# Stability and functionality of cysteine-less $F_OF_1$ ATP synthase from *Escherichia coli*

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**Abstract All 21 native cysteines in the *Escherichia coli* $F_OF_1$ ATP synthase were replaced by alanines. In isolated *E. coli* membranes, ATP-dependent proton pumping, turnover of ATP hydrolysis and steady-state transition state thermodynamic parameters of the cysteine-less enzyme were similar to wild-type. The cysteine-less enzyme was solubilized in *n*-octyl β-D-glucopyranoside, purified by affinity chromatography, and reconstituted into pre-formed liposomes made from *E. coli* lipids. The properties of the reconstituted, purified enzyme were not significantly different from the membranous enzyme. These data demonstrate that cysteine-less $F_OF_1$ is biochemically stable and has functionality similar to wild-type.**



*Key words:* ATP synthase; Bioenergetics; Cysteine; Proton pumping; Purification; Reconstitution

## 1. Introduction

The $F_OF_1$ ATP synthase is a large multi-subunit complex. In the case of *Escherichia coli*, there are eight different subunits in a stoichiometry of $ab_2c_{9-12}\alpha_3\beta_3\gamma\delta\varepsilon$. The first three subunits, *a*, *b* and *c* are integral membrane proteins which are not well understood structurally (see [1–3] for reviews). With realization of a rotational catalytic mechanism (see [4] for a review), the ability to analyze the structure of the membranous subunits and molecular dynamics at selected positions within the multi-subunit complex has become even more critical. Chemical modification of cysteines by spectroscopic probes provides a powerful tool for this type of analysis. Towards this end, we have changed the 21 native cysteines in the *E. coli* $F_OF_1$ synthase complex to alanines, thus creating a background lacking modifiable residues. In addition, an epitope tag was added to one of the subunits to facilitate purification of solubilized complex. We report here that the cysteine-depleted enzyme complex remains stable during detergent solubilization and purification, can be reconstituted into pre-formed liposomes with uniform orientation, and has functional properties similar to the wild-type enzyme.

## 2. Materials and methods

### 2.1. Strains and plasmids

The *unc*-deleted strain, DK8 was previously described [5]. The expression plasmid pACWU1.2 incorporates the entire *E. coli unc* or *atp* operon into modified pACYC117 [6]. The $Kan^R$ gene on pACYC177 was removed from *Nhe*I to *Bam*HI and replaced by a linker containing *Hin*dIII and *Xba*I restriction sites. The *unc* operon from pBWU13.4 [7] from *Hin*dIII (154 bp upstream of the *uncB* open reading frame; the first base of the *Hin*dIII site is used as base 1 for the operon) to *Xba*I (324 bp downstream of the *uncC* open reading frame) was moved into the modified pACYC177.

Some key modifications of the *unc* operon in pACWU1.2: (1) The *Xba*I site at the 3′ end of the *unc* operon was introduced into pBWU13.4 by picking up the *Xba*I site from a genomic subclone (*Eco*47III to *Bgl*II) in vector pUC18. This was done by eliminating the sequence between the *Dra*I site 320 bp downstream of the *uncC* open reading frame and the vector *Hin*cII site just prior to the *Xba*I site. (2) The *Bam*HI site in *uncB* at base 241 was eliminated by site-directed mutagenesis using the oligonucleotide, GTGGATCaACCAGCGAGAATG (mutation in lower case) creating a silent mutation. (3) The *Hin*dIII site in *uncG* at base 4439 was removed by site-directed mutagenesis using oligo ACAATGTAAAGtTTGTCCAGACGGCCTTC creating a silent mutation.

### 2.2. Molecular biology procedures

Molecular biology procedures were performed according to manufacturers' instructions or as detailed by Sambrook et al. [8]. Restriction and DNA modifying enzymes were from Boehringer Mannheim, Gibco BRL, New England Biolabs, and Promega. Site-directed mutagenesis was carried out using the Stratagene Chameleon Kit or by PCR methodologies [9] with Pfu polymerase (Stratagene). Oligonucleotides were synthesized by Oligos Etc. (Wilsonville, OR, USA) or Integrated DNA Technologies (Coralville, IA, USA).

### 2.3. Introduction of the Flag epitope tag

Sequence encoding the Flag epitope [10] was introduced at the 5′ end of the *uncD* open reading frame (β subunit) by PCR. The oligonucleotide primer, GGTTAACTAGTAGAGGATTTAAGATGgactacaaagacgacgatgacaagGCTACTGGAAAGATTGTCC-3′ (Flag epitope sequence in lower case) inserted the Flag epitope (DYKDDDDK) directly after the N-terminal methionine without altering any other residues. This construct is similar to that of Zhou et al. [11].

To assist mutagenesis and cloning, several subclones were used. pBH-RI was derived from pBluescriptII KS+ (Stratagene) and contained the *unc* operon from *Bam*HI (at base 859) to *Eco*RI (at base 2358; most of *uncB* and all of *uncE*, *F* and *H*). pUCαShK was derived from pUC18 and contained a portion of *uncA* from *Sph*I to *Kpn*I. pBγKS contained the entire *uncG* gene from *Kpn*I to *Spe*I and was described previously [7]. pBKSβSS subclone was derived from pBluescriptII KS+ and contained a portion of *uncD* from *Spe*I to *Sac*I. Mutagenesis was performed in subclones and moved back to the expression vector after sequence conformation by a combination of manual sequencing (Sequenase Kit, Amersham) and automated sequencing at the University of Virginia Biomolecular Research Facility. Mutagenic oligonucleotides used for changing cysteines to alanines are listed in Table 1.

### 2.4. Growth of strains and membrane preparations

Strain DK8 carrying derivatives of the pACWU1.2 were grown on supplemented minimal medium as previously described [7]. For determination of oxidative phosphorylation-dependent growth, 0.4% sodium succinate was used as a sole carbon source. For preparation of $F_OF_1$-containing membrane vesicles, *E. coli* were grown in minimal medium supplemented with 0.4% glucose at 37°C and harvested during mid-logarithmic growth. Membranes were prepared as described previously [12]. The membrane vesicles were finally suspended at 20–40 mg/ml in a buffer consisting of 5 mM Tris-Cl, 70 mM KCl, 0.5

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mM DTT and 55% glycerol at pH 8.0. Protein concentrations were determined by the method of Lowry et al. [13].

*2.5. Affinity purification of solubilized $F_OF_1$*

Membrane protein (25–35 mg) was diluted approximately four-fold with 10 mM Tris-Cl, 140 mM KCl and 10% glycerol, pH 8.0, and centrifuged at 541 000×*g* for 30 min at 4°C. The pellet was resuspended at a protein:detergent ratio of 1:4 (w/w) in solubilization buffer consisting of 2 or 2.5% (depending on the quantity of protein) *n*-octyl β-D-glucopyranoside, 20 mM MOPS, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 1 mg/ml para-aminobenzamidine, and 20% glycerol adjusted to pH 7.0 at 20°C with NaOH. Diisopropylfluorophosphate was added at 1 mM to prevent proteolysis. The suspension was centrifuged again to remove insoluble material.

Anti-Flag M2 affinity gel (Kodak) was pre-washed as prescribed by the manufacturer. The supernatant containing the solubilized proteins was directly mixed with the gel with moderate agitation for 30–60 min at room temperature (subsequent steps were also carried out at room temperature). Approximately 30 mg of protein saturated 0.5 ml of M2 antibody gel. After packing, the column was washed with 10–15 column volumes of the solubilization buffer with the exceptions that *n*-octyl β-D-glucopyranoside was now 1.4% and 3.6 mg/ml *E. coli* lipids (Avanti Polar Lipids) were added. $F_OF_1$ complex was eluted with four sequential washes of one column volume each of 0.25 mg/ml Flag peptide (DYKDDDDK) in wash buffer. Batch elutions were incubated with the gel for 5–10 min each wash. On average, approximately 80% of $F_OF_1$ retained in the column was recovered in the elutions and 1 ml of affinity gel yielded approximately 1 mg of purified complex.

*2.6. Reconstitution into pre-formed liposomes*

Twenty mg of *E. coli* lipids (Avanti Polar Lipids) were dried in a rotary evaporator. The lipids were resuspended in 1 ml of reconstitution buffer (10 mM HEPES, 300 mM KCl, 5 mM $MgCl_2$, adjusted to pH 7.5 with KOH) with a bath sonicator until the suspension cleared (approximately 1 min). Pre-formed liposomes were mixed with purified enzyme at a protein:lipid ratio of 1:5 (w/w) at room temperature for 30 min. The enzyme was then diluted with 50 volumes of reconstitution buffer and the liposomes collected by ultracentrifugation at 20°C. The amido black assay [14] was used to determine the concentration of reconstituted protein.

*2.7. Determination of $F_1$ content*

Determination of $F_1$ content in membrane preparations was performed by a quantitative immunoblot analysis described previously [15] using purified $F_1$ as a reference standard.

*2.8. Enzymatic assays*

Formation of electrochemical gradients of protons was assayed by monitoring acridine orange fluorescence quenching as previously described [16]. For Arrhenius analysis, temperature dependence of maximal steady-state rates of ATP hydrolysis was measured in buffers whose pH was adjusted at the appropriate temperature. The concentration of free $Mg^{2+}$ and Mg•ATP was calculated using the algorithm of Fabiato and Fabiato [17].

Table 1
Synthetic oligonucleotides for site-directed replacement of cysteines with alanines

| Mutation | Oligonucleotide sequence[a] |
|---|---|
| bC21A | 5′-CATACGTACTTCATGgcGAACAGAACGA-3′ |
| δC64A | 5′-GAGTCGTTTATCGCAGTTgcTGGTGAG-3′ |
| δC140A | 5′-TAAGCTGAATgcCAAAATCGATAAGTCTGTA-3′ |
| αC46A | 5′-CTGGCCGATgcTATGCAGGGTGAAATG-3′ |
| αC89A | 5′-GAAAGTTAAGgcTACTGGaCGTATCCTGGA-3′ |
| αC192A | 5′-TTCCGGTATCAAAgcTATCTATGTCGC-3′ |
| αC242A | 5′-CGTATGCCGGTgcCGCCATGGGTGAATACTTCCGTG-3′ |
| γC87A | 5′-CGACCGACCGTGGTTTGgcgGGTGGTTTGAAC-3′ |
| | 5′-GTTCAAACCACCcgcCAAACCACGGTCGGTCG-3′ |
| γC112A | 5′-GGACCGACAAAGGCGTTCAAgcCGACCTCGCAATG-3′ |
| | 5′-CATTGCGAGGTCGgcTTGAACGCCTTTGTCGGTCC-3′ |
| βC138A | 5′-CAAAGTTATCGACCTGATGgccCCGTTCGC-TAAGGGCGGT-3′ |
| | 5′-CCTTAGCGAACGGggcCATCAGGTCGATAACTTTG-3′ |

[a]Lower case letters denote altered bases.

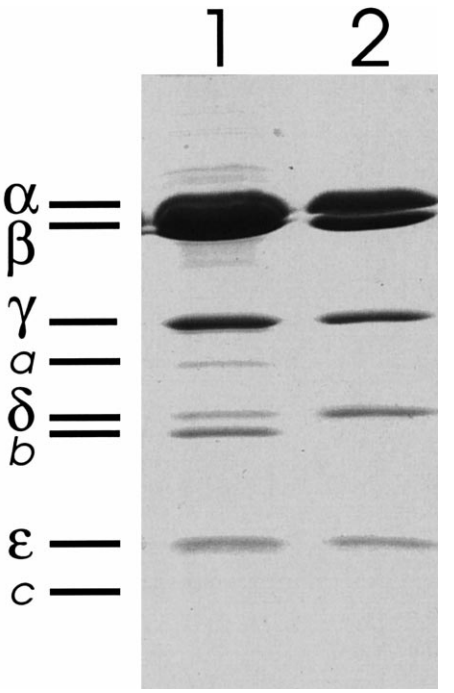


Fig. 1. Coomassie-stained gel of Flag affinity gel purified $F_OF_1$. Twenty μg of $F_OF_1$ after the M2 anti-Flag affinity column (lane 1), or 10 μg of purified $F_1$ [15] (lane 2) were separated on a 15% polyacrylamide gel. Subunits are indicated. Note that *c* subunit does not stain with Coomassie.

## 3. Results

*3.1. Overexpression of an epitope-tagged $F_OF_1$ complex*

The *unc* operon was moved to the low copy number plasmid, pACYC177 [6] in an effort to reduce the amount of $F_OF_1$ complex in the membrane. Previously, we reported [7] that the $F_OF_1$ complex accounted for 25–40% of membrane protein in strain DK8/pBWU13 [18]. ATP hydrolysis and synthesis activities associated with membrane vesicles isolated from this strain were partially uncoupled from proton translocation. Because we are concerned with energy coupling, we decided that a lower expression level would be more appropriate. From the *unc* operon borne on pACWU1.2 described in Section 2, the amount of wild-type complex was routinely found to be approximately 10% of membrane protein (Table 2). Furthermore, proton pumping and ATP synthesis rates were similar to those achieved in strains containing a haploid complement of the operon (i.e. KF10rA/pBWG15, [15], and Ketchum, C.J. and Nakamoto, R.K., unpublished results).

Because of the lower expression levels, an affinity tag to facilitate purification became necessary. The Flag epitope was added to the amino-terminus of the β subunit, resulting in a construct similar to that used by Zhou et al. [11]. The presence of the additional eight or nine amino acids (the N-terminal Met is likely not removed during biogenesis [11]) did not affect any properties of the enzyme (Table 2).

*3.2. Cysteine-less $F_OF_1$ complex retains functionality*

In the pACWU1.2/βFlag construct, all ten encoded cysteine residues were replaced by alanines (Table 1). The resultant plasmid named pACWU1.2/βFlag/ΔCys, conferred oxidative phosphorylation-dependent growth to strain DK8 (Δ*unc*).

Growth yields of DK8/pACWU1.2/βFlag/ΔCys on sodium succinate, a non-fermentable carbon source which makes functional $F_OF_1$ ATP synthase required for growth, were only slightly less than wild-type (Table 2).

Inner membrane vesicles were prepared from strain DK8/pACWU1.2/βFlag/ΔCys, and the levels of enzyme determined by a quantitative immunoblot assay using a polyclonal anti-α subunit antibody [15]. Expression levels of the cysteine-less complex were reduced at average of 20% compared to Flag-tagged wild-type, but more significantly, turnover rates of steady-state ATP hydrolysis of the cysteine-less and wild-type enzymes were similar, 337 $s^{-1}$ versus 344 $s^{-1}$ at 30°C, respectively (Table 2). Furthermore, vesicles containing the mutant enzyme generated an electrochemical gradient of protons comparable to wild-type (Fig. 2A).

Previously, we have used differences in transition state thermodynamic parameters of the steady-state ATP hydrolytic reaction as a sensitive indicator of mechanistically significant structural perturbations [7,15,19]. In the case of the cysteine-less enzyme, the transition state activation energy or $\Delta H^{\ddagger}$ was slightly higher than wild-type (Table 2) indicating that additional energy was required to achieve the catalytic transition state, probably due to a structural perturbation. Compared to previous analyses of mutants, this change of activation energy was relatively small [7]. Importantly, there is little difference in the transition state free energy ($\Delta G^{\ddagger}$) indicating that the cysteine-less enzyme utilizes the same reaction pathway as wild-type [19].

### 3.3. Stability of cysteine-less $F_OF_1$ during detergent solubilization and reconstitution

The stability of the cysteine-less Flag-tagged $F_OF_1$ was demonstrated by the ability to purify solubilized complex. Membranes were solubilized in 2 or 2.5% *n*-octyl β-D-glucopyranoside similar to that done by Moriyama et al. [18]. In addition, we added ATP, para-aminobenzamidine and 20% glycerol to help stabilize the complex. The complex was rapidly purified using batch methods with the anti-Flag M2 affinity gel. While on the affinity gel, the complex was particularly susceptible to dissociation unless a minimum of 20% glycerol and lipids in the column wash buffer were present. The eluted enzyme was nearly homogeneous as judged by Coomassie staining (Fig. 1).

Reconstitution of purified cysteine-less enzyme with the addition of pre-formed liposomes produced vesicles capable of establishing an ATP-driven proton gradient as measured by acridine orange fluorescence quenching (Fig. 2) and with $F_1$ uniformly oriented outside as judged by susceptibility to trypsin digestion (data not shown). Reconstitution without addition of pre-formed liposomes was also possible because of the presence of lipids in the elution buffer. Arrhenius analysis of the reconstituted enzyme indicated that the enzymatic properties of the reconstituted complex were essentially the same as in the native membrane (Table 2).

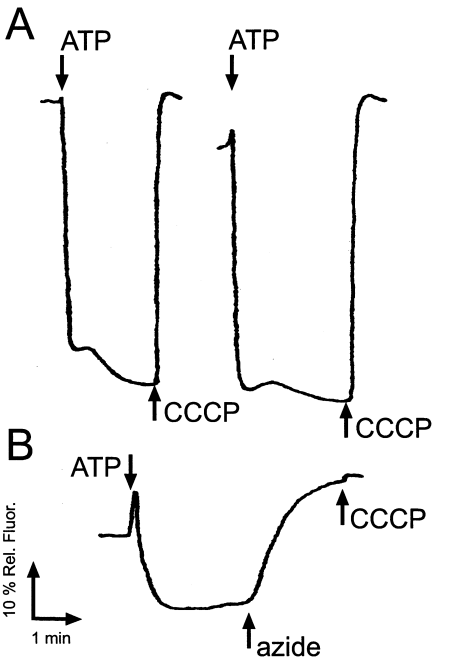


Fig. 2. Acridine orange fluorescence traces indicating ATP-driven proton pumping in membrane vesicles (A) or liposomes reconstituted with cysteine-less $F_OF_1$ (B). A: 0.10 mg of membrane protein was suspended in 10 mM HEPES, 300 mM KCl, 5 mM $MgCl_2$ and 1 μM acridine orange. The left trace is wild-type membranes, and the right is mutant membranes. One μg/ml valinomycin in ethanol was added. The reactions were started with addition of 1 mM ATP and terminated with 1 μM of the protonophore carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP). B: 10 μg of Flag tag purified and reconstituted cysteine-less $F_OF_1$ (1:5 protein:lipid (w/w)) in the same buffer as in A. In this case, the reaction was terminated by addition of 1 mM sodium azide, an ATPase inhibitor.

## 4. Discussion

We demonstrated that replacement of all 21 native cysteine residues in the *E. coli* $F_OF_1$ resulted in only minor effects on enzyme assembly and stability. Equally important, the cys-

Table 2
Kinetic and thermodynamic characteristics of β-Flag-tagged, cysteine-less $F_OF_1$ expressed from DK8/pACWU1.2

| Strain | Growth on succinate (% of wild-type) | % $F_1$ in membrane[a] | Turnover of ATP hydrolysis ($s^{-1}$) | Transition state thermodynamic parameters (kjoules/mol)[b] | | |
|---|---|---|---|---|---|---|
| | | | | $\Delta H^{\ddagger}$ | $T\Delta S^{\ddagger}$ | $\Delta G^{\ddagger}$ |
| (wt) | 100 | 10.0 | 337 | 34.6 | −25.0 | 59.6 |
| $\Delta F_OF_1$ (pBR322) | 0 | 0 | | | | |
| β-Flag | 100 | 10.0 | 344 | 35.2 | −24.4 | 59.6 |
| β-Flag/ΔCys (membranous) | 91 | 7.9 | 337 | 47.6 | −12.1 | 59.7 |
| β-Flag/ΔCys (reconstituted) | – | 72.0[c] | 301 | 47.6 | −12.3 | 59.9 |

[a]Percent of protein in membranes that is $F_1$ complex determined by quantitative immunoblot as described in Section 2.
[b]Transition state thermodynamic parameters at 30°C are derived from Arrhenius analysis of the listed enzyme preparation. ATP hydrolytic rates were determined in $V_{max}$ conditions between 20 and 40°C as described in Section 2.
[c]Enzyme was assumed to be 100% pure.

teine-less enzyme retained full functionality including ATP-driven proton pumping and ATP synthesis as indicated by the ability to support oxidative phosphorylation-dependent growth. We have recently described the use of Arrhenius analysis of steady-state activity and the derived transition state thermodynamic parameters as a highly sensitive and reliable indicator of structural perturbations that affect enzyme mechanism [15,19]. The cysteine-less enzyme did have an increased activation energy (Table 2); however, this effect was not accompanied by a large reduction in catalytic turnover nor an altered reaction pathway. Furthermore, catalysis of ATP hydrolysis and synthesis remained tightly coupled to proton translocation.

The addition of an epitope tag on the β subunits allowed for fast and easy purification of the complex even from the relatively low level expression system described above. The yields were limited by the M2 anti-Flag affinity gel and were easily scaled up to increase the production of purified complex. The purified, reconstituted, and cysteine-less enzyme complex which retained near wild-type levels of functionality will prove most useful in techniques requiring unique cysteine residues. In particular, this system will allow the use of methodologies such as site-directed placement of spin labels [20] to probe the molecular structure and dynamics of this large and complex enzyme.

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