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Enhanced Electron Transfer Activity of Photosystem I by Polycations in Aqueous Solution

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The use of proteins in advanced nanotechnological applications requires extended stabilization of the functional protein conformation and enhanced activity. Here we report that simple cationic poly(amino acid)s can significantly increase the activity of the multidomain protein supercomplex Photosystem-I (PS-I) in solution better than other commonly used chemical detergents and anionic poly(amino acid)s. We carried out a systematic analysis using a series of poly(amino acid)s (i.e., poly-L-tyrosine, poly-L-histidine, poly-L-aspartic and poly-L-glutamic acid, poly-L-arginine, and poly-L-lysine). Our results show that the polycations poly-L-lysine and poly-L-arginine significantly enhance the photochemical activity of PS-I, whereas negatively charged and hydrophobic poly(amino acid)s did not increase the PS-I functionality in solution. Furthermore, we show that poly-L-lysine can stabilize highly active PS-I in the dry state, resulting in 84% activity recovery. These simple and inexpensive poly(amino acid)s will likely make significant contributions toward a highly active form of the PS-I membrane protein with important applications in nanotechnology and biotechnology.

Introduction

The ability to preserve protein conformation and maintain or even increase its activity in solution during a chemical reaction is of utmost importance for biotechnological applications. To this end, a number of strategies have been employed including the addition of sugars,[1] lipids,[2] chemical detergents,[3] alcohols,[4] peptides,[5] proteins,[6] polysaccharides,[6] and synthetic polymers.[7] Knowledge of how these agents act on solubilized proteins can provide alternative routes to design new and more efficient technologies for the stabilization and functionalization of proteins. Many proteins are not stable outside their natural environment. This is also true for membrane proteins, which, when removed from the cell membrane bilayer, tend to unfold and aggregate with a subsequent loss of activity.

Photosystem I (PS-I) is a thylakoid transmembrane protein complex that is associated with one of the first steps of the photosynthetic process.[8] The photocatalytic properties of PS-I, which can be used for the production of hydrogen, have sparkled a vigorous research toward the development of strategies to stabilize and increase the activity of PS-I. To date, there has been modest success in developing technologies and the construction of devices that employ PS-I to harvest light energy and convert it to chemical energy.[9,10] Current technologies that integrate PS-I in solid-state electronics cannot parallel the efficiency of the molecular circuitry and organization found in nature.

The crystal structure of PS-I from the thermophilic cyanobacterium *Thermosynechoccus elongatus* was solved by Jordan et al.[11] The trimeric protein complex has a molecular weight of 1.07 MDa and consists of 36 proteins with 381 noncovalently attached cofactors. Each monomer consists of 12 proteins, 9 of which feature a network of 34 transmembrane α-helices (for a total of 102 helices in the trimer) that are buried within the lipid bilayer (Figure 1). The large number of transmembrane helices and extensive interactions with the thylakoid membrane 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 at high concentrations of detergent to remove extraneous membrane components, the soluble membrane protein molecules still need small quantities of detergent to avoid aggregation and denaturation.

Previously, we tested the efficiency of designer seven-residue peptides with surfactant properties for their ability to enhance the photoinduced activity of the PS-I membrane protein from *T. elongatus*.[5] Surfactant-like peptides are ca. 2.5 nm long, similar to biological phospholipids. On the basis of the conclusions of the previous work about the type of charge and charge distribution of an efficient molecule toward a stable and active PS-I, herein we tested several poly(amino acid)s mixed with the PS-I complex in solution. The photochemical activity of functionalized PS-I was measured using an electrochemical reaction in which the reaction was monitored by the decrease in the dissolved oxygen. However, the photon-induced electron transport activity of PS-I can be easily transferable to produce H_2,[10,12−16] which is an important fuel. Herein, we propose a strategy for the stabilization of PS-I to be used for the conversion of light energy into chemical energy and the production of hydrogen fuel.

Materials and Methods

PS-I Purification. The PS-I complex was extracted from the thylakoid membranes of the thermophilic cyanobacteria *T. elongatus*. Bacterial growth was followed by incubation with 0.25% (w/v) lysozyme for 2 to 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,

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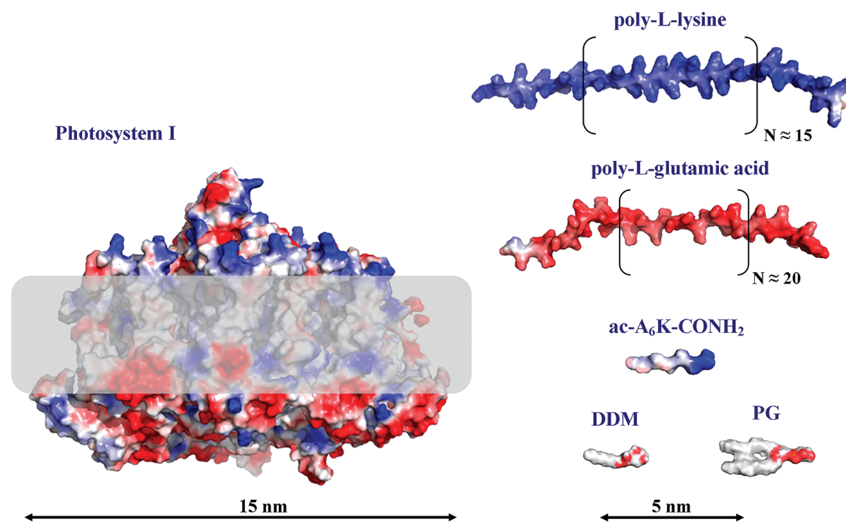

Figure 1. Comparative schematic representation of the PS-I monomer in which the transmembrane domain is shaded. Models of poly-L-lysine, poly-L-glutamic acid, and the chemical surfactants DDM and PG are also shown. Electrostatic potential models were generated by PyMol; blue and red represent positive and negative charges, respectively.

and membranes were collected at 20 000 rpm. The membranes were washed and solubilized as in Fromme and Witt[17] 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, 1 mM, *n*-dodecyl-*β*-D-maltopyranoside, DDM) for 18 h at 100 000*g* and at 4 °C. The PS-I band was collected, pooled, and stored at −20 °C. Purity was confirmed by Tris-tricine SDS-PAGE gel electrophoresis.[18] The chlorophyll content of the PS-I sample was measured by the method of Porra.[19]

Chemicals and Poly(amino acid)s. The chemical surfactant *n*-dodecyl-*β*-D-maltopyranoside (DDM) was purchased from Anatrace (Maumee, OH). L-Lysine, poly-L-lysine hydrobromide (MW 15 000−30 000), poly-L-arginine hydrochloride (MW 15 000−70 000), poly-L-histidine (MW 5000−25 000), poly-L-tyrosine (MW 10 000−40 000), poly-L-aspartic acid sodium salt (MW 15 000−50 000), poly-L-glutamic acid sodium salt (MW 15 000−50 000), tricine, methylviologen (MV), 2,6-dichloroindophenol (DCIP), sodium ascorbate, and 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea (DCMU) were purchased from Sigma Aldrich. 1,2-Dioleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) sodium salt (PG) was obtained from Avanti Polar Lipids (Alabaster, AL). Pure chlorophyll was purchased from Sigma Aldrich and was used for control experiments.

Oxygen Consumption Measurements. PS-I functionality was determined by a method that is routinely employed to study PS-I functionality.[20,21] In the presence of PS-I, the O_2 consumption in solution was monitored with an oxygen-specific electrode according to Tjus et al.[22] The working solution with volume totaling of 3.5 mL contained 40 mM tricine, 167 μM MV, 0.1 mM DCIP, 1 mM sodium ascorbate, 10 mM NH_4Cl, and 10 μM DCMU at pH 7.5. The PS-I concentration corresponds to 5.6 μM chlorophyll. To determine the activity and functionality of PS-I, we monitored the course of an electrochemical reaction, which involved electron flow through PS-I using as electron donor and acceptor, DCIP and MV, respectively.[22] 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 the dissolved O_2. The decrease in the latter was measured by an O_2 electrode model ISO2 (World Precision Instruments, 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.[23] The electrode was standardized before and after each set of measurements with air-saturated water (20.4% at 24 °C) according to the instrument 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 of 30 W and luminous intensity of 107 600 cd sr/m^2 (which corresponds to ca. 1800 μmol of photons $m^{-2}\cdot s^{-1}$). All measurements were performed at 24 °C in 5 mL of poly(methyl methacrylate) (PMMA) closed cuvettes under continuous stirring.

Oxygen depletion from the solution in the presence of PS-I was recorded after a stable O_2 concentration reading was achieved in the air-saturated working solution. Upon illumination of the PS-I sample, the O_2 concentration was monitored every minute. The PS-I activities were determined from the initial slopes of the plots of O_2 consumption as a function of time. In all cases, the standard deviation ($n = 3$) was <2.4%. Before and after each experiment, a series of blank tests were performed.

Western Blots. The structural integrity of the PS-I supercomplex was analyzed by Western blot using specific antibodies for the PsaC and PsaD subunits of PS-I (Agrisera, Vännäs Sweden).[24] To verify that PsaC and PsaD were not removed from the PS-I in the medium used for the activity tests, we vigorously vortexed and then centrifuged PS-I samples at 100 000*g* for 30 min at 4 °C. The supernatant was 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).

Atomic Force Microscopy (AFM). AFM studies were carried out using a SII SPA400 (Seiko Instruments, Japan) operated in tapping mode. Soft silicon probes were used (SI-DF20, Seiko Instruments) with tip radius <10 nm mounted on a single-beam cantilever. Cantilever deflections were recorded with a cantilever frequency of 119 kHz, horizontal scan rate of 1.2 Hz, and 512 samples per line. PS-I sample (2 μL) in 40 mM tricine buffer was sonicated for 50 s and deposited on freshly cleaved muscovite mica (Agar Scientific, Stansted, Essex, U.K.). The 2 μL sample was allowed to interact with the mica surface for 30 s; then, it was rinsed with Milli-Q water and dried in a gentle stream of nitrogen gas, and the AFM images were acquired immediately. Imaging was performed in air at temperature 23 °C and relative humidity 21%. For the data analysis, the instrument's image processing software was employed to obtain height patterns, cross sections, and rms. For each experimental condition, AFM images were collected from two different samples and at random spot surface sampling (at least five spots). The images were reproducible.

Stabilization of PS-I in the Dry State. PS-I alone and in the presence of poly-L-lysine was freeze-dried (Lyph Lock 4.5, Labconco, Kansas City, MO) at 50 × 10^{-3} mbar for 15 h. Freeze-dried PS-I

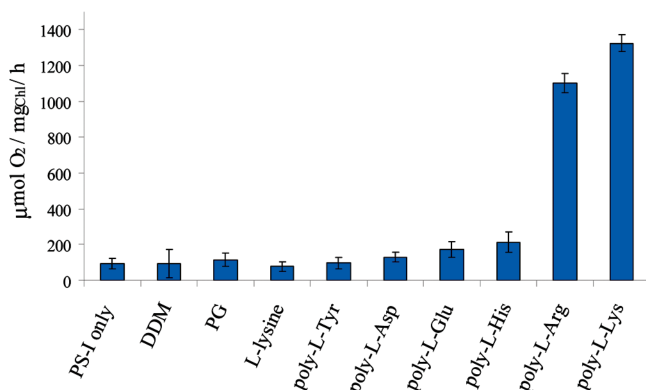

Figure 2. Comparative analysis of the PS-I activities in the presence of 0.28 mg/mL of chemical surfactants and poly(amino acid)s. The PS-I concentration corresponds to 5.6 μM of chlorophyll. All data points are the average of $n = 3$.

samples with and without 0.28 mg/mL of the stabilizing additive were stored at 4 °C for a period of up to 1 week prior to analyses. Subsequently, the PS-I samples were rehydrated, and their activity was tested in triplicate using the O_2 consumption assay, as previously described.

Results and Discussion

Enhanced PS-I Activity in the Presence of Poly(amino acid)s. We systematically studied the effect of poly(amino acid)s on PS-I electron transfer activity using a well-established assay that is routinely employed to evaluate PS-I functionality. The activity, as determined by electron transfer phenomena, is sensitive to light, as indicated by control experiments in which the consumption of O_2 in the dark was negligible regardless of the presence of PS-I and protein stabilization additives. In the presence of PS-I, illumination of a solution containing ascorbate and DCIP as electron donors and MV as electron acceptor resulted in O_2 consumption, which was measured by an oxygen electrode. The PS-I photoactivity was investigated in the presence of the chemical surfactants DDM and PG at concentrations above and below their critical micelle concentration (CMC) or in 0.28 mg/mL of basic, acidic, and hydrophobic poly(amino acid)s (Figure 2).

The decrease in the dissolved O_2 concentration could not be attributed to (i) the presence of chemical surfactants or poly(amino acid)s because control experiments without PS-I using different concentrations of the additives did not show measurable consumption of O_2; (ii) the presence of plastocyanin (in vivo electron donor) in the PS-I samples because in control experiments in the absence of DCIP (in vitro electron donor) we did not measure O_2 consumption (if PS-I samples were contaminated with plastocyanin, O_2 consumption would be observed even in the absence of DCIP); (iii) the dissociation of the PS-I supercomplex and release of chlorophyll molecules because Western Blot analysis of PS-I in the solution used for the activity measurements did not show release of the stroma subunits PsaC and PsaD, which are loosely coupled to the transmembrane subunits of PS-I (Figure 3); (iv) uncoupled and core-antenna chlorophylls that could possibly have been released from the PS-I complex because activity tests performed at different concentrations of free chlorophyll molecules did not result in measurable consumption of O_2 in the reaction solution; and (v) singlet oxygen production because the reaction solution contains ascorbate that would have reacted with singlet oxygen and had produced H_2O_2.[25]

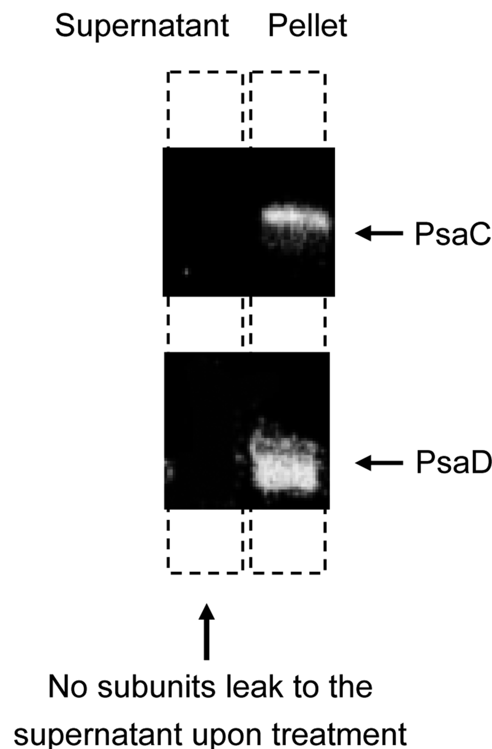

Figure 3. Western blotting of PsaC and PsaD subunits of the PS-I sample used for the activity measurements after vortexing and centrifugation at 100 000*g*. The blots show the absence of PsaC and PsaD in the supernatant.

Control experiments were performed in which the O_2 consumption was measured in the absence of light or in PS-I free working solutions containing DDM or PG chemical surfactants or poly(amino acid)s only. In these controls, 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. Experiments were also performed using pre-denatured, unfolded PS-I in which we did not observe O_2 consumption, which suggests that chlorophyll molecules of the PS-I complex did not affect our measurements. In control experiments performed in the absence of ascorbate and DCIP, we did not observe O_2 consumption, which suggests that the PS-I sample did not contain cytochrome c_6 or plastocyanin, which are natural electron donors to PS-I.

The presence of different concentrations of the negatively charged DDM or PG (phosphatidylglycerol) above and below their CMC did not show any measurable effect on PS-I photoactivity (Figure 2). This suggests that the stabilization of PS-I by PG, which is the natural lipid of the chloroplasts, although necessary in nature, is not sufficient for an active PS-I in vitro.

Previously, we showed that the addition of 0.28 mg/mL of positively charged ac-A_6K-$CONH_2$ in the PS-I reaction solution resulted in a nine-fold increase in the initial rate of PS-I activity compared with the activity of PS-I alone. Herein, basic (poly-L-lysine, poly-L-arginine, and poly-L-histidine), acidic (poly-L-aspartic acid and poly-L-glutamic acid), and hydrophobic (poly-L-tyrosine) poly(amino acid)s were tested at a concentration of 0.28 mg/mL for their efficiency to enhance the activity of PS-I. Poly-L-lysine and poly-L-arginine significantly accelerated the O_2 consumption of PS-I up to 14 and 12 times, respectively (Figure 2), whereas poly-L-histidine, which is also a positively charged poly(amino acid), showed a mere two-fold increase in PS-I activity. The effect of the negatively charged poly-L-

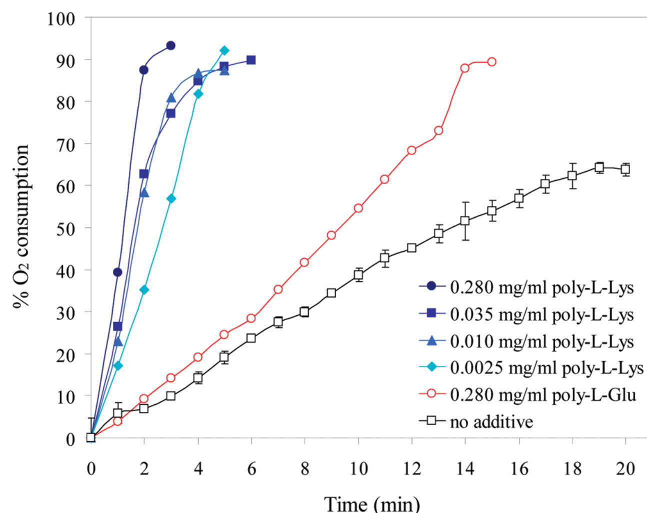

Figure 4. Stability kinetics of PS-I as a function of different concentrations of poly-L-lysine and 0.28 mg/mL poly-L-glutamic acid in aqueous medium. All data points are the average of $n = 3$, and the error is <2.4%. The PS-I concentration corresponds to 5.6 μM chlorophyll. Percentages on the y axis represent O_2 consumption relative to the maximum amount of O_2 in the solution, which corresponds to a solution saturated with O_2.

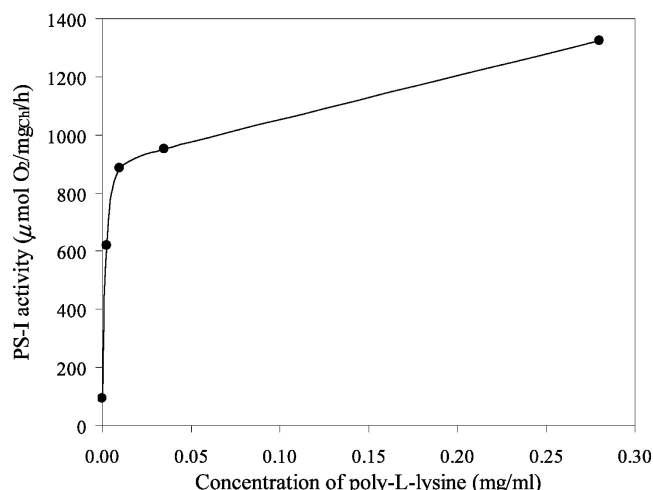

Figure 5. Light-induced activity of PS-I alone and at different concentrations of poly-L-lysine.

aspartic acid or poly-L-glutamic acid on the PS-I electron transfer activity was small. These results agree with our previous work, where we showed that negatively charged amphiphilic peptides did not enhance the PS-I activity.[5] The hydrophobic poly-L-tyrosine did not increase the PS-I functionality.

The effect of different poly-L-lysine concentrations on the PS-I activity is shown in Figures 4 and 5. The O_2 consumption rate in the presence of PS-I increased with increasing poly-L-lysine concentration and until reaching a plateau at poly-L-lysine concentrations >0.28 mg/mL. Even trace concentrations of poly-L-lysine (i.e., 0.0025 mg/mL, which is 100 times lower than that used in the experiments shown in Figure 2) resulted in a significant seven times higher PS-I activity compared with that of PS-I alone.

To determine whether the observed effect of poly-L-lysine was simply due to charge interactions, as previously hypothesized, we performed a control experiment using the L-lysine monomer at a concentration identical to that of the poly-L-lysine (i.e., 0.28 mg/mL) and tested the activity of PS-I. As seen in Figure 2, the presence of the basic amino acid L-lysine did not exhibit an increase in PS-I activity compared with that of PS-I alone. This null finding suggests that the positive charge alone of the L-lysine monomers is a not sufficient condition to enhance the PS-I electrochemical activity. Instead, it seems that the interaction of the poly-L-lysine polymer with the PS-I protein supercomplex occurs at the macromolecular level and likely involves a higher order organization of the protein−polycation system, as previously suggested for the functionalization effect of peptide surfactants on PS-I.[5]

Previous reports appear to be controversial, suggesting that the addition of poly-L-lysine has either an inhibitory[26,27] or activating[28] effect on the PS-I activity. However, these studies were performed in whole chloroplasts or their fragments. Therein, the effect of polylysine on PS-I activity was attributed to the interaction of poly-L-lysine with plastocyanin, a copper-containing protein that is an electron relay that transfers electrons from cytochrome b_6f to PSI-I in vivo. In our experiments, we tested poly-L-lysine interacting with pure PS-I samples, and we observed that the interaction of poly-L-lysine with PS-I significantly enhances the photochemical activity of PS-I more than any other agent used so far.

AFM Imaging. The morphology of PS-I alone and in the presence of additives was studied by tapping mode AFM (Figure 6). The bare surface of mica is smooth, that is, rms is 0.4 nm, which is small compared with the size of protein−additive assemblies. 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, whereas the PS-I monomer has a diameter of ca. 15 nm (Figure 1).[11] AFM image analysis of a sample of freshly isolated PS-I alone revealed the presence of particles with 5 to 6 nm height and diameter between 30 and 60 nm. Because of tip broadening effects, the actual diameters of the individual molecules and structures are smaller than those measured by AFM.[29] Therefore, the structures observed in the AFM images correlate well with the presence of a mixture of monomers, dimers, and trimers in the PS-I sample. AFM imaging of PS-I mixed with DDM revealed a surface pattern similar to that of PS-I alone, suggesting that DDM did not induce notable structural changes in PS-I. (The surface topology of DDM on mica could not be distinguished from the bare mica surface; data not shown.)

The surface pattern of PS-I in the presence of poly-L-lysine shows particles with diameter between 40 and 60 nm (Figure 6). A similar pattern was observed upon AFM inspection of PS-I mixed with poly-L-arginine with particle diameter between 50 and 60 nm (Figure 6). Conversely, mixing PS-I with poly-L-glutamic acid resulted in smaller particles between 20 and 45 nm. From the preceding AFM analysis and the O_2 consumption tests, we may conclude that mixing PS-I with poly-L-lysine or poly-L-arginine results in the stabilization of PS-I protein supercomplex in the trimeric form, the active PS-I conformation. However, the interaction of PS-I with poly-L-glutamic acid resulted in destabilization of the PS-I supercomplex (Figure 2) and induced notable structural and morphological changes in the PS-I sample. When PS-I was mixed with poly-L-glutamic acid, we observed smaller particles with sizes that correspond to PS-I monomers and to few dimers (Figure 6), which are not functional for the electrochemical reaction of O_2 consumption in solution. The poly-L-lysine, poly-L-arginine, and poly-L-glutamic acid polymers alone on the surface of mica had a different morphology (Figure 6), featuring particles with a height between 11 and 28 nm and a clearly distinguishable topology compared with the PS-I sample and that of PS-I mixed with the poly(amino acid)s.

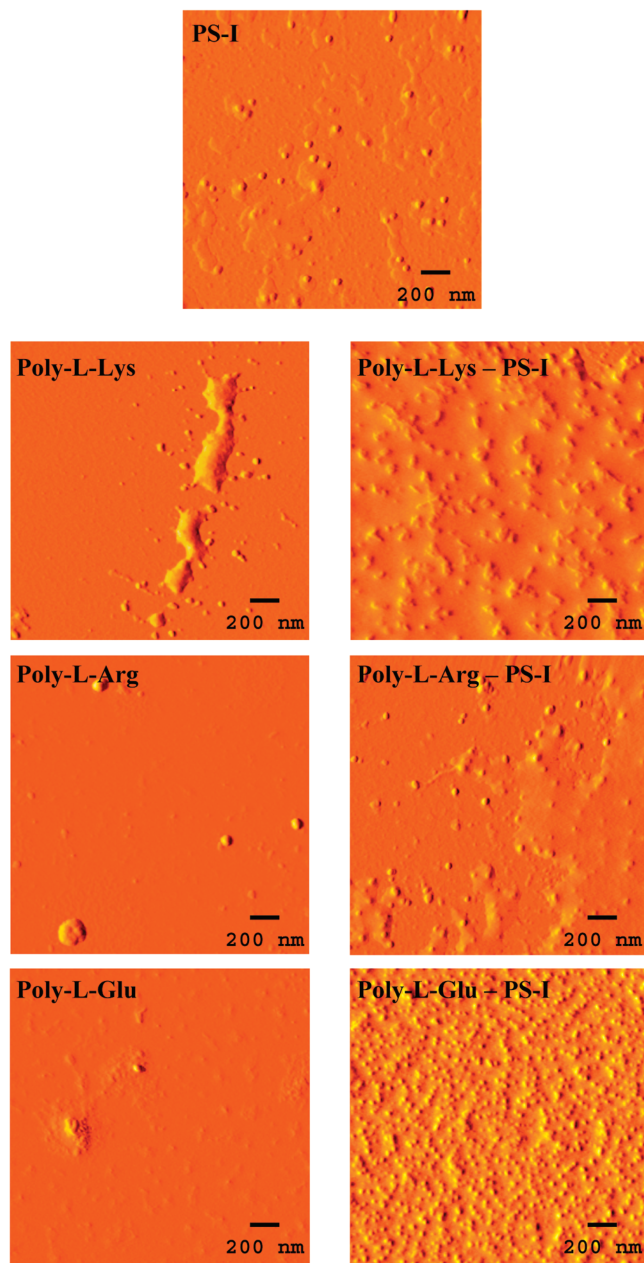

Figure 6. Tapping mode AFM images of PS-I stabilized by poly-L-lysine, poly-L-arginine, and poly-L-glutamic acid. The PS-I multiprotein complex alone appears as a dimer or trimer with diameter 30–50 nm. The surface topology of PS-I mixed with poly-L-lysine shows particles between 40 and 60 nm. In the case of PS-I mixed with poly-L-arginine, the particles are of similar size between 50 and 65 nm. Smaller particles between 30 and 40 nm are observed when PS-I is mixed with a poly-L-glutamic acid solution. Scale bar is 200 nm.

PS-I Stabilization by Poly(amino acid)s during Freeze-Drying. The PS-I from the thermophilic microorganism *T. elongatus* is a thermostable protein and therefore is a good candidate for technological applications such as solar energy harvesting. To this end, it is essential to find ways to preserve the activity of functional PS-I in the dry state. Previously, sugars such as sucrose and trehalose and polysaccharides such as dextran have been used to protect the functional integrity of proteins and enzymes in the dry state.[1,6] Herein, we tested the PS-I longevity upon freeze-drying treatment and prolonged storage in the dry state. Despite the stresses to which the multisubunit protein PS-I is subjected during freeze-drying and dehydration, we found that poly-L-lysine provided a great degree of protection. Following resolubilization in water, previously freeze-dried PS-I in the presence of poly-L-lysine showed only a small drop in activity; the remaining activity was still 84% of that observed prior to freeze-drying. Furthermore, the stabilization effect of poly-L-lysine on freeze-dried PS-I did not affect the protein's solubility, which is often an issue with other dry-state stabilization agents.

Conclusions

Although a significant amount of work has been performed on the spectroscopic characterization of PS-I, studies aimed at the stabilization of a highly active PS-I are scarce. The challenging nanotechnological applications and the promise of an efficient, bioinspired system for energy conversion has led to renewed interest in developing methodologies to increase the stability and light-induced electron transfer activity of PS-I. From a bioengineering perspective, the functional organization and stabilization of PS-I in plants is striking and represents a paradigm of design efficiency and condition optimization. However, the stabilization of PS-I in a functional form in vitro remains elusive.

In this study, we have investigated the effect of poly(amino acid)s on the PS-I functionality. Mixing PS-I with the positively charged poly(amino acid)s poly-L-lysine and poly-L-arginine resulted in significantly enhanced PS-I activity compared with PS-I alone, with 14- and 12-fold increases, respectively. Poly-L-histidine, which has a weak basic side chain, showed a smaller effect of up to two-fold. Conversely, the negatively charged poly-L-aspartic acid and poly-L-glutamic acid as well as the hydrophobic poly-L-tyrosine did not increase PS-I activity compared with PS-I alone. The charge effect and the macromolecular assembly mechanism of PS-I stabilization by poly(amino acid)s that we observed here may become a useful tool to engineer novel highly potent molecules for stabilizing and maintaining the activity of native PS-I. Our data show a correlation between increased PS-I electron transfer activity and stabilization of PS-I trimers, which is facilitated by the presence of polycations. The precise nature by which structural and charge aspects of the polycations affect trimerization and the mechanism by which polycations stabilize the PS-I trimers still needs to be determined. The results presented here suggest that the inexpensive poly-L-lysine has the potential to be a good material for the stabilization of highly active PS-I for applications in photovoltaic devices to harvest light energy and other biotechnological applications such as biosensors.

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