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Focus on the aggregation processes of Photosystem II complexes

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Abstract

In this work the effect of temperature and *n*-dodecyl-β-D-maltoside (DM) on PSII complexes organization was investigated. An aggregation process of PSII monomers and dimers was documented at different temperatures and low DM concentration by steady-state fluorescence, absorption, circular dichroism, Rayleigh and dynamic light-scattering experiments. Measures of oxygen evolution enabled us to estimate the change in photoactivity of PSII during the aggregation. This process was found to be extensively reversed by increasing DM concentration as proved by means of steady-state fluorescence and dynamic light-scattering experiments.

Keywords: Photosystem II; Fluorescence quenching; Protein aggregation; Dynamic light scattering

1. Introduction

Photosystem II (PSII) is a large supramolecular pigment–protein complex which converts light energy into the electrochemical energy required for the water oxidation. It is embedded in the lipid matrix of the thylakoid membrane and it is constituted by more than 25 proteic subunits [1,2]. At least nine of them interact in the reduction process of plastoquinone and in the oxygen evolution: the main subunits are D_1, D_2, the cytochrome b559 dimer, CP47 and CP43. The remaining three extrinsic polypeptides having molecular masses of 33, 23 and 17 kDa constitute the "oxygen evolving complex" (OEC) together with the manganese cluster. Other subunits act as antenna to harvest light in cooperation with the LHCII complex, which binds about 50–65% of the total amount of chlorophylls a and b (Chl a and b) and other pigments such as carotenoids [1,3]. All these pigments work as light collectors transferring light energy to the PSII reaction centre (RC).

Several methods have been optimised to extract and purify PSII complexes from higher plants and it is widely accepted that PSII can exist, in vivo, both in a monomeric and in a dimeric aggregation state [4–6]. These two states are both functional and active, and there are evidences that for spinach the dimeric form is more efficient [7]. The stability and the functionality of the dimeric state can be affected by several factors: for example in literature data report the importance of phosphatidylglicerol (PG) [4], of the extrinsic 33 kDa protein, of phosphorylation [6], as well as of osmo-protectors [7] in stabilizing PSII dimers. Moreover it is largely documented [8–15] that several membrane complexes are functionally active in their oligomeric form and that aggregation processes can be influenced by temperature and detergent treatment [16,17].

Studies on the factors involved in photosynthetic protein aggregation and stabilization are therefore important in order to understand the specific photosynthetic mechanisms and to find biotechnology applications in environmental and energetic fields [18–21]. In this paper an aggregation process of PSII monomers and dimers obtained from spinach leaves was evidenced. This phenomenon resulted to be thermally activated and affected by concentration of *n*-dodecyl-β-D-maltoside (DM) detergent, used for the isolation of PSII core monomers and dimers.

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In particular macroaggregates with dimension of about 1 μm were obtained in less than 2 h both for PSII monomers and dimers at low DM concentration, as clearly documented by dynamic light-scattering measures. Oxygen evolution rate experiments evidenced that PSII aggregates retained about 30% of their initial activity, indicating that the oxygen evolving complex was not completely damaged during the aggregation process, which was found to be extensively reversed by increasing DM concentration, as proved by means of steady-state fluorescence and dynamic light-scattering experiments.

2. Materials and methods

2.1. Isolation of chloroplasts, thylakoids, membrane fractions enriched in PSII (BBYs), OG–core complexes, PSII core monomers and dimers

Chloroplasts, thylakoids, membrane fractions enriched in PSII (BBYs), OG–core complexes (that is PSII complexes stabilized by *n*-octyl-β-D-glucopyranoside), as well as purified monomeric and dimeric PSII core complexes, were isolated from market spinach according to Hankamer's procedure [22–25]. Briefly, chloroplasts were obtained from spinach leaves by differential centrifugation and thylakoids from chloroplasts by their rupture in a hypotonic solution. Thylakoids were solubilized with Triton X-100 (Sigma) in order to extract PSII-enriched membranes (BBYs). These last membranes were solubilized with *n*-octyl-β-D-glucopyranoside (Sigma) to detach the LHCII proteins from the PSII core (OG–core complexes). Sucrose gradients, supplemented with *n*-dodecyl-β-D-maltoside (Sigma), were employed to obtain oxygen evolving PSII core monomers and dimers which lack of the 23 and 17 kDa extrinsic proteins and the CP29, CP26, CP24 chlorophyll binding proteins. The PSII samples were kept at 4 °C.

PSII monomer and dimer samples obtained from spinach leaves were diluted in an aqueous buffer (MNCB) composed of [2-*N*-morpholin]ethane-sulfonic acid (MES) 25 mM, NaCl 10 mM, $CaCl_2$ 5 mM, $NaHCO_3$ 10 mM; the concentration of PSII monomer and dimer samples was estimated as Chl mg/ml [26]. In this study the concentration of chlorophyll used for absorption, fluorescence, RLS and DLS measurements was 0.015 mg/ml, while for CD experiments was 0.005 mg/ml.

2.2. Absorption spectra measurements

Visible absorption spectra were recorded using a Varian CARY/3 spectrophotometer using 1 cm path length quartz cells.

2.3. Steady-state fluorescence and Rayleigh light-scattering measurements

Fluorescence measurements were realized using a Varian Cary Eclipse spectrofluorimeter. Resonance light-scattering (RLS) spectra were obtained on a Jasco mod. FP-750 spectrofluorimeter, according to a synchronous scan protocol with a right angle geometry [27].

For the experiments, 1 cm path length quartz cells were used; in case of highly scattering samples, neutral density filters were set in the excitation path (optical density 0.8). The emission and resonance light-scattering spectra were not corrected for the absorption of the samples.

2.4. CD measurements

Circular dichroism spectra were recorded on a JASCO J-810 spectropolarimeter investigating the range 200–800 nm and using 1 cm path length quartz cells.

2.5. DLS measurements

DLS experiments were performed by means of a 25 mW polarized laser source Nd:YAG (532 nm). The scattered light was collected at 90°, in a self-beating mode, through an optical fiber matched with Hamamatsu R942 photomultiplier cooled at −30 °C. The signal was sent to a Malvern 4700 submicrometer particle analyzer system and the intensity–intensity correlation function $g_2(t)$ was measured in the range 0.1 ms$<\tau<$1 s, using a typical acquisition time of 200 s. The investigated samples were placed in a Burchard cylindrical quartz cell (Hellma) and thermostated by a homemade water-circulating system.

Information on particle size in solution can be obtained measuring the diffusion coefficient D by means of the scattered electric field correlation function $g_1(t)$:

$$g_1(t) \propto \sqrt{g_2(t)-1} = \exp(-t/\tau)$$

with $\tau = 1/(Dq^2)$.

The hydrodynamic radius is calculated using the Einstein–Stokes relation [28]:

$$R_H = (K_B T)/(6\pi\eta D_c)$$

with η the solvent viscosity, T the temperature, K_B the Boltzmann constant and q the exchanged momentum of the scattered light.

In the case of a polydisperse sample, the correlation function is a superposition of exponential functions with different decay rates.

$$g_1(t) = \int \tau A(\tau)\exp(-t/\tau)d(\ln\tau)$$

The distribution of these decay rates can be obtained through a Laplace inversion of the field correlation function $g_1(t)$. To perform this inversion procedure we used a discrete multi-exponential nonnegative least-squares fit to (NNLS).

2.6. Oxygen evolution rate measurements

Oxygen evolution rates were obtained by means of a composite oxygraphic device by Rank Brothers. Experiments were conducted under nitrogen flux at room temperature, by adding 0.04 mmol of the oxidizing reagent 2,5-dichloro-*p*-benzoquinine (DCBQ) per mg of chlorophyll to samples and illuminating by an optical fiber with a lamp at 150 Watt (lamp

temperature 3 200 K). Values of about 400 μmol O_2/h mg chl were obtained for freshly prepared PSII complexes.

3. Results and discussion

Fig. 1 shows the time evolution of steady-state emission spectra of PSII monomer complexes in the course of 2 h. The emission spectra show a characteristic band at 683 nm and a shoulder at 740 nm, which most probably originated from a vibrational sublevel as reported in literature [29–31]. For PSII monomer sample, a decrease in fluorescence intensity with time at 25 °C is observed. Dimers show the same emission spectra than monomers but a more rapid decrease in 683 nm fluorescence intensity with time (inset Fig. 1). In fact after 2 h the fluorescence intensity is reduced by about 35% for monomers and about 60% for dimers.

Since chlorophyll a fluorescence quenching has been reported to take place exclusively in the LHCII aggregates [32], our results could suggest that an aggregation process is occurring in PSII samples and that this process is favoured for dimer preparations. Rayleigh light-scattering measurements confirm the presence of aggregates. Fig. 2 shows that PSII monomer Rayleigh scattering peak intensity (515 nm) increases as a function of time, because of the increasing dimension of scattering objects, due to the occurring of an aggregation process [17,33,34]. PSII dimers produce the same behaviour of PSII monomers (data not shown), suggesting that also PSII dimers are involved in aggregation processes. In both cases after 2 h no significant changes in the RLS spectra occur suggesting that the aggregation processes reach its completeness by this time interval.

Measurements of dynamic light-scattering gave information about the time evolution of the hydrodynamic radii for both PSII monomers and dimers. Hydrodynamic radii were calculated by the analysis of the correlation function. These experiments were performed at different temperatures. From Fig. 3 it is evident that increasing of temperatures enhances the

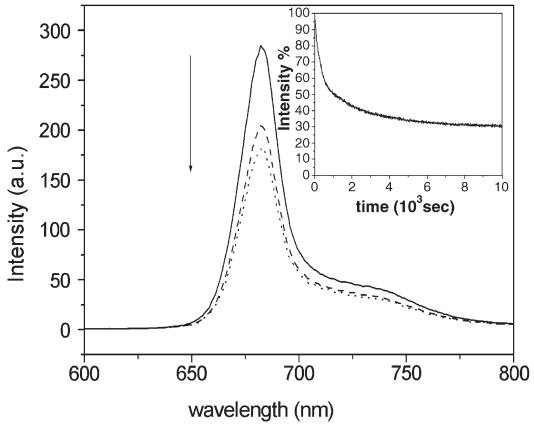

Fig. 1. Decrease in PSII monomer fluorescence intensity with time at 25 °C. Continuous line represents the fluorescence emission spectrum at zero-time (when samples reach 25 °C); the dashed line is the spectrum 1 h later; the dotted line is the spectrum 2 h later. The inset shows the fluorescence emission intensity % of PSII dimers at 683 nm, as a function of time, at 25 °C; the values are normalized with respect to the initial intensity.

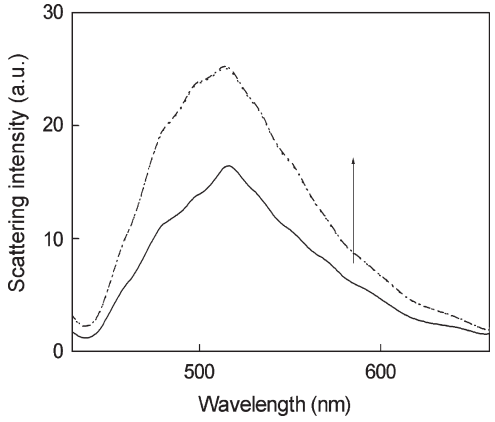

Fig. 2. Time evolution of PSII monomer Rayleigh light-scattering at 25 °C: it is evident an increase of the 515 nm peak intensity. Continuous line represents the fluorescence emission spectrum at zero-time; the dashed line is the spectrum 2 h later; the dotted line is the spectrum 3 h later.

aggregation process and that at a fixed temperature the aggregation kinetic is faster for dimers than for monomers as evidenced also by fluorescence experiments. After about 2 h, aggregates of micrometric dimensions are obtained. All these results confirm the existence of the aggregation process and indicate that it is somehow thermo-activated.

In general protein aggregation processes require either domain rearrangements or partial unfolding of the protein environment [17,35]. Therefore, in order to gain additional structural information about PSII aggregates, absorption and circular dichroism measurements were performed. PSII monomer visible absorption spectra are reported in Fig. 4. The spectra show typical absorption peaks at 674 and 436 nm contributed by chlorophyll a and shoulders in the range 450–550 nm originated from carotenoids and luteine [36–40]. As shown in Fig. 4a a slight decrease in amplitude of absorption peaks with time, together with a small blue shift (about 1 nm) of the chlorophyll a red band, is observable. PSII dimers behave as monomers (Fig. 4b), although the ratio A_{674}/A_{436}, constant for monomers, decreased by 11% in time for dimer samples.

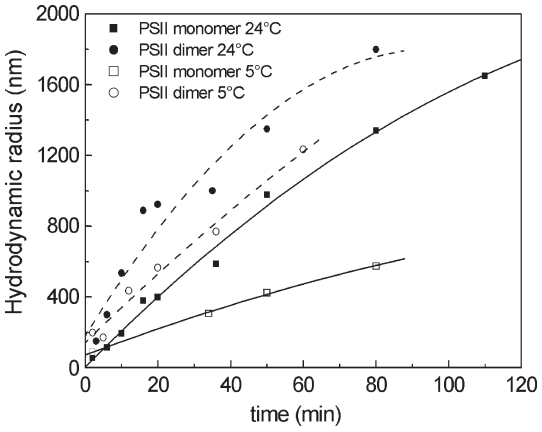

Fig. 3. Time evolution of calculated hydrodynamic radii for PSII monomers and dimers from DLS experiments at 5 °C and 24 °C (lines are guides to read the graph).

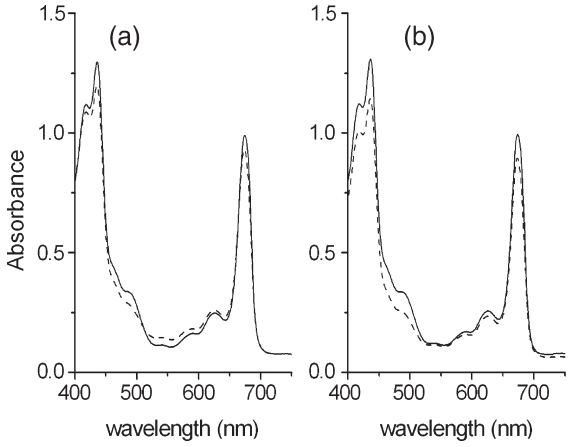

Fig. 4. Absorption spectra of PSII monomer (a) and dimer (b) samples at zero-time (continuous line) and 2 h later (dashed line).

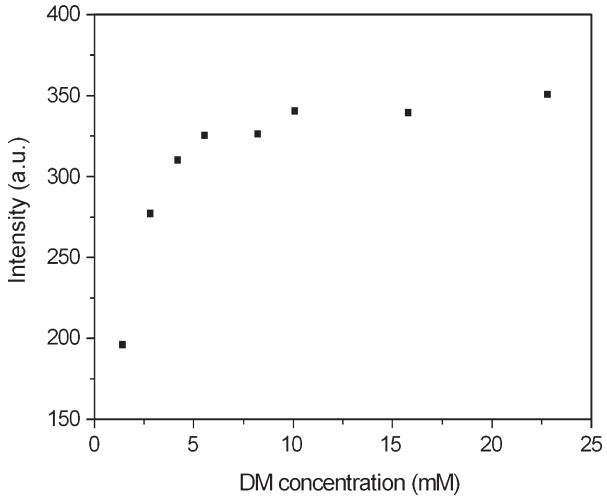

Fig. 6. Fluorescence intensity at 683 nm of PSII monomer aggregates at 25 °C, as a function of DM concentration.

Moreover the blue shift, observed for monomers, is absent for dimer samples. Generally slight blue shifts of chlorophyll a absorption peaks are related to small microenvironment changes around the pigment molecules [14]. In our cases the small change in absorbance maxima both for monomers and dimers suggests that little chlorophyll a molecular structure modification occurs during aggregation process. Moreover, the reduction of absorption intensity of carotenoids shoulder, more pronounced for PSII dimer samples, could be related to a loose of the PSII carotenoids or to a change in the microenvironment around PSII carotenoids, as confirmed by circular dichroism spectra of PSII (Fig. 5).

The visible CD spectroscopy is very useful to probe the pigment–pigment and/or pigment–protein interactions [7,41,42]. In Fig. 5, the CD spectra of PSII monomer (a) and dimer (b) samples are reported. For both samples, in the 660–700 nm region a doublet signal is evident, with a negative peak at about 684 nm and a positive peak at about 671 nm, which is due to the excitonic coupling interaction of chlorophyll a in PSII [14,36]. In the Soret region the two negative signals at about 439 and 454 nm are due to chlorophyll a and β-carotene respectively, while the positive ones at about 386 and 411 nm are related to chlorophyll a [14,36]. In the CD spectrum of PSII dimer at zero-time, a positive peak at about 507 nm is also visible. Literature reports assign it to carotenoids [14,36]. This suggests that very little differences in the microenvironment around carotenoids occur in PSII dimer preparation with respect to monomers, whose CD spectrum does not show the signal at 507 nm. Moreover little differences are present also in the red region of the spectra. In fact a more symmetrical splitting of the two bands and their slight red shift (with negative peak at 686 nm and positive ones at 672 for dimers at zero-time) is observable for dimer. This seems to indicate the presence of a slightly higher and uniform chlorophyll–chlorophyll dipole coupling in PSII dimer preparations. As reported before conformational structures are important in aggregative

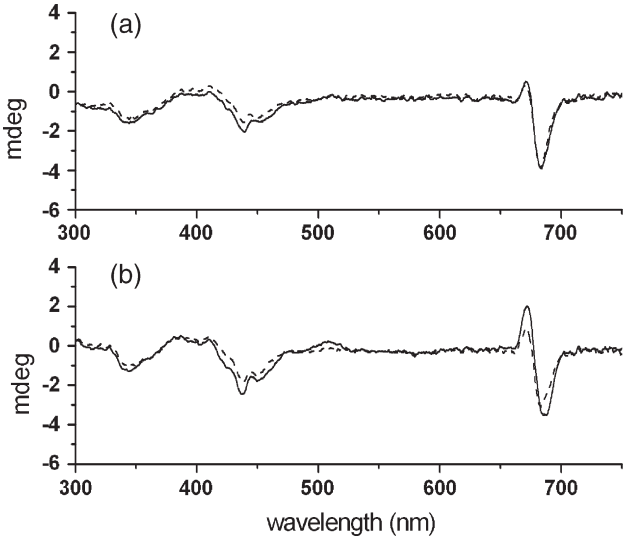

Fig. 5. CD spectra of PSII monomer (a) and dimer (b) samples at zero-time (continuous line) and 2 h later (dashed line).

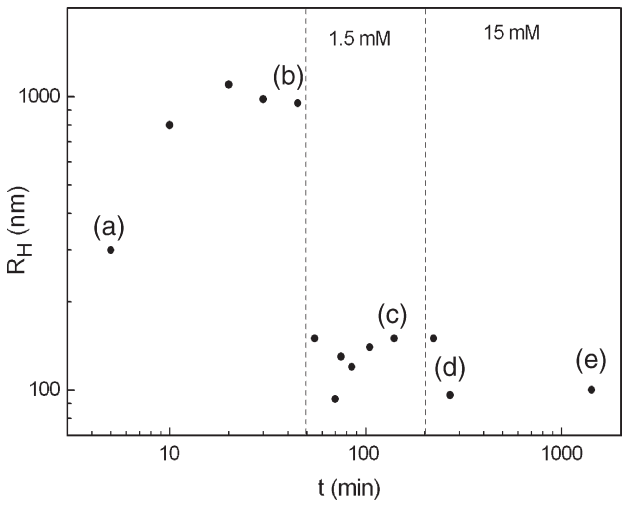

Fig. 7. Average hydrodynamic radius of the PSII aggregates before and after the addition of DM.

processes, so the little conformational differences observed in the case of dimers could facilitate their aggregation process and therefore, explain the faster dimer assembly growth, as evidenced by fluorescence and DLS measurements (Figs. 1 and 3).

However such CD differences disappear in time. The dimer CD spectra recorded after 2 h show in fact a slight blue shifted non-symmetrical splitting with a negative peak at 683 nm and positive peak at 671 nm, which is similar to the monomer ones recorded after 2 h. Therefore the pigment–pigment and pigment–protein interactions seem to be the same in both aggregates which show little chlorophylls and carotenoids microenvironment modifications. Such slight structural rearrangements may affect the efficiency of energy transfer among pigments and justify the fluorescence quenching as discussed by Grudzinski et al. [32].

Measures of oxygen evolution rates evidenced that during the aggregation process PSII complexes retained about 30% of their activity. This indicates that although some damage can occur to PSII subunits during the growth of the aggregates, the assemblies retain part of their activity and functionality.

In this work, as previously reported, PSII core dimers and monomers were isolated from thylakoid membranes by sucrose density gradient centrifugation in the presence of DM. However since PSII samples were subjected to dilutions DM concentration cannot be known exactly. Reports refer to the importance of DM in affecting the oligomeric state of LHCII complexes [32], so it is plausible that DM plays an important role in the case of PSII aggregation.

In order to investigate the effect of *n*-dodecyl-β-D-maltoside (DM) on PSII aggregation process, samples containing aggregates of PSII were treated with DM and then steady-state emission spectra were recorded at different detergent concentrations (Fig. 6). As a result an increment of fluorescence intensity at 683 nm was observed increasing DM concentration both for monomers and dimers. This suggests that DM has a disaggregating effect on PSII assemblies.

The effect of DM on PSII aggregation state was then observed by means of a dynamic light-scattering experiment in which PSII aggregate growth process was followed in about 2 h; then an injection of DM was realized on the sample under investigation, obtaining a concentration of 1.5 mM (lower than cmc value of 1.7 mM). As a result it can be observed that a drastic decrease of the average hydrodynamic radius of PSII aggregates occurs after DM addition (Fig. 7). Further addition of DM at concentration 15 mM brings about a slight reduction of complexes dimensions. Fig. 8 reports the hydrodynamic radius distribution for the scattering objects. At the beginning of the aggregative process it is possible to observe three distribution peaks, so different populations of aggregates are present. After about 2 h 92% of the DLS spectrum corresponds to aggregates with an average hydrodynamic radius of about 1 μm. The DM addition (1.5 mM) causes a decrease of this super-aggregate and an increase of aggregates with smaller dimensions (about 130 nm), present in little amount at the start of the aggregative process. Subsequent addition of DM (15 mM) promotes further disaggregating processes, as

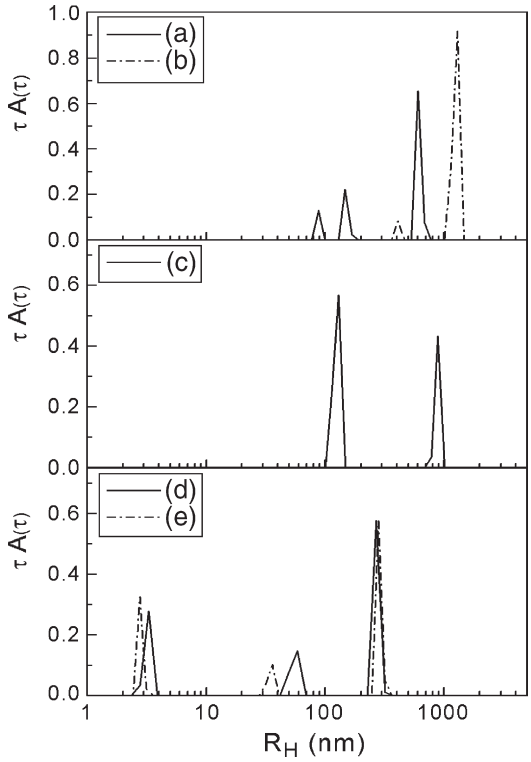

Fig. 8. Amplitude of the spectral radius distributions of the samples indicated by letters in Fig. 7.

evidenced by the relative distribution peaks. The peak at 3 nm, present in the distribution curves (d) and (e) of Fig. 7, is the contribution from DM micelles.

4. Conclusions

In this work data show that for PSII monomer and dimer complexes at room temperature and at low DM concentration, an aggregation process takes place. Such process resulted faster for dimeric preparations and favoured by temperature. Spectroscopic data showed that the protein–pigment and pigment–pigment interactions are the same for the aggregates obtained from both PSII monomer and dimer preparations, which reach micrometric dimensions retaining about 30% of oxygen evolving capability.

The mechanism of this process could be an interesting argument to deal with in future works and certainly it implicates some conformational changes and involves the detergent used to solubilize PSII monomers and dimers in the buffer. The aggregation process resulted in fact reversible by adding DM as proven by the rise of steady-state fluorescence intensity and by the prompt reduction of aggregate hydrodynamic radius after its addition.

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