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Designer Peptide Surfactants Stabilize Functional Photosystem-I Membrane Complex in Aqueous Solution for Extended Time[⊥]

Kazuya Matsumoto,[‡,§] Michael Vaughn,[||] Barry D. Bruce,[||] Sotirios Koutsopoulos,*[,‡] and Shuguang Zhang*[,‡]

Center for Biomedical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139-4307, Catalysis Science Laboratory, Mitsui Chemicals, Inc., 1144 Togo, Mobara-shi, Chiba 297-0017, Japan, and Center for Environmental Biotechnology and Department of Biochemistry, Cellular and Molecular Biology, University of Tennessee, Knoxville, Tennessee

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Detailed structural analyses of membrane proteins as well as their uses in advanced nanobiotechnological applications require extended stabilization of the functional protein conformation. Here we report that a new class of designer surfactant like peptides can significantly increase the activity and stabilize the functional form of the multidomain protein complex Photosystem-I (PS-I) in solution better than other commonly used chemical detergents. We carried out a systematic analysis using a series of such peptides to identify the chemical and structural features that enhance the photochemical activity of PS-I. We observed that peptide surfactant amphiphilicity is necessary but not sufficient to stabilize PS-I in its functional form. A number of factors are essential for designing the optimal peptide including amino acid sequence, N-terminal acetylation and C-terminal amidation. Furthermore, we showed that the polarity and number of charges on the hydrophilic head are important as well as hydrophobicity and size of the amino acid side groups in the hydrophobic tail play an important role. The best performing peptides for the stabilization of functional PS-I are, in order of effectiveness, ac-I_6K_2-$CONH_2$, ac-A_6K-$CONH_2$, ac-V_6K_2-$CONH_2$, and ac-V_6R_2-$CONH_2$. These simple and inexpensive peptide surfactants will likely make significant contributions to stabilize the functional form of diverse and currently elusive membrane proteins and their complexes with important applications.

Introduction

The production and release of atmospheric O_2 is a byproduct in green plants, algae and cyanobacteria. In the presence of light, water is oxidized on the thylakoid membranes through a number of electrochemical reactions which result in oxygenic photosynthesis. Photosystem I (PS-I) is a thylakoid transmembrane protein complex that is associated with one of the first steps of the photosynthetic process.[1] The crystal structure of PS-I from the thermophilic cyanobacterium *Thermosynechoccus elongatus* was solved by Jordan et al.[2] The trimeric protein complex has a molecular weight of 1.07 MDa and consists of 36 proteins to which 381 cofactors are noncovalently attached. Each monomer consists of 12 proteins and 9 of these proteins per monomer feature a network of 34 transmembrane α-helices (for a total of 102 helices in the trimer) that are buried within the lipid bilayer. The large number of transmembrane helices and extensive interactions with the thylakoid membranes has been problematic in developing protocols for the efficient purification, solubilization and crystallization of the native PS-I supercomplex, complete with its associated antenna pigments and cofactors. Following solubilization in high concentrations of detergent to remove extraneous membrane components, the soluble membrane protein molecules still need small quantities of detergent not only to avoid aggregation and denaturation but also to maintain the functional conformation.

There is not a single detergent that can be universally used for all membrane proteins. A wide spectrum of chemicals, lipids, peptides and peptide−chemical hybrid detergents have been designed and tested for membrane protein stabilization.[3−9] However, our understanding of the interaction between detergents and proteins is still limited and up to date the choice of a detergent is purely empirical.

We have shown previously that specific peptide sequences stabilized PS-I in the dry state for up to 3 weeks,[10,11] the integral membrane glycerol-3-phosphate dehydrogenase (GlpD) from *E. coli*,[12] the soluble redox flavoenzyme, NADH peroxidase (Npx) from *Enterococcus casseliflavus*[12] and the G-protein coupled receptor (GPCR) bovine Rhodopsin in solution[13] more effectively than other commercial detergents.

In this work, we designed and studied a new class of short peptides (7−8 residues) with surfactant properties for their ability to stabilize the functional conformation of the PS-I membrane protein from *T. elongatus*. These peptides have been characterized and it was shown that depending on the concentration, their critical micelle concentration (CMC), and the type of electrolyte in the medium they form stable nanotubes, nanovesicles or micelles similar to lipids and other chemical detergents.[14−17] We here report the long-term stabilization of functional PS-I complex in aqueous media using peptide surfactant molecules with specific amino acid sequences. On the basis of our results, we propose a mechanism by which designer peptide surfactants interact with the protein complex.

* Corresponding authors. S.K.: tel, 617-324-7612; fax, 617-258-5239; e-mail, sotiris@mit.edu. S.Z.: tel, 617-258-7514; fax, 617-258-5239; e-mail, shuguang@mit.edu.

‡ Massachusetts Institute of Technology.

§ Mitsui Chemicals, Inc.

|| University of Tennessee.

⊥ Abbreviations: PS-I, photosystem I; DDM, *n*-dodecyl-*β*-D-maltopyranoside; FC-14, Fos-choline-14; DG, Digitonin; CMC, critical micelle concentration; DLS, dynamic light scattering; AFM, atomic force microscopy; MV, methylviologen; DCIP, 2,6-dichloroindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CF, 5,6-carboxyfluorescein.

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Experimental Section

PS-I Purification. The PS-I complex was extracted from the thylakoid membranes of the thermophilic cyanobacteria *T. elongatus*. Briefly, the purification protocol included bacterial growth followed by incubation with 0.25% (w/v) lysozyme for 2−3 h at 37 °C under gentle agitation. Cells were lysed with the French press; whole cells were removed at 3000*g* for 5 min and membranes were collected at 20000 rpm. The membranes were washed and solubilized as in Fromme and Witt[18] with the exception that in the final wash 3 M NaBr was used. Then the supernatant was loaded on a 10−40% linear sucrose gradient (20 mM MES pH 7.0, 10 mM $MgCl_2$, 10 mM $CaCl_2$ and 0.05% w/v, i.e., 1 mM, *n*-dodecyl-β-D-maltopyranoside, DDM) for 18 h at 100000*g* and 4 °C. The lower green band was collected, pooled and stored at −20 °C. Purity was confirmed by Tris−tricine SDS-PAGE gel electrophoresis.[19] The chlorophyll content of PS-I was measured by the method of Porra.[20]

Chemicals and Peptide Surfactants. *n*-Dodecyl-β-D-maltopyranoside (DDM), Fos-choline-14 (FC-14), and Triton X-100 were purchased from Anatrace (Maumee, OH). Digitonin (DG) was purchased from EMD Biosciences (San Diego, CA). Peptide surfactants, acetyl-AAAAAAK-NH_2 (ac-A_6K-NH_2), acetyl-AAAAAAK-OH (ac-A_6K-OH), acetyl-AAAAAAD-OH (ac-A_6D-OH), acetyl-IIIIIIKK-NH_2 (ac-I_6K_2-NH_2), KAAAAAA-NH_2 (KA_6-NH_2), DAAAAAA-NH_2 (DA_6-NH_2), acetyl-VVVVVVKK-NH_2 (ac-V_6K_2-NH_2), acetyl-VVVVVVRR-NH_2 (ac-V_6R_2-NH_2), and acetyl-VVVVVVDD-NH_2 (ac-V_6D_2-NH_2) were custom synthesized, purified by HPLC and further characterized by Biopolymers Laboratory at Massachusetts Institute of Technology. Tricine, methylviologen (MV), 2,6-Dichloroindophenol (DCIP), sodium ascorbate, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 5,6-carboxyfluorescein (CF) were purchased from Sigma Aldrich. Free chlorophyll was also purchased from Sigma Aldrich and it was used for control experiments.

Molecular Modeling. Peptide structures were generated by modeling simulations using the CHARMM software.[21] The most stable structures were evaluated by molecular dynamics simulations which included an energy minimization procedure of 20000 steps with the steepest decent (SD) method followed by 20000 steps of adapted basis Newton−Raphson (ABNR) method and dynamic simulations using the generalized Born with simple switching (GBSW) implicit solvent model.[21,22] The model is formulated in such a way to yield numerically stable electrostatic solvation forces between the peptides and virtual water molecules. Graphic illustrations were generated by VMD software.[23]

Oxygen Consumption Measurements. PS-I functionality was determined by a method that is based on the consumption of dissolved O_2 in the presence of PS-I. This method is widely used to study PS-I activity.[24] The working solution with a total volume of 3.5 mL contained 40 mM tricine, 167 μM MV, 0.1 mM DCIP, 1 mM sodium ascorbate, 10 mM NH_4Cl, 10 μM DCMU at pH 7.5. To determine the activity and functionality of PS-I, we monitored the course of an electrochemical reaction that involved electron flow through PS-I using as electron donor and acceptor DCIP and MV, respectively.[24] DCIP provides electrons from sodium ascorbate and reduces PS-I, which in turn transfers electrons to MV. The latter is easily oxidized by the dissolved O_2 in the solution. Illumination of the reaction cell triggered a light-catalyzed electrochemical reaction cascade, which lead to consumption of dissolved O_2. The decrease of the latter was measured by an O_2 electrode (Model ISO2, World Precision Instruments, Inc. Sarasota, FL). To avoid electron transfer from traces of PS-II that may be present in the working solution, as a result of incomplete purification, we added DCMU, which is a potent inhibitor of PS-II.[25] The electrode was standardized before and after each set of measurements with air saturated water (20.4% at 24 °C) according to the manufacturer's specifications. As a light source we used a fiber optic illuminator Model 9745-00 (Cole Palmer Instrument Company, Chicago, IL) with lamp power 30 W and luminous intensity of 107600 cd sr/m^2 (which corresponds to ca. 1800 μmol of photons m^{-2} s^{-1}). All measurements were performed at 24 °C in 5 mL of poly(methylmethacrylate), PMMA, closed cuvettes under continuous stirring.

Upon illumination of the PS-I sample the O_2 concentration was monitored every 1 min after a stable reading was achieved in the air-saturated working solution. Before and after each experiment, a series of blank tests were performed. The O_2 consumption was also measured in the absence of light and in working solutions that contained the commercial detergent or the peptide surfactant only. In all cases of blank experiments, it was shown that the kinetics of O_2 consumption were similar to that of the buffer, which corresponds to the O_2 consumption by the electrode alone. The PS-I activities were determined from the initial slopes of the plots of O_2 consumption as a function of time. Standard deviations were calculated using erro propagation techniques ($n = 3$).

For the time course experiment, PS-I samples were incubated at 4 °C in the presence and in the absence of ac-A_6K-NH_2, ac-A_6K-OH or DDM. At each time point up to 2 months, a sample was transferred into the reaction cell, which contained the O_2-saturated working solution, and O_2 consumption was measured as before. The final composition of the reaction mixture was identical to that of the previous O_2 consumption tests, i.e., 5 μg Chl of PS-I/mL and 0.47 mM of the peptide or DDM surfactant.

In all solutions, the concentration of the peptide surfactants and of the conventional detergents was adjusted to 0.47 mM which was higher than each surfactant's respective CMC. To exclude the possibility that the low activity of PS-I in the presence 0.47 mM of the chemical detergents was due to PS-I unfolding, we performed activity tests using 0.05 mM of the chemical detergents and compared the results. Furthermore, we tested the protein conformation as a function of temperature using fluorescence spectroscopy. The data suggested that at 24 °C, 0.47 mM of FC-14, DDM, DG or Triton X-100 did not result in protein unfolding. Control experiments using predenatured, unfolded PS-I, were also performed to exclude the possibility that the observed O_2 consumption was due to the presence of free plastocyanine (in vivo electron donor).

Western Blots. The structural integrity of PS-I was analyzed by Western blots using specific antibodies for the PsaC and PsaD subunits of PS-I (Agrisera, Vännäs Sweden) according to the protocol by Minai et al.[26] Briefly, to show that the PsaC and PsaD subunits were not removed from the PS-I protein complex, PS-I samples in the presence and in the absence of the ac-A_6K-NH_2 peptide were vigorously vortexed and centrifuged at 100000*g* for 30 min at 4 °C. The supernatant was examined for the presence of PsaC and PsaD subunits. The supernatants and precipitants were collected, loaded on Novex 18% Tris−glycine gel (Invitrogen, Carlsbad, CA) and transferred on 0.2 μm nitrocellulose membranes (BIO RAD, Hercules, CA). The membranes were incubated with rabbit serum PsaC or PsaD polyclonal antibodies (1:5000 dilution) and developed using goat antirabbit horseradish peroxidase (ECL chemiluminescence kit, GE Healthcare).

Critical Micelle Concentration (CMC) of the Surfactants. To determine the CMC of the peptide surfactants and of the conventional detergents dynamic light scattering (DLS) experiments were performed in a PDDLS/Batch setup (Precision Detectors, Franklin, MA). Peptide and detergent samples were prepared at different concentrations in a medium with a composition identical to that used for the PS-I activity measurements (i.e., 40 mM tricine, 167 μM MV, 0.1 mM DCIP, 1 mM sodium ascorbate, 10 mM NH_4Cl, 10 μM DCMU in Milli-Q water at pH 7.5). The molecular weight of Triton X-100 was taken as 647 g/mol according to the manufacturer's specifications. All solutions were filtered through 0.2 μm pore size filters prior to measuring light scattering. Scattered light was detected at 90° angle and the number of photons reaching the avalanche photodiode was recorded. The solvent viscosity and the refractive index of the buffer were taken as 0.894 cP and 1.33, respectively, at 20 °C. Data were acquired and displayed by Precision Deconvolve program. Triplicates were recorded for each sample.

Thermal Stability of PS-I in the Presence of Peptide Surfactants. The thermal stability of the PS-I protein complex with and without peptide surfactants and DDM detergent was studied by fluorescence spectroscopy using a Perkin-Elmer LS-50B spectrophotometer between 30 and 85 °C. The temperature was controlled by a water bath. Protein samples were incubated for 20 min prior to recording fluorescence emission spectra between 600 and 800 nm using 1 cm path length quartz cuvettes. The excitation wavelength was 442 nm to excite the chlorophyll molecules. The excitation and emission slit widths were set at 5.0 and 2.5 nm, respectively.

Atomic Force Microscopy (AFM) Imaging. AFM studies of peptide surfactants and commercial detergents alone and mixed with PS-I were performed using Nanoscope IIIa (Digital Instruments, Santa Barbara, CA) operated in tapping mode. Soft silicon probes were chosen (FESP, Veeco Probes) with a tip radius of <10 nm, mounted on a single-beam cantilever. AFM images of a 2 μL sample deposited onto freshly cleaved mica surfaces were recorded in air at room temperature. Each sample was allowed to interact with the mica surface for 30 s and then it was rinsed with Milli-Q water. Samples on the mica surface were then air-dried and images were acquired immediately. Cantilever deflections were recorded with a cantilever frequency of 250 Hz, horizontal scan rate of 1.2 Hz and 512 samples per line.

For the data analysis, the Nanoscope image processing software was employed by providing height patterns, cross sections and root-mean-square roughness (rms) of PS-I in the presence and in the absence of peptide surfactants or commercial detergents. AFM images were collected from two different samples and at random spot surface sampling (at least five spots). The images were reproducible.

Peptide Vesicle Stability by Fluorescence Spectroscopy. The stability of the peptide vesicle assemblies was studied using the method proposed by Senior and Gregoriadis.[27] Briefly, the peptide vesicle structural integrity was assessed by measuring the release of the fluorescent probe 5,6-carboxyfluorescein (CF) through the peptide bilayer. In the presence and in the absence of PS-I, the peptide surfactants and DDM were mixed with CF in a solution with composition identical to that used in the activity measurements. Nonencapsulated CF was removed by centrifugation at 11000 rpm for 25 min using an Eppendorf microcentrifuge and Microcon YM-10 membrane tubes (10 kDa cutoff). Then the vesicles were resuspended in the buffer (40 mM tricine, 10 mM NH_4Cl in Milli-Q water at pH 7.5) and incubated for 1 h at 20 °C. The CF released during incubation was collected by centrifugation, through the dialysis membrane and the fluorescence intensity was measured. This process was repeated every 1 h and a graph was created to show the release kinetics of CF as a function of time. Encapsulated CF is strongly quenched, and therefore, only the released CF will contribute to the fluorescence intensity signal. All measurements were carried out in a Perkin-Elmer LS-50B spectrophotometer at 20 °C using quartz cuvettes of 1 cm path length. The excitation wavelength was at 470 nm and the emission maximum was observed at 520 nm. The excitation and emission slit widths were set at 5.0 and 2.5 nm, respectively.

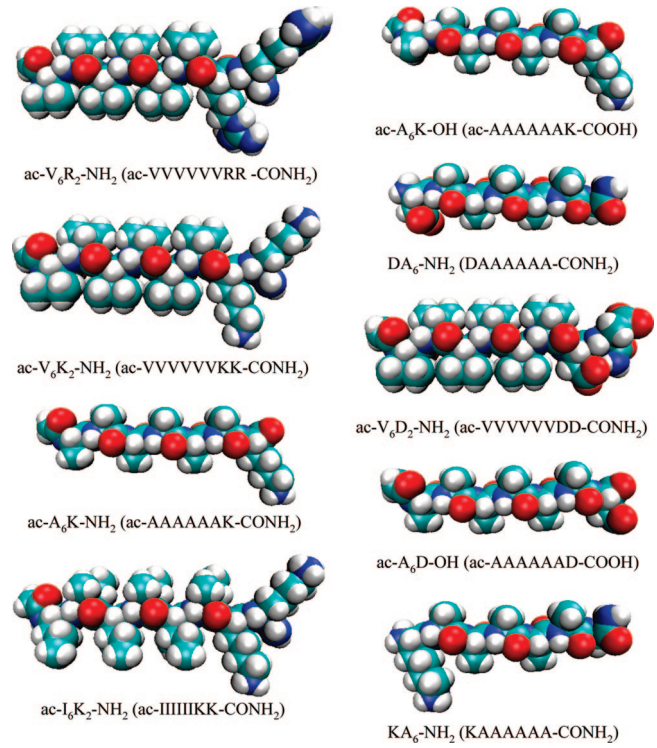


Figure 1. Molecular modeling of peptide surfactants and commercial detergents. At pH 7.5, aspartic acid (D) is negatively charged and arginine (R) and lysine (K) are positively charged. The hydrophobic tails of the peptide surfactants consist of alanine (A), valine (V) or isoleucine (I). Each peptide is ca. 2.5 nm long, similar to biological phospholipids. Color code: cyan, carbon; red, oxygen; blue, nitrogen; white, hydrogen.

Results and Discussion

Enhanced PS-I Activity in the Presence of Peptide Surfactants. We systematically studied the effect of conventional detergents and designer peptide surfactants on PS-I electron transfer activity using a well-established assay. The PS-I functionality was sensitive to light exposure as indicated by blank control experiments (Figure 3A). In the absence of light, O_2 consumption was negligible regardless of the presence of PS-I, peptide surfactants or other chemical detergents that are commonly used for membrane protein stabilization. In the presence of PS-I, illumination of a solution containing ascorbate and DCIP as electron donors and methylviologen as acceptor, resulted in O_2 consumption. The kinetics of O_2 consumption were measured using an oxygen electrode.

The O_2 consumption observed in the presence of PS-I in the reaction solution and upon illumination of the sample could not be attributed to (i) the presence of the peptide molecules alone because control experiments using different concentrations of

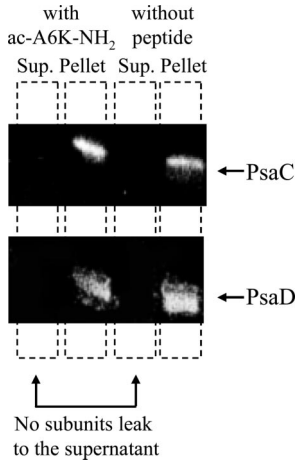


Figure 2. Western blot immunodetection of the PsaC and PsaD component subunits of the PS-I protein complex in the presence and in the absence of the ac-A6K-NH_2 peptide. Samples were tested after vortexing and centrifugations at 100000*g* in both the supernatant and the pellet showing the absence of subunit removal.

peptide surfactants in the working solution did not show measurable consumption of O_2; (ii) the presence of free plastocyanine (in vivo electron donor) in the PS-I samples because in control experiments in the absence of DCIP (in vitro electron donor), PS-I did not show O_2 consumption activity (if PS-I samples were contaminated with plastocyanine they would consume O_2 even in the absence of DCIP); (iii) the dissociation of the PS-I supercomplex and release of chlorophyll molecules because Western Blot analysis of the PS-I samples in the presence of the ac-A_6K-NH_2 peptide did not show release of the stroma subunits PsaC and PsaD which are coupled with the transmembrane subunits of PS-I (Figure 2); (iv) uncoupled and released core-antenna chlorophylls released from the PS-I complex because activity tests performed in the presence of different concentrations of free chlorophyll molecules did not show any measureable consumption of O_2 in the reaction solution; (v) singlet oxygen production because in the reaction solution we use ascorbate which would react with singlet oxygen and produces H_2O_2.[28]

Furthermore, the low activity of PS-I in the presence of the chemical detergents FC-14, DDM, DG and Triton X-100 was not due to protein denaturation. Protein destabilization is often observed at high detergent concentrations. Activity tests performed using 0.05 mM (below the CMC) and 0.47 mM (above the CMC) of the chemical detergents resulted in similar O_2 consumption activities of PS-I (Figure 3B). It should be noted, however, that the purification and stabilization of PS-I for crystallographic analyses included steps where PS-I interacted with 3 mM of Triton X-100 and 1 mM of DDM without damage of the protein complex conformation.

The effect of different ac-A_6K-NH_2 concentrations on the PS-I activity is shown in Figure 4. The CMC of ac-A_6K-NH_2 in the reaction solution is 0.26 mM (Table 1). PS-I activity showed a strong dependency on the ac-A_6K-NH_2 concentration from 0.017 mM (~15 times less than the CMC) to 0.47 mM (~1.8× CMC). The addition of 0.47 mM of ac-A_6K-NH_2 resulted in a significant 9-time increase of the initial rate of PS-I activity as compared to the activity of PS-I alone (Figure 3A). Further increase in the concentration of ac-A_6K-NH_2 did not result in further increase of the PS-I activity: PS-I stabilized by 0.47 mM and 0.94 mM of ac-A_6K-NH_2, i.e., 1.8× CMC and 3.6× CMC, respectively, showed essentially the same activity indicating saturation of the system with the peptide surfactant.

We then tested the effect of various peptide sequences on PS-I activity. We designed a number of peptides with a hydrophobic tail and a hydrophilic head. The ac-$I_6K_2-NH_2$, ac-V_6K_2-NH_2 and ac-V_6R_2-NH_2, peptide surfactants accelerated the O_2 consumption of PS-I up to 8, 10 and 12 times, respectively (Figure 5). All peptides and commercial detergents were tested at the same concentration, i.e., 0.47 mM, which was higher than their respective CMC and the values presented in Figure 5 were determined from the slope of O_2 consumption curves similar to those presented in Figures 3A,B and 4. The PS-I activity in the presence of the best performing peptide surfactants was superior compared to that of standard chemical detergents that are used for the stabilization of membrane proteins. Positively charged amphiphilic peptides significantly enhanced the activity of PS-I (Figures 1 and 5). Sequences containing negative charges including the ac-A_6K-OH (zwitterionic), ac-A_6D-OH (anionic) and ac-V_6D_2-NH_2 peptide (anionic, with net charge one negative because the $-NH_2$ group is positively charged at the experimental conditions used for the activity measurements) did not improve the PS-I activity although they had amphiphilic properties.

PS-I Stabilization by Peptide Surfactants for Extended Time. The stabilization of functional PS-I by peptide surfactants was also studied as a function of time. The initial activity of PS-I mixed with the ac-A_6K-NH_2 or ac-A_6D-OH peptides, alone or in combination with the chemical detergent DDM was measured over a period of more than 2 months. In the presence of ac-A_6K-NH_2, PS-I showed an initial decrease of activity within the first two days and reached a plateau after 1 week. However, even after 2 months in the presence of 0.47 mM ac-A_6K-NH_2, PS-I was highly active, namely, ~5 times more active than PS-I alone or PS-I in the presence of 0.47 mM DDM (Figure 6A).

For structural analyses and for applications in biosensors, it is crucial to stabilize membrane proteins for extended time. The time course experiments showed that PS-I stabilized by ac-A_6K-NH_2 remained highly active even after 2 months. The fact that PS-I mixed with peptide surfactants retains enhanced activity for prolonged periods of time suggests that stabilization of the functional conformation of PS-I complex by the peptides is not temporary. It was shown previously that PS-I was stabilized by peptides for 3 weeks in the dry state on the surface of a semiconductor.[11] The current study not only confirms the earlier results but also shows that stabilization can be maintained for more than 2 months in aqueous media, an environment in which membrane proteins tend to aggregate more readily.

Thermal Stability Experiments. The structural stability of PS-I alone or mixed with peptide surfactants and DDM was investigated at different temperatures using fluorescence spectroscopy. Upon excitation at 442 nm the emission profile of PS-I is sensitive to the relative position of the chlorophyll molecules in the three-dimensional structure of the protein. Therefore, monitoring the chlorophyll fluorescence may be used to study the structural stability of the PS-I complex. The wavelength of maximum emission (λ_{max}) was monitored as a function of temperature between 20−85 °C (Figure 6B). A blue shift of the λ_{max} was observed at a temperature representing denaturation of the protein.

Peptide surfactants, regardless of their ability to increase the PS-I activity did not improve the thermal stability of PS-I. On the contrary, the presence of peptides resulted in denaturation of PS-I at temperatures which were ~15 °C lower than those of PS-I alone (containing 0.03 mM DDM to prevent aggregation). Plotting the wavelength of maximum emission (λ_{max}) of

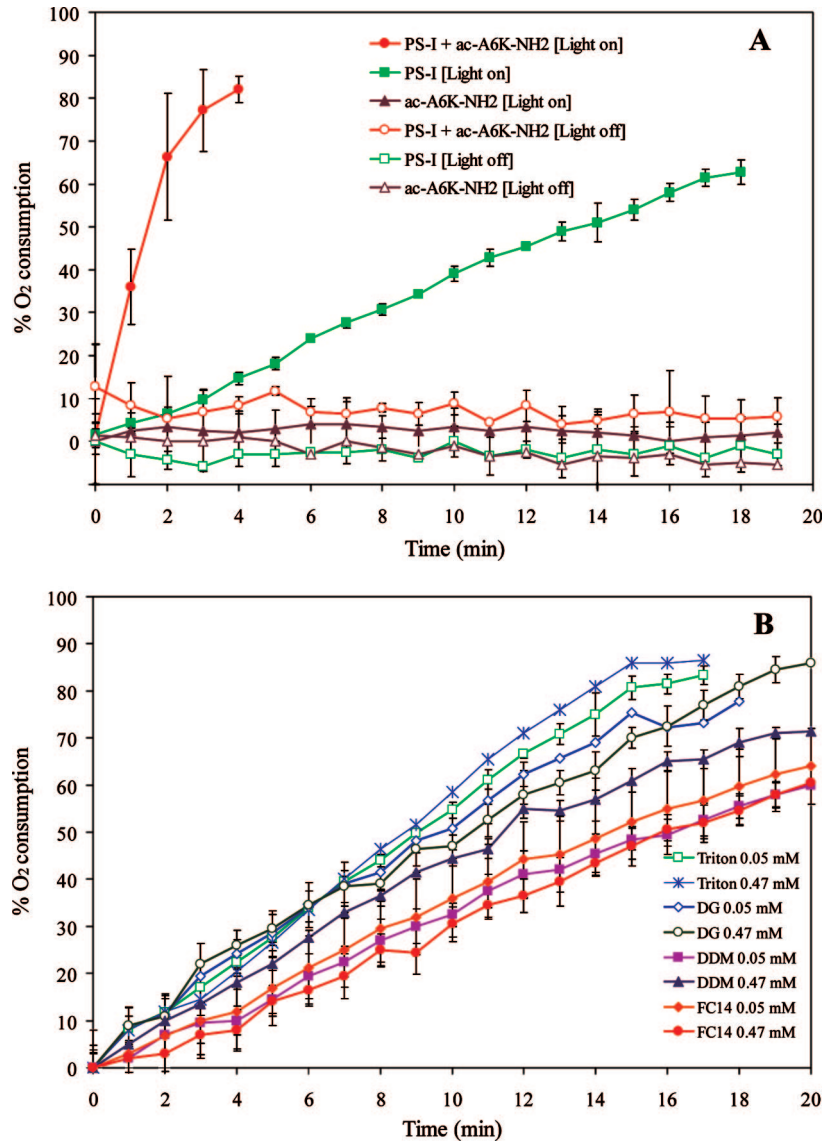


Figure 3. Functional enhancement of PS-I interacting with different amphiphilic peptides and with commercial detergents. (A) The PS-I activity was 9 times higher in the presence of 0.47 mM ac-A6K-NH_2 relative to that measured in the absence of the peptide. The PS-I functionality depends on light which initiates an electrochemical reaction cascade that results in the consumption of the O_2 dissolved in the solution. (B) Control experiments of the PS-I activity in the presence of the commercial detergents FC-14, DDM, DG, Triton X-100 at concentrations of 0.47 mM, which was used in the experiments presented in Figure 4, and 0.05 mM, which is below the CMC of the detergents; these measurements were performed to confirm that the observed low activity of PS-I in the presence of 0.47 mM of the commercial detergents is not due to destabilization of the PS-I protein.

PS-I mixed with peptide surfactants as a function of temperature revealed a transition at ca. 66 °C. The PS-I from the thermophile *T. elongatus* is a thermostable molecule, and therefore, it is not surprising to observe denaturation at such high temperatures. Testing the PS-I activity after thermal treatment showed that all samples were inactive for the O_2 consumption electrochemical reaction, suggesting that upon heat treatment PS-I was irreversibly denatured. These experiments suggest that peptide surfactants increased the activity of the membrane protein by a mechanism which does not involve thermal stabilization. It is also suggested that the peptide surfactants do not bind to the protein in a tight and irreversible mode.

Heating PS-I in the presence of 0.47 mM DDM, as in the oxygen consumption experiments, did not reveal a clear denaturation transition but a gradual wavelength shift, suggesting that DDM destabilizes the three-dimensional structure of the PS-I complex above room temperature. At 24 °C, PS-I was active in the presence of 0.47 mM DDM (i.e., 2.7× above the CMC). However, above room temperature this concentration of DDM resulted in notable changes in the tertiary structure of the protein complex. It is likely that the presence of 0.47 mM DDM or peptide surfactants results in decreased thermal stability of PS-I, by a similar mechanism involving dissociation of the PS-I subunits. At elevated temperatures, the energy given to the system and the surfactant properties of DDM or the peptides loosen the intersubunit interactions and facilitate the PS-I complex denaturation. DDM, which possesses stronger surfactant properties compared to those of the peptide surfactants, had a more pronounced effect upon PS-I thermal destabilization. From these experiments we can also hypothesize that the increased activity measured observed at room temperature upon mixing peptide surfactants with PS-I could not be due to destabilization of PS-I and release of chlorophyll molecules because this phenomenon occurs at higher temperatures.

AFM Imaging. We also studied the topological structure of PS-I, with and without surfactants, on the surface of mica using

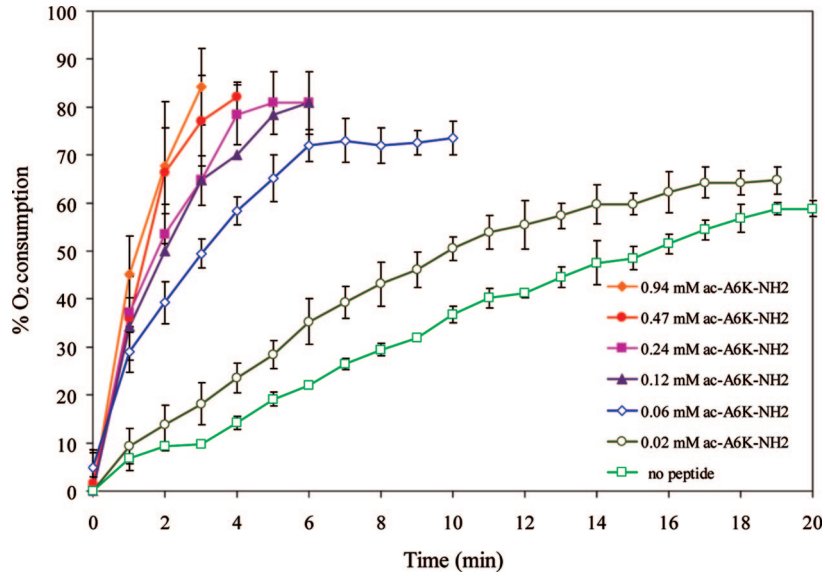


Figure 4. PS-I activity at different concentrations of ac-A6K-NH_2. All data points are average of $n = 3$. The PS-I concentration corresponds to 5.6 μM of chlorophyll. Percentages on y-axis denote O_2 consumption relative to the maximum amount of O_2 in the solution which corresponds to solution saturation with O_2.

TABLE 1: Critical Micelle Concentration (CMC) of the Lipid Like Peptide Surfactants in the Reaction Solution Used for the O_2 Consumption Measurements

surfactant	type	CMC in the reaction solution (mM)
ac-V_6R_2-NH_2	cationic	0.08
ac-V_6K_2-NH_2	cationic	0.09
ac-A_6K-NH_2	cationic	0.26
ac-I_6K_2-NH_2	cationic	0.10
KA_6-NH_2	cationic	0.13
ac-A_6K-OH	zwitterionic	0.11
DA_6-NH_2	zwitterionic	0.09
ac-V_6D_2-NH_2	anionic	0.10
ac-A_6D-OH	anionic	0.22
DDM	nonionic	0.17
Triton X-100	nonionic	0.29
DG	nonionic	0.19
FC-14	zwitterionic	0.09

tapping mode AFM. The bare surface of mica is smooth, i.e., rms 0.4 nm, which is small compared to the dimensions of PS-I and of the peptide surfactant assemblies. Figure 7a shows PS-I molecules with an average height of 5−6 nm and a diameter between 40−60 nm. Because of tip broadening effects the actual

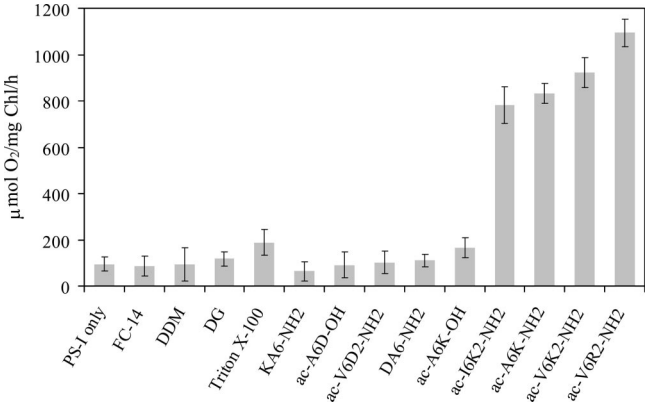
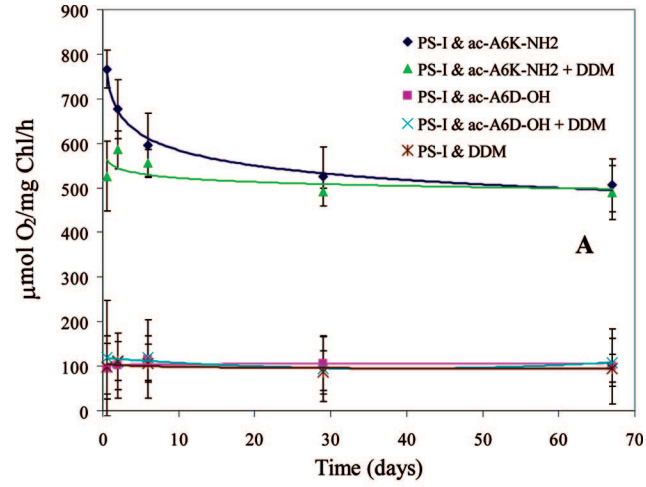


Figure 5. Comparative analysis of the PS-I activities in the presence of commercial detergents that are commonly used for the stabilization of membrane proteins and of peptide surfactants. The error bar in the O_2 consumption of PS-I stabilized by ac-A6K-NH_2 is 6.7%.



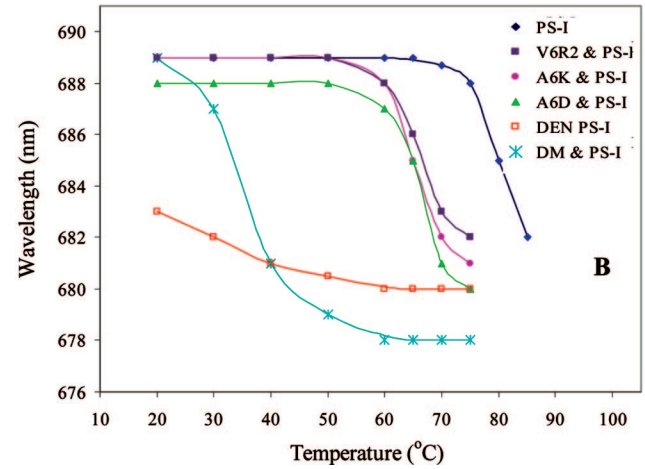


Figure 6. (A) Stability kinetics of PS-I interacting with ac-A_6K-NH_2 or ac-A_6D-OH amphiphilic peptides alone or mixed with the DDM detergent. In the time course experiment, the activity of PS-I was measured at 20 °C over a period of 2 months. (B) The thermal stability of PS-I was determined by monitoring the wavelength of maximum emission as a function of temperature (excitation was at 442 nm). Measurements were carried out in the presence of 0.47 mM of amphiphilic peptide or DDM.

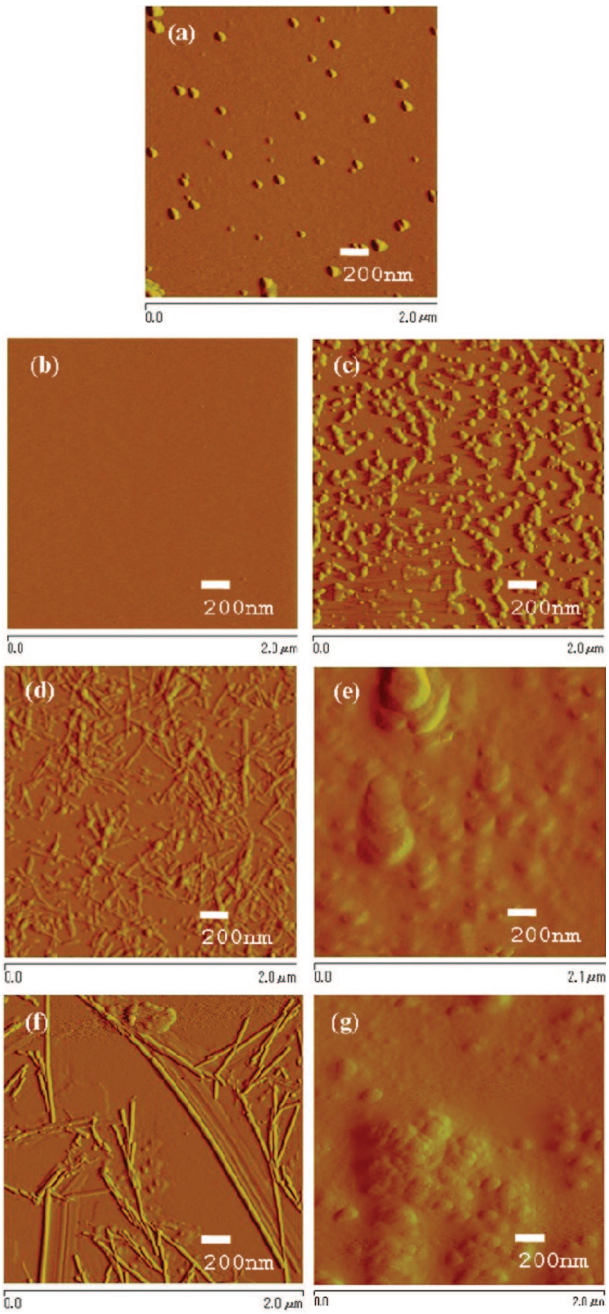


Figure 7. Tapping mode AFM images of PS-I stabilized by ac-A_6K-NH_2 peptide and by DDM or Triton X-100 detergent. (a) the PS-I multiprotein complex natively appears as dimer or trimer with a diameter between 30−50 nm, (b) DDM detergent on mica surface, (c) DDM mixed with PS-I, (d) Triton X-100 on mica surface, (e) PS-I mixed with Triton X-100, (f) ac-A_6K-NH_2 peptide forms nanotubes approximately 100 nm long, (g) PS-I in the presence of ac-A_6K-NH_2 (h) ac-A_6D-OH forms long nanotubes more than 500 nm long, (i) PS-I in the presence of ac-A_6D-OH. DDM and Triton X-100 dispersed PS-I to small particles whereas PS-I mixed with ac-A6K-NH_2 or ac-A_6D-OH resulted in large and small vesicles, respectively. Scale bar is 200 nm.

width of individual molecules and structures are smaller than those measured.[29] On the basis of crystallographic data, the PS-I trimer complex has a calculated height of 6 nm and a diameter of ca. 50 nm. Therefore, the structures observed in the AFM topographical images correlate well with the PS-I trimers.

When PS-I was mixed with 0.47 mM DDM (Figure 7c), showed surface patterns similar to that of PS-I alone (Figure 7a), which suggests that 0.47 mM DDM did not induce notable

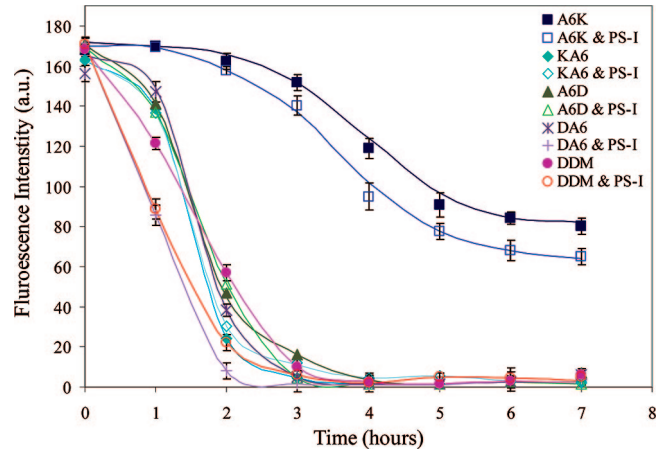


Figure 8. Retention of encapsulated carboxyfluorescein, CF, in peptide- and DDM-based vesicles upon incubation at 20 °C in the presence and in the absence of PS-I.

structural changes in PS-I. The surface topology of DDM on mica could not be distinguished from the bare mica surface (Figures 7b).

The ac-A6K-NH_2 peptides alone formed nanotubes of more than 100 nm long (Figure 7d) featuring a morphology similar to that observed for other types of amphiphilic peptides.[14,17] However, the (PS-I)-(ac-A_6K-NH_2) mixtures featured a two-dimensional "island" topology of large vesicles (Figure 7e). Cross-sectional analysis of the surface topography showed that the clusters have a step height of 8−15 nm and an average diameter of 160 nm with particle sizes ranging between 110 and 350 nm. Similar morphology but different vesicle size was observed upon mixing PS-I with other peptide surfactants including ac-A_6D-OH (e.g., Figure 7g).

Peptide Vesicle Stability. The stability and structural integrity of the peptide vesicles in the presence and in the absence of PS-I was determined by measuring the release kinetics of the fluorescent probe CF. Release experiments were performed by testing vesicles that consisted of positively ac-A_6K-NH_2 and KA_6-$CONH_2$ (the former increased the PS-I activity but the latter did not) and negatively charged peptides ac-A_6D-OH and DA_6-$CONH_2$ (which did not enhanced the PS-I activity). As may be seen in Figure 8, the peptide vesicles consisting of ac-A_6K-NH_2 were more stable compared to other peptide surfactant vesicles and DDM and retained the fluorescent probe entrapped in the vesicle for prolonged periods of time. Under the harsh conditions employed in the release experiment (i.e., centrifugation and resuspension of the vesicles) the CF release through the ac-A_6K-NH_2 peptide vesicle reached a plateau after 7 h at a concentration 50% less than the initial loading of the vesicle. In the case of vesicles composed of KA_6-$CONH_2$, ac-A_6D-OH and DA_6-$CONH_2$ peptides and DDM detergent CF was completely released within 3−4 h.

In all cases, the presence of PS-I resulted in faster release kinetics of CF through the vesicles, which suggests that the protein complex affects the structural integrity of the vesicle probably by integration in the vesicle bilayer. This is commonly observed upon addition of lipophilic molecules in liposome systems.[30]

Important Features for Enhancing PS-I Activity. In this study we showed the stabilization of functional PS-I complex using designer peptide surfactants. We measured the light-induced electron transfer activity of PS-I upon interaction with peptide surfactants and chemical detergents. Although the latter are commonly used for the stabilization, purification, and

TABLE 2: Amino Acid Sequence and Charge Distribution of the Peptides Used for PS-I Stabilization[a]

Surfactant	Peptide sequence and charge distribution	Initial O_2 turnover (μmol/mg$_{Chl}$/h)	Activity increase
ac-V_6R_2-NH_2	acetyl-Val-Val-Val-Val-Val-Val-Arg-Arg-$CONH_2$	1094	11.6
ac-V_6K_2-NH_2	acetyl-Val-Val-Val-Val-Val-Val-Lys-Lys-$CONH_2$	923	9.8
ac-A_6K-NH_2	acetyl-Ala-Ala-Ala-Ala-Ala-Ala-Lys-$CONH_2$	833	8.9
ac-I_6K_2-NH_2	acetyl-Ile-Ile-Ile-Ile-Ile-Ile-Lys-Lys-$CONH_2$	781	8.3
ac-A_6K-OH	acetyl-Ala-Ala-Ala-Ala-Ala-Ala-Lys-COO^-	166	1.8
DA_6-NH_2	$^+NH_3$-Asp-Ala-Ala-Ala-Ala-Ala-Ala-$CONH_2$	110	1.2
ac-V_6D_2-NH_2	acetyl-Val-Val-Val-Val-Val-Val-Asp-Asp-$CONH_2$	103	1.1
ac-A_6D-OH	acetyl-Ala-Ala-Ala-Ala-Ala-Ala-Asp-COO^-	92	1.0
KA_6-NH_2	$^+NH_3$-Lys-Ala-Ala-Ala-Ala-Ala-Ala-$CONH_2$	64	0.7

[a] Highlighted domains represent amino acids with positive charge (blue), negative charge (red) and hydrophobic side chains (gray). The activity increase was calculated with regards to the initial activity of PS-I alone containing traces of DDM [i.e., 94 (μmol/mg$_{Chl}$)/h].

crystallization of membrane proteins, they did not improve the PS-I activity. The peptide surfactants ac-I_6K_2-NH_2, ac-A_6K-NH_2, ac-V_6K_2-NH_2 and ac-V_6R_2-NH_2 significantly enhanced the activity of PS-I. Our results suggest that peptide surfactants may interact with the membrane protein in such a way that the conformational characteristics of the peptides may be complementary to that of PS-I. Although all peptides were designed with a hydrophobic tail and a hydrophilic head, other factors were shown to be important for ensuring high PS-I functional activity.

A comparative analysis of the data showed that positive charges on the C-terminal end of the peptide (e.g., ac-V_6K_2-NH_2 and ac-A_6K-NH_2) are essential for maintaining high PS-I activity. Peptides carrying a negative charge in the C-terminus did not enhance the PS-I activity. Negatively charged peptide sequences such as ac-V_6D_2-NH_2, ac-A_6D-OH and DA_6-NH_2 where lysine (K) was substituted by aspartic acid (D) had essentially no effect toward a functional PS-I.

Furthermore, although ac-A_6K-NH_2 and ac-A_6K-OH have the same amino acid sequence, differing only in the capping group of the C-terminal amino acid, lysine, ac-A_6K-NH_2 is very effective in increasing the activity of PS-I for prolonged times whereas ac-A_6K-OH has no significant effect (Figures 5 and 6A). Both peptides are acetylated at their N-terminal and the ϵ-amine group of lysine is positively charged, i.e., $-CH_2-NH_3^+$. However, at pH 7.5 the amidated C-terminus of ac-A_6K-NH_2, i.e., $-CONH_2$, is not charged, but the C-terminus ac-A_6K-OH is negatively charged, i.e., $-COO^-$. Hence, the net charge of ac-A_6K-OH is neutral (Figure 1 and Table 2).

The amino acid sequence, which defines the position of the charges on the peptide, is also important for designing an efficient surfactant. Although the interaction of ac-A_6K-NH_2 with PS-I significantly increased the protein activity, the KA_6-NH_2 peptide did not. The nonacetylated N-terminal side of KA_6-NH_2 has a free amine group, which is positively charged at pH 7.5 (Figure 1 and Table 2). Hence, the charge distribution of KA_6-NH_2 differs from that of the ac-A_6K-NH_2.

The importance of the sequence was also identified in the cases of the ac-I_6K_2-NH_2, ac-A_6K-NH_2, ac-V_6K_2-NH_2 and ac-V_6R_2-NH_2 peptides, which significantly enhanced the activity of PS-I. It was shown that increasing the hydrophobicity of the side chain, namely, alanine < valine < isoleucine,[31,32] of the hydrophobic tail amino acids was not crucial for PS-I activity. Although the 6 isoleucines in the ac-I_6K_2-NH_2 sequence render the peptide tail more hydrophobic compared to ac-V_6K_2-NH_2, the latter is more effective toward an active PS-I. Furthermore, when the PS-I activities in the presence of ac-V_6K_2-NH_2 and ac-V_6R_2-NH_2 peptides were compared, it was shown that the two positively charged arginines (the most hydrophilic amino acid at pH 7.5) at the C-terminus are better than the two lysines (Figure 5).

Proposed Interaction. On the basis of our observations, we propose here that chemical and structural compatibility between designer peptide surfactants and PS-I are crucial for the activity enhancement and stabilization of PS-I. AFM analysis revealed the formation of large vesicles for all of the peptide surfactants mixed with PS-I. However, not all the peptides are effective in enhancing the activity of PS-I. This suggests that the formation of vesicles is not a necessary condition to determine the peptide surfactant effectiveness.

The release profiles of the fluorescent probe CF through the vesicles suggested that the structural integrity of the peptide vesicles depends on the type of peptide. Although all peptide vesicles seem to maintain their macroscopic organization, they fail to retain the encapsulated material. The fact that the CF leak was faster when PS-I was mixed with the peptide surfactants suggests that the continuous hydrophobic vesicle bilayer was marginally disrupted upon incorporation of the large transmembrane PS-I protein complex in the bilayer. The intrinsic structural instability of the vesicle bilayer and the weak interactions between the peptide surfactants and the protein results in leakage of the PS-I complex outside of the vesicle environment. This mechanism may be responsible for the decreased activity of PS-I when mixed with some of the peptides.

The higher rigidity of the vesicles consisting of ac-A_6K-NH_2 correlates well with the PS-I activity tests (Figure 4). It was shown that the interaction of ac-A_6K-NH_2 with PS-I resulted in increased protein activity as compared to that observed when PS-I was mixed with the KA_6-$CONH_2$, ac-A_6D-OH, DA_6-$CONH_2$ peptides or DDM.

The higher rigidity and packing order of the vesicles consisting of ac-A_6K-NH_2 correlates well with the PS-I activity tests (Figure 4). It was shown that the interaction of ac-A_6K-NH_2 with PS-I resulted in increased protein activity as compared to that observed when PS-I was mixed with the KA_6-$CONH_2$, ac-A_6D-OH, DA_6-$CONH_2$ peptides or DDM. The fact that ac-A_6K-NH_2 based vesicles maintained (i) their structural integrity

for prolonged periods of time under extreme handling conditions (i.e., repetitive cycles of centrifugation and resuspension) and (ii) the PS-I activity for extended periods of time (Figure 6A) suggests that the stabilization effect of the ac-A_6K-NH_2 peptides on PS-I is significant and has the potential to be used for applications in bioengineering.

Although standard chemical surfactants substitute the membrane lipid bilayer by aligning in the vicinity of the trans-membrane region, the peptide surfactants not only have surfactant properties but also self-assemble into larger structural architectures that can encapsulate the PS-I complex into macro-structures. Therefore, peptide surfactants may act on two levels: (i) at the molecular level they surround and stabilize the PS-I trans-membrane protein complex and (ii) macroscopically they form a three-dimensional network that protects a system of many PS-I molecules for prolonged time.

Other Important Features. In this study, the light-induced electron transfer activity of PS-I was measured in aqueous media in the presence of peptide surfactants. Our results showed that the peptide surfactant's amphiphilic properties alone are not sufficient to stabilize the trans-membrane complex PS-I in a functional state and that the conformational characteristics of the surfactant peptides are important. It was shown that the amino acid sequence is crucial, and thus, we propose here a guide for designing new peptide surfactants for the stabilization of active PS-I. The best peptide surfactant should have: (i) acetylated N-terminus, (ii) a short hydrophobic tail with 6 consecutive hydrophobic amino acids: valines showed optimal PS-I stabilization, probably because they are short and very hydrophobic, (iii) 1 or 2 polar positively charged amino acids such as lysine or arginine in the C-terminus; these amino acids do not increase PS-I activity when present in the N-terminal, and (iv) amidated C-terminus.

It is believed that these simple, short, inexpensive (less than $45 per gram) and versatile peptides will offer new possibilities to study complex and previously intractable membrane proteins. Molecular engineering of the designer peptides at the single amino acid level is an enabling technology that will likely play an increasingly important role both in studying elusive membrane proteins in the coming decades and in exploring their use for applications in protein-based nanodevice for solar energy device, biosensors and nanoelectronics.

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