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Tethering polypeptides through bifunctional PEG cross-linking agents to probe protein function: Application to ATP Synthase

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Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; CBD, chitin binding domain; EDTA, ethylenediamine tetraacetic acid; DEAE, diethylaminoethyl; DTT, dithiothreitol; MBP, maltose binding protein; NEM, N-ethyl maleimide; PEG, polyethylene glycol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

ABSTRACT

Chemical cross-linking mediated by short bifunctional reagents has been widely used for determining physical relationships among polypeptides in multi-subunit proteins, but less often for functional studies. Here we introduce the approach of tethering polypeptides by using bifunctional reagents containing a lengthy, flexible PEG linker as a form of cross-linking especially suited to functional analyses. The rotary molecular motor ATP synthase was used as a model subject. Single cysteine residues were introduced into selected positions of ATP synthase ε subunit, a component of the rotor subcomplex of the enzyme, and the unrelated maltose binding protein (MBP), then the two purified recombinant proteins were cross-linked by means of a dimaleimido-PEG cross-linking agent. Following purification, the ε-PEG-MBP was incorporated into membrane-bound ATP synthase by reconstitution with ε-depleted F_1-ATPase and membrane vesicles that had been stripped of endogenous F_1. ATP synthase reconstituted using ε-PEG-MBP had reduced ATP hydrolytic activity that was uncoupled from the pumping of H^+, indicating the physical blockage of rotation of the $\gamma\varepsilon c_{10}$ rotor by the conjugated MBP, whereas enzyme reconstituted with ε-PEG was normal. These results directly demonstrate the feasibility of studying mechanistic features of molecular motors through PEG-based conjugation of unrelated proteins. Since tethering polypeptides provides a means of maintaining proximity without directly specifying or modifying interactions, application of the general method to other types of protein functional studies is envisioned.

INTRODUCTION

Chemical cross-linking has been used for many years for the structural analysis of multisubunit proteins, as it provides a probe of subunit stoichiometry and arrangement.[1-5] The methodology has continually developed, and applications of this type are again resurgent as methods for rapidly identifying sites of cross-linking through fragmentation coupled with mass spectrometric analysis are improved.[6,7]. Chemical cross-linking has also been used for stabilizing proteins, for altering their solubilities, for targeting them to specific sites, and in some cases for basic functional analysis.[3,8-11] Examples of the latter application include trapping an intermediate state in a conformational change,[12] restricting the ability of a polypeptide to adopt more than one of multiple known conformational states,[13] preventing major subunit rearrangements,[14,15] for creating a dual-function protein[16] or for investigating the location of active sites in an enzyme.[17,18] The potential for using chemical cross-linking to investigate function has been substantially increased by the use of site-directed mutagenesis to direct one end of the reagent to a specific site and to reduce the possibility of modifying multiple sites. Nevertheless, the use of traditional short bifunctional reagents for functional analyses is limited by their potential for severely restricting normal protein dynamics and introducing steric hindrance to important interactions. The goal of the current project was to cross-link polypeptides in a manner that would be unlikely to compromise subunit interactions, conformational changes or otherwise interfere with function through direct mechanisms. To achieve this we used a bifunctional reagent, dimaleimidyl-PEG[1], with a flexible linker containing about 75 ethylene glycol units.

The subject of our study is *Escherichia coli* ATP synthase (Figure 1), a membrane-bound multi-subunit enzyme that couples the energy stored in a transmembrane proton gradient to the synthesis of ATP from the precursors ADP and P_i. ATP synthase is composed of two molecular motors that work together in an unusual rotational coupling mechanism. See recent reviews.[19-22] The enzyme can be physically separated into a membrane-peripheral F_1 sector, an ATP-driven motor composed of five different polypeptides in a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$, and the membrane-integral F_0 sector, an ion flux-driven motor composed of three different subunits with a stoichiometry of ab_2c_{10}. The γ and ε subunits of F_1 rotate relative to $\alpha_3\beta_3\delta$ during ATP hydrolysis/synthesis, while the c_{10} ring rotates relative to ab_2 as protons cross the membrane. The $\gamma\varepsilon c_{10}$ subassembly acts as a joint rotor, held together by non-covalent interactions, so that either motor can drive the other in reverse. A peripheral stator stalk composed of $b_2\delta$ links the sectors to prevent $\alpha_3\beta_3$ from simply rotating along with $\gamma\varepsilon c_{10}$ during proton flow.

Previously we engineered forms of ATP synthase with unrelated polypeptides fused to either the N- or C-terminus of ε.[23,24] Since turning the rotor would require dragging the fused protein "cargo" through the small port between $\gamma\varepsilon$ and b_2 (see Fig. 1A), we expected such fusions to produce an enzyme lacking significant ATP synthetic or hydrolytic activity. We found that the port can accommodate a C-terminally fused protein with dimensions 23 X 24 X 48 Å, but not proteins that are 24 X 37 X 48 Å or 30 X 35 X 55 Å in size.[24] The inability of the larger

constructs to allow growth on nonfermentable carbon sources indicated a lack of oxidative phosphorylation, supporting operation of the rotational mechanism *in vivo*. Surprisingly, however, membrane vesicles prepared from all such forms retained significant hydrolytic activity. This residual activity was completely uncoupled from proton pumping, suggesting that coupling in the enzyme is intrinsically loose. The fusion proteins, however, might perturb the normal interactions of the terminal segments of ε in the enzyme complex and thus could be the source of the uncoupling. To broaden the range of positions where the added protein "cargo" might be attached, we have taken a chemical cross-linking approach to tether the 40-kDa (36 X 40 X 63 Å) maltose binding protein (MBP) to ε at a different location where effects on the ability of the polypeptide to interact with other parts of the enzyme are unlikely. By cross-linking MBP to ε through a long flexible PEG-based linker, rotation and proton pumping have been blocked, while again the enzyme retained substantial uncoupled ATPase activity. We believe that this methodology of cross-linking through flexible and lengthy linkers will be useful in investigating mechanistic features of other proteins, including molecular motors and reversibly associating complexes.

MATERIALS AND METHODS

General methods and reagents

A PEG-based cross-linker (average molecular weight of 3,400 Da) containing maleimide groups on each end (Mal-PEG-Mal), was purchased from Nektar (San Carlos, CA). Aqueous stocks containing 25 mM Mal-PEG-Mal were made in ddH_2O. All other laboratory chemicals were of the highest grade. Recombinant DNA techniques were performed by standard methods. Protein determinations were performed using the Bradford method[25] for soluble proteins, and by the Lowry method[26] for membrane samples. SDS-PAGE was carried out using 15% separating gels with the Laemmli buffer system[27], and gels were stained using Coomassie Brilliant Blue R-250. Western blotting to polyvinylidene difluoride membranes was performed using the carbonate buffer system,[28] and probed with antibodies that were labeled with ^{125}I using the IODO-GEN method.[29] The anti-ε monoclonal antibody (ε-1) has been described.[30,31] The anti-α monoclonal antibody (α-II)[32,33] was a generous gift from Drs. Robert Aggeler and Rod Capaldi of the University of Oregon. Preparation of *E. coli* inner membrane vesicles depleted of F_1-ATPase, as well as measurements of ATP hydrolysis, H^+-pumping, and ε inhibition were performed as described by Cipriano *et al.*[24] The *unc* deletion mutant strain DK8,[34] carrying a plasmid encoding an inactive but properly assembled ATP synthase due to an αR376A point mutation, was used for preparation of the F_1-depleted membranes. This plasmid was kindly provided by Dr. Robert Nakamoto of the University of Virginia.

Plasmid construction and mutagenesis

Plasmid pLH1[35] encoding wild type maltose binding protein was the parent used for construction of MBP containing a single, surface-exposed cysteine residue. An A21C substitution was introduced using polymerase chain reaction with mutagenic primers. The 5' end of the MBP coding sequence was amplified using primers GCAACATTCATGATGGG and

GACCTGCTTCACAGAGACCGTTATAGCC (mutagenic codon underlined) and cut with PflMI and BsaI. The 3' end was amplified using primers GCAGGGTCTCTGTGAAGTCGGTAAGAAATTCGAG (mutagenic codon underlined) and GTCTTTAATGTCGCCATACTTGAACGCATAACCCCCGTC and cut with BsaI and BsiWI. These two DNA fragments were ligated into pLH1 that had been linearized with PflMI and BsiWI in a three-part ligation to produce pDC37. Plasmid pES2[36], encoding wild type ε was the parental plasmid for the ε_{H56C} mutation. The PCR product obtained using primers CTGGTATGATTCGCATCGTGAAACAGTGCGGTCACGAAGAG (mutagenic codon underlined) and GCGATGTAACACCGGCTTGA was cut with BsaBI and SgfI and ligated into pES2 that had been cut with the same enzymes to produce pSD174. Plasmid constructions were confirmed by restriction endonuclease mapping and DNA sequencing.

Protein purification

ε_{H56C} was expressed from pSD174 in *E. coli* strain MM294[37] and the growth and purification was performed as described for wild type ε[24], except that 1 mM DTT was included in buffers. MBP_{A21C} was expressed from pDC37 in strain HS3309[35] at 37 °C with vigorous shaking in 1 liter of 2xYT media until cultures reached stationary phase. Periplasmic proteins were extracted by osmotic shock,[38] dialyzed into 50 mM Tris-HCl, pH 8.0, 1 mM DTT and loaded onto a 35-ml column of DEAE Sepharose Fast Flow (GE Healthcare). The column was washed with 5 volumes of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and eluted with a 0-500 mM linear gradient of NaCl in 8 column volumes of the same buffer. Fractions containing MBP_{A21C} were pooled, concentrated, and subjected to gel filtration chromatography using a Sephacryl S200 (GE Healthcare) column that had been equilibrated with 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT.

To obtain ε-depleted F_1-ATPase, we first purified F_1-ATPase containing an N-terminally fused and C-terminally truncated form of ε, CBD-$\varepsilon_{88\text{-stop}}$, expressed from pDC46[23] in DK8[34] cells, using described methods.[31] ε-depleted F_1 was prepared essentially as described[39] but using F_1 containing CBD-$\varepsilon_{88\text{-stop}}$ as the starting material and a chitin affinity column in place of the immunoaffinity column containing the ε-4 monoclonal antibody.

Synthesis and purification of ε-PEG-MBP

Two samples, one containing 22 ml of 73 μM ε_{H56C}, and another containing 22 ml of 73 μM MBP_{A21C}, were independently reduced with 1 mM DTT and dialyzed against 2 litres of 10 mM MOPS-NaOH, pH 7.0, 1 mM EDTA. After 8 hours the buffer was changed and the samples were dialyzed against an additional 2 litres for a further 10 hours. An aliquot of each was removed and diluted into SDS-gel sample buffer containing 15 mM N-ethylmaleimide (NEM) and saved for SDS-PAGE analysis. The two samples were mixed, and an additional aliquot was removed and treated with NEM for SDS-PAGE. Cross-linking was performed at room temperature by the addition of Mal-PEG-Mal to 36.6 μM in 10 equal aliquots, with 2 minutes between successive additions. The reaction was incubated at room temperature for 2 hours

before addition of 1 mM NEM to block any remaining thiol groups. Finally, 10 mM β-mercaptoethanol was added to quench any residual maleimide groups.

ε-PEG-MBP was purified from the product mixture using two consecutive precipitations with 80% saturated ammonium sulfate. The final pellet was suspended in 50 mM HEPES-KOH, pH 7.0, 1 mM EDTA, 50 mM NaCl, and the remaining ammonium sulfate was removed by dialysis into the same buffer. The sample was then loaded onto a column containing 5 grams of hydroxylapatite (Biorad), and eluted with a linear gradient of 0-200 mM sodium phosphate, pH 7.0. As controls, ε cross-linked to only a PEG molecule, and ε labeled with NEM were also prepared. A 10-fold excess of the Mal-PEG-Mal cross-linker was added to ε_{H56C} alone and quenched with β-mercaptoethanol to produce ε-PEG. The ε-PEG was then purified from unreacted ε_{H56C} and ε-PEG-ε using DEAE anion exchange chromatography.

Reconstitution of ε constructs into ATP synthase

150 μg of ε-depeleted F_1 and 7.5 μg of purified δ were mixed with either 6 μg of ε, ε-NEM, ε-PEG, or with 21.7 μg of ε-PEG-MBP in a total volume of 200 μl of 50 mM HEPES-KOH, pH 7.5, 5 mM $MgCl_2$, 300 mM KCl. After incubation at 37 °C for 10 minutes, 3 mg of stripped membrane vesicles were added, followed by an additional 10 minute incubation at 37 °C. The sample was then diluted into 10 ml of 10 mM MOPS-NaOH, pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 10% methanol, and centrifuged for 1.5 hours at 38,000 RPM in a Beckman 50-Ti rotor. The resultant pellet was suspended in 200 μl of the same buffer by gentle homogenization.

RESULTS

Strategy for blocking rotation

Previous attempts to block rotation in ATP synthase by genetically fusing large polypeptides to either the N-[23] or the C-terminus[24] of ε resulted in the uncoupling of residual ATP hydrolysis from proton pumping. These findings implied that either the ATP synthase is naturally partially uncoupled, or else that the added protein masses at the extreme ends of the ε molecule perturbed its natural structure and/or function, resulting in uncoupling. Here we overcame the limitation of fusion at only the ends of the polypeptide chain by taking a chemical approach to tether a large globular protein to a position internal in the ε sequence, but at the periphery of the protein structure (Figure 1), so that effects on the structure, function, or interactions of ε are unlikely.

The overall approach, outlined in Figure 1B, was to cross-link ε to the 40-kDa maltose binding protein (MBP) of *E. coli in vitro*, then to use the known biochemical properties of ATP synthase to reconstitute cross-linked ε back into the membrane-bound enzyme. To achieve cross-linking through specific sites, cysteine residues were introduced by site-directed mutagenesis into specifically chosen positions of the two polypeptides, neither of which contain endogenous cysteines. ε residue His-56 was chosen as the site for chemical linkage because in the bovine F_1 crystal structure[40] the corresponding residue of the homologous subunit is far-removed from other subunits (Figure 1C), and it has previously been mutated to cysteine and

labeled with maleimide fluorophores with no apparent effect on function.[41] Utilizing the high resolution structure of MBP[42] we selected the surface-exposed position Ala-21 of the mature protein. Following expression and purification, the polypeptides were cross-linked using 3.4 kDa Mal-PEG-Mal, a cross-linker with maleimido moieties on each end of a PEG chain that contained on average 75 ethylene glycol units, providing a 250-Å tether between the proteins.

Synthesis, purification and inhibitory activity of ε-PEG-MBP

Plasmid-borne ε_{H56C} and MBP_{A21C} were expressed in *E. coli* and purified using standard methods. Cross-linking of a mixture of the two proteins using Mal-PEG-Mal was carried out as described under Experimental Procedures, yielding a mixture of seven different protein species: MBP-PEG-MBP, ε-PEG-MBP, MBP-PEG, unreacted MBP, ε-PEG-ε, ε-PEG, and unreacted ε (Figure 2A). Assignment of the bands was based on apparent molecular weights and by comparison to products seen in control reactions in which only one of the proteins was treated with Mal-PEG-Mal.

The ε-PEG-MBP was purified from the mixture utilizing differences in the solubility and adsorption to hydroxylapatite as described under Experimental Procedures. This and other forms of ε used for reconstitution of the ATP synthase are shown on the SDS-PAGE in Figure 2B. A small amount of MBP-PEG remained in the ε-PEG-MBP sample, but this would not be expected to interfere with the reconstitution of ε-PEG-MBP into the ATP synthase. Control proteins used in the studies included ε_{H56C} that was reacted with an excess of N-ethylmaleimide to modify the cysteine residue (ε-NEM), and ε_{H56C} that was labeled with a single PEG molecule (ε-PEG) and purified by DEAE chromatography.

One concern with any method of adding one protein to another is that the added protein may alter the normal interactions of the targeted protein. Since ε is a dissociable inhibitor of ATP hydrolysis by soluble F_1,[43] we determined the effect of the PEG-MBP linkage on this functional property. It should be noted that the mechanism by which ε subunit inhibits ATP hydrolysis by isolated F_1 is quite different from that outlined in Figure 1A, as it depends on direct interactions of its C-terminal domain with $\alpha_3\beta_3\gamma$[43-45] and is independent of the peripheral stalk. The wild type ε and ε-PEG-MBP forms inhibited ATPase activity to similar extents and with similar affinities (Figure 2C), indicating that cross-linking ε_{H56C} to MBP_{A21C} with Mal-PEG-Mal did not perturb the normal interactions of ε with F_1. In contrast, previous results showed that fusing globular proteins to the C-terminus of ε, even through a linking peptide, completely abolished this inhibition,[24] implying that the C-terminal fusions did perturb the normal interactions of the C-terminal domain of ε with $\alpha_3\beta_3\gamma$.

Reconstitution of ε-PEG-MBP into ATP synthase

The ATP synthase reconstitution procedure (Figure 1B) takes advantage of the facts that F_1 reversibly dissociates from the membrane-embedded F_0 under conditions of low-ionic strength[46] and that ε is bound reversibly to F_1 such that it can be removed and replaced.[43] A previously described method to produce ε-depleted F_1[39] using anti-ε immunoaffinity chromotography was adapted here using an F_1-ATPase carrying a C-terminally truncated form of

the ε subunit fused N-terminally to a chitin binding domain (CBD-$\varepsilon_{88\text{-stop}}$).[23] The CBD acts as an affinity tag, allowing the substitution of a chitin column for the anti ε-4 immunoaffinity column originally used in the ε-depletion procedure[39] while the C-terminal deletion is expected to reduce the affinity of ε for F_1. As seen in Figure 2D, the chitin column effectively removed the CBD-$\varepsilon_{88\text{-stop}}$ (apparent molecular weight 16 kDa) from F_1. This procedure provides a method for depleting F_1-ATPase of ε without the need for monoclonal antibodies, and could be adapted to remove any protein subunit that reversibly dissociates from a multi-subunit protein complex.

Incubation of membrane bacterial vesicles in buffer of low ionic strength lacking Mg^{2+} releases the F_1 sector of ATP synthase, leaving the membrane-integral F_0 sector in these F_1-depleted, or "stripped", vesicles. The enzyme may be reconstituted by incubation of the stripped vesicles with added F_1-ATPase in the presence of Mg^{2+}; this reconstitution depends on both the δ and ε subunits.[47,48] During the ε depletion procedure, the δ subunit was degraded (Figure 2D), giving rise to the δ' fragment (residues 1-134) described previously;[49,50] intact recombinant δ[51] was therefore added to mediate reconstitution. After assembly of the various ε constructs into F_1, and rebinding F_1 to membrane-bound F_0, the vesicles were subjected to a wash step to remove any unbound F_1, and then analyzed for assembly of ATP synthase complexes by assaying for ATPase activity (Table 1) and by western blotting using antibodies against the α and ε subunits (Figure 3A).

Reconstituted membrane samples were assayed for ATP hydrolysis activity in two ways[24]. In the "Membrane-bound ATPase" assay, samples are continually in the presence of Mg^{2+}, which maintains the integrity of ATP synthase in the membranes. In the "Released ATPase" assay, the membranes are initially diluted into Mg^{2+}-free, low ionic strength buffer, promoting the release of F_1 from membrane-integral F_0. Due to the high level of dilution, ε subunit dissociates from F_1 and the enzyme reaches its maximal activity, providing a measure of the total amount of F_1 in the sample. We call the ratio of membrane-bound to released activity the "normalized activity"; for wild-type ATP synthase the normalized activity is in the range of 0.4-0.5, reflecting differences between the assays in conditions and the modest inhibitory effect of the ε subunit within intact ATP synthase. Blockage of rotation (Fig. 1A) specifically affects the ATPase activity of the membrane-bound enzyme, resulting in lower normalized activity.[23,24]

For these reconstitution reactions we used stripped membranes prepared from a strain carrying the catalytically inactive αR376A mutant[52] so that any residual F_1 would not contribute to measured activities. The stripped membranes contained only 0.01 to 0.02 U of ATPase activity per mg of protein (see Table 1, footnote b). The low level of ε seen by western blot in the sample reconstituted with only ε-depleted F_1 (Figure 3A) reflects traces of residual, inactive ATPase from the membranes, since any remaining ε in the ε-depleted F_1 would be the larger CBD-$\varepsilon_{88\text{-stop}}$ construct. A low level of F_1 was bound independently of ε, as seen by the modestly higher level of α subunit, relative to ε, in the sample reconstituted with ε-depleted F_1. This ε-independent F_1 binding (less than 0.3 U of ATPase per mg of membrane protein, see Table 1, footnote c) was about one quarter of the ε-dependent binding seen in the other samples. The

nonspecific nature of the binding seen in the absence of ε is also reflected by the high (>0.8) normalized activity of this sample.

Membranes reconstituted with F_1 containing either ε-PEG or ε-PEG-MBP showed levels of α and ε similar to controls (Figure 3A) and normal levels of released ATPase activity (Table 1), indicating that the abilities of ε-PEG and ε-PEG-MBP to mediate binding of F_1 to stripped membranes were uncompromised. As expected in samples that are blocked in rotation by the peripheral stalk, the membrane-bound ATPase activity of the ε-PEG-MBP sample was specifically suppressed, lowering the normalized activity to 0.21. It is notable, however, that membranes reconstituted with ε-PEG-MBP still retained a substantial level of ATP hydrolysis, as previously seen with samples bearing large N-terminal ε fusions.[23]

Proton pumping is blocked by tethered MBP

The reconstituted membrane vesicles were assayed for ATP-dependent proton pumping as a measure of the coupled state of the enzyme (Figure 3B). In this assay, the quenching of fluorescence of ACMA, which is accumulated by the vesicles in response to a proton gradient, is monitored to provide a semiquantitative indication of the ATP-dependent pumping of protons into the lumen of the vesicles. Subsequent addition of FCCP, an uncoupler that dissipates the proton gradient and allows redistribution of ACMA across the membrane, distinguishes quenching due to vesicular uptake from that attributable to direct quenching by nucleotides. As seen in Figure 3B (upper panel), vesicles reconstituted with wild-type F_1-ATPase (trace c) exhibited typical ATP-dependent proton pumping, while the stripped vesicles, or those with the non-specifically bound ε-depleted F_1, did not. In Figure 3B (lower panel) it can be seen that when the ε-depleted F_1 was mixed with either wild-type ε, ε-NEM, or ε-PEG, to promote reconstitution onto membranes, proton pumping was restored. These results indicate that neither simple modification of position 56 nor attachment of the lengthy PEG chain to that position directly affected function. In contrast, reconstitution with ε-PEG-MBP failed to restore discernible ATP-dependent proton pumping (trace d) despite the significant levels of membrane-bound ATP hydrolysis (Table 1).

DISCUSSION

In recent work, we have explored the effects of attaching an unrelated globular protein to the ATP synthase rotor subunit ε in such a way that rotational catalysis would be blocked. In our previous studies polypeptides were fused to the N- or C-termini of ε through a recombinant DNA approach. The gene fusion strategy has many advantages, but the terminal regions of a polypeptide may have critical functions that could be disrupted by the fusions. In the case of ε, fusions at the C-terminus resulted in high levels of uncoupled ATPase activity, while N-terminal fusions caused lower, but still significant, levels. In order to confirm that the uncoupled activity is inherent to rotation-blocked ATP synthase, we considered additional ways to attach the protein "cargo". Internal protein fusions are possible but difficult,[53] and these would seem to be more likely than terminal fusions to have direct functional consequences.

We therefore adopted the protein chemical strategy described here. One of the advantages of this approach is that nearly any surface position can be selected as the site of attachment. Direct addition of another polypeptide through an intermolecular cystine linkage or a short cross-linking agent might be expected to directly hinder some aspects of protein function, such as conformational changes, so we used the lengthy (250 Å) and flexible dimaleimido-PEG. The validity of this approach was confirmed by the lack of functional impairment of enzyme reconstituted with ε that had bound only the PEG molecule. This control experiment allowed us to conclude that the functional impairment seen for enzyme reconstituted with ε-PEG-MBP was due to the blockage of rotation by MBP. It should be noted that by linking MBP to the ATP synthase, the effective local concentration of MBP around the synthase will be high. Potentially, this could interfere with the ATPase activity and coupling, but we feel this possibility to be unlikely. *In vivo*, ATP synthase functions despite macromolecular crowding[54] that presents the enzyme with a plethora of proteins and nucleic acids that could also have this effect. The local MBP concentration is also minimized by using a long linker (250 Å in this case). The fact that tethering MBP to ε results in an ATP synthase with the same properties as those in which ε was genetically linked to two non-related proteins[23,24] validates this expectation. Together, these results provide substantial support for our hypothesis that rotation-blocked ATP synthase possesses residual, uncoupled ATP hydrolysis activity.

It is unclear at this point whether the intrinsic uncoupled ATPase activity we observe serves a physiological purpose, *e.g.* in dissipating excessive protonmotive force, or if it reflects the challenges inherent in achieving a perfectly coupled system. The latter would require the exact co-ordination of substrate binding, catalysis, product release, rotation, and proton translocation. As we noted previously,[23] an uncoupled event will occur if the rotor turns in the direction of ATP synthesis when ADP and phosphate are not bound at the active site. In the chloroplast and mitochondrial ATP synthase, a role for ε as a coupling factor has been well established.[55,56] It will be interesting to see if direct observation of this slippage may be possible using single molecule techniques.[57,58] The conformation of the C-terminal domain has been shown to change in response to different nucleotides[59] and a regulatory role has been proposed.[59-61]

PEGs are commercially available in various lengths and with a number of different reactive groups at their ends. Currently, Mal_2-PEG reagents can be obtained in 2K, 3.4K, 6K, 8K and 10K molecular weights from SunBio Corporation. Multi-branched PEG derivatives are also available. One of the major uses of activated PEG reagents is the PEGylation of proteins that are injected for therapeutic purposes;[62-65] PEGylation enhances the lifetimes of such proteins *in vivo*, increasing their therapeutic effectiveness. In the current work we required a reagent to cross-link two proteins through specific, defined sites, so we used mal-PEG-mal, which reacts specifically with sulfhydril groups at each end. Cysteine is among the least common of amino acids in most proteins, and here, as in many cases, there were no endogenous cysteines to remove before engineering them into the sites of our choice. Furthermore, since cysteine has a relatively hydrophobic sidechain, endogenous cysteines in many proteins are

buried rather than on the surface where they would react readily. Nevertheless, due to the random nature of the cross-linking reaction it was necessary to purify the desired product ε-PEG-MBP from the product mixture. However, increasingly specific ways of modifying proteins, or of introducing uniquely reactive groups into them, are under ongoing development and we anticipate that the specificity of cross-linking proteins through such sites will increase in the future.

CONCLUSION

Elucidating the structure and function of a protein may require application of a wide range of techniques, each with its own strengths and limitations. Polypeptide fusions are frequently useful, but may disrupt structure if the terminus is buried in the protein structure, or may directly affect function, as we saw for the C-terminal ε fusions.[24] While traditional cross-linking methodology using relatively short spacers has numerous variations that are useful and appropriate for studies of the spatial relationships of polypeptides, its application to questions of function have been limited. The use of derivatized PEG molecules for protein cross-linking offers a number of advantages. The substantial length of the PEG makes localized steric effects due to the proximity of globular proteins unlikely and avoids the distortion of protein structure that often accompanies the engineering of disulfide linkages directly between introduced cysteine residues. The hydrophilic nature of PEG reduces the potential for nonspecific interactions with protein surfaces. To our knowledge, the method we have developed is novel, but a number of types of applications can be envisaged. In the present work we have used this approach to block rotation of the ATP synthase and show that the enzyme is loosely coupled. In the field of molecular motors, the attachment of cargo molecules to motor proteins can be useful in studying rotational aspects, as we have done, or in quantifying force generation. More generally, flexible PEG linkers can maintain physical proximity between two molecules, fostering their interaction without dictating its molecular details. Ordinarily, then, one would expect that native-like interactions would be formed. This property makes the technique suitable for flexibly and artificially linking individually expressed domains so that the functional importance of the polypeptide linkage normally connecting them might be determined. We also see general applicability to proteins whose function requires the reversible assembly of complexes at the site of action, such as type I restriction endonucleases and transcription factor complexes. Finally, the method could be adapted to tag one subunit of an *in vitro*-assembled complex for purposes such as quantitation or affinity isolation.

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Table I. ATPase activity of reconstituted membrane vesicles[a]

Additions to reconstitution				Activity		
Stripped Membranes	WT F_1	ε-depleted F_1	ε type	Membrane-bound ATPase activity[b,c]	Released ATPase activity[b,c]	Normalized activity[d]
				units/mg	*units/mg*	
+	-	+	None	0.00 ± 0.01	0.00 ± 0.02	N/A
+	+	-	None	0.52 ± 0.02	1.18 ± 0.03	0.44
+	-	+	WT ε	0.42 ± 0.01	1.11 ± 0.05	0.38
+	-	+	ε-NEM	0.50 ± 0.02	1.22 ± 0.02	0.41
+	-	+	ε-PEG	0.60 ± 0.04	1.63 ± 0.05	0.37
+	-	+	ε-PEG-MBP	0.24 ± 0.05	1.13 ± 0.02	0.21

[a]Membranes vesicles were diluted into ATP hydrolysis assays under conditions that either maintain F_1 bound to membranes or cause its release. Data shown are the average of triplicate assays ± standard deviation. 1 Unit of activity is defined as 1 μmol product formed per min.

[b]Stripped membranes alone showed 0.02 ± 0.01 and 0.01 ± 0.01 U/mg in the bound and released assays respectively (uncorrected).

[c]Activity values were corrected for background activity of 0.23 U/mg (bound) and 0.28 U/mg (released) due to unbound F_1 detected in the sample reconstituted with only ε-depleted F_1.

[d]Membrane-bound activities were normalized to the activity released from membranes and expressed as the fraction of units bound per unit released.

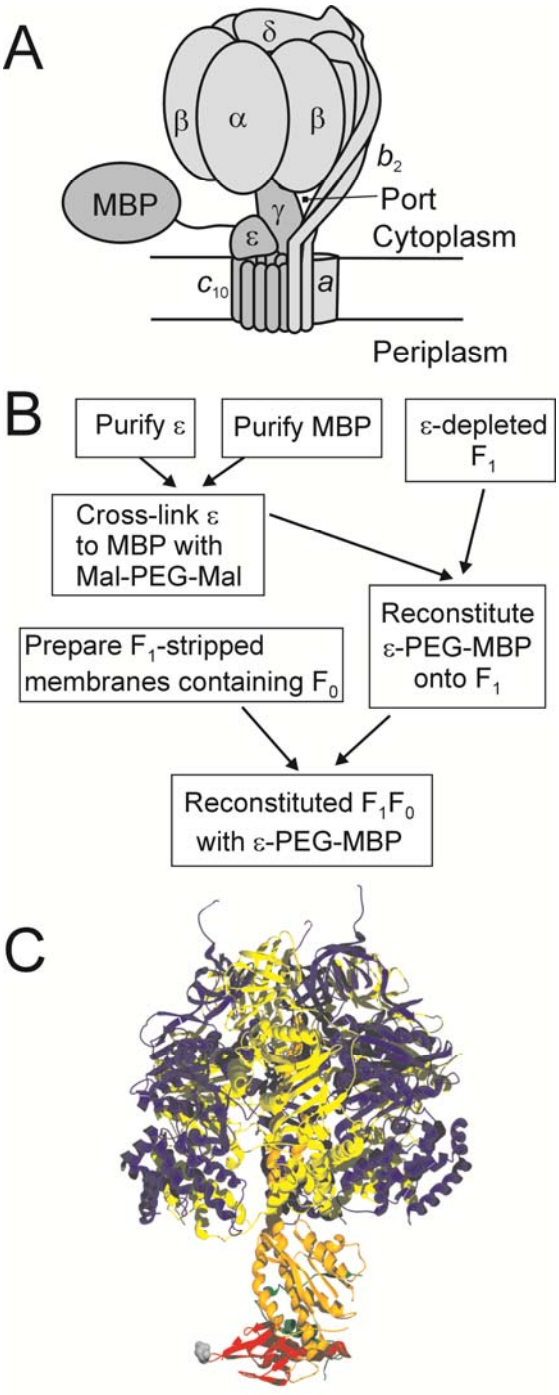

Figure 1. Tethering MBP to the rotor of ATP synthase. Schematic diagram (A) outlines the subunit composition of ATP synthase and the strategy employed to block rotation of the $\gamma\varepsilon c_{10}$ rotor (shown in dark grey) by chemically linking the 40-kDa maltose binding protein (MBP) to the ε subunit. Rotation would drag the MBP between the central and peripheral stalks, but it is too large to pass through the port. The dimensions of the enzyme are shown but the drawing is not exactly to scale. Flow diagram (B) outlines the reconstitution process to assemble ATP synthase complexes with ε-PEG-MBP. Crystal structure (C) of the bovine F_1 complex (PDB # 1E79) shows the homologue of bacterial ε in red with residue Glu-69 (the position homologous to His-56 in the *E. coli* ε sequence) in grey spacefill. The rotor subunit γ is in orange. The short polypeptide shown in green, located behind γ, is not present in the bacterial enzyme.

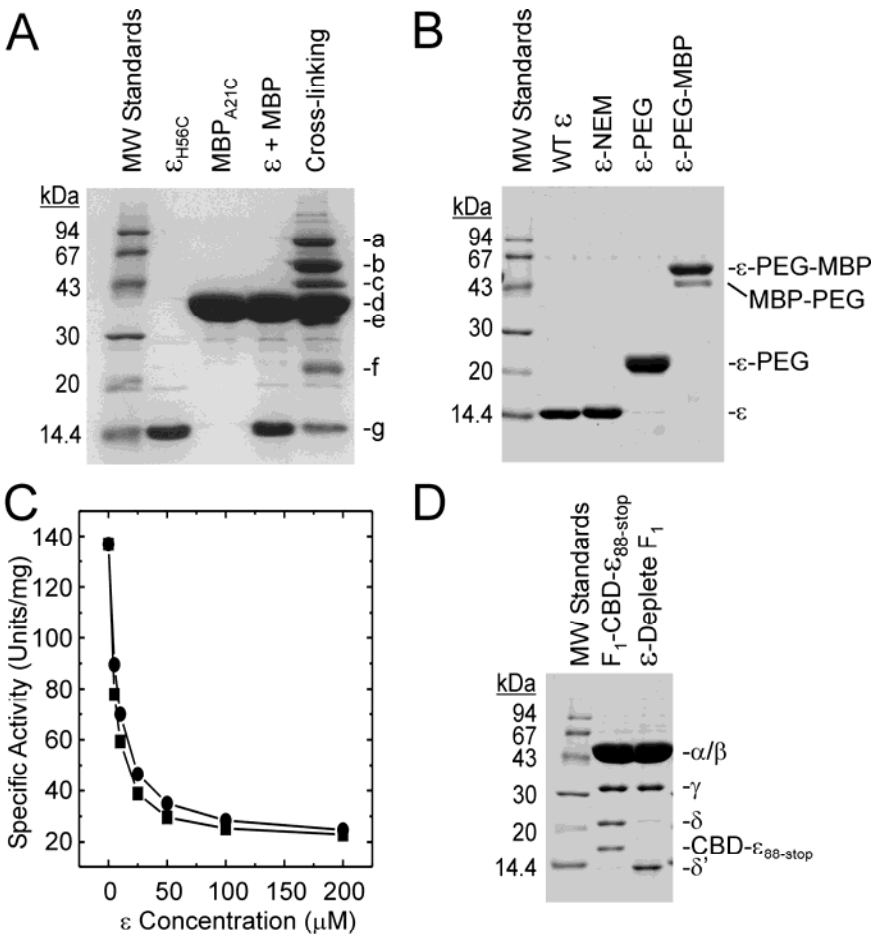

Figure 2. Cross-linking, preparation and characterization of samples. (A) Non-reducing SDS-PAGE analysis illustrating the cross-linking of MBP to ε. Samples of the individual purified proteins, a mixture at a 1:1 molar ratio before cross-linking, and the product mixture obtained after cross-linking with the PEG-bis-maleimide reagent are shown. The bands were assigned as: a, MBP-PEG-MBP; b, ε-PEG-MBP; c, MBP-PEG; d, unreacted MBP; e, ε-PEG-ε; f, ε-PEG; and g, unreacted ε, based on cross-linking experiments using the polypeptides and PEG-bis-maleimide at varying ratios (data not shown). (B) The purity of ε derivatives prepared in this study was analyzed by SDS-PAGE. (C) The effect of various concentrations of wild type ε (squares) and ε-PEG-MBP (circles) on the ATPase activity of F_1 was determined. (D) Purified F_1-ATPase containing CBD-ε_{88stop} (left) was depleted of CBD-ε_{88stop} by chitin affinity chromatography (right).

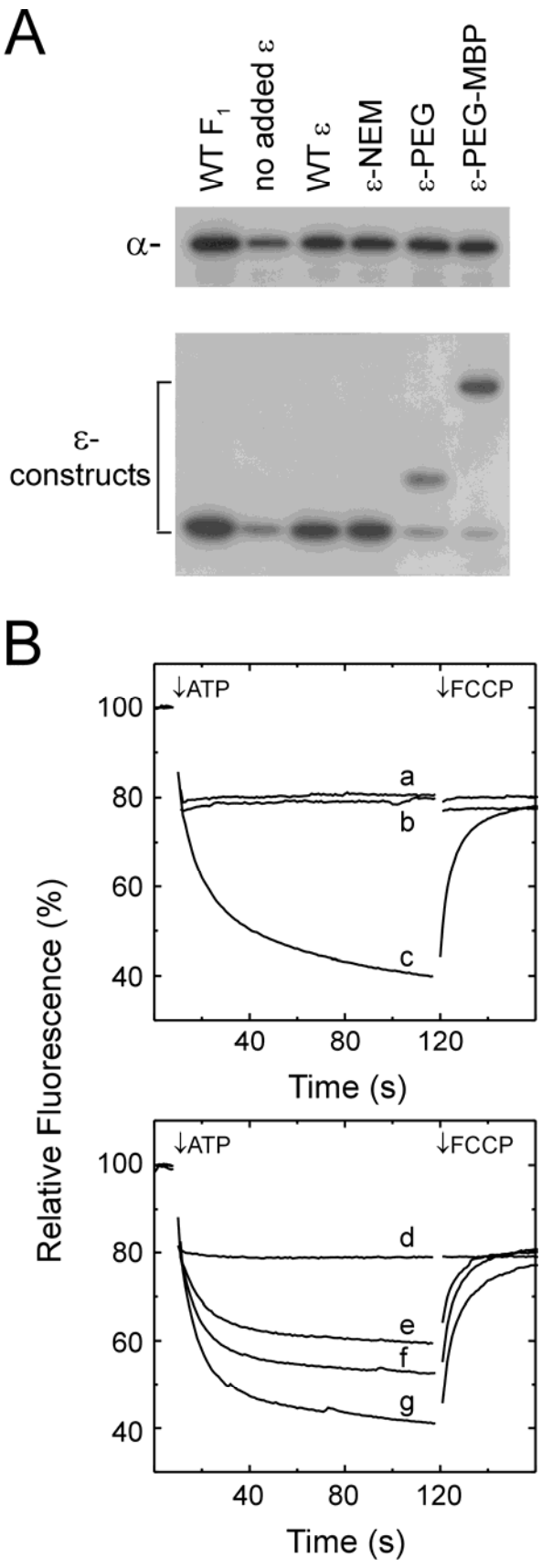

Figure 3. F_1-Depleted membrane vesicles were reconstituted with either wild-type F_1-ATPase (control) or ε-depleted F_1-ATPase in conjunction with the indicated ε-derivatives. (A) Samples of membrane protein (2 μg) carrying reconstituted ATP synthase were analyzed by western blotting. Blots were probed with ^{125}I labeled antibodies specific to α or ε. (B) ATP-Dependent H^+ pumping by reconstituted membranes was analyzed by ACMA fluroescence quenching. At the times indicated, ATP was added to 2 mM and the uncoupler carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) was added to 2 μM. Upper Panel: control experiments with traces representing H^+ pumping by membrane vesicles reconstituted with ε-depleted F_1, (a); no F_1 (b); and wild-type F_1, (c). Lower Panel: membranes carrying ATP synthase reconstituted with ε-depleted F_1-ATPase in conjunction with ε-PEG-MBP (d); wild-type ε, (e); ε-NEM, (f); and ε-PEG, (g).