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Over-expression and refolding of β-subunit from the chloroplast ATP synthase

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We established a bacterial system for high-level over-expression of the spinach chloroplast *atpB* gene which encodes the ATP synthase β subunit. Upon induction, *atpB* was expressed as at least 50% to 70% of total cell protein. Although the over-expressed β polypeptide formed insoluble inclusion bodies, more than fifty percent of it was restored to a functional form by solubilizing the inclusion bodies with 4 M urea and slowly removing the urea by stepwise dialysis. The resulting β subunit exhibited specific and selective nucleotide binding properties identical to those of the native β subunit.

ATP synthase; Over-expressed (chloroplast) β subunit

1. INTRODUCTION

The cloroplast ATP synthase utilizes the energy of the light-driven transmembrane proton gradient to synthesize ATP. The enzyme is comprised of two parts, CF_0, a membrane-spanning proton channel, and CF_1, which is peripheral to the membrane and which contains the catalytic site(s) for ATP synthesis and hydrolysis. CF_1 is composed of five different types of subunit designated α to ε in order of decreasing molecular weight. The most probable subunit stoichiometry is 3α, 3β, 1γ, 1δ, and 1ε [1–4].

Photoaffinity labeling of CF_1 [5,6] and other F_1 enzymes (see [1,4] for reviews) with nucleotide analogs have indicated that the nucleotide-binding, catalytic sites of the enzyme reside either entirely on the three β subunits or at interfaces between the β subunits and the α subunits. Three nucleotide binding sites have been identified on CF_1 and mapped with respect to each other and with respect to several other specific sites on the enzyme using fluorescence resonance energy transfer distance mapping [7–9]. Similar mapping studies have also been attempted with the isolated β subunit of CF_1. The single cysteine residue, C63, of the β subunit was derivatized with coumarinyl maleimide and the distance between C63 and the fluorescent nucleotide analog trinitrophenyl-ATP (TNP-ATP), located at the single nucleotide binding site on β [10], was determined. The technique of distance mapping by fluorescence energy transfer is thus providing to be a very useful method of obtaining detailed structural information for CF_1, but it is limited by the availability of sites on the protein surface which can be specifically labeled with fluorescent probes. To this end we have sought to introduce new labeling sites into CF_1 by way of site-specific mutation of the encoding DNA.

In this paper we report over-expression of a full-length clone of the DNA encoding the β subunit of CF_1. The β subunit thus synthesized was recovered from *E. coli* inclusion bodies in a form which binds nucleotides with an affinity and specificity identical to that of the native β subunit.

2. MATERIALS AND METHODS

2.1. *Materials*

CF_1 was prepared from fresh market spinach [11–13] and stored as an ammonium sulfate precipitate. The β subunit was prepared from CF_1 lacking the δ and ε subunits as described elsewhere [14]. Trinitrophenyl ATP (TNP-ATP) was purchased from Molecular Probes Inc. All other reagents were of the highest purity available.

2.2. *Recombinant DNA methods*

Most methods have been described elsewhere [15]. Subcloning was accomplished by ligation of purified insert and vector DNAs in melted NuSieve GTG agarose (FMC). Transformation protocols were as described elsewhere [16]. The presence of the correct insert was verified by restriction mapping and hybridization of a Southern blot with the *atpB*-specific oligodeoxyribonucleotide **β2C** (see below).

Plasmid DNA for sequencing was prepared by alkaline-SDS lysis and PEG precipitation [15]. Double-strand sequencing was performed with modified T7 DNA polymerase (Sequenase 2.0, US Biochemicals).

Abbreviations: CF_1, chloroplast couling factor 1; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)ATP; Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl) glycine; Tris, tris(hydroxymethyl)aminomethane; IPTG, isopropyl-β-D-thiogalactoside.

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2.3. *Mutagenesis*

Oligonucleotides used for in vitro mutagenesis or for hybridization and sequencing were designed manually and with the aid of computer programs PRIMER and RNAFOLD (Scientific and Educational Software) and SeqAidII. Sequences were selected to exhibit no self-complimentarity, not to hybridize with non-target sequences, and to contain a 3'-terminal 'clamp' of 1 to 2 G-C basepairs. In addition, the target sequence for mutagenesis was analyzed (with RNAFOLD) for possible selfcomplementarity which would compete with oligonucleotide annealing. The following oligonucleotides were used: **βS3.Nde**, 5'-CTT TAC TAT ATc aTA TGA GAA TC (bases −14 to +9 with respect to the *atpB* coding sequence); **β310C**, 5'-CGT CCA AGA GTC GGT CC (bases +326 to +310); and **β2C**, 5'-CAA GTG TGG AAA CCC C (bases +46 to +31). These oligonucleotides were synthesized via β-cyanoethyl phosphoramidite chemistry on a Biosearch 8600 synthesizer operated by the University of Kansas Biochemical Research Services Laboratory, and were desalted over Sephadex G25 before use. Oligonucleotide **T7** was obtained from Stratagene (cat. no. 300302), and is complimentary to the bottom strand of the T7 promoter in the pBS(−)β and pET3 vectors.

Oligonucleotide-directed mutagenesis [17] was accomplished by the approach of Kunkel [18] using reagents supplied by Bio-Rad and following the supplier's protocol. Briefly, phagemid DNA was transformed into *E. coli* CJ236 and single-stranded uracil-containing DNA was prepared after infection with helper phage M13K07 [19]. Approximately 0.6 pmol ssDNA was annealed with ~0.18 pmol of phosphorylated oligonucleotide **βS3.Nde**. After elongation with phage T4 DNA polymerase in the presence of DNA ligase, DNA was transformed into *E. coli* XL1-Blue (Stratagene). Miniprep plasmid DNA from twelve colonies was screened for the presence of a second NdeI site; all were positive. One clone was further confirmed, by mapping and DNA sequencing, as containing the desired mutation. This plasmid was designated pBS(−)βXE2.

2.4. *Construction of the over-expression clone of atpB*

Plasmid pSCXD005, generously provided by W. Gruissem, contains an *Xba*I fragment of spinach (*S. oleracea*) chloroplast DNA bearing the *atpB* gene encoding the ATP synthase β subunit and the overlapping *atpE* gene encoding the ε subunit [20]. Fig. 1A shows this gene arrangement. An *Xba*I to *Eco*RI fragment containing *atpB* and 16 codons of *atpE* was subcloned into the correspondingly cleaved phagemid vector pBS(−) (Stratagene), giving plasmid pBS(−)βXE0. An *Nde*I restriction site was introduced at the initiator ATG as described above, giving plasmid pBS(−)βXE2. The *Nde*I to *Eco*RI fragment of pBS(−)βXE2 was then excised and inserted, along with 60 pmol of non-phosphorylated double-stranded *Eco*RI–*Bam*HI adaptor DNA (New England Biolabs cat. no. 1105 and no. 1106) into expression plasmid pET3a [21] which had been cleaved with *Nde*I and *Bam*HI. DNA was transformed into *E. coli* DH5α (Bethesda Research Labs). One insert-containing clone was kept and designated pET3a-βNE2. To reduce the extent of insert requiring resequencing, a derivative clone, pET3a-βNE3, was made in which the *Sac*I to *Eco*RI fragment containing the distal ~four-fifths of *atpB* was replaced with the corresponding fragment of wild-type DNA from plasmid pBS(−)βXE0. The structure of this plasmid is depicted in Fig. 1B. The remaining proximal *Nde*I–*Sac*I portion of the *atpB* insert (in pET3a-βNE2), was sequenced on both strands using primers **T7** and **β310**. The corresponding control 'untreated' *atpB* sequence was obtained from plasmids pSCXD005, using primer **βS3.Nde**, and from pBS(−)βXE0, using primers **β2C** and **β310C**. These sequences were compared to the published sequence [20] obtained from the GenBank database [22]. Plasmid pET3a-βNE3 was finally transformed into the expression host *E. coli* BL21(DE3)/pLysS. To reduce basal T7 transcription and eventually to facilitate cell lysis, this host also contains a cloned phage T7 lysozyme gene [23].

2.5. *Solubilization and refolding of the atpB gene product*

E. coli cells containing the *atpB* gene were grown at 37°C in LB medium [15] containing L-ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). In mid-exponential phase growth, cells were induced with 0.1 mM IPTG for up to 5 h. Cells were harvested by centrifugation at 4,000×*g* for 10 min, washed once with TE50/2 buffer (50 mM Tris-HCl/2 mM EDTA, pH 8.0) and resuspended in a small volume (10–15 ml) of TE50/2. Cells were lysed by one to three cycles of freezing (at −70°C or on dry ice) and thawing [23]. DNA was then sheared by sonication with a Branson 250 sonifier for 4×15 s at an output of 4, and a duty cycle of 10. Inclusion bodies, together with some cell debris, were sedimented at 4,000×*g* for 10 min. The pellet, containing mostly insoluble β polypeptide, was washed three times with 25 ml of TE50/2.

The insoluble β polypeptide, suspended to ~0.5 mg/ml in TE50/2, was solubilized by dialysis against 50 vols. of a solution containing 4 M urea/50 mM Tris-HCl (pH 8.0)/2 mM EDTA/2 mM dithiothreitol for 2 h at 4°C. Urea was removed by three sequential steps of dialysis against buffered solutions containing decreasing amounts of urea. The first step was dialysis for 7 h at 4°C against 100 vols. of 50 mM Tris-HCl (pH 8.0)/2 mM EDTA/2 mM dithiothreitol/ 1 mM ATP/5% (v/v) glycerol/3 M urea. The second step was dialysis for 10 h at 4°C against 100 vols. of the same solution containing 10% (v/v) glycerol and 1.5 M urea. The last step was dialysis for 7 h at room temperature against 125 vols. of the same solution but with 20% (v/v) glycerol and no urea. The protein was concentrated by pressure dialysis using an Amicon YM30 membrane filter.

2.6. *Spectroscopic measurements*

Absorbance measurements were made using a Beckman DU-70 spectrophotometer. Protein concentrations were determined by the method of Bradford [24]. Binding of TNP-ATP to the β subunit was determined by monitoring the increase in fluorescence emission of TNP-ATP at 550 nm (415 nm excitation) [10,25]. The dissociation constant (K_D) and binding site occupancy (n) for TNP-ATP were determined by least squares non-linear regression curve-fitting of the standard single-site binding equation (below) to the plot of corrected fluorescence vs. total [TNP-ATP]. Curve fitting was accomplished using ENZFITTER Software (R.J. Leatherbarrow, distributed by Biosoft, Cambridge, UK).

The binding equation is:

$$F = \{(K_D + n[E_t] + [L_t]) - ((K_D + n[E_t] + [L_t])^2 - 4n[E_t][L_t])^{1/2}\}/2c$$

where F is the corrected fluorescence, $[E_t]$ is the total concentration of β polypeptide, $[L_t]$ is the total concentration of TNP-ATP, and c is the fluorescence correction factor determined previously. The equation was derived directly from the equilibrium definition of K_D by substituting [bound TNP-ATP] = $c \cdot F$ and solving for F as a function of $[L_t]$.

3. RESULTS

3.1. *Deduced amino acid sequence of the spinach chloroplast atpB gene*

Following construction of a recombinant plasmid which could express the spinach chloroplast *atpB* gene, we resequenced the portion of *atpB* which had been subjected to the in vitro mutagenesis procedure described below and in section 2. The same region of untreated *atpB* DNA was also sequenced. Both sequences were identical to each other but differed at two positions from that previously published [20] and entered in the GenBank database (accession J01441). First, nucleotide 94 of the *atpB* coding region is G rather than A. This changes codon 32 from AAC (Asn) to GAC (Asp). The predicted amino acid sequence was confirmed by direct peptide sequencing of the native β subunit (manuscript in preparation). The resultant β subunit amino acid sequence is not at all surprising

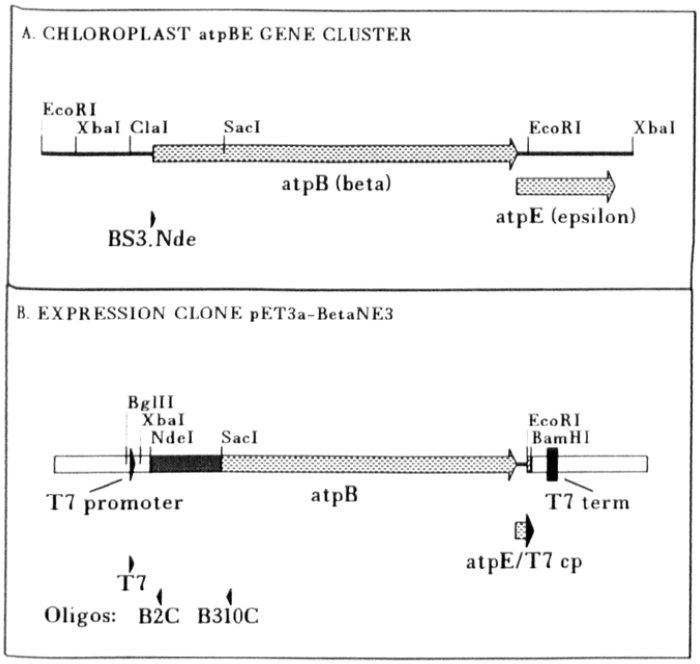

Fig. 1. Expression clone of the chloroplast *atpB* gene. A. Structure of the spinach chloroplast *atpBE* gene cluster. B. Structure of the relevant region of the expression clone pET3a-βNE3. The chloroplast DNA insert extends from the *Nde*I site to the *Eco*RI site. Stippled boxes represent native chloroplast sequences from plasmid pBS(−)βXEΦ; the shaded region denotes the portion of *atpB* from plasmid pBS(−)βXE2 which has been subjected to in vitro mutagenesis. The T7 promoter and T7 transcription terminator (T7 term) from the vector pET3 are labeled, as is the *Eco*RI–*Bam*HI adaptor (see text). The positions and orientations of synthetic oligonucleotide primers are indicated. Depicted below *atpB* is the translational fusion of *atpE* to the adaptor and the 3′ end of phage T7 gene 10 (capsid protein) present in pET3a [25].

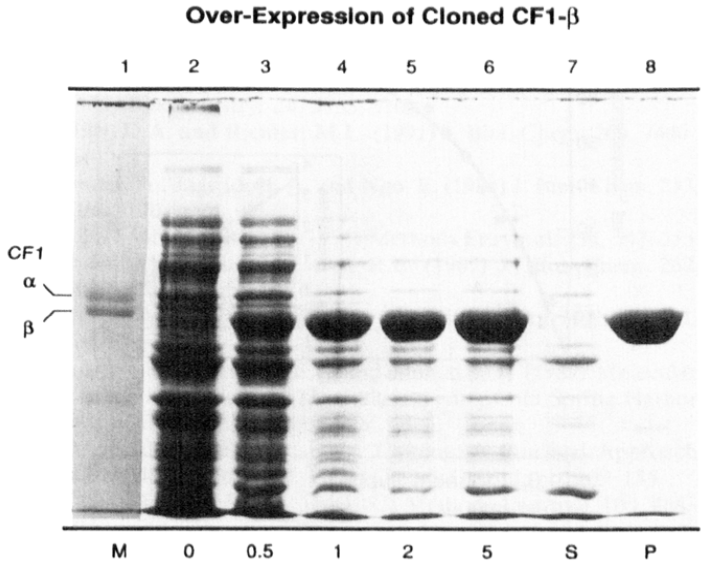

Fig. 2. Bacterial synthesis of chloroplast ATP synthase β subunit. A culture of strain BL21(DE3)/pLysS/pET3a-βNE3 was diluted 1:100 into 10 ml of LB medium containing ampicillin and chloramphenicol (see section 2). After 3 h at 37°C, IPTG was added to 0.1 mM. One ml samples were taken at the times indicated. Cells were precipitated, resuspended in 50–100 μl 0.5 M Tris-HCl (pH 8.0), lysed by freeze-thawing (3×) and subjected to electrophoresis on 8% polyacrylamide gels in the presence of sodium dodecylsulfate. The gel was stained with Coomassie blue. Lane 1, CF1 α and β subunits purified from spinach chloroplasts; lane 2, total lysate of uninduced cells; lanes 3–6, total lysates of cells induced with IPTG for 0.5, 1, 2 and 5 h; lane 7, supernatant fraction; and lane 8, pellet fraction of the initial lysate of cells induced for 5 h.

because Asp is found at the homologous position in all other chloroplast *atpB* sequences entered in the GenBank database (Release 64.0). The second change, an A rather than a G at nucleotide 246, is a silent third position change in a Thr codon.

3.2. *Over-expression of the chloroplast atpB gene and recovery of the* β subunit

To obtain milligram amounts of the β protein needed for physical biochemical studies and ultimately for crystallization trials, we employed the bacteriophage T7 RNA polymerase/promoter system of Studier and colleagues [21,23,27]. Since our goal was to express a full-length native-sequence β subunit, we constructed a non-fusion clone in which translation started at the native *atpB* initiation site. The details of construction of expression plasmid pET3a-βNE3 are described in section 2 and shown diagramatically in Fig. 1.

Expression of *atpB* was induced by addition of IPTG to mid-exponential phase cultures of *E. coli* BL21(DE3)/pLysS/pET3a-βNE3. Cells were harvested and lysed as described in section 2. Fig. 2 illustrates the time course of β subunit synthesis by these cells. Little if any β subunit could be detected in uninduced cells, whereas after induction, β polypeptide was detected within 0.5 h, and accumulated within 2–5 h to a conservatively estimated 70% of total cell protein. The over-expressed β protein was found exclusively as insoluble inclusion bodies. Lanes 7 and 8 of Fig. 2 demonstrate that β-sized material was found only in the pellet and not in the supernatant when induced cells were lysed and separated into soluble and insoluble fractions (see methods). The protein was authentic β subunit as determined by its electrophoretic mobility and its strong reactivity with monoclonal antibody raised against native β polypeptide (data not shown). Growth of cells at lower temperatures (~20°C) did not yield soluble β polypeptide.

The β subunit recovered from purified inclusion bodies was solubilized with 4 M urea and the urea was removed by the lengthy dialysis protocol described in section 2. Attempts to remove the urea more rapidly resulted in the loss of nucleotide binding capacity.

3.3. *Nucleotide binding studies*

The nucleotide-binding capacity of the cloned β subunit was assessed using the fluorescent nucleotide analog TNP-ATP as described elsewhere [10] for the native β subunit. Fig. 3 demonstrates that the binding affinity for TNP-ATP was essentially indistinguishable from that of native β subunit when the two proteins

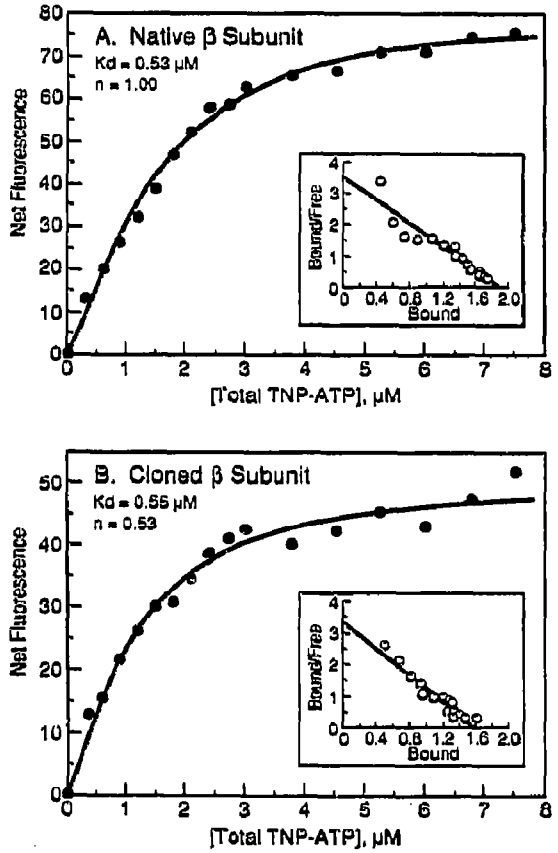

Fig. 3. Binding of TNP-ATP to cloned and native β subunits. TNP-ATP was added as indicated to either native (1.87 μM) or cloned (2.23 μM) β subunit in 50 mM Tricine-NaOH (pH 8.0). Net fluorescence (fluorescence enhancement) at 550 nm (415 nm excitation) was determined as described in section 2. Two representative experiments are shown. The curve represents the best fit of the data to the single-site binding equation (see section 2) from which K_D and n were determined. In the inset the data are reported in linearized form.

were titrated side by side. The experimental data best fit a single-site binding model. The dissociation constant for TNP-ATP binding to the cloned β subunit (mean of two independent determinations = 0.46±0.12 μM) is identical to that for native β subunit (mean = 0.46±0.1 μM). The binding stoichiometry for TNP-ATP varied between 0.4 and 0.6 mol TNP-ATP per mol of β (mean = 0.47 ± 0.08) for several different preparations of the cloned β subunit, compared with values close to unity (mean = 1.1±0.12) for the native β subunit. This suggests that approximately half of the β subunit recovered from inclusion bodies had been correctly folded into a conformation fully active in nucleotide binding, and that the rest were incapable of binding nucleotide.

The specificity of TNP-ATP binding to the β subunit was verified by nucleotide competition experiments. Fig. 4 shows that ATP effectively competed with TNP-ATP for binding to the cloned β subunit. GTP and ITP were less effective than ATP in displacing TNP-ATP whereas CTP was essentially ineffective. This specificity is identical to that observed earlier [10] for the native β subunit, and it mimicks the normal substrate specificity for ATP hydrolysis by CF_1 [28]. As an additional test of the authenticity of the nucleotide binding site on the cloned β subunit, we examined the effect of heating the protein at 40°C for 10 min. Under these conditions nucleotide binding to the native β subunit is completely lost [10]. Indeed, this treatment also totally abolished nucleotide binding to the cloned β subunit (data not shown).

4. DISCUSSION

Over-expression, recovery and reconstitution of F_1 subunits from thermophilic bacterium PS3 have recently been demonstrated [29,30]. The results presented in this communication represent the first time to our knowledge that a full-length β subunit of an ATP synthase from a eucaryotic source has been obtained in large quantities and in a functional conformation from an over-expression system. In one recent study [31] a clone of a mitochondrial F_1 β gene was linked to an alkaline phosphatase leader sequence, thus targeting the fusion protein to the periplasmic space of the *E. coli* host cells. A fragment of the β subunit containing residues 122 through 479 was solubilized from the cytoplasmic membrane fraction and shown to bind TNP-ATP. It will be of interest to see if the specificity of nucleotide binding to that fragment resembles that of our full-length β polypeptide. Similarly, a mitochondrial α subunit (residues 15 to 510) has been over-expressed in *E. coli* and recovered in a form which binds TNP-ATP [32].

Our ability to obtain large quantities of the chloroplast β subunit have made it feasible to attempt structural and conformational mapping studies of the β subunit by introducing labeling sites for fluorescent probes on the β polypeptide using site-specific mutagenesis. In order to proceed with these studies it is important to establish that the cloned β subunit is folded into the same conformation as the native protein. A variety of

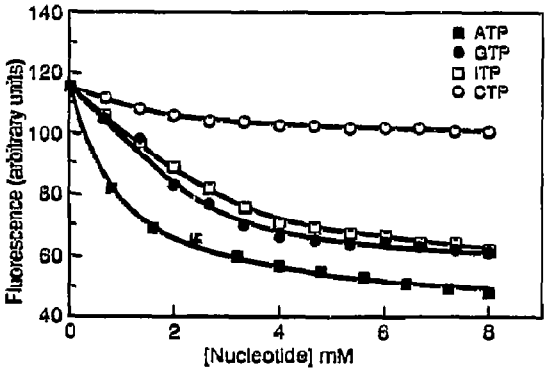

Fig. 4. Nucleotide selectivity of the nucleotide binding site on the cloned β subunit. Assay mixtures contained 50 mM Tricine-NaOH (pH 8.0), approximately 3 μM β subunit, and 1 μM TNP-ATP. ATP, GTP, ITP, or CTP were added from concentrated stock solutions in water to the concentrations indicated. The amount of bound TNP-ATP was determined as described in section 2.

genetic and chemical modification studies [1,4] have indicated that several regions of the β polypeptide, which are distant from each other in the linear amino acid sequence, come together in three-dimensional space to form the nucleotide binding domain. This domain probably represents a substantial portion of the folded β polypeptide [33] and it is likely that only one unique conformation can satisfy the nucleotide-binding requirements [34]. The fact that the binding site for nucleotides on the cloned β subunit is the same in every measured respect to that of the native β subunit strongly argues that the cloned β subunits which bind nucleotides are indeed correctly folded.

We recently introduced a derivative of pET3a-βNE2 into *E. coli* strain JP17 (a kind gift of Dr. Alan Senior) which has a partial deletion of the *uncD* gene encoding the β subunit [35]. The ability of the transferred strain to grow on succinate-containing medium indicated that the expressed chloroplast β subunit was incorporated into an active hybrid ATP synthase. This result, which is the subject of a forthcoming communication, confirms that our chloroplast *atpB* gene construct can encode a functional β subunit, and also provides a facile method for screening in vitro *atpB* mutants for their ability to fold an assemble correctly.

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