Integration of $b$ Subunits of Unequal Lengths into $F_{1}F_{0}$-ATP Synthase*

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$F_{1}F_{0}$-ATP synthases provide the bulk of cellular energy production in both eukaryotes and prokaryotes (1–3). Enzymes in this family utilize the electrochemical gradient of protons across membranes in order to synthesize ATP from ADP and inorganic phosphate in a coupled reaction (4). In *Escherichia coli*, $F_{1}F_{0}$-ATP synthase is a complex enzyme composed of approximately 22 polypeptides with the stoichiometry of $\alpha_3\beta_3\gamma_2\delta_3$, $\epsilon_1$ (3, 5). The $F_{1}$ portion is composed of the subunits $\alpha_3\beta_3\gamma_2\delta_3\epsilon_1$, and $\epsilon_1$ is responsible for enzymatic catalysis. The $F_{0}$ portion of the enzyme consists of the $ab\gamma\delta\varepsilon$ subunits and is responsible for the translocation of protons through the membrane.

Electron microscopy has shown that the $F_{1}$ and $F_{0}$ sectors are linked by two slender stalk structures (6). During ATP synthesis proton translocation drives the rotation of the central stalk, which consists of subunits $\gamma\delta$ within the $\alpha_3\beta_3$ hexamer held stationary by the peripheral stalk. This rotation propagates the conformational changes in the active sites located at the $\alpha\beta$ interfaces driving catalytic activity (1, 7–11). The $\delta$ subunit of $F_{1}$ and a dimer of two identical $b$ subunits from $F_{0}$ comprise the peripheral stalk acting as the stator. The $\delta$ subunit has been visualized seated at the top of the $F_{1}\alpha_3\beta_3\gamma_2\delta_3$ hexamer (12). However, recent evidence has suggested that the $\delta$ subunit may be positioned slightly to the side of $F_{1}$ in association with a single $a$ subunit (13–16). The $C$-terminal region of the $\delta$ subunit is in direct contact with the extreme $C$-terminal end of the $b$ dimer (1, 17–20). The $b$ subunit dimer constitutes the majority of the peripheral stalk stretching from within the membrane to near the top of $F_{1}$ (21).

Dimerization of the $b$ subunits is required for the normal assembly and function of $F_{1}F_{0}$-ATP synthase (22). The two $b$ subunits are believed to exist in parallel as an extended structure spanning from the periplasmic side of the membrane near the top of $F_{0}$ to near the bottom of $F_{1}$, a dimerization domain, and a $\delta$-binding domain (23). The ability of $b$ to bind to $F_{1}$ is proportional to the ability of $b$ to form dimers, suggesting the necessity of the $b$ dimer formation before the binding of $F_{1}$ to the complex (22). Presently, there is no high-resolution structure of the entire $b$ subunit. A model polypeptide of the first 34 residues of the $N$ terminus has been solved by NMR, revealing a hydrophobic membrane-spanning $\alpha$-helix (24). A crystal structure of a monomeric dimerization domain, consisting of residues 62–122, has been solved and refined to 1.55 Å (25). Dunn and co-workers (25) have constructed a model in which the two $\alpha$-helices of the $b_{62-122}$ region form a right-handed coiled coil. Much of the structural information on the $b$ dimer has been gleaned from classical biochemical approaches such as CD spectroscopy, cross-linking, and sedimentation experiments (26–32). These studies revealed that the overall structure of the $b$ subunit dimer is a highly extended conformation with $\sim80\% \alpha$-helix.

Previous studies have shown that $b$ subunits with deletions of up to 11 amino acids and insertions of up to 14 amino acids, corresponding to residues 16 and 21, respectively, formed functional $F_{1}F_{0}$ complexes (33, 34). When $b$ subunits with either a seven amino acid deletion or an insertion, $b_{-7}$ or $b_{+7}$, respectively, were incorporated into the $F_{1}F_{0}$-ATP synthase complex, the properties of the enzymes were essentially wild type. These observations suggested that the role of the $b$ dimer is more of a flexible structural feature. However, it was not known whether this flexibility extended to the dimerization of two $b$ subunits of unequal lengths and their incorporation into an enzyme complex.

In *Escherichia coli* the peripheral stalk of $F_{1}F_{0}$-ATP synthase consists of a parallel dimer of identical $b$ subunits. However, the length of the two $b$ subunits need not be fixed. This led us to ask whether it is possible for two $b$ subunits of unequal length to dimerize in a functional enzyme complex. A two-plasmid expression system has been developed that directs production of $b$ subunits of unequal lengths in the same cell. Two $b$ subunits differing in length have been expressed with either a histidine or V5 epitope tag to facilitate nickel-affinity resin purification (Ni-resin) and Western blot analysis. The epitope tags did not materially affect enzyme function. The system allowed us to determine whether the different $b$ subunits segregate to form homodimers or, conversely, whether a heterodimer consisting of both the shortened and lengthened $b$ subunits can occur in an intact enzyme complex. Experiments expressing different $b$ subunits lengthened and shortened by up to 7 amino acids were detected in the same enzyme complex. The V5-tagged $b$ subunit shortened by 7 amino acids ($b_{35,37,39}$) was detected in Ni-resin-purified membrane preparations only when coexpressed with a histidine-tagged $b$ subunit in the same cell. The results demonstrate that the enzyme complex can tolerate a size difference between the two $b$ subunits of up to 14 amino acids. Moreover, the experiments demonstrated the feasibility of constructing enzyme complexes with non-identical $b$ subunits that will be valuable for research requiring specific chemical modification of a single $b$ subunit.

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into an F1F0-ATP synthase complex. Here, we demonstrate that the F1F0-ATP synthase complex can tolerate b subunits with a size difference of at least 14 amino acids.

EXPERIMENTAL PROCEDURES

Materials—Molecular biology enzymes and oligonucleotides were obtained from Invitrogen, New England Biolabs, and Stratagene. Reagents were obtained from Sigma, Bio-Rad, and Fisher. Western blotting reagents and high performance chemiluminescence film were purchased from Amersham Biosciences. Polyclonal antibodies against SDS-denatured subunit b (35, 36) were kindly provided by Dr. Karlheinz Altendorf (Universität Osnabrück, Osnabrück, Germany). Monoclonal antibodies against the V5 epitope were purchased from Invitrogen.

Strains and Media—The wild type b subunit expression plasmid, pKAM14, and plasmids used to express shortened or lengthened b subunits have been described previously (28, 33, 34). The plasmids encoding the uncF(b) gene were used to complement E. coli strain KM2 (∆b) carrying a chromosomal deletion of the gene. All strains were grown in Luria broth supplemented with 25 μg/ml ampicillin (AP) or chloramphenicol (Cmr) (33) or pKAM14 (b, Apr) (33) or pAUL19 (b-, Apr) (34) were used to construct the epitope-tagged b subunits. Epitope tags were inserted into each of the plasmids using the Stratagene QuikChange kit. A histidine epitope tag was inserted at the N terminus by mutagenesis between the first and second codons of the uncF(b) gene to express bH6 or b2-H6 (Fig. 1A). All of the recombinant histidine-tagged b subunit plasmids were then digested with PstI and NdeI and subsequently ligated into a plasmid containing the chloramphenicol resistance gene and the pUC18 origin of replication (Table I). A V5 epitope tag was added to the C terminus by site-directed mutagenesis before the termination codon of the uncF(b) gene to express bV5 or b2-V5 (Fig. 1A). The recombinant V5-tagged b subunit plasmids included the ampilicillin resistance gene and the pUC18 origin of replication. Unique restriction enzyme sites SphI and NdeI were constructed near the histidine and V5 epitope tag sequence, respectively, for an initial detection of the insertions, and then the nucleotide sequence was subsequently confirmed by automated sequencing in the core facility of the University of Florida Interdisciplinary Center for Biotechnology Research.

Mutagenesis and Strain Construction—Plasmids pKAM14 (b, Apr) (28), pUL3 (b, Apr) (33) or pUL19 (b-, Apr) (34) were used to construct the epitope-tagged b subunits. Epitope tags were inserted into each of the plasmids using the Stratagene QuikChange kit. A histidine epitope tag was inserted at the N terminus by mutagenesis between the first and second codons of the uncF(b) gene to express bH6 or b2-H6 (Fig. 1A). All of the recombinant histidine-tagged b subunit plasmids were then digested with PstI and NdeI and subsequently ligated into a plasmid containing the chloramphenicol resistance gene and the pUC18 origin of replication (Table I). A V5 epitope tag was added to the C terminus by site-directed mutagenesis before the termination codon of the uncF(b) gene to express bV5 or b2-V5 (Fig. 1A). The recombinant V5-tagged b subunit plasmids included the ampilicillin resistance gene and the pUC18 origin of replication. Unique restriction enzyme sites SphI and NdeI were constructed near the histidine and V5 epitope tag sequence, respectively, for an initial detection of the insertions, and then the nucleotide sequence was subsequently confirmed by automated sequencing in the core facility of the University of Florida Interdisciplinary Center for Biotechnology Research. Throughout this paper, the insertion or deletion and the epitope tag are indicated after the gene and the pUC18 origin of replication. Unique restriction enzyme sites SphI and NdeI were constructed near the histidine and V5 epitope tag sequence, respectively, for an initial detection of the insertions, and then the nucleotide sequence was subsequently confirmed by automated sequencing in the core facility of the University of Florida Interdisciplinary Center for Biotechnology Research.

Preparative Procedures—Inverted membrane vesicles from KM2 (∆b) strains expressing the desired b subunits were prepared essentially as described previously (33). Protein concentrations were determined by the bicinchoninic acid (BCA) assay (37). Ni-Resin1 purification was achieved using the high capacity nickel chelate affinity matrix purchased from Sigma. A total of 5 mg of membrane protein was brought up to 1 ml with final concentrations of 0.2% teganemone WS-35, 0.15 M NaCl, and 1 mM imidazole. The purification procedure was accomplished using the batch method as described by the manufacturer. To control for possible enzyme disruption during the solubilization and purification procedures, the tagged wild-type length b subunits were either mock treated or treated with the homobifunctional cross-linker bis(sulfo-succinimidyl) suberate (BS3) after the addition of detergent. Membrane preparations were chemically cross-linked by treating with 1 mM BS3 for 30 min at room temperature. The cross-linking reaction was stopped by the addition of 100 mM ethanolamine HCl, pH 7.5, for 10 min.

Assay of F1F0-ATP Synthase Activity—Growth on a minimal succinate medium was used as an initial in vivo assay for enzyme viability. ATP hydrolysis activity was assayed by the acid molybdate method (38). Membrane preparations were assayed in buffer (50 mM Tris-HCl, pH 9.1) to determine the linearity with respect to time and enzyme concentration. Membrane energization was detected by the fluorescence quenching of 9-amin-6-chloro-2-methoxyacidine (ACMA) (39).

Immunoblot Analysis—Proteins were loaded on a 15% tris-glycine SDS gel and transferred onto nitrocellulose by electroblot. The subunit antibody incubation was performed essentially as described previously by Tamarappoo et al. (40) using a 1:25,000 dilution of the anti-b subunit antibodies. Secondary antibody incubation was performed with horseradish peroxidase-linked donkey anti-rabbit antibody (1:50,000), and the antibody was detected by enhanced chemiluminescence. The V5 subunit antibody incubation was performed as described by the manufacturer followed by a secondary antibody incubation with horseradish peroxidase-linked sheep anti-mouse antibody (1:10,000). Signals were visualized on high performance chemiluminescence film using a Kodak X-OMat.

RESULTS

Construction and Growth Characteristics of Mutants—To investigate whether it is possible for two b subunits of unequal length to dimerize to form the peripheral stalk in a functional enzyme complex, a collection of plasmids expressing histidine or V5 epitope-tagged b subunits was generated by site-directed mutagenesis. The epitope tags were needed to facilitate enzyme purification and subunit detection, respectively, on a Western blot. To express two different b subunits in the same cell, we developed a two-plasmid expression system (Fig. 1B). A total of four plasmids were constructed expressing bwt-his (Cmr), b7-his (Cmr), b7-V5 (Ap), or b7-V5 (Ap) (Table I). The deletion removed the segment from Leu254 to Ser370 and the insertion resulted in duplication of the same series of amino acids.

A previous analysis of F1F0-ATP synthase complexes with b subunits shortened and lengthened by 7 amino acids found the mutants to be essentially wild type (33, 34). However, it was possible that the epitope tags would affect enzyme assembly or function. This was particularly a concern at the C terminus where small deletions at the extreme C-terminal end of the subunit had been shown to inhibit F1F0-ATP synthase function (41, 42). Addition of the 14 amino acid V5 epitope tag to the C terminus of b might have impinged on enzyme assembly. The effects of the added epitope tags were studied by the ability of the plasmids to complement the E. coli strain KM2 (∆b) (43). Growth on succinate minimal medium was used as an initial qualitative gauge of enzyme activity in vivo because E. coli strains lacking F1F0-ATP synthase cannot derive energy from nonfermentable carbon sources. In each case, the strains expressing the epitope-tagged b subunits grew comparably to the wild-type strain (Table I). Hence, even though deletion of as few as 2 amino acids affected the ability of the b dimer to interact with the F1 subunit (41, 42), the addition of 14 amino acids to the C terminus did not interfere with the interaction of the b and subunits.

Effects of Epitope Tags—Because F1 has little affinity for the membrane in the absence of intact F0, total membrane-associated F1F0-ATP hydrolase activity was used as a test of F1F0-ATP synthase complex assembly. Under conditions of high pH, F1 can be released from the influence of F0 (38), so the amount of ATPase activity in the solution was used as a measure of the

1 The abbreviations used are: Ni-resin, nickel-affinity resin (purification); BS3, bis(sulfo-succinimidyl) suberate; wt, wild type; MOPS, 4-morpholinepropanesulfonic acid.
amount of intact enzyme complex located in the membrane vesicles. The V5 epitope tag did not have a significant affect on the activity in membrane vesicles prepared from the epitope-tagged mutants was used as an indication of coupled activity. Acidification of inverted membrane vesicles was examined by the fluorescence of 9-amino-6-chloro-2-methoxyacridine (Fig. 2). The level of NADH-driven fluorescence quenching was monitored for all membrane preparations to demonstrate that the vesicles were intact and closed. The levels of NADH-driven fluorescence quenching were strong and directly comparable in every case (data not shown). Membranes isolated from cells with a V5 epitope tag incorporated onto the $b_{\text{wt}}$ or $b_{\text{77}}$ subunit, KM2/pTAM46 or KM2/pTAM47, respectively, displayed a very slight reduction in coupled activity. The reduction in coupled activity correlated very closely with the minor reduction in $F_{\text{r}}$-ATP hydrolysis activity. A larger reduction in coupled activity, of about 20–25%, was observed in membrane vesicles isolated from cells when a histidine epitope tag was incorporated onto the $b_{\text{wt}}$ or $b_{\text{77}}$ subunit, KM2/pTAM37 or KM2/pTAM35, respectively. The decrease in coupled activity also paralleled the reduction seen in $F_{\text{r}}$-ATP hydrolysis activity. Moreover, the coupled activities observed in membranes isolated from cells coexpressing histidine-tagged and V5-tagged $b$ subunits were, as expected, intermediate between the V5-tagged species and the histidine-tagged species. The larger reduction seen in the cells expressing a histidine epitope tag arose in part from the plasmid vector. The moderate-copy plasmid vector pACYC184 was used to express the histidine-tagged $b$ subunits. When histidine-tagged $b$ subunits were expressed from the pUC18 plasmid vector, both coupled activity and ATP hydrolysis activity were similar to untagged $b$ subunits (data not shown). Hence the reductions in activity observed in membranes from the histidine-tagged strains reflected reduced amounts of intact $F_{\text{r}}F_{\text{p}}$-ATP synthase incorporated into the membranes. For the purposes of this study, the important parameters were the presence of an intact and functional $F_{\text{r}}F_{\text{p}}$-ATP synthase enzyme complex and the ability to distinguish the two differently tagged $b$ subunits via Western blot.

Detection of Heterodimers—Dimerization of the $b$ subunits is thought to be an early event in enzyme assembly, making the requirement for immediate dimerization of $b$ via co-translation of a single $b$ subunit transcript, feasible. Therefore, it was necessary to establish whether a two-plasmid expression system could be utilized to direct production of two different $b$ subunits that will dimerize to form an intact $F_{\text{r}}F_{\text{p}}$ enzyme complex. Two wild-type-length $b$ subunits, expressed from plasmids pTAM37 ($b_{\text{wt,his}}$, Cm$^r$) and pTAM46 ($b_{\text{wt, V5}, A p^r}$), or pTAM35 ($b_{\text{V5,his}}$, Cm$^r$), and pTAM47 ($b_{\text{V5, V5}}, A p^r$) were developed for coexpression experiments. C. diagram of the different $b$ subunits that were coexpressed in the same cell. Interactions between $b_{\text{wt,his}}/b_{\text{wt, V5}}, b_{\text{77,his}}/b_{\text{wt, V5}},$ and $b_{\text{V5,his}}/b_{\text{V5, V5}}$ were investigated.
subunit dimer was stable during solubilization with tegamin-
oxide WS-35, taurodeoxycholate, and lauryldimethylamine
oxide, but not with SDS (data not shown).

As an added precaution to ensure there was no nonspecific
aggregation of b subunits after membrane solubilization or
during Ni-resin purification, two independent membrane prepa-
rations were mixed together (Fig. 3, lanes 9 and 10). Mem-
brane vesicles derived from strains KM2/pTAM37 (b<sub>wt-his</sub>) and
KM2/pTAM46 (b<sub>wt-V5</sub>) were mixed, and the membranes were
solubilized with 0.2% tegamin-oxide, allowed to incubate at
room temperature for 30 min, and then treated with BS<sub>3</sub>
before Ni-resin purification was performed. Immunoblot analysis
with anti-b antibodies showed normal levels of histidine-tagged b
subunit upon Ni-resin purification (Fig. 3A, lanes 9–10). No V5
epitope was present after Ni-resin purification, indicating that
nonspecific aggregation was not a factor in the observed results
(Fig. 3B, lanes 9–10). Therefore, any observed interactions of b
subunits must have been because of heterodimers integrating
within an intact F<sub>H</sub>F<sub>0</sub>-ATP synthase complex in a cell expressing
both b subunits. Importantly, immunoblotting using the
anti-V5 antibody clearly detected V5 epitope-tagged b subunits
in Ni-resin-purified samples when the two were coexpressed
(Fig. 3B, lanes 11 and 12).

Formation of Mixed Length b Subunits in F<sub>H</sub>F<sub>0</sub>-ATP Syn-
Thase—The expression system was used to study whether unequal
length b subunits could form a dimer or, alternatively,
whether the $b$ subunits dimerize and incorporate into enzyme complexes in a segregated manner. To determine whether there is a direct protein-protein interaction between the different length $b$ subunits, we investigated the ability of $b_{7-V5}$ to interact with $b_{wt-V5}$ or $b_{7-his}$ subunits following Ni-purification (Fig. 4). All membranes prepared from strains expressing a $b$ subunit were readily detectable and distinguishable by size on an immunoblot (Fig. 4A, lanes 1–7). Only $b$ subunits with a histidine tag were retained by Ni-resin purification (Fig. 4A, lanes 8–12). Immunoblot analysis using an anti-V5 antibody was performed on the membrane preparations and Ni-purified products (Fig. 4B). The V5-epitope tag was detected only in membrane vesicles derived from KM2 strains expressing either the V5-tagged $b$ subunit or the coexpressed V5-tagged and his-tagged $b$ subunits (Fig. 4B, lanes 4–7). As expected, upon Ni-resin purification, the V5 epitope was not detected in samples containing only histidine-tagged $b$ subunit (Fig. 4B, lane 8). Likewise, samples containing only the V5-tagged $b$ subunits were not detected upon Ni-resin purification, signifying that they were efficiently removed from the resin during wash steps (Fig. 4B, lanes 9–10). Finally, we investigated the ability of $b_{7-his}$ to dimerize to form intact $F_1F_0$-ATP synthase complexes with $b_{wt-V5}$ or $b_{7-V5}$. The $b_{wt-V5}$ was detected with anti-V5 antibodies and was readily observed to dimerize with the $b_{7-his}$, indicating a $b_{7-his}/b_{wt-V5}$ interaction (Fig. 4B, lane 11). To a much lesser extent, the $b_{7-V5}$ subunit was also distinguishable with anti-V5 antibodies (Fig. 4B, lane 12). The data indicated that the $F_1F_0$-ATP synthase enzyme complex could tolerate $b$ subunit heterodimers with a size difference of at least 14 amino acids.

Detection of $b$ subunit heterodimers led directly to two additional questions. How many $b$ subunit heterodimer $F_1F_0$-ATP synthase complexes were formed? Were they active? We have not yet purified a heterodimeric complex to homogeneity, and so the direct assay has not been performed. However, an indication of activity could be assessed based on the percentage of homodimeric and heterodimeric $F_1F_0$ complexes. Therefore membranes were prepared from strains KM2/pTAM37/pTAM46 ($b_{wt-his}/b_{wt-V5}$) and KM2/pTAM35/pTAM47 ($b_{7-his}/b_{7-V5}$). Membranes prepared from strains expressing an epitope-tagged $b$ subunit were readily detectable and distinguishable by size on an immunoblot of a 15-cm 15% SDS-PAGE membrane.
Unequal b Subunits

F₁F₀-ATP synthase complexes (Fig. 5B). Relative amounts of the b₁₇-his and b₁₅-V5 subunits were also determined from Ni-resin-purified samples coexpressing both b subunits. Upon Ni-resin purification, all of the b₁₅-V5 subunit seen on a Western blot must necessarily be incorporated into the enzyme complex as a heterodimer along with a b₁₇-his subunit. Hence, when the two different wild-type-length b subunits were coexpressed, there was at least 15% heterodimer formation (Fig. 5B). It is possible that further manipulation of subunit expression and optimization of the purification procedure might result in a higher percentage of heterodimers being found in the membranes. Somewhat lower percentages of heterodimer formation were found when b₁₅-V5 was coexpressed with b₁₇-his or b₁₅-V5 (Fig. 5B). Based on the percentage of heterodimer formation and the activities observed when the different b subunits were coexpressed in the same cell (Fig. 5, Table I), it is likely that the heterodimeric F₁F₀-ATP synthase species are functionally active.

DISCUSSION

In the present work, we have developed an expression system that facilitates the production, purification, and detection of b subunits of unequal lengths within E. coli. The experiments involved an epitope tag system that allowed us to determine whether the different b subunits segregated into homodimers, or alternatively, whether a heterodimer of long and short b subunits can be incorporated into an F₁F₀-ATP synthase complex. The histidine and V5 epitope tags introduced into the b subunits did not appreciably affect enzyme assembly or function. Expression of two different wild-type-length b subunits led to three distinct F₁F₀-ATP synthase complexes in the same cell; 1) a homodimer of histidine-tagged b subunits, 2) a homodimer of V5-tagged b subunits, and 3) a heterodimer consisting of a histidine-tagged b and a V5-tagged b subunit.

More importantly, three different F₁F₀-ATP synthase complexes were present even when the b subunits were of identical length. We observed dimerization of b subunits between b₁₇-his and both b₁₅-V5 and b₁₅-V5. This demonstrates that b subunits that differ in length by at least 14 amino acids can be incorporated into an enzyme complex. Given that the tether domain is likely an α-helix, the difference in length between the b subunits would be 21 Å. Dimerization could occur in two ways. First, assuming that the transmembrane domains were in parallel, the hydrophilic domains could be out of register. Alternatively, we favor a conformation in which both the transmembrane domains and the dimerization domains as defined by Dunn et al. (23) exist in parallel. This would require that a section of the tether domain in the longer b subunit be out of contact with the shorter b subunit (Fig. 6). It is likely that a parallel alignment of the dimerization domain is required for enzyme assembly. Recent electron microscopy and NMR studies have revealed a distinctive 20° bend in the b dimer within the tether domain (24, 30, 44). The research presented here suggests the possibility of straightening or further bending of the two b subunits within the peripheral stalk and lends support to the concept of a flexible peripheral stalk.

This raises the question of why the tether domain should be so flexible as to allow insertions, deletions, and dimerization of b subunits of unequal lengths. If one views the peripheral stalk to be a rope-like structure linking F₁ to F₀, then its position holding F₁ against the rotation of the central stalk would not be expected to be the same for counterclockwise and clockwise rotation. Flexibility of the tether domain might facilitate the reorientation of the peripheral stalk, to act as a stator for rotation in either direction during ATP synthesis and ATP hydrolysis.

The ability to generate and purify F₁F₀ complexes with two genetically different b subunits provides a potentially useful experimental tool. It is now feasible to specifically label a single b subunit within a purified complex. This will facilitate biochemical modification experiments and the use of physical methods.

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