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Rapid Estimation of Membrane Protein Orientation in Liposomes

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The topological organization of proteins embedded in biological membranes is crucial for the tight interplay between these enzymes and their accessibility to substrates in order to fulfil enzymatic activity. The orientation of a membrane protein reconstituted in artificial membranes depends on many parameters and is hardly predictable. Here, we present a convenient approach to assess this important property independent of the enzymatic activity of the reconstituted protein. Based on cysteine-specific chemical modification of a target membrane protein with a cyanine fluorophore and a corresponding membrane-impermeable fluorescence quencher, the novel strategy allows rapid evaluation of the distribution of the two orientations after reconstitution. The assay has been tested for the respiratory complexes bo_3 oxidase and ATP synthase of *Escherichia coli* and the results agree well with other orientation determination approaches. Given the simple procedure, the proposed method is a powerful tool for optimization of reconstitution conditions or quantitative orientation information prior to functional measurements.

Introduction

Biological membranes are two-dimensional barriers that separate cellular compartments with potentially different chemical properties. These membranes are packed with proteins that organize and mediate communication and molecule transport between the two sides of the membrane. A membrane protein (MP) can span the membrane in two possible orientations – right-side out as found in the intact cell or inside-out as found e.g. in inverted membrane vesicles. The orientation of a MP has direct consequences for the access to substrates and ligands and is therefore critical for its functionality. In cells, insertion is co-translational and mediated by the translation machinery,[1] and usually unidirectional. Insertion of MPs into artificial membranes such as liposomes, however, occurs in the absence of any auxiliary enzymes leading to more random orientation. The topological organization in liposomes is specific for each MP and depends strongly on the reconstitution method and conditions,[2] and it is hardly predictable. Highly unidirectional reconstitution has rarely been described, while a preference for one orientation is often observed.[3–8] Knowledge of the orientation distribution of the protein is highly desirable to design proteoliposome experiments and their quantitative analysis. In the past, relative orientation of MPs has been measured with functional assays if the substrate is membrane-impermeable, and enzyme activity is readily determined (e.g. ATPase activity,[9] NADH-dehydrogenase I activity[10]). If this is not possible (e.g. light-driven enzymes, membrane-permeable substrates, elaborate functional assays or non-enzymatic MPs), alternative methods have been applied. Functionally independent methods include side-selective protease digestion of reconstituted MPs and subsequent analysis via SDS-PAGE[5,11] or site-specific biotinylation of cysteine residues followed by Western Blot analysis.[12] Both methods are rather time-consuming and proteolysis approaches are likely to be limited to small proteins as band pattern complexity increases for larger proteins (for recent review on the topic, see Ref. [3]).

In the present work, we set out to establish a straightforward and rapid assay to measure MP orientation in liposomes independent of function and applicable to a wide variety of MPs (Figure 1A). The assay is based on the recent observation that certain cyanine fluorophores (e.g. Cy5 or DY647P1) can be rapidly and selectively quenched by the membrane-impermeable chemical Tris(2-carboxyethyl)phosphine (TCEP), a widely used reductant.[13] We have selectively labeled single-cysteine mutants of the *E. coli* multi-subunit respiratory enzymes bo_3 oxidase and ATP synthase with the fluorescent dye DY647P1 based on maleimide chemistry. The labeled protein was reconstituted into liposomes and fluorescence of DY647P1 was monitored before and after addition of TCEP to determine the amount of fluorophore accessible to the quencher. Finally, liposomes were solubilized, allowing TCEP to quench the remainder of fluorophores, and orientation was calculated as the ratio of initial to total quench.

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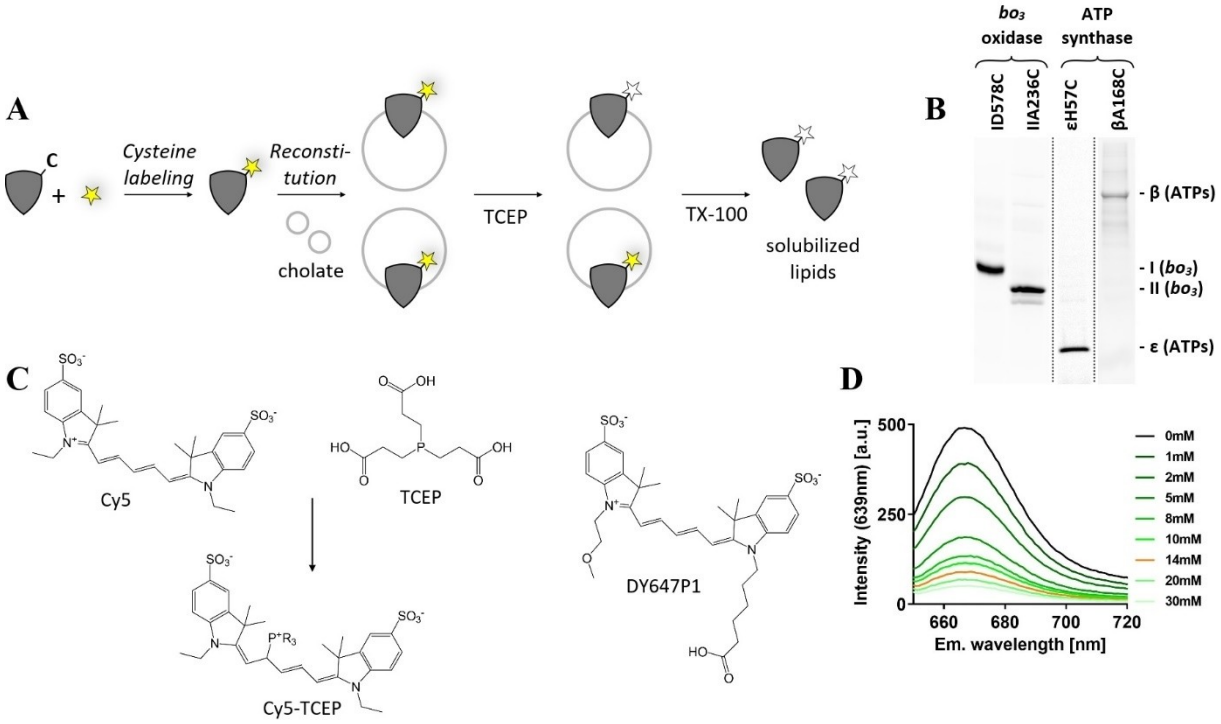

Figure 1. A) Scheme of the TCEP-based orientation determination assay. A single-cysteine MP is labeled with DY647P1 via maleimide chemistry and reconstituted into liposomes partially solubilized with cholate. The two possible orientation populations are sequentially quenched. In a first step, outwards oriented dye is quenched by TCEP addition, while residual fluorophores are quenched upon solubilization of liposomes by Triton X-100 in a second step. B) Fluorescence image of SDS-PAGE from different single-cysteine bo_3 oxidase-DY647P1 and ATP synthase-DY647P1 mutants. bo_3 oxidase subunits I and II as well as ATP synthase subunits β and ε are indicated on the right side. C) Chemical structures of cyanine dye Cy5 and quencher TCEP as well as Cy5-TCEP adduct, and DY647P1. D) Emission scan (λex = 639 nm) of TCEP titration to 100 nM DY647P1-labeled bo_3 oxidase in 250 mM Tris-HCl pH 8.5 containing 0.05% Triton X-100.

Results

Protein labeling and setup of experiment

The motivation to develop such an assay is our interest in terminal quinol oxidases, such as bo_3 and *bd* oxidase of *E. coli*. They react with membrane-embedded ubiquinol and reduce molecular oxygen to water, coupled to proton pumping. Thereby, they establish a directed proton motive force (*pmf*) across biological membranes, fueling ATP synthesis by the F_1F_0 ATP synthase or many other *pmf*-driven processes such as nutrient uptake by secondary transport proteins. As ubiquinol binding happens in the transmembrane part of the enzyme,[14] both populations are activated in the presence of ubiquinol and cancel each other's *pmf* out, complicating quantitative interpretation of proton transport or ATP synthesis measurements. The recent observation of Vaughan et al. that Cy5, but not Cy2 or Cy3, is rapidly quenched by TCEP (Figure 1C) via 1,4-addition at the γ-carbon of the polymethine bridge[13] prompted us to exploit this property. The overall scheme of the experiment is depicted in Figure 1A. Purified bo_3 oxidase labeled with Cy5 on a single-cysteine mutant is reconstituted into liposomes, yielding proteoliposomes with an unknown orientation distribution of bo_3 oxidase. Addition of a sufficiently high TCEP concentration quenches selectively fluorophores located on the outside of the liposomes, as TCEP cannot readily cross membranes.[15] Further addition of a minimal but sufficient amount of detergent destabilizes the liposomes, allowing TCEP to reach the remainder of the fluorophores located on the inside. Several closely related dyes to Cy5 are commercially available and due to its slightly higher solubility and lower price, we have settled for DY647P1 (Figure 1C) in our experiments.

Consistent with,[13] efficient quenching was reached with the deprotonated phosphorus form of TCEP ($pK_a = 7.66$[16]) and experiments were thus performed in high buffer at pH 8.5.

The eight natural cysteines in bo_3 oxidase (except C25 on subunit II, which is palmitoylated[17]) were replaced by alanine using molecular biology techniques and a single cysteine was introduced either at position D578 in subunit I (*N*-side of bo_3; see Figure 2A) or at position A236 in subunit II (*P*-side of bo_3). Care was taken to choose sites at the distance from the membrane with good aqueous accessibility. Cysteines were selectively labeled via maleimide derivatization[18] and specificity of the labeling towards the respective subunits was confirmed using SDS-PAGE and fluorescence detection (Figure 1B). The lack of a fluorescent band for subunit II in the ID578C sample shows the inaccessibility of the remaining C25 for DY647P1 labeling due to palmitoylation.

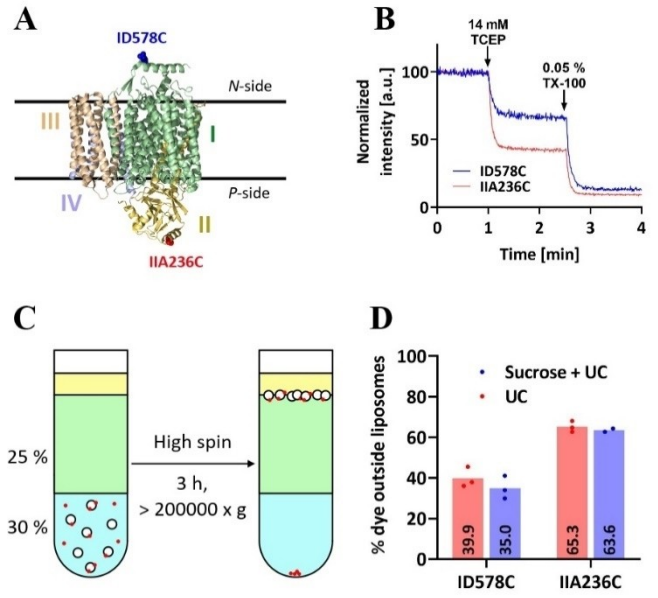

Figure 2. A) Cartoon representation of *E. coli* bo_3 oxidase structure (PDB 6WTI) with subunit I (pale green), II (yellow), III (pale orange), IV (light blue). Residues ID578C and IIA236C used for single cysteine labeling are depicted in blue and red spheres, respectively. B) Raw data of a typical TCEP-based orientation measurement. After monitoring a baseline, TCEP is added, yielding a rapid drop in fluorescence, indicating quenching of the accessible fluorophores by TCEP. A second quench is obtained after addition of detergent to solubilize liposomes, rendering all fluorophores accessible to TCEP quenching. C) Principle of liposome flotation assay to separate non-reconstituted MPs from proteoliposomes. Reconstituted liposomes are mixed with a final concentration of 30% sucrose (blue) and layered in an ultracentrifugation tube with 25% sucrose solution (green) and finally with buffer (yellow). After centrifugation (3 h, $>200{,}000\times g$) in a fixed-angle rotor, liposomes floating in the interface of the two top layers are collected while non-reconstituted or aggregated MP is pelleted. D) Percentage of dye oriented to the outside of liposomes of bo_3 oxidase mutants ID578C and IIA236C after reconstitution followed by ultracentrifugation (UC) or liposome flotation assay (Sucrose + UC), respectively. Inside-out and right-side out oriented bo_3 oxidase populations are determined by ID578C and IIA236C, respectively.

To find an optimal TCEP concentration for our quenching experiments, DY647P1-labeled bo_3 oxidase solubilized in detergent solution was titrated with TCEP up to 30 mM. For the remainder of the experiments, 14 mM was used which resulted in >85% quenching (Figure 1D). Linear dilution effects by addition of aqueous quencher and detergent solutions were considered during data evaluation.

Orientation measurement of bo_3 oxidase

Raw data from a typical TCEP-based orientation determination measurement is depicted in Figure 2B. Liposomes (10–100 µL) containing ID578C-labeled bo_3 oxidase were suspended in measurement buffer (1.4 mL) and a fluorescence baseline was recorded (corresponding to 100% fluorescence: Figure 2B blue line). After ~1 min, 14 mM TCEP was added from a 1 M stock solution and a rapid fluorescence decrease to ~70% was observed. Addition of 0.05% Triton X-100, allowing TCEP to access and quench dyes located on the inside of liposomes, decreased fluorescence to 5–10% of the starting value. Orientation, or rather distribution of the two enzyme populations, was calculated from the ratio of the first to the total quench (Figure S1). Similar results were obtained if liposomes were solubilized with 0.05% DDM instead of Triton X-100, indicating that the second quench can be induced also by other detergents. The experiment displayed in Figure 2B yields a ~40% inside-out orientation of bo_3 oxidase, well in agreement with estimations made from single molecule studies (~70% right-side out[8]).

To validate our method, we repeated the experiment with bo_3 oxidase labeled at position IIA236 on subunit II, being on the opposite side of the membrane than ID578 (Figure 2A). Convincingly, the results are inverted, and the first quench was now larger than observed with ID578 mutant (Figure 2B red line), yielding a ~65% orientation right-side out. Theoretically, the relative values of the first quench of the two experiments should add up to 100%. Data from three independent measurements however yielded a slightly higher value of 105–110% (Figure 2D, red bars), which indicates an overestimation of the first quench. A possible reason for an overestimation is non-reconstituted MP, either aggregated or soluble, that will contribute to the first quench. We have addressed this problem using an additional liposome purification step termed liposome flotation assay (Figure 2C). Here, the liposome suspension is deposited on a two-step sucrose gradient and centrifuged for 3 h. Non-incorporated or aggregated proteins have a higher density and will be pelleted on the bottom of the tube, while the lighter proteoliposome fraction floats on top of the upper sucrose layer.[19,20] The results from these experiments indeed affected the orientation ratio in the expected direction and the total sum was now close to 100% (Figure 2D, blue bars; for more detail see Figure S2).

Recently, Yue et al.[21] and Huang et al.[22] have used a related approach to verify calculations that predict peptide insertion into membranes. Peptides were labeled using TAMRA and fluorescence quenching was titrated by tryptophan addition to the solution. Using the Stern-Volmer equation describing the quenching behavior, the relative orientation of the peptides in the membrane was calculated.

We have adapted this approach and titrated liposomes containing reconstituted bo_3 oxidase (labeled with DY647P1 either at ID578 or IIA236) with different amounts of TCEP (Figure 3A). Although quenching of DY647P1 by TCEP is based on a covalent bond formation, the concentration dependent quenching followed the Stern-Volmer relationship (Supplementary Figure S3A). We therefore applied the same equation as Yue et al.[21] used for tryptophan-TAMRA quenching. The apparent Stern-Volmer quenching constant for the different enzymes was determined in the absence of liposomes but in presence of 0.05% Triton X-100 (Equation (1), see Experimental Section; Figure S3A). In a second step, TCEP was titrated to reconstituted liposomes and the data was fitted with Equation (2) (see Experimental Section; Figure 3A, S3B), allowing calculation of bo_3 oxidase orientation in liposomes. As shown in Figure 3B, the values obtained with the rapid method above

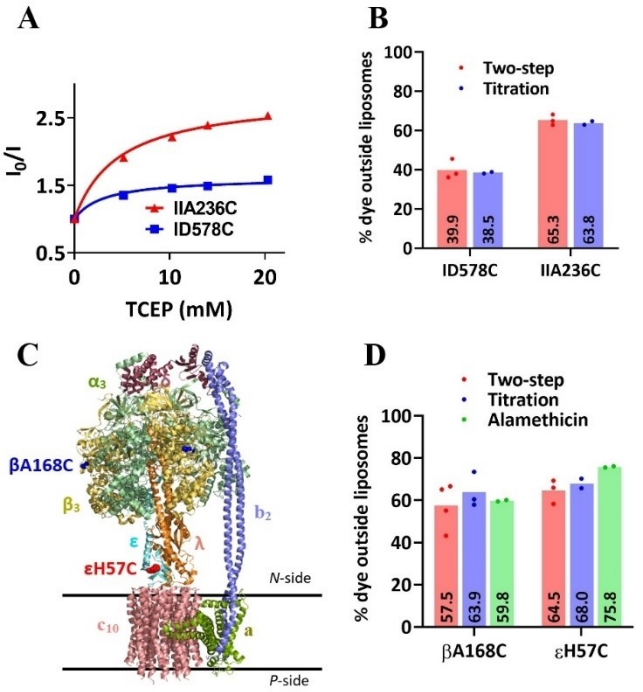

Figure 3. A) Fluorescence quenching titration assay.[21,22] TCEP was titrated to liposomes reconstituted with DY647P1-labeled bo_3 oxidase mutants ID578C and IIA236C for orientation determination by fitting the data to Equation (2) (see Experimental Section). The apparent Stern-Volmer constant was measured for both mutants in detergent solution (see Figure S3B). B) Comparison of two step and titration quenching method. bo_3 oxidase orientation was determined either by the here presented TCEP-based method (Two-step) or by fluorescence titration (Titration)[21,22] for both ID578C and IIA236C mutants (see text for details). C) Overall structure of the *E. coli* ATP synthase (PDB 6OQU). βA168 and εH57 are shown in blue and red spheres, respectively, and the membrane is depicted by lines. D) Orientation of *E. coli* ATP synthase was determined by the here presented TCEP-based method (Two-step), by fluorescence quenching titration (Titration)[21,22] or by a function-based alamethicin assay (see Figure S4)[9] for both mutants βA168C and εH57C. For the fluorescence quenching titration assay, the Stern-Volmer constant was measured for both mutants in detergent and the data were fitted with Equation (2) (see Experimental Section; Figure S3).

(red bars) correlated well with the more laborious titration experiments (blue bars).

Orientation measurement of ATP synthase

The ATP synthase is a highly conserved enzyme that uses the *pmf* generated by terminal oxidases such as bo_3 oxidase to catalyze ATP synthesis from ADP and phosphate by its rotational mechanism. The enzyme harbors a relatively small membrane part (F_0) and a large mushroom-like extra-membrane part (F_1 part, consisting of $\alpha_3\beta_3\gamma\varepsilon\delta$ and harboring ADP/ATP binding site; Figure 3C). To determine the orientation of reconstituted ATP synthase, its reversible reaction of ATP hydrolysis is exploited typically in an ATP regenerating assay.[9,23,24] As ATP is not membrane-permeable, its addition only leads to ATP hydrolysis from the enzymes with F_1 heads oriented towards the outside. After addition of alamethicin, a membrane pore-forming agent,[25] ATP can also access the internally located ATP binding sites and total ATP hydrolysis can be determined (Figure S4). To compare this activity-based method with the two fluorescence-based methods, two single-cysteine variants in the F_1 part were constructed. Single cysteines either located in subunit β at position A168 or at position H57 of subunit ε were labeled with DY647P1 using maleimide chemistry as described above. In contrast to labeling at subunit ε, labeling at subunit β was poor, despite the presence of three cysteine copies per enzyme (see discussion). Figure 3D shows that the three different assays yielded similar results for each mutant. Interestingly, however, a significant discrepancy of the orientation distribution between the two mutants was observed that is discussed in detail below.

Discussion

Reconstitution of purified MPs is a powerful technique to investigate the function and reaction mechanism of an enzyme, and is widely used for respiratory enzymes, primary and secondary transporters, and receptors. The best-known reconstitution technique was established by Rigaud[26] and many other colleagues and is based on formation of a ternary complex of preformed liposomes, detergent and MP. After an appropriate incubation time, the detergent is removed by one of several established techniques. Here, we have employed sodium cholate as a detergent and have destabilized preformed liposomes before the MP was added. The small cholate micelles were then removed by a Zetadex-25 gel filtration column. Liposome reconstitution is accompanied by an unpredictable distribution of the two possible enzyme orientations after the ternary complex has been formed and the detergent is removed. For a variety of reasons, it is interesting to determine the relative protein orientation, but no general and convenient methods exist.[3] Here, we took advantage of the membrane-impermeability of TCEP to quench Cy5 related fluorophores site-specifically attached to MPs to distinguish the inside-out from right-side out oriented population by quenching the fluorescence of the two populations sequentially.

Care has to be taken that labeling of the MP occurs specifically and that uncontrolled labeling is suppressed. We have chosen maleimide-based labeling of single-cysteine variants of the enzymes that occurs specifically with reasonable reaction rates. Further strategies are *N*-terminal amine labeling (at lower pH) or fusion of SNAP or HALO Tag to the protein of interest. Alternatively, fast and specific labeling can be achieved by bioorthogonal reactions if an unnatural, click-chemistry competent amino acid is introduced in the protein using amber tRNA suppression methods (e.g. amino acids containing alkine, azide, *trans*-cyclooctene handle).[27] Complete labeling of the enzyme population is not necessary, but a sufficient fluorescence signal is desirable for reliable orientation determination. If downstream experiments are planned with the same liposomes, the labeled protein should be tested for activity (all mutants tested here retained protein function). We suggest a location of the cysteine at the protein surface to ensure good accessibility to the aqueous environment, allowing rapid label-

ing and efficient quenching by TCEP. While the bo_3 oxidase mutants ID578C and IIA236C showed similar labeling efficiencies, ATP synthase mutant βA168C was significantly less labeled than εH57C. For optimal results, several single-cysteine variants of the protein of interest should be tested. The protocol described here was not tested with fluorophores attached to protein residues close to the membrane surface, but we would not recommend such constructs. Although not described for Cy5, fluorophores typically react highly sensitively to their local environment, and accessibility of a negatively charged compound as TCEP might also be affected by the charged (negative or zwitterionic) surface of a lipid bilayer.

Fluorescence of DY647P1 was measured at excitation and emission wavelengths 649 nm and 672 nm, respectively. In an effort to minimize light scattering effects, we blueshifted the excitation to 580 nm, while keeping emission at 672 nm, yielding a lower starting fluorescence that required more liposomes per measurement. No difference of orientation values was observed between the two settings and we therefore suggest to use $\lambda_{ex} = 649$ nm to minimize sample volume.

TCEP quenching is most efficient if the solution pH is above the phosphorous pK_a of TCEP (7.66[16]) and membrane impermeability is warranted by the three deprotonated carboxylic acid side chains. If one or more of the acids is esterified, the pK_a decreases to <5 for the triply esterified version, however at the expense of membrane permeability.[15] Measurement conditions of pH 8.5, however, are unproblematic and liposome integrity is not affected. High buffer capacity was used to avoid any changes on pH after addition of the acidic TCEP stock solution. In our experiments, we settled for 14 mM TCEP, showing $>85\%$ quenching and allowing a rapid experimental procedure.

We have verified the working principle of our method using two bo_3 oxidase mutants that are labeled on opposite sides of the membrane. In these, the first quench reflects thus either the fraction of inside-out or the fraction of right-side out oriented MPs. In theory, the sum of the two experiments should add up to 100%, but it was found to be slightly above.

A likely explanation is that incorporation of protein into liposomes is not complete and that a remainder is dissolved or suspended as protein or protein-lipid aggregates. The fluorescence of these non-incorporated proteins will be quenched after addition of TCEP (first quench), leading to an overestimation of the outwards oriented fluorophore population. These aggregates are neither removed during reconstitution nor during the following ultracentrifuge collection. Non-continuous density gradients have been used to separate liposomes from non-incorporated protein or even differentiate between empty liposomes and liposomes containing protein, but these require centrifugation times >12 hours. A more rapid approach is the liposome flotation assay,[19,20] in which proteoliposomes are mixed with a 30% sucrose solution and layered with an equal volume of 25% sucrose and a thin buffer layer and subjected to ultracentrifugation for three hours. The liposome fraction devoid of non-incorporated protein migrates to the top of the gradient and can be conveniently collected and used for orientation measurements or activity measurements. We found that the procedure indeed decreases the first quench in all our measurements and is therefore recommended for more exact results. However, a solid estimation of MP orientation is also possible without this additional step, providing the method to be a fast and easy assay to estimate the orientation of MPs prior to other experiments or for reconstitution optimization.

We further compared our rapid two-step method with the results obtained by a fluorescence quenching titration assay[21,22] and found very similar results. In the latter, it is not necessary to solubilize the liposomes and the fraction of outwards oriented fluorophore is calculated using the Stern-Volmer equation. The method, however, relies on the Stern-Volmer constant that must be determined prior to orientation determination in the absence of liposomes. Finally, we tested our method with reconstitution of the *E. coli* ATP synthase and compared it with another method based on enzyme activity. Here, membrane-impermeable ATP is added to liposomes and ATP hydrolysis is first assessed on the outside, before pore-forming alamethicin is added to allow ATP to reach also the catalytic sites of the inwardly-oriented ATP synthases. It is preferred to not use detergents in functional assays, as these often affect turnover number of enzymes (typically increasing) by uncoupling them.[3,9,28] Results of this function-based assay supported the accuracy of our novel method.

We were surprised to see the relatively random orientation of the *E. coli* ATP synthase (60 to 75% with the F_1 head towards the outside). In an earlier work, our group found an almost unidirectional orientation of $>90\%$ with the same enzyme using Western Blotting, and a recent publication reports around $75 \pm 20\%$.[9] While the enzyme used in these experiments was always the *E. coli* ATP synthase, the purification protocols and the reconstitution conditions were not identical. In the experiments with highest level of orientation, purified protein was reconstituted into liposomes of high density (30 mg/mL), while here and in the recent report, only 10 mg/mL or 5 mg/mL were used, respectively. It is beyond the scope of the present manuscript to discuss the possible implications in detail, but such differences in liposome concentration can lead to different levels of liposome solubilization and thus a different kinetics of protein insertion into liposomes can be envisioned as discussed in impressive detail for lacS.[6] We also attribute the observed difference in orientation with the ATP synthase labeled either at βA168C or εH57C to similar phenomena. Not only were they purified using two different purification protocols (see Experimental Section), but the former was also less efficiently labeled, yielding a larger enzyme volume used for reconstitution for βA168C than for εH57C. This increases also the detergent concentration during reconstitution and likely affects the properties of ternary complex and the kinetics of detergent removal, two important properties that influence orientation.[3,6,26,29] These findings underline the importance and usefulness of a rapid assay that assesses the influence of several parameters during reconstitution, independent of the protein's function, which is often sensitive to experimental parameters (e.g. lipid composition, detergent used, kinetics of detergent removal).

In conclusion, we provide methodology and testing of a robust and straightforward estimation of MP orientation after reconstitution into liposomes. Although it is generally applicable, it must be optimized for every single protein by finding an appropriate position for labeling. However, once this is done, the method is rapid and powerful, and determination of orientation can be performed before every measurement. To increase the accuracy of the determination, we suggest performing a liposome flotation assay to remove non-incorporated protein. This additional procedure will further allow to determine the efficiency of the reconstitution process by comparing total fluorescence before and after the flotation assay. Here, it is important to critically monitor the exact volumes of the different fractions and correct for dilution effects during the procedure. This is not critical for the determination of the orientation, however, since a ratiometric value is obtained that is independent of the liposome yield in the flotation assay.

Experimental Section

Expression of ATP synthase

ATP synthase variants βA168C and εH57C were constructed from cysteine-free plasmid pFV2[30] using standard molecular biology techniques and constitutively expressed in *E. coli* DK8 cells (lacking the whole ATP operon). Cells were grown in LB medium containing 100 μg/mL ampicillin and 1 mM $MgCl_2$ from precultures for at least 4–5 h in a LEX48 system (epiphyte3) at 38 °C.

Purification of ATP synthase variant βA168C

Cells were harvested by centrifugation and broken by 3 passes through MAXIMATOR (HPL6 High-Pressure Homogenizer, Maximator AG) at 1200 bar at 2 °C in Buffer A (50 mM HEPES pH 8, 100 mM NaCl, 5% glycerol) containing DNase I (spatula tip) and protease inhibitors PMSF (0.1 mM) and Pefabloc (spatula tip; Biomol). After removal of cell debris (centrifugation at 5000×*g* for 0.5 h, 4 °C), membranes were pelleted by ultracentrifugation (175,000×*g*, 1.5 h, 4 °C) and resuspended in 10 mM Tris-HCl pH 7.5 (1 mL per g of wet cells). For solubilization, homogenized membranes were diluted with 2× solubilization buffer S (50 mM HEPES pH 7.5, 100 mM KCl, 250 mM sucrose, 20 mM imidazole, 40 mM 6-aminohexanoic acid, 15 mM P-aminobenzamidin, 5 mM $MgSO_4$, 0.1 mM Na_2-EDTA, 0.2 mM DTT, 0.8% soy bean type_II asolectin, 1.5% *n*-octyl β-D-glucopyranoside, 0.5% sodium deoxycholate, 0.5% sodium cholate, 2.5% glycerol) in a ratio of 1:1 and incubated at 4 °C for 1.5 h while stirring. Non-solubilized material was removed by ultracentrifugation (200,000×*g*, 30 min, 4 °C) and the supernatant was looped on a prepacked 5 mL HisTrap column (GE Healthcare) equilibrated with buffer S at 4 °C for 2 h. The column was washed with 4 column volumes (cv) of buffer S containing 40 mM imidazole and 2 cv of buffer S containing 90 mM imidazole. Purified protein was eluted with buffer S containing 250 mM imidazole and fractions containing ATP synthase were identified by ATP regenerating assay[23] and pooled. The pooled fraction was divided into aliquots, frozen in liquid nitrogen and stored at −80 °C.

Purification of ATP synthase variant εH57C

Cells were harvested and membranes were prepared as described above in Buffer B (50 mM MOPS/NaOH pH 8, 100 mM NaCl, 5 mM $MgCl_2$, 5% glycerol). Pelleted membranes (2 mL per g of wet cells) were resuspended in Buffer C (50 mM MOPS/NaOH pH 8, 100 mM NaCl, 5 mM $MgCl_2$, 30 g/l sucrose, 10% glycerol). For solubilization, LMNG (Anatrace) was added to a final concentration of 2% from a 5% stock solution. After the suspension was stirred for 30 min at room temperature and 30 min at 4 °C in presence of 1 mM PMSF, 5 mL of Buffer C was added per g of membranes and non-solubilized material was removed by ultracentrifugation (200,000×*g*, 0.5 h, 4 °C). The supernatant was loaded onto a prepacked 5 mL HisTrap column (GE Healthcare) in presence of 10 mM imidazole via loop loading for 2 h at 4 °C. Bound protein was eluted via gradient elution from 20 mM to 400 mM imidazole in Buffer C containing 0.005% LMNG. Fractions containing ATP synthase were identified by ATP regenerating assay,[23] pooled and concentrated with a 100 kDa MWCO Amicon Ultra-15 filter (Merck Millipore). The pooled fraction was divided into aliquots, frozen in liquid nitrogen and stored at −80 °C.

Expression and purification of cytochrome bo_3 oxidase variants

bo_3 oxidase mutants were expressed in *E. coli* strain C43Δcyo[31] cells containing the appropriate plasmid based on pETcyo[32] encoding for the entire *cyo* operon. Cells were grown either in M63 minimal medium (3 g/l KH_2PO_4, 7 g/l K_2HPO_4, 0.5 mg/l $FeSO_4$, 100 μg/mL ampicillin, 1 mM $MgSO_4$, 100 mg/l thiamine, 10 μM $CuSO_4$, 0.2% glucose, 0.2% NH_4Cl) containing 100–200 μg/mL ampicillin in a LEX48 system at 38 °C. Expression was induced at an OD_{600} of 0.5–1 with 1 mM IPTG (Santa Cruz) followed by an additional incubation at 38 °C for at least 4–5 h. Cells were harvested by centrifugation, resuspended in Buffer D (50 mM HEPES pH 8.3, 5 mM $MgCl_2$) containing DNase I and protease inhibitors PMSF (1 mM) and Pefabloc (spatula tip; Biomol) and lysed by 3–4 passes through MAXIMATOR (HPL6 High-Pressure Homogenizer, Maximator AG) at 2 °C. After cell debris was removed by centrifugation (8000×*g*, 0.5 h, 4 °C), membranes were harvested by ultracentrifugation (200,000×*g*, 1 h, 4 °C) and resuspended in Buffer E (50 mM K_2HPO_4, pH 8.3) containing 5 mM imidazole. Solubilization was performed with 1% DDM (Glycon Biochemicals GmbH) for 2 h at 4 °C, followed by ultracentrifugation (200,000×*g*, 45 min, 4 °C). Solubilized protein was loaded on prepacked 5 mL HisTrap columns (GE Healthcare), washed with buffer E containing 0.05% DDM and 35 mM imidazole and eluted with the same buffer containing 100 mM imidazole. Fractions containing bo_3 oxidase were pooled and concentrated with a 100 kDa MWCO Amicon Ultra-15 filter (Merck Millipore). The pooled fraction was divided into aliquots, frozen in liquid nitrogen and stored at −80 °C.

Site-specific labelling with DY647P1-maleimide

Labeling was essentially performed as described.[18] Purified protein (20–40 μM for bo_3 oxidase mutants and 2–5 μM for ATP synthase mutants) was diluted in a ratio of 1:5 with maleimide reaction buffer (20 mM HEPES pH 6.5, 100 mM KOAc, and either 0.05% DDM (bo_3 oxidase) or 0.005% LMNG (ATP synthase)) to adjust the pH. Cysteines were reduced by the addition of 0.4 mM TCEP and the samples were incubated overnight at 4 °C (end-over-end rotation) with a 10-fold excess of DY647P1-maleimide (Dyomics GmbH). Excess dye was removed by gel filtration using CentriPure P10 column (emp Biotech GmbH) and three cycles of diluting and concentrating with an Amicon Ultra-15 filter (Merck Millipore).

Liposome preparation

Lipids were dissolved in chloroform and mixed in a PC/DOPG (LIPOID E PC S; LIPOID PG 18:1/18:1) ratio of 60/40% (w/w). Chloroform was evaporated overnight in a desiccator and lipids resuspended in liposome buffer L (50 mM MOPS-BTP, pH 6.75) at a concentration of 40 mg/mL. The liposomes were made unilamellar by 7 cycles of freezing (liquid nitrogen), thawing (at 29.4 °C) and 10 s vortexing. Liposomes were aliquoted, frozen in liquid nitrogen and stored at −80 °C. Directly before using liposomes, an aliquot was thawed at 29.4 °C, diluted with buffer L to 10 mg/mL and extruded 21 times through a Whatman polycarbonate membrane (Sigma-Aldrich) with a 100 nm pore size.

Reconstitution of membrane proteins

Reconstitution of ATP synthase or bo_3 oxidase was performed as described by von Ballmoos et al.[33] Briefly, liposomes were partially solubilized by 0.4% sodium cholate (from a 30% stock solution) before enzymes were added. We used varying amounts of protein to adjust for fluorescence signal (3–5 proteins per 100 nm liposome; lipid : protein ratios (w/w) were = 120–140 for bo_3 oxidase and 25–100 for ATP synthase). The mixture was incubated for 30 min at 4 °C with occasional flicking of the tube, followed by removal of the detergent by gel filtration (CentriPure P10, emp Biotech GmbH). Equilibration and elution usually were done with Reconstitution buffer (100 mM MOPS pH 7.5, 25 mM K_2SO_4 1 mM $MgCl_2$). Liposomes were either pelleted by ultracentrifugation (Ti 70.1 rotor, 200,000 × *g*, 1 h, 4 °C) or subjected to a liposome flotation assay (see below).

Liposome flotation assay

Non-reconstituted enzyme was separated from the liposome mixture by a sucrose gradient after reconstitution as described[19,20] with minor adaptations. Briefly, 1.2 mL of P10 eluate after reconstitution was mixed homogenously with 1.6 mL 60% sucrose (dissolved in Reconstitution buffer) and 400 µL Reconstitution buffer to get a final concentration of 30% sucrose. In a Beckman Type 70.1 Ti rotor tube, the mixture was then carefully layered first with 4 mL 25% sucrose and then with 800 µL Reconstitution buffer. All solutions were precooled to 4 °C to improve layering experience. Sucrose gradients were centrifuged in the fixed-angle Beckman Type 70.1 Ti rotor at 200,000 × *g* for 3 h at 4 °C, setting acceleration and deceleration (coast) to the minimum. The liposome layer was removed from the sucrose gradient (~1 mL) and liposomes were pelleted by ultracentrifugation at 200,000 × *g* for 45 min at 4 °C to remove sucrose. Pelleted liposomes were resuspended in 200 µL Reconstitution buffer.

Orientation determination by TCEP-based assay

For the TCEP-based orientation determination assay, 10–100 µL liposomes was diluted in 1.4 mL 250 mM Tris-HCl, pH 8.5 and fluorescence of DY647P1 was monitored (excitation 649 nm or 580 nm, emission 672 nm) on a Cary Eclipse Fluorescence Spectrometer (Agilent Technologies). After the baseline was stable (~1 min), 14 mM TCEP was added from a 1 M stock solution, leading to a first quenching plateau. After ~2.5 min, 0.05% Triton X-100 from a 20% stock solution was added and fluorescence monitored until the signal was stable (~5 min). The orientation was determined by calculating the ratio between the first and the total quench. Dilution effects from TCEP and Triton X-100 adding were considered during calculation, assuming a linear decrease of fluorescence proportional to dilution of fluorophore.

Fluorescence quenching titration assay

Fluorescence quenching titration experiments were performed as described in Yue et al.[21] First, ~100 nM labelled bo_3 oxidase or ATP synthase solubilized in 250 mM Tris-HCl, pH 8.5, 0.05% Triton was mixed with increasing TCEP concentrations (0–20 mM) and fluorescence quenching was monitored as described above. The apparent Stern-Volmer constant (K_{sv}) was obtained from fitting the obtained fluorescence ratios to the Stern-Volmer equation (Equation (1)) (Prism software, GraphPad).

$$\frac{Io}{I} = 1 + Ksv\ [TCEP] \tag{1}$$

As described in Yue et al.,[21] the percentage of outside oriented dye (x) in liposomes can be determined with various fluorescence ratios obtained with increasing TCEP concentration using the previously calculated Ksv according to the following formula:

$$\frac{Io}{I} = \frac{1 + Ksv^*[TCEP]}{(1-x)^*(1 + Ksv^*[TCEP]) + x} \tag{2}$$

Alamethicin assay

ATP hydrolysis based orientation measurements were performed according to Biner et al.[9] In brief, ATP hydrolysis of proteoliposomes was measured with ATP regenerating system that couples ATP regeneration to NADH oxidation[23] in presence of 100 nM valinomycin and 2 µM FCCP to prohibit the buildup of an inhibitory *pmf*. ATP was added (2.5 mM) from a 250 mM stock solution and NADH oxidation was followed spectrometrically at 340 nm. After a few minutes, the pore-forming substance alamethicin from *T. viride* (20 ug/mL; 5 mg/mL stock solution in DMSO) was added and NADH oxidation was monitored. The orientation was calculated by dividing the NADH oxidation slope before and after alamethicin addition (Figure S4). A linear range of at least 1 minute was chosen to calculate the slope of ATP hydrolysis.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: fluorescence quenching · liposomes · membrane proteins · orientation determination · site-specific modification of proteins

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