Avai fragment, or duplicate Avai fragments, from plasmid pPJc2 back into plasmid pPJc2 DNA that was partially digested with Avai and dephosphorylated with alkaline phosphatase. The subcloning was confirmed by restriction mapping. This generated plasmids pPJc3 and pPJc4.

**Construction of Cys Mutants in Subunit c**—Double Cys substitutions were constructed in plasmid pNOC as described previously (15). Plasmid pPJc1-11/75 was generated so that Cys now occupied positions 11 and 75 in the NMR model (Ref. 14). In the c$_{2}$ dimers, helices 1 and 2 are postulated to pack preferentially to the right of helices 1 and 2. This preference would account for observed cross-linked multimers in the 21/65 c$_{2}$ dimer and lack of multimer formation in the 20/66 c$_{2}$ dimer. The regions around residues 11 and 75 must be somewhat mobile to account for the observed dimer and multimer formation discussed in the text.

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$^{1}$ The unc DNA numbering system corresponds to that used by Walker et al. (34).

$^{2}$ The abbreviations used are: PCR, polymerase chain reaction; ACMA, 9-amino-6-chloro-2-methoxyacridine; CuP, Cu(II)-(1,10-phenanthroline)$_{3}$; Tricine, N’-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] glycine.
TABLE I

| Growth of cells expressing c₂ and c₃ fusion proteins by oxidative phosphorylation on succinate minimal medium |
|---------------------------------------------------------------|
| Version of Subunit index | Growth on succinate (colony size) |
|--------------------------|----------------------------------|
| c₁ (wild type)
| c₂
| c₃
| c₄
| c₁-M11C/M75C
| c₂-M11C/M75C
| c₃-M11C/M75C
| c₁-A20C/I66C
| c₂-A20C/I66C
| c₁-M21C/M65C
| c₂-M21C/M65C
| c₃-M21C/M65C |
| mm                  | Colony size after 72-h incubation at 37°C |
|---------------------|------------------------------------------|
| 2.4                 | Colony size after 72-h incubation at 37°C |
| 2.2                 | Colony size after 72-h incubation at 37°C |
| 2.0                 | Colony size after 72-h incubation at 37°C |
| 1.0                 | Colony size after 72-h incubation at 37°C |
| 0.0                 | Colony size after 72-h incubation at 37°C |
| 2.0                 | Colony size after 72-h incubation at 37°C |
| 1.5                 | Colony size after 72-h incubation at 37°C |
| 0.5                 | Colony size after 72-h incubation at 37°C |
| 0.8                 | Colony size after 72-h incubation at 37°C |
| 2.0                 | Colony size after 72-h incubation at 37°C |
| 1.5                 | Colony size after 72-h incubation at 37°C |
| 1.8                 | Colony size after 72-h incubation at 37°C |
| 1.0                 | Colony size after 72-h incubation at 37°C |

* Subunit c derivatives or fusion proteins were expressed from a pNOC-like plasmid in ΔuncBEFHΔ(a, b, c) chromosomal background strain JWP109 (recA4), except as noted. Colony size after 72-h incubation at 37°C. * Expressed in strain JWP109 (ΔuncBEFH, recA4).

mid. Because the AnaI site falls in the coding sequence for amino acids 31 and 32, subcloning of the AnaI fragment from plasmid pPJ2 into plasmids pPJ1-11/75, pPJ1-20/66, and pPJ1-21/65 should generate dimers and trimers with one Cys in the first helix and one Cys in the last helix (Fig. 1A).

Expression—The chromosomal uncBEFH deletion strain, JWP109 (pyrE41, entA603, ArgH1, rpsL109, supE44, ΔuncBEFH) (27), was made recA by cotransduction of recA56 with strT:10 from strain CP242 (+pX174) strain anaA1, anaB22, thi-l, uuc106, recA56, strT:10 (28) resulting in strain JWP109. Transduction was confirmed by UV sensitivity (28). Strain JWP109 was transformed with plasmids pNOC, pPJ2C, pPJ3C, pPJ4C, and their Cys-substituted derivatives. Complementation was tested by transferring transformant colonies to minimal medium 63 plates (29) containing 22 mM succinate, 2 mg/liter thiamin, 0.2 mM uracil, 0.2 mM L-arginine, 0.02 mM dihydroxybenzoic acid, and 100 μg/liter ampicillin.

Biochemical Assays and Cross-link Analysis—ATPase and ATP-driven ACMA quenching assays were carried out as described (30) with membranes prepared from cells disrupted by passage through a French press. Cross-linking was carried out as described previously (15) using membranes in TMG buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 10% (v/v) glycerol) and 1.5 mM Cu(II)-(1,10-phenanthroline)₂ (CuP) as an oxidant. Reactions were terminated by addition and incubation for 10 min with 50 mM EDTA and 25 mM N-ethylmaleimide and then mixed with an equal volume of 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, and 0.02% bromophenol blue) containing 20 mM EDTA. The solubilized membrane proteins were separated by SDS-polyacrylamide gel electrophoresis with a Tris-Tricine buffer system (31), and proteins were transferred from the gel electrophoretically onto a polyvinylidene difluoride membrane (32). Immunostaining was performed using rabbit antiserum to subunit c and then onto a polyvinylidene difluoride membrane (32). Immunostaining was performed using rabbit antiserum to subunit c and then membranes were probed with 50 mM EDTA and 25 mM N-ethylmaleimide and then mixed with an equal volume of 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, and 0.02% bromophenol blue) containing 20 mM EDTA. The solubilized membrane proteins were separated by SDS-polyacrylamide gel electrophoresis with a Tris-Tricine buffer system (31), and proteins were transferred from the gel electrophoretically onto a polyvinylidene difluoride membrane (32). Immunostaining was performed using rabbit antiserum to subunit c and then.
Pressing wild type respectively. Also shown are the migration of unsubstituted scence by membranes from cells expressing protein, substitutions fall in the first and last transmembrane helices of each protein, i.e. at position 11/75, 11/75', and 11/75'' of c1, c2, and c3, respectively. Also shown are the migration of unsubstituted c1, c2, and c3 (left three lanes) and the singly substituted A11C and M75C c1 monomer. CuP-treated membranes are indicated by + and untreated membranes by −. Membranes (25 μg of protein/lane) in panel A were treated with CuP for 1.5 h at 22 °C and electrophoresed on a 12.5–15% gradient gel. Membranes (25 μg of protein/lane) in panel B were treated with CuP for 2 h at 37 °C and electrophoresed on a 12% gel. Subunits were detected by immunoblotting.

results suggests that there is considerable bias as to how the second half of the subunit c dimer packs with respect to the first half. The results can be explained if the second half of the molecule packs to the right side of the first half when viewed from the F1 binding side (Fig. 1B). In such a case, the 20/66 c2 subunit should only form intramolecular cross-links. For this reason, only the 21/65 double Cys substitution in the c3 trimer was constructed for further analysis. On cross-linking by oxidation the 21/65 double Cys c3 trimer showed products corresponding to c9 and c9 oligomers (Fig. 3, A and B) and on prolonged oxidation a faint band corresponding to a c12 oligomer (Fig. 3B).

To maximize oligomer formation, double Cys substitutions were introduced at positions 11 and 75 in c1, c2, and c3 versions of the subunit. Single Cys substitutions at these positions form high yield cross-linked homodimers (Ref. 15; Fig. 4A). The residues are thought to lie in flexible regions at the N and C termini of the molecule since Cys substitutions in residues immediately adjacent, i.e. in positions 8–11 and 73–75, also form high yield dimers (15). We reasoned that the 11/75 double Cys mutants might allow maximal oligomerization during CuP treatment. This proved to be the case as shown in Fig. 4, A and B. Beginning with the 11/75 c1 monomer in Fig. 4A and using the multimeric ladders formed on oxidation of 11/75 c9 dimers and 11/75' c3 trimers as calibration standards, note that the 11/75 c1 forms distinct cross-linked species corresponding to oligomers at the c2, c3, c4, c5, c6, c7, c9, c10, and c12 positions. Also note that cross-linking stops at the position of c12. Distinct cross-linked products of the 11/75 c1 dimer, corresponding to c4, c6, c9, c10, and c12 oligomers, are also obvious (Fig. 4, A and B). For the 11/75' c3 trimer in Fig. 4A, obvious products corresponding to c6, c9, and c12 oligomers are observed, but the cross-linking results are confounded by traces of higher molecular weight material in this lane. From a series of experiments with 11/75' c3, we believe that the traces of material seen on some gels at positions greater than c12 (e.g. as in Fig. 4A) are
likely due to aggregates not dissociated by SDS treatment. In the second set of experiments shown in Fig. 4B, cross-linking of the 11/75′ c3 trimer clearly stops at the position of the c12 oligomer. The c12 band seen in Fig. 4B for 11/75′ c3 appears as a distinct doublet. Conceivably, one band of the doublet could correspond to the linear form of the c12 oligomer and the other band to the entirely cross-linked c12 ring.

**DISCUSSION**

The experiments described here, when correlated with previous information, provide compelling evidence for arrangement of subunit c in F0 as a c12 oligomer. First, both the fused c2 dimers and c3 trimers of subunit c were active on incorporation into F0. This evidence by itself argues that the total number of monomeric subunit c in F0 must be a multiple of 2 and 3, i.e. 6, 12, 18, etc., if one assumes that the number of subunits c in F0 is fixed and constant. Twelve is the only multiple of 2 and 3 within the range of the stoichiometry determined by labeling in the experiments of Foster and Fillingame (17). We note the experimental values of the ratio of subunit c normalized to 3(α + β) was 10 ± 1 for purified F1F0 and 12 for crude membranes in which F1F0 was overproduced by 6-fold, the latter estimate being done only once. Using similar methods, von Meyenburg et al. (18) have independently estimated the subunit c content of crude membranes after overproduction of F1F0 and reported 12.5 ± 1.5 subunits per 3 α subunits.

The disulfide cross-linking experiments reported here with doubly Cys-substituted subunits clearly indicate c12 as the maximum sized multimer. The experiments extend and clarify the previous report of Jones et al. (15) where cross-linking of doubly Cys-substituted monomeric subunits was examined in detail. In that report, the oligomeric content of the highest molecular weight multimers was uncertain due to lack of calibration standards. Here the multimers of the c2-fused dimer and c3-fused trimer could be used as calibration standards. Multimers of the 21/65 monomer do tend to maximize at c10 (Fig. 3, A and B). However, cross-linking of the 21/65′ trimer clearly shows maximum sized multimers corresponding to c12 (Fig. 3B). The results may indicate that two of the monomeric subunits c in F0, perhaps those associated with subunit α, may be in different conformations that are not subject to cross-linking. We emphasize here that both the 11/75 c1 monomer and 11/75′ c2 dimer form multimers that maximize at a size corresponding to c12 (Fig. 4). This seems to eliminate the possibility that the c12 multimers formed by cross-linking of the 21/65′ and 11/75′ c3 trimers are a structural aberration caused by the protein fusion.

A stoichiometry of 12 subunits c per F0 leads to a prediction of an H+/ATP = 4 if the rotary mechanism of coupling is correct. If the same subunit c stoichiometry holds for mitochondrials F0, an H+/ATP stoichiometry of 4 would lead to predictions of a P/O ratio of 2 for NADH-linked substrates, if the consensus stoichiometry of 10 H+/2e− is correct for NADH oxidation (33). In the V-type ATPase, the number of carboxyl groups (equivalent to Asp-61) in the subunit c rotor is half that in F0 due to lack of a carboxylate residue in the first of the two pairs of transmembrane helices. This is presumed to relate to a lower mechanistic H+/ATP pumping ratio for the vacuolar enzyme (24). In the future we plan to test this hypothesis with c2 derivatives of the E. coli enzyme. We should also be able to test whether each subunit c requires a competent coupling region in the polar loop for linkage to ATP synthesis.

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5 The subunit c aggregates are most commonly observed on immuno blotting, perhaps because they bind more avidly to the blotting membrane. Aggregate formation is promoted by heat treatment in SDS sample buffer or by freeze/thaw cycles. For that reason all samples shown here were prepared immediately before use by incubation in SDS sample buffer for 1 h at room temperature, a treatment that may in some cases be insufficient for disaggregation of all complexes.