Solvent-induced changes in photochemical activity and conformation of photosystem I particles by glycerol

Xiaohua Ren1,2, Zhenle Yang1,* and Tingyun Kuang4
1 Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China
2 Graduate School of the Chinese Academy of Sciences, Beijing 100049, China
*Corresponding author
e-mail: yangzhl@ibcas.ac.cn

Abstract
It has been shown that a large number of water molecules coordinate with the pigments and subunits of photosystem I (PSI); however, the function of these water molecules remains to be clarified. In this study, the photosynthetic properties of PSI from spinach were investigated using different spectroscopic and activity measurements under conditions of decreasing water content caused by increasing concentrations of glycerol. The results show that glycerol addition caused pronounced changes in the photochemical activity of PSI particles. At low concentrations (<60%, v/v), glycerol stimulated the rate of oxygen uptake in PSI particles, while higher concentrations of glycerol cause inhibition of PSI activity. The capacity of P700 photooxidation also increased with glycerol concentrations lower than 60%. In contrast, this capacity decreased at higher glycerol concentrations. On the other hand, glycerol addition considerably affected the distribution of the bulk and red antenna chlorophyll (Chl) forms or states, with the population of red-shifted Chl forms augmented with increasing glycerol. In addition, glycerol-treated PSI particles showed a blue shift of the tryptophan fluorescence emission maximum and an increase in their capacity to bind the hydrophobic probe 1-anilino-8-naphthalene sulfonate, indicating a more non-polar environment for tryptophan residues and increased exposure of hydrophobic surfaces.

Keywords: conformational change; fluorescence; glycerol; oxygen uptake; P700 photooxidation; photosystem I.

Introduction
Photosystem PSI (PSI) is a multi-subunit protein supramolecular complex that functions as the light-driven plastocyanin-ferrrodoxin oxidoreductase in the thylakoid membranes of cyanobacteria and chloroplasts of higher plants. It contains five different electron carriers: Aq (primary electron acceptors of PSI), A1 (secondary electron acceptors) and Fx (iron-sulfur center X) located in the core reaction center PsA/B, and F6 and F8 (iron-sulfur centers A and B, respectively) located in the subunit of PsAC – a peripheral protein on the stromal side of PSI complexes (Chitnis, 2001; Fromme et al., 2001). A peculiar property of PSI is that it contains a significant amount of red-shifted spectral Chl forms with lower energy absorption than the primary electron donor P700, a Chl a dimer (Brody, 1958; Butler, 1961, 1978).

The 2.5-Å crystal structure of the PSI reaction center from the cyanobacterium Thermosynechococcus elongatus has a composition of 12 protein subunits and 127 co-factors, including 96 chlorophylls, two phylloquinones, three Fe4S4 clusters, 22 carotenoids, four lipids, a putative Ca2+ ion and 201 water molecules (Jordan et al., 2001). The existence of a large number of water molecules incorporated into PSI complexes is very interesting, with almost twice as many water molecules as chlorophylls. These water molecules seem to be of importance to PSI function. For example, it was shown that one water molecule forms hydrogen bonds with Chl a in P700. Several subunits, such as Psaj, Psak and Psal, coordinate the Mg2+ ions of antenna Chl a molecules directly or through water molecules (Jordan et al., 2001). These facts clearly indicate that bound water molecules are integral components and may stabilize the structure of PSI complexes. It was reported that the concentration of water molecules near the electron-transfer cofactors A1 and Fx is particularly high, suggesting a crucial role for water molecules in the PSI electron transfer reactions. Recent investigations revealed that the rate of P700 reduction and the interactions of P700 with the protein environment were affected by changes in hydration (Kim et al., 2001; Dashdorj et al., 2004; Sacksteder et al., 2005). However, the physiological functions of these water molecules are still not completely understood.

Polyols (e.g., glycerol, ethylene glycol) are commonly used as stabilizing agents or cryoprotectants for pigment-protein preparations. Polyol molecules contain hydrocarbon chains with hydroxyl groups that allow the hydrogen to bond with water. Thus, they may influence the binding state of water molecules around proteins, leading to changes in protein conformation and modifications of protein function (Kornblatt and Kornblatt, 2002; Robinson and Sligar, 1995, 1998). For example, it was found that under very high concentrations of ethylene glycol (>95%), PSI particles show a loss of primary photochemistry (Hillier et al., 1997). It was suggested that the inhibitory effects were due to reduced water concentrations, which caused irreversible protein denaturation.

In the present work, we investigated the effects of glycerol on PSI under conditions of increasing concentrations of glycerol. Glycerol is very useful because it does not lead to significant extraction of the chlorophyll pigments and is completely compatible with water. The photosynthetic behavior of PSI, followed by treatment
with different concentrations of glycerol, revealed that glycerol exhibited biphasic effects on the PSI activity. In other words, PSI-dependent electron transfer [ascorbate-dichlorophenolindophenol (DCPIP) to methyl violo- gen (MV)] and the photochemical reaction capacity of P700 can be markedly affected by glycerol, with a stimulatory and an inhibitory concentration range. Moreover, room-temperature fluorescence emission studies of the intrinsic fluorescence of tryptophan residues, 1-anilino-8-naphthalene sulfonate (ANS) binding to the protein surfaces and Chl fluorescence emission provided evidence that there was a conformational change in PSI proteins.

Results

Changes in oxygen uptake in PSI particles

The rate of oxygen uptake in PSI particles was measured as the PSI-mediated electron transfer from reduced DCPIP as an artificial electron donor to MV as electron acceptor. Figure 1 shows changes in the rate of light-saturated oxygen uptake of PSI particles in the presence of increasing glycerol at 25.0±0.2°C. In this figure, the control samples were PSI preparations that were not incubated with glycerol. The average 100% oxygen uptake in PSI corresponded to 420 μmol O2/mg Chl per h. It is evident from Figure 1 that the activity of PSI particles was affected in the presence of glycerol at both low (0–50%) and higher concentrations (60–80%).

A stimulatory effect of glycerol on the PSI activity occurred at concentrations of 0–50%. Furthermore, this stimulatory effect was dependent on the glycerol concentration. For example, there was a rapid increase in oxygen uptake as the glycerol content increased from 0 to 10%. At 10% glycerol, oxygen uptake was stimulated to a maximum that was almost double that of the control. At higher PG concentrations, the rate of oxygen uptake started to decline gradually, but still exceeded the activity of PSI without glycerol. A further increase in glycerol content (>50%) gave rise to a decrease in the PSI activity. In this region, inhibition of PSI activity became more pronounced as the glycerol content increased. For example, at a very high concentration of 80% glycerol, 75% of the activity was lost.

In this investigation, reversible glycerol effects occurred in the range 0–60% glycerol. At glycerol concentrations above 60%, the effects of glycerol were irreversible.

Alterations in P700 photooxidation

To explore the effect of glycerol on the photochemical activity of PSI, the reaction center P700 of PSI was analyzed by measuring absorbance changes at 830 nm (ΔA830). Figure 2 shows the changes in absorbance at 830 nm of PSI particles exposed to varying concentrations of glycerol at room temperature. These changes could reflect the redox states of the P700 reaction center and the photochemical reaction capacity of PSI. As shown in Figure 2, the magnitude of ΔA830 increased for 0–60% glycerol, indicating enhanced ability of P700 to undergo reversible redox changes. However, a higher glycerol content (70–80%) led to a progressive decrease in ΔA830, indicating that the total amount of photooxidizable P700 decreased in the presence of high glycerol content.

Changes in Chl fluorescence emission at room temperature

Steady-state emission spectra at room temperature for the PSI complexes with and without glycerol are shown in Figure 3. The Chl fluorescence emission spectra of untreated PSI particles showed a characteristic peak at 683 nm and a broad shoulder around 715 nm. The quite intense fluorescence emission at 680–690 nm is associated with the bulk antenna Chls, while the peak at 710–720 nm is characteristic of the red spectral Chl forms or states absorbing at energies close to or lower than the primary donor P700 (Croce et al., 1996). As
Effects of glycerol on photosystem I

The inset shows changes in the fluorescence intensity (expressed as $F_{715}/F_{683}$) measured at different glycerol concentrations. The Chl concentration was 10 $\mu$g/ml.

shown in Figure 3, treatment with glycerol significantly affected the fluorescence emission of PSI complexes. The intensity of Chl fluorescence at 680–690 nm decreased following an increase in glycerol concentration and the peak became only a shoulder at higher glycerol concentrations (70–80%). On the other hand, glycerol caused an increase in the fluorescence intensity of the red spectral chlorophyll forms at 710–720 nm. At high glycerol concentrations, this shoulder tended to become a prominent peak. The changes in fluorescence emission are particularly evident from the $F_{715}/F_{683}$ ratio (inset of Figure 3). These results clearly indicate that glycerol led to significant changes in the Chl fluorescence emission of PSI particles, which could be related to a conformational change in the PSI particles (Rajagopal et al., 2003).

Changes in tryptophan fluorescence emission

The intrinsic fluorescence of proteins caused by the presence of aromatic amino-acid residues (tryptophan, tyrosine, and phenylalanine) is very sensitive to the electronic environment and can be used to monitor changes in emission induced by protein conformational changes and anything that affects the local environment surrounding these residues (Lakowicz, 1999).

Figure 4A shows the room-temperature emission spectra of fluorescent tryptophan residues in PSI particles treated with varying concentrations of glycerol. The untreated PSI particles show a main peak at 327 nm and a shoulder around 350 nm, indicating heterogeneity in the emission of tryptophan residues in PSI particles. In general, three discrete spectral classes may exist in tryptophan residues of proteins, depending on whether they are buried in non-polar regions of proteins (maximum at $\sim$330 nm), completely exposed to the aqueous environment (maximum at $\sim$350 nm), or located in an environment of intermediate polarity (maximum at $\sim$340 nm) (Brustein et al., 1973). As indicated in Figure 4, there are at least two tryptophan residue classes in PSI proteins located in hydrophobic and polar environments, respectively, with those in the non-polar environment predominant.

On treatment with increasing concentrations of glycerol, tryptophan fluorescence emission gradually decreased in intensity, which was accompanied by a blue shift of the maximum peak (inset in Figure 4A). This blue shift is indicative of a more hydrophobic environment for tryptophan residues, as it is known that the fluorescence of the indole group shows blue or red shifts when the polarity of the microenvironment decreases or increases, respectively (Lakowicz, 1999). The change in polarity of the tryptophan environment seems to occur with conformational changes, since the observed decrease in PSI fluorescence intensity produced by glycerol treatment could arise from increased static quenching caused by the conformational change. Conformational changes of proteins were also reflected by the change in fluorescence emission of tryptophan residues induced by glycerol. Tryptophan fluorescence spectra normalized to the maximum emission (Figure 4B) clearly demonstrate that
the intensity at 350 nm increased and the shoulder tended to become a peak as the glycerol content increased. For example, the $F_{350}/F_{320-330}$ ratio increased from 0.49 for untreated PSI particles to 0.88 for particles treated with 80% glycerol.

**ANS binding**

An additional method for revealing glycerol-induced conformational changes in PSI particles involves analysis of the accessibility of hydrophobic protein surfaces to ANS, an efficient fluorescence hydrophobic probe. It has a low fluorescence yield in polar environments, while the fluorescence intensity increases when ANS binds to hydrophobic protein surfaces. ANS binds non-covalently to proteins and its fluorescence varies with changes in the probe environment. Thus, ANS is very useful for the detection of conformational changes in proteins associated with solvent exposure of hydrophobic regions. Both an increase in fluorescence intensity and a decrease in emission maximum (blue shift) are usually observed upon binding of ANS to exposed hydrophobic regions (Slavik, 1982; Matulis and Lovrien, 1998; Matulis et al., 1999).

In this study, binding experiments with PSI particles at pH 7.8 in the absence and presence of glycerol were performed using 20 $\mu$M ANS solution to detect changes in the hydrophobic parts of protein surfaces. The ANS fluorescence spectrum exhibited a wide band of very low intensity and an emission maximum of $\lambda_{max}$ 526 nm on addition to PSI particles (Figure 5A), which is similar to pure ANS in the same buffer (10 mM NaCl, Tricine-NaOH, pH 7.8) without PSI particles (data not shown). Th lack of significant differences in the $\lambda_{max}$ value (526 nm) and the fluorescence intensity of ANS indicates the absence of ANS binding to PSI protein under these conditions, and probably reflects a lack of exposure of the hydrophobic surfaces in PSI particles and a fully compact state of PSI proteins. However, in the presence of glycerol, changes were observed. Figure 5B shows the ANS fluorescence intensity at the emission maximum as a function of glycerol concentration. The increase in fluorescence intensity and the significant blue shift of $\lambda_{max}$ (inset in Figure 5B) on the addition of glycerol indicate that the hydrophobic regions of PSI proteins were exposed.

**Discussion**

A previous study showed that the redox property of the electron acceptor F$_{x}$ in spinach PSI is different in the presence of glycerol (Evans and Heathcote, 1980). In the present work, results indicate that the activity of PSI-dependent electron transfer was significantly affected by glycerol and this effect was highly concentration-dependent (Figure 1). Glycerol stimulated the rate of oxygen uptake in the range from 0 to 50%, with maximal PSI activity at approximately 10% glycerol. However, at higher concentrations, glycerol inhibited the activity of PSI particles.

A biphasic stimulatory/inhibitory effect of glycerol was also observed on the photochemical reaction capacity of PSI particles (Figure 2). The amount of photooxidizable P700 was increased with glycerol concentrations below 60%, while exposure of PSI particles to high levels of glycerol (70–80%) resulted in the loss of P700 ability to undergo reversible redox changes. Compared with untreated PSI, photochemical activity remained high even at glycerol concentrations above 60%. For example, PSI activity approached 80% of the native activity in 80% glycerol solutions. These results show that PSI activities were stable in organic systems. The change in P700 photooxidation capacity induced by glycerol could result from changes in distance or mutual orientations between the primary P700 donor and its electron acceptors and/or between antenna Chls and the reaction center. In the presence of stimulatory concentrations of glycerol, the distance between P700 and its primary acceptor or between P700 and the neighboring Chl molecules responsible for transferring excitation energy to the reaction center may become more appropriate to facilitate P700 photooxidation (Rajagopal et al., 2003).

Glycerol was previously shown to have diverse effects on PSII. It was recognized that low concentrations of glycerol could stabilize PSII activity and structure. For example, 4 M glycerol (~30% v/v) protected PSII from...
the destructive effects of intense white light, high NaCl concentrations, and the detergent Triton X-100 in the cells of Dunaliella salina (Khristin and Simonova, 1998). Moreover, 30% glycerol stabilized a 33-kDa protein from PSII against pressure-induced unfolding (Ruan et al., 2003). At low concentrations, glycerol and other osmolytes increased the peak temperature of thermoluminescence bands of PSII (Krieger et al., 1998).

A biphasic glycerol effect was also observed on PSII, as for other polyols (Hillier et al., 1997). In a previous study, glycerol addition stimulated the steady-state rate of PSII oxygen evolution in the range from 0 to 30%, while it was inhibitory at higher concentrations. Sucrose has similar biphasic stimulatory/inhibitory effects on PSII. It has been suggested that these effects are due to preferential hydration, which could produce structural changes at the active site in the water oxidizing complex in PSII (Halverson and Barry, 2003).

Glycerol can induce osmotic stress between the bulk solvent and the hydration water around a protein, therefore lowering the water activity and changing the hydration state of the protein (Davis-Searles et al., 2001; Kornblatt and Kornblatt, 2002). According to the mechanism proposed by Timasheff (2002), the effects of glycerol on proteins are interpreted as a consequence of preferential exclusion phenomena – solvophobic effects whereby contact between water and non-polar regions of the protein is more favorable than contact between the non-polar regions of the protein and the glycerol-water mixture. As a result, glycerol molecules have an affinity for the polar regions of proteins and move away from the non-polar regions into the bulk solvent, leading to preferential hydration of the protein. This preferential exclusion increases the chemical potential of the protein, which is proportional to the surface area exposed to solvent.

Changes in hydration can potentially alter the protein conformation. In this study, the resulting conformational changes were most likely responsible for the biphasic stimulatory/inhibitory effects of glycerol on PSI activity. In other words, the stimulatory effects of glycerol were attributed to the preferential hydration of PSI, which in turn led to the increased activity of the catalytic structure, as suggested by Halverson and Barry (2003). Any greater increase in hydration could cause local destabilization of critical structural elements, thereby leading to the inhibition of PSI activity.

Alternatively, the loss of PSI activity in very high glycerol concentrations (>60%) may be due to much lower water concentrations. It is well known that glycerol molecules contain hydrocarbon chains with hydroxyl groups that allow hydrogen bonding with water. Consequently, water can be removed from the protein (Robinson and Sligar, 1995, 1998; Kornblatt and Kornblatt, 2002). For many proteins, water molecules serve as direct ligands, with some being very tightly bound, such as the PSI core complex, which was reported to contain 201 water molecules (Jordan et al., 2001). If the tightly bound fraction is removed, the protein may denature and lose its activity. Thus, at a very high concentration of glycerol, there may be a loss of tightly bound water through mass action, resulting in conformational changes that alter the PSI activity (Hillier et al., 1997).

Nevertheless, it was noted that glycerol treatment did not result in gross dissociation of the PSI complex, as revealed by protein SDS-PAGE (data not shown). This means that the inhibition of PSI activity induced by high concentrations of glycerol was not the result of the decay of PSI protein, but probably of conformational changes in PSI.

Conformational changes induced by glycerol indeed occurred in PSI particles, as characterized by changes in intrinsic fluorescence for tryptophan residues and ANS binding to the protein surfaces of PSI particles. The change in room-temperature Chl fluorescence was also the result of a conformational change in the PSI particles (Rajagopal et al., 2003).

Glycerol addition altered the Chl fluorescence at room temperature. The fluorescence spectra of PSI samples were characterized by emission bands attributed to the bulk (680–690 nm) and red (710–720 nm) antenna Chl forms. As indicated in Figure 3, 0–60% glycerol slightly enhanced the F_710/F_730 intensity ratio. However, higher concentrations of glycerol (70–80%) led to a considerable increase in fluorescence intensity. The results clearly indicate that glycerol affected the distribution of the bulk and red antenna chlorophyll forms; the population of red-shifted Chl forms increased following an increase in glycerol concentration. It is most likely that with increasing glycerol content, more energy is trapped in the red forms, which subsequently emit more fluorescence.

The results for fluorescence spectra of tryptophan residues in PSI particles indicate that there are at least two tryptophan residue classes in different microenvironments, revealing heterogeneity in the emission of tryptophan residues in PSI proteins. The predominant tryptophan residues are buried more deeply within the protein matrix and are located in a hydrophobic environment (fluorescence at ~330 nm), while the other residues are more exposed to solvent (fluorescence at ~350 nm). In fact, analysis of the amino acid sequences of cyanobacterial or eukaryotic PSI proteins showed that at least 60% of the tryptophan residues in PSI protein complexes are contained in the core Psaa/Psab reaction center. For example, there are approximately 28 and 33 out of a total of 75 tryptophan residues per P700 in cyanobacterial Thermosynechococcus elongatus PsaaA and Psab, respectively. It has been suggested that some tryptophan residues in PsaaA/Psab are involved in the electron transfer process of PSI complexes (Guergova-Kuras et al., 2001; Muhiuddin et al., 2001; Purton et al., 2001; Agalarov and Brettel, 2003; Ben-Shem et al., 2003; Ishikita and Knapp, 2003). The high content of tryptophan residues in the heterodimer PsaaA/Psab protein complex therefore reflects the intrinsic fluorescence emission of PSI proteins, where the maximum at 327 nm is blue-shifted in comparison to that of free tryptophan in solution (~350 nm).

Obvious changes occurred in the intrinsic fluorescence following the addition of glycerol. The fluorescence intensity of PSI particles decreased as the glycerol content increased, which was accompanied by a blue shift in the maximum emission wavelength. The quantum yield of buried tryptophan residues is mainly controlled by static quenching processes, which depend on the proximity...
and orientation of quencher groups such as disulfide, thi-ol and amide (Casals et al., 1993). Thus, the observed decrease in fluorescence intensity of PSI particles treated with glycerol could arise from increased static quenching caused by the resulting conformational changes. This was further confirmed by acrylamide quenching analysis (data not shown), which showed that Stern-Volmer plots became more upward with increasing glycerol concentration, indicating an increased static-quenching component in PSI particles. On the other hand, changes in the $F_{320}/F_{330}$ ratio on addition of glycerol also demonstrated the modification of PSI proteins. Accompanying these conformational changes was a polarity change in the environment of tryptophan residues, since the observed blue shift in the position of fluorescence emission maxima clearly reflected a more hydrophobic environment for tryptophan residues.

Moreover, the solvent-induced conformational changes in PSI complexes on glycerol treatment were manifested by the ANS binding behavior. PSI particles seem to have a small proportion of hydrophobic groups exposed to the solvent, as suggested by the tryptophan fluorescence emission in Figure 4 and the ANS binding data in Figure 5. However, greater exposure of the hydrophobic groups of PSI particles could be triggered by glycerol. As shown in Figure 5B, on addition of glycerol the fluorescence emission intensity of ANS increased, along with a blue shift of the emission maximum, indicating increased exposure of the hydrophobic groups of PSI particles to the solvent. It was likely that, on interaction with glycerol, the PSI particles became less compact than untreated PSI, and a greater number of hydrophobic binding sites were accessible to ANS.

In conclusion, our study reveals a biphasic pattern of stimulatory/inhibitory effects of glycerol on PSI-dependent electron transfer and P700 photooxidation. The effects of glycerol also resulted in significant changes in PSI protein conformation, as revealed by changes in the intrinsic fluorescence of tryptophan residues, ANS binding to the protein surfaces and Chl fluorescence emission.

Materials and methods

Preparation of PSI particles

PSI particles were prepared from the fresh leaves of spinach using the methods of Mullet et al. (1980) and Bassi and Simpson (1987), with a modification as described in our previous study (Yang et al., 2005). The final PSI particles were suspended in a buffer containing 10 mM NaCl and 20 mM Tricine-NaOH (pH 7.8), supplemented with 200 μM DCPIP and 5 mM ascorbate as an electron donor couple, and 0.2 mM MV with 1 mM sodium azide as an electron acceptor couple. The photosynthetic photon flux density of the white light was ca. 1000 μmol/m²s and the temperature was held steady at 25.0 ± 0.2°C. The final Chl concentration of the PSI sample was 10 μg/ml. Before measurements, samples were incubated for 5 min at 25.0 ± 0.2°C in the dark.

It was noted that the background (in the absence of MV) was also affected by glycerol, and thus the data for oxygen consumption by PSI samples were calculated by subtraction of the background.

To examine the reversibility of the glycerol effect on activity, glycerol-treated PSI samples were pelleted by centrifugation at 40,000 g and then resuspended in the standard buffer medium (10 mM NaCl, 20 mM Tricine-NaOH, pH 7.8) at the same Chl concentration. Then activity in the PSI samples this investigation, the reversible glycerol effects occurred in the range 0–60% glycerol. At glycerol concentrations above 60%, the effects were irreversible.

Measurements of the absorbance changes at 830 nm

Absorbance changes at 830 nm ($ΔA_{830}$) represent the oxidation and reduction of P700. Changes in the redox state of P700 were evaluated by measuring the light-induced absorbance changes at 830 nm using a dual-wavelength ED-P700DW unit connected to a pulse amplitude modulation (PAM) chlorophyll fluorimeter (model PAM101, Walz, Effeltrich, Germany). Absorbance values were recorded using a PDA-100 PAM data acquisition system. Saturating far-red light (730 nm, 15 W/m) emitted by a far-red diode (102-FR) was applied to oxidize P700 for 1 min. The light intensity was strong enough to completely oxidize P700 (Bukhov et al., 2001). The Chl concentration of PSI samples in suspension buffer (10 mM NaCl, 20 mM Tricine-NaOH, pH 7.8) was 20 μg/ml during measurement. Ascorbate (200 μM) was added to PSI samples prior to $ΔA_{830}$ measurements to ensure complete reduction of P700 in the dark.

Recording of fluorescence emission spectra

Room-temperature fluorescence measurements were performed on PSI samples containing different concentrations of glycerol (0–80%) at pH 7.8 (10 mM NaCl, 20 mM Tricine-NaOH) using a Hitachi F-4500 fluorescence spectrophotometer. Chl fluorescence spectra of the samples were obtained with excitation at 440 nm. The fluorescence emission spectra were measured between 650 and 780 nm. The concentration of Chl in PSI samples was 10 μg/ml.

Intrinsic fluorescence measurements were performed using an excitation wavelength of 295 nm and the emission spectra from 300 to 400 nm were recorded. The Chl concentration of PSI samples was adjusted to 20 μg/ml for measurement.

ANS binding fluorescence measurements were carried out using an excitation wavelength of 360 nm and the emission
spectra from 380 to 600 nm were recorded. The concentration of ANS used in this study was 20 μM.

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References


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