The lateral distance between a proton pump and ATP synthase determines the ATP-synthesis rate

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We have investigated the effect of lipid composition on interactions between cytochrome bo3 and ATP-synthase, and the ATP-synthesis activity driven by proton pumping. The two proteins were labeled by fluorescent probes and co-reconstituted in large \((d \approx 100 \text{ nm})\) or giant \((d \approx 10 \text{ µm})\) unilamellar lipid vesicles. Interactions were investigated using fluorescence correlation/cross-correlation spectroscopy and the activity was determined by measuring ATP production, driven by electron-proton transfer, as a function of time. We found that conditions that promoted direct interactions between the two proteins in the membrane (higher fraction DOPC lipids or labeling by hydrophobic molecules) correlated with an increased activity. These data indicate that the ATP-synthesis rate increases with decreasing distance between cytochrome bo3 and the ATP-synthase, and involves proton transfer along the membrane surface. The maximum distance for lateral proton transfer along the surface was found to be \(~80 \text{ nm}\).
the same with the different lipids, the decrease in ATP production was explained in terms of a kinetic effect, indicating that protons were transferred along the membrane surface. However, the data from these earlier studies could not explain the observed effect at a molecular level. In the present study we investigated a possible link between the lipid head group composition, the average protein-protein distance and ATP-synthesis activity using fluorescence correlation spectroscopy (FCS) and fluorescence cross-correlation spectroscopy (FCCS). We labeled cyt. bo$_3$ and ATP-synthase with fluorescent probes that were used both to detect their interactions in an essentially planar unilamellar membrane, and also to promote protein-protein interactions via contacts between the hydrophobic probes. Changes in average distance were correlated with changes in average activity. The data indicate that the average distance between the proton pump and ATP-synthase depends on lipid composition, and the ATP-synthesis rate increases with decreasing distance between cyt. bo$_3$ and ATP-synthase. The maximum average distance for proton diffusion at the membrane surface was ~80 nm.

### Results

**Protein reconstitution in giant unilamellar vesicles.** To investigate protein-protein interactions in lipid membranes, cyt. bo$_3$ and ATP-synthase were co-reconstituted in giant unilamellar lipid vesicles (GUVs) with a diameter of ~10μm, using a modified protocol of Dezi et al.$^{28}$ (for review, see ref. 29). Here, the GUVs were formed in a sucrose solution from dried lipid films placed on gold-covered glass plates by applying a voltage across the liquid (usually referred to as electroformation). The proteins, solubilized in detergent, were incorporated by dilution of the detergent (described in detail in the “Materials and Methods” section). The diameter of these vesicles is such that the membrane surface is essentially planar in the measuring area of the confocal FCS setup. The microscope set-up allows for detection of two fluorophores at a time, and thus a cross correlation analysis of cyt. bo$_3$ (labeled with either ATTO 647N or Abberior STAR 635) and ATP synthase (labeled with ATTO 594), i.e. both diffusion and co-diffusion of the protein complexes could be studied.

Figure 2 shows confocal laser scanning microscope images of a GUV with co-reconstituted cyt. bo$_3$ labeled with ATTO 647N (panel A, red) and ATP synthase labeled with ATTO 594 (panel B, green). In panel C (yellow), an overlay of panels A and B is shown. Using a water-immersion objective, the focal plane could be
put in solution on top of the vesicle (Fig. 2D). In this essentially planar section of the membrane we measured fluorescence-intensity fluctuations in different lipid environments.

**Protein diffusion in the lipid bilayer.** The diffusion of the proteins in the membrane was measured using FCS in two sets of experiments. In one experiment, only cyt. bo$_3$ was present in the vesicles, but half of the population was labeled with ATTO 647N and the other half with ATTO 594. In the other experiment, cyt. bo$_3$, labeled with either ATTO 647N or Abberior STAR 635, and ATP synthase, labeled with ATTO 594, were co-reconstituted in the GUVs. The stoichiometry of the proteins was kept constant between the different experiments, with a 2–3-fold excess of cyt. bo$_3$ compared to the ATP synthase. In cases where only cyt. bo$_3$ was present in the membrane the stoichiometry of the two fluorescent dyes was kept approximately the same, i.e. a 2–3 fold excess of ATTO 647N or Abberior STAR 635 compared to ATTO 594. Auto-correlation curves G(τ) calculated from the detected fluorescence intensity time traces (see "Materials and Methods" and ref. 30) are shown for the co-reconstituted proteins in DOPC vesicles (Fig. 3A,B) or DOPC:DOPG(5%) vesicles (Fig. 3D,E). These curves were fitted with a two-dimensional diffusion model in order to estimate the average number of particles in the detection area (N) and the average diffusion time of the fluorescent molecules through this area, $\tau_D$:

$$G(\tau) = \frac{1}{N(1 - T)} \left[ 1 + \frac{\tau}{\tau_D} \right]^{-1} \left[ 1 - T + T e^{-\frac{\tau}{\tau_T}} \right]$$

(1)

It is assumed that the fluorophores exhibit a "dark"; non-fluorescent triplet state of fraction T with a relaxation time, $\tau_T$. The diffusion coefficient D was calculated from the diffusion time $\tau_D$, using the known lateral 1/e$^2$ radius of the detection area (w):

$$\tau_D = \frac{w^2}{4D}$$

(2)

The measured diffusion coefficients of cyt. bo$_3$ and ATP-synthase, labeled with two different ATTO dyes are shown in Fig. 4 (the dyes are indicated on the right-hand side schemes). The following observations were made:

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**Figure 3.** Auto-correlation data measured with GUVs containing co-reconstituted cyt. bo$_3$ and ATP synthase. GUVs were composed of either 99% DOPC (A–C) or 94% DOPC and 5% DOPG (D–F), with the addition of 1% DPPE functionalized with a biotinyl head group. Measurements were done at pH 7.4 in 10 mM HEPES supplemented with 10 mM NaCl and 100 mM glucose. (A,D) FCS was used to study samples where cyt. bo$_3$ was labeled with either ATTO 647N (red trace) or ATTO 594 (green trace). (B,E) samples with cyt. bo$_3$ labeled with ATTO 647N and ATP-synthase labeled with ATTO 594. The dashed lines represent best fits of the data using a single component with planar two-dimensional diffusion and a triplet state fraction. The amplitude of the diffusional component obtained from the fit of the data with the ATTO 594-labeled protein has been set to unity (amplitude at ~10$^{-4}$ s) to facilitate comparison of the traces. The autocorrelation function for FCCS was calculated in all cases and the normalized cross correlation amplitudes are compared in panels (C) and (F).
(i) The diffusion coefficient of cyt. bo$_3$ was, within experimental error, independent on the labeling dye (ATTO 647 N or ATTO 594), and similar in both DOPC and DOPC:DOPG(5%) membranes (Fig. 4A).

(ii) The diffusion coefficient of cyt. bo$_3$ was a factor of two lower upon co-reconstitution of cyt. bo$_3$ with the ATP-synthase in a DOPC membrane (Fig. 4B, two left-hand side bars, cyt. bo$_3$ diffusion is slowed to the same value as that of the ATP-synthase).

(iii) The diffusion coefficient of cyt. bo$_3$ was unaltered upon co-reconstitution with ATP synthase when 5% DOPG was present in the membrane (compare the red bars in panels A and B in Fig. 4 for DOPC:DOPG(5%) membranes), while the diffusion coefficient of the ATP synthase remained the same as in the GUVs prepared from only DOPC.

(iv) When cyt. bo$_3$ was labeled with the dye Abberior STAR 635 (more hydrophilic than the dyes used in the measurements described in points (i)–(iii) above), the diffusion coefficient of cyt. bo$_3$ was unaltered upon introduction of the ATP synthase (Fig. 4C), also when measured in a membrane composed of only DOPC (compare to the data in Fig. 4B).
addition of ATP-synthase (labeled with ATTO 594) to membranes containing cyt. bo3. Fig. 5B shows that only a moderate increase in the normalized cross-correlation amplitudes could be noted upon the DOPC membranes. Hydrophilic Abberior STAR 635 dye. The amplitudes were slightly lowered upon addition of 5% or 15% DOPG to above). Direct protein-protein interactions (and use the same conditions as those in the FCS-measurements discussed in these specific measurements only to investigate the effect of introducing hydrophobic probes that promote charge separation and proton pumping in the presence of O2 (Fig. 6A). It should be noted that the dyes were used in these specific measurements only to investigate the effect of introducing hydrophobic probes that promote direct protein-protein interactions (and use the same conditions as those in the FCS-measurements discussed above).

As seen in Fig. 6B, when ATTO 594 labeled ATP-synthase and ATTO 647N labeled cyt. bo3 were co-reconstituted, the coupled activity was about a factor of five higher than that measured with unlabeled proteins (c.f. “control” in Fig. 6B). This increase in coupled activity was much smaller (less than a factor of two) when cyt. bo3 was instead labeled with the more hydrophilic dye Abberior STAR 635 (ATP-synthase was still labeled with ATTO 594). Similarly, the cyt. bo3-ATP-synthase interactions were lost upon addition of DOPG when cyt. bo3 was labeled with the hydrophobic dye ATTO 647N.

To investigate whether or not the diffusion of cyt. bo3 and ATP-synthase were correlated, we measured the cross-correlation of the fluorescence intensity fluctuations from two different dyes attached to cyt. bo3 and ATP-synthase, respectively, using FCCS (measured simultaneously with FCS). In Fig. 3C and F, the normalized (see “Materials and Methods” section, Equation 6) cross-correlation amplitudes are shown. We compare GUVs with only cyt. bo3 (dotted lines, two populations of cyt. bo3, each labeled with either ATTO 647N or ATTO 594) to those with both cyt. bo3 and ATP-synthase (solid lines, cyt. bo3 and ATP-synthase labeled with ATTO 647N and ATTO 594, respectively) in membranes composed of only DOPC (panel C in Fig. 3) or DOPC: DOPG(5%) (panel F). In pure DOPC the normalized cross-correlation amplitude measured with membranes containing only cyt. bo3 was found to be a factor of ~3 lower than that measured in membranes containing both cyt. bo3 and ATP-synthase (Fig. 3C). No such amplitude differences could be observed upon introduction of 5% DOPG into the membrane (Fig. 3F). Furthermore with 5% DOPG, the normalized cross-correlation amplitude for the cyt. bo3 and ATP-synthase was about the same as that for cyt. bo3 only (two populations of cyt. bo3, each labeled with a different dye, summarized in Fig. 5A). Furthermore, the data in Fig. 5B shows that only a moderate increase in the normalized cross-correlation amplitudes could be noted upon addition of ATP-synthase (labeled with ATTO 594) to membranes containing cyt. bo3, labeled with the more hydrophilic Abberior STAR 635 dye. The amplitudes were slightly lowered upon addition of 5% or 15% DOPG to the DOPC membranes.

**Coupled enzymatic activity.** To investigate a possible link between the average protein-protein distance and activity, we investigated the rates of ATP-synthesis, driven by proton pumping by cyt. bo3 (referred to as the “coupled activity”), under similar conditions to those used in the studies described above. The fluorophore-labeled cyt. bo3, and ATP-synthase where co-reconstituted in large (diameter ~100 nm) unilamellar vesicles. The protein concentration was adjusted to approximately five proteins of each per vesicle, i.e. the same as that used in our earlier studies (see refs 26 and 27). The coupled cyt. bo3-ATP synthase activity was measured by monitoring the ATP-production rate upon addition of ubiquinol Q1/DTT, which reduces cyt. bo3, leading to transmembrane charge separation and proton pumping in the presence of O2 (Fig. 6A). It should be noted that the dyes were used in these specific measurements only to investigate the effect of introducing hydrophobic probes that promote direct protein-protein interactions (and use the same conditions as those in the FCS-measurements discussed above).

As seen in Fig. 6B, when ATTO 594 labeled ATP-synthase and ATTO 647N labeled cyt. bo3 were co-reconstituted, the coupled activity was about a factor of five higher than that measured with unlabeled proteins (c.f. “control” in Fig. 6B). This increase in coupled activity was much smaller (less than a factor of two) when cyt. bo3 was instead labeled with the more hydrophilic dye Abberior STAR 635 (ATP-synthase was still labeled with ATTO 594).

Next, we studied the coupled activity as a function of the lipid composition, i.e. with increasing amounts of DOPG, (0–40%, Fig. 6C) added to vesicles composed of DOPC. As observed previously26, 27, there was a clear...
Figure 6. Coupled cyt. bo$_3$-ATP synthase activity. (A) ATP production by the ATP synthase, driven by an electrochemical gradient generated by cyt bo$_3$ (as shown in the scheme). ATP synthesis was measured as a change in luminescence from the luciferin-luciferase couple over 3 × 30 s. The reactions were started by the addition of ubiquinol Q$_1$H$_2$ (20 μM final concentration) in the presence of 2 mM DTT and 80 μM ADP. Measurements were done at pH 7.5 in 20 mM Tris-PO$_4$ buffer supplemented with 2.5 mM MgCl$_2$. Rates were calculated from the average slopes, calibrated by addition of well-defined amount of ATP (5 pmol, see mark at 30 s). The trace shown was obtained with 100 nm, 100% DOPC liposomes. (B) ATP-synthesis rates measured in DOPC vesicles with cyt. bo$_3$ labeled with either ATTO (A) 647N (and unlabeled ATP synthase) or Abberior STAR 635 (and ATP-synthase labeled with ATTO 594). Rates are compared to those obtained with proteoliposomes with unlabeled cyt. bo$_3$ and ATP-synthase in the presence (2.5 μM DDM, Detergent ctrl., see text for explanation) or absence (No label) of detergent. (C) Normalized ATP-synthesis rates of DOPC:DOPG vesicles with cyt. bo$_3$ and ATP-synthase labeled with ATTO 647N and ATTO 594 respectively (blue) or vesicles with unlabeled protein (green). The rates are normalized to that obtained with 100% DOPC vesicles to facilitate comparison. At 100% DOPC the activity was a factor of ~5 larger with the labeled than with the unlabeled proteins (c.f. panel B). Error bars is the standard deviation from measurements with four samples (except Detergent ctrl., two samples).

decrease in the ATP-synthesis rate with increasing concentrations of DOPG. However, upon labeling cyt. bo$_3$ with ATTO 647N and the ATP-synthase with ATTO 594, the dependence on lipid composition was significantly less pronounced. Upon decreasing the fraction of DOPC from 100% to 60%, the ATP-synthesis rate dropped to ~60% for the labeled proteins, as compared to ~10% for the unlabeled proteins (Fig. 6C).

In the experiments discussed here, the detergent was removed by gel chromatography during vesicle reconstitution, while during reconstitution in GUVs the detergent concentration was decreased below CMC by dilution. To investigate whether or not the presence of remaining small amounts of detergent (~2.5 μM DDM) altered the coupled enzymatic activity, the ATP production was measured also in the presence of the detergent DDM at the same low concentration (Fig. 6B, "Detergent ctrl."). As seen in the Figure, the coupled activity was about the same with and without detergent.

A few relevant controls should be mentioned. Binding of the dyes did not alter the activities of the two enzymes alone. The activity of cyt. bo$_3$ was measured using a Clark-type electrode and was found to be about the same with (460 ± 50 e^-/s) and without (440 ± 60 e^-/s) the label ATTO 647 N. The data in Fig. 6B show that the coupled cyt. bo$_3$-ATP-synthase activity was about the same for unlabeled proteins as upon labeling cyt. bo$_3$ with Abberior STAR 635 and ATP-synthase with ATTO 594, i.e. labeling of these proteins with the non-interacting dyes did not alter the activity indicating that the activity of the ATP-synthase was unaltered upon labeling with ATTO 594. Furthermore, the coupled activity was essentially unaltered upon labeling cyt. bo$_3$ with ATTO647N leaving the ATP-synthase unlabeled. Taken together, these data indicate that neither labeling of cyt. bo$_3$ nor of ATP-synthase did result in altering the activity of these enzymes. Furthermore, because the coupled cyt. bo$_3$-ATP-synthase activity was insensitive to labeling of one of the proteins at a time or with non-interacting dyes, the data suggest that neither the relative orientation nor proton leaks were influenced by the labeling.

Discussion

As outlined in the Introduction section, in a recent study we found that the coupled enzymatic activity of cyt. bo$_3$ and ATP synthase (Fig. 1) was dependent on the lipid composition of the vesicles; the activity dropped by a factor of ~10 upon addition of e.g. 40% DOPG to DOPC vesicles with a diameter of 100 nm. On the basis of these findings we concluded that the coupled reaction involves proton transfer along the lipid membrane. However, in the earlier study we could not discriminate between lipid-dependent changes in the lateral proton-transfer rate or changes in protein-protein interactions. Here, we investigated direct interactions between the two membrane-bound proteins and found that these interactions correlated with the coupled ATP-synthesis activity.

We used two different combinations of protein-attached fluorophores; cyt. bo$_3$ was labeled with either ATTO 647N or Abberior STAR 635 while the ATP-synthase was always labeled with ATTO 594. The ATTO 647N dye is hydrophobic while Abberior STAR 635 and ATTO 594 are more hydrophilic. These properties were used to address questions related to dye-mediated interactions between the protein complexes.
We note that upon labeling of one population of cyt. bo\textsubscript{3} with ATTO 647N (Fig. 4A), the diffusion was a factor of ~2 slower than when using Abberior STAR 635 (compare to data in Fig. 4C). This observation is consistent with earlier reports indicating that the hydrophobicity/polarity of the dye could have an effect on the diffusion coefficient.\textsuperscript{32} The presence of ATTO 647N also affected the apparent diffusion time of the cyt. bo\textsubscript{3} population that was labeled with ATTO 594 in the same sample (Fig. 4A). This observation indicates some degree of interaction between the two protein populations, as also observed when using FCCS (see below).

The ATP synthase displayed a factor of ~2 slower diffusion than cyt. bo\textsubscript{3} (which cannot be attributed to the dye). This difference in diffusion constants between cyt. bo\textsubscript{3} and the ATP-synthase, in combination with the dye-dependent differences in interactions between the proteins, was used here as a tool to investigate interactions between the two proteins. The origin of the slower ATP-synthase diffusion compared to that of cyt. bo\textsubscript{3} is outside the scope of this work (it will be followed up in a future study) and we only discuss the effect briefly. Results from a recent study indicate that the lateral diffusion of a membrane protein is primarily determined by protein-induced deformation of the membrane, rather than the size of the protein.\textsuperscript{34, 35} Thus, even though the ATP-synthase is larger than cyt. bo\textsubscript{3} and has a significantly larger domain penetrating into solution outside of the membrane, the observed effect is more likely to originate from the fact that the ATP-synthase bends the membrane. The mitochondrial ATP-synthase dimer induces a curvature of ~90° in the membrane, while the monomeric form is thought to induce a bending of ~45°.\textsuperscript{36} In bacteria, there are so far no reports of a dimeric form of ATP-synthase, but because also the monomeric form is expected to induce membrane bending, the slower diffusion could be explained in terms of bending. It is also interesting to note that under conditions where cyt. bo\textsubscript{3} and the ATP-synthase do interact (see e.g. Fig. 4B, left-hand side) the diffusion coefficient of the cyt. bo\textsubscript{3}-ATP-synthase complex is about the same as that of ATP-synthase alone, which suggests that the larger size of the cyt. bo\textsubscript{3}-ATP-synthase multi-protein complex does not slow the diffusion.

The FCCS data showed that there was a cross-correlation amplitude larger than zero, indicating some degree of protein-protein interactions. No cross-correlation was observed in an experiment using labeled DPPE lipids (i.e. ATTO 647N and ATTO 594), incorporated in GUVs at similar concentrations (data not shwon), which indicates that the fluorophores alone do not bind each other and that the contribution from spectral cross-talk between the detection channels to the FCCS amplitudes can be neglected. We normalized the data to the maximum theoretical cross-correlation amplitude, which was set to unity (Figs 3C,F and 5, see also Materials and Methods section for details). However, there are several reasons why the maximum observed amplitude may be lower than the theoretical value. For example, protein-protein interactions are likely to depend on the relative orientation of the interacting proteins in the membrane such that only proteins in the correct relative orientation would give rise to a cross correlation. Furthermore, we used a lower concentration of one of the fluorophores (i.e. ATTO 594) to eliminate spectral cross talk in the FCCS, but this also resulted in a lower maximal cross-correlation amplitude in the experiment. Thus, the absolute fraction of proteins that interact could not be determined accurately. Nevertheless, a comparison of amplitudes between samples is relevant, which is the basis of the conclusions drawn here.

The cross-correlation analysis shows that the cyt. bo\textsubscript{3}-ATP-synthase interaction in a DOPC membrane was three-fold larger than the cyt. bo\textsubscript{3}-cyt. bo\textsubscript{3} cross correlation (Fig. 5A, blue bars). This difference was not observed upon addition of 5% DOPG to the DOPC membrane (Fig. 5A, green bars), which indicates that introduction of DOPG into the DOPC membrane resulted in weakened cyt. bo\textsubscript{3}-ATP-synthase interactions. This conclusion is also supported by the data in Fig. 4 showing that the diffusion coefficient of cyt. bo\textsubscript{3} decreased significantly upon introduction of ATTO synthase into the membrane.

When using Abberior STAR 635 as a label for cyt. bo\textsubscript{3} in DOPC membranes (Fig. 5B, left), there was a smaller difference in cross correlation between samples containing cyt. bo\textsubscript{3}-cyt. bo\textsubscript{3} and those containing cyt. bo\textsubscript{3}-ATP synthase than when using ATTO 647N (Fig. 5A, left), respectively. Moreover, the effect of introducing negatively charged lipids (DOPG) was also less pronounced when cyt. bo\textsubscript{3} was labeled with Abberior STAR 635 (Fig. 5B, right). Both the cross-correlation data (Fig. 5) and the diffusion data (Fig. 4) indicate that the interactions between cyt. bo\textsubscript{3} and the ATP-synthase were more pronounced with the ATTO 594/ATTO 647N pair of dyes than with the ATTO 594/Abberior STAR 635 pair of dyes. In other words, the interactions between cyt. bo\textsubscript{3} and ATP-synthase could be promoted when the hydrophobic ATTO 647N dye was used. Measurements of the ATP synthesis activity (Fig. 6) show that the dye-promoted interactions result in an increase in the coupled activity. The data show that the activity was three times larger when using the ATTO 594/ATTO 647N pair of labels than when using the ATTO 594/Abberior STAR 635 pair (Fig. 6B). Furthermore, when using the ATTO 594/ATTO 647N pair of labels the activity was five times higher than for that measured with unlabeled proteins. Importantly, the lipid dependence was significantly less pronounced in the ATTO 674N/ATTO 594 sample than with unlabeled proteins (Fig. 6C). In other words, the interactions between cyt. bo\textsubscript{3} and the ATP-synthase, promoted by the dyes, were resistant towards introduction of DOPG into the membrane.\textsuperscript{33} It should be noted also that in the GUVs, used for the FCS and FCCS experiments, the protein density in the membrane was ~10 times lower (~30 proteins/μm²) than in the functional studies using 100-nm vesicles (~320 proteins/μm²). Therefore, we can only do qualitative comparisons of the data from these two sets of experiments. Furthermore, we note that in our earlier studies we found that a decrease in the protein density from ~320 proteins/μm² to ~80 proteins/μm² resulted in significant loss of the lipid dependence of the activity. Consequently, we propose that the reason why we observe interactions in the GUVs (FCS experiments) even though the protein density is lower than ~80 proteins/μm² is the ability of the ATTO 647N dye to promote interactions between the cyt. bo\textsubscript{3} and the ATP-synthase, i.e. we would not expect to see interactions between cyt. bo\textsubscript{3} and ATP-synthase in the GUVs without the use of the dyes. In other words, as already mentioned above, the ATTO dye itself is a tool to promote and modulate protein-protein interactions, which allowed us to study effects of lipid composition on the coupled activity also with the lower protein density in the GUVs (than in the large unilamellar vesicles). These dyes most likely promote interactions between any membrane proteins, depending on the site of labeling.
and number of labels. Therefore, the effect should be considered when using these dyes in other studies aimed at investigating protein-protein interactions.

The distribution of the distances between cyt. bo$_3$ and ATP-synthase range up to ~80 nm for the 100-nm vesicles (Fig. 7), i.e. under conditions where we did observe a lipid dependence of the coupled activity the maximum distance between the membrane-bound proteins was ~80 nm. This experimentally estimated distance of ~80 nm is consistent with the surface area of ~10$^4$ nm$^2$ (radius $\cong$ 60 nm) at which protons ejected from a single proton pump spread before being released to bulk solution$^{38}$. The value is also in agreement with data from earlier experimental and theoretical studies$^{5, 22, 25, 39–41}$.

We may also ask whether or not the mechanism has a functional role in the living cell. In mitochondria, the respiratory chain (i.e. the proton pumps and transporters) and the ATP-synthases are physically separated in the cristae (reviewed in ref. $^{42}$). The size of the cristae is of the same order as the maximum average distance between cyt. bo$_3$ and ATP-synthase in the 100-nm vesicles, i.e. ~80 nm (Fig. 7). Consequently, it is possible that protons that are pumped or translocated by components of the respiratory chain are transferred along the membrane surface to the ATP-synthase$^{42, 43}$.

A system in which proton transfer along membrane surfaces is particularly important to ATP synthesis is in alkaliphilic bacteria as outlined in the introduction section. In the native system where membrane proteins diffuse more freely, direct interactions could be promoted by regulatory small, membrane-bound proteins$^{44–47}$, which would have a similar role to the hydrophobic probe used here. The findings from this study point to a mechanism by which the cell could exercise such a regulation of the energy-conversion rate (rate of ATP-synthesis) by altering the distance between the two proteins.

Unrelated to the present study, but potentially interesting in studies where protein-protein interactions are investigated, is the effect of the hydrophobic dyes to promote protein-protein interactions. In other words, care should be exercised when interpreting results from studies of interactions when these fluorophores are used to detect the proteins of interest.

Summary
As outlined above, results from earlier studies indicate that the membrane surface is involved in proton transfer between membrane proteins. The data presented in this work show that in GUVs (where the proteins are diluted in the plane of the membrane by the large membrane surface area) direct interactions between cyt. bo$_3$ and ATP synthase were promoted by binding of hydrophobic fluorescent dyes (which were also used to study the interactions). Binding of these dyes also resulted in an increase in the ATP-synthesis rate. Both the physical protein-protein interactions and the coupled activity dropped upon introduction of the negatively charged DOPG into the DOPC vesicles. In other words, we found a link between average lateral distance and the coupled activity. Consequently, the earlier observed lipid dependence of the coupled activity can be explained by changes in the average protein-protein distance. We also found that lateral proton transfer along the membrane occurs over distances ranging up to ~80 nm.

Materials and Methods
Protein expression and purification. F$_{1}$F$_{0}$ ATP synthase was expressed from plasmid pBWU13-βHis in E. coli strain DK8 and purified as described$^{48}$. The quinol-type oxidase cyt. bo$_3$, was expressed from plasmid pETcyo in E. coli strain C43 and purified as described (ref. 49, see also ref. 50).
Protein labeling. The proteins were labeled with either of the three thiol-reactive fluorophores ATTO 594, ATTO 647N (ATTO TEC GmbH), or Abberior STAR 635 (Abberior GmbH). ATP synthase was labeled with ATTO 594 by adding a 3-fold molar excess of the dye and the sample was incubated while gently shaking for 1.5 h at room temperature. Cyt. bo$_2$, was labeled with ATTO 647N by adding a 1.3-fold molar excess dye or with Abberior STAR 635 by adding a 5-fold molar excess of dye in the presence of 1/20 volume of NaHCO$_3$ (pH 9.0), and incubated as described above. Unbound dye was removed using a pre-packet gel filtration column (PD-10, GE Healthcare) equilibrated with a 10 mM phosphate buffer (pH 7.4) supplemented with 100 mM sucrose, 10 mM KCl and 1 mM DDM. The proteins were stored in the same buffer at $-70^\circ$C until use.

The labels were attached to cysteine residues using thiol-reactive dyes. The degree of labeling was estimated using both absorbance spectroscopy and so-called fluorescence antibunching$^{5,2}$, where the number of independently emitting fluorophores within a molecule is determined; cyt. bo$_2$ was on average labeled with four fluorophores per protein whereas ATP synthase was labeled with three. From a simple solvent accessibility analysis, using a probe radius of 1.4 Å, six of the seven cysteine residues found in the structure of cyt. bo$_2$ (PDB id: 1FFT) are surface exposed. The F$_1$ part of the ATP synthase has 17 cysteines (PDB id: 1OAA), of which three are available at the surface.

Luminescence assay. Vesicles were prepared from 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG) purchased from Avanti Polar Lipids Inc. The lipids were stored in chloroform at $-20^\circ$C until use. The lipid stock solutions were mixed at specific ratios (see Figure legends) and the chloroform was evaporated under nitrogen followed by vacuum evaporation. The lipid mixture was re-suspended at a 5 mg/ml lipid concentration in a buffer containing 10 mM HEPES pH 7.4, 2.5 mM MgCl$_2$, 50 µl sucrose and subjected to 6 freeze-thaw cycles (one minute in liquid nitrogen, then at 30°C until thawed, followed by 30s vortexing). Finally, vesicles were formed by extrusion (>20 times) of the mixture through 100 nm Nucleopore membranes (Whatman Ltd). Enzymes were reconstituted as previously described$^{26}$. Briefly, a solution of 0.37 µM ATP synthase (unlabeled or labeled with ATTO 594) and 0.37 µM cyt. bo$_2$ (unlabeled or labeled with either ATTO 647N or Abberior STAR 635) was mixed with 0.07 µM vesicles in the presence of 0.4% sodium cholate. Samples were incubated on ice for 30 minutes. The detergent was removed using a pre-packed gel filtration column (PD-10, GE healthcare). Coupled cyt. bo$_2$-ATP synthase activity was measured as described earlier$^{26,27}$. Briefly, to 460 µl of measuring buffer (20 mM Tris-PO$_4$ pH 7.5, 2.5 mM MgCl$_2$, 2 mM DTT, 80 µM ADP) we added 20 µl of a 10 mg/ml luciferase/luciferin solution (CLSI1, Roche) and 20 µl of the proteoliposome solution. A baseline was recorded. An addition of 2 µl ATP (2.5 µM) was made and a new baseline was recorded for calibration purposes. The reaction was started by addition of 1 µl ubiquinol Q$_1$ (10 mM) and ATP synthesis was recorded for 3 × 30 s.

Preparation of giant unilamellar vesicles. The GUVs were prepared by electroformation using a modified version of the method described in ref. 28. The stock solutions of lipids (Avanti Polar Lipids), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG), were mixed at different ratios to a final concentration of 1 mM in chloroform. In all samples 1% (0.01 mM) 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl) (DPPE-biotinyl) was supplemented to the lipid mixture. The lipid mixtures were spread on glass plates (25 mm diameter) in the image plane, split by a dichroic mirror and then detected by two single fiber-coupled, pulsed (20 MHz) diode lasers emitting at 637 nm (LDH-D-C, PicoQuant AG, Berlin) and 594 nm (Abberior Instruments) were used for excitation (alternating mode, with the excitation pulses of the two lasers out of phase with each other to minimize cross-talk). The two laser beams were overlapped and focused by a water immersion objective (Olympus, UPLSAPO 60XO, NA 1.2). The fluorescence was collected through the same objective, separated from the excitation path via a dichroic mirror, passed through a motorized confocal pinhole (MPH16, ThorLabs, set at 50 µm diameter) in the image plane, split by a dichroic mirror and then detected by two single
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