Biochemistry (Moscow), Vol. 69, No. 12, 2004, pp. 1372-1378. Translated from Biokhimiya, Vol. 69, No. 12, 2004, pp. 1683-1690.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-111, June 27, 2004.

Low Sodium Dodecyl Sulfate Concentrations Inhibit Tobacco Mosaic Virus Coat Protein Amorphous Aggregation and Change the Protein Stability

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Received May 6, 2004
Revision received June 1, 2004

Abstract—Effects of low SDS concentrations on amorphous aggregation of tobacco mosaic virus (TMV) coat protein (CP) at 52°C and on the protein structure were studied. It was found that SDS completely inhibits the TMV CP (11.5 μM) unordered aggregation at the detergent/CP molar ratio of 15 : 1 (0.005% SDS). As judged by fluorescence spectroscopy, these SDS concentrations did not prevent heating-induced disordering of the large-distance part of the TMV CP subunit, including the so-called "hydrophobic girdle". At somewhat higher SDS/protein ratio (40 : 1) the detergent completely disrupted the TMV CP hydrophobic girdle structure even at room temperature. At the same time, these low SDS concentrations (15 : 1, 40 : 1) strongly stabilized the structure of the small-distance part of the TMV CP molecule (the four α-helix bundle) against thermal disordering as judged by the far-UV (200-250 nm) CD spectra. Possible mechanisms of TMV CP heating-induced unordered aggregation initiation are discussed.

Key words: tobacco mosaic virus coat protein, amorphous aggregation, sodium dodecyl sulfate

Amorphous (unordered) aggregates of different proteins have been recently implicated in pathogenesis of many important human diseases [1-3]. But structural studies of such aggregates are greatly hampered by their large size, transient character, and heterogeneity. Probably because of this, up to now, as far as we know, only one detailed model of mechanism of amorphous protein aggregation has been proposed. It is the Goldberg–Wetzel model [4, 5] according to which unordered aggregates are formed by *inter*molecular interactions of those domains of partly disordered protein molecules, which in the native state were involved in *intra*molecular interactions between the same domains.

Tobacco mosaic virus (TMV) coat protein (CP) is well known for its ability to produce ordered assemblies [6]. It is well known that at room temperature, pH values in the range from 7.5 to 9.0, and ionic strengths in the range from 10 to 100 mM, the TMV CP exists as a dynamic mixture of pentamers and trimers, with a minor amount of monomers. This mixture is known as the 4S-protein. At pH of about 7.0 and ionic strength of about 100 mM, 60-80% of the TMV CP is in the form of the 20S-aggregate (two-layer disk or short helix), with the other 40 to 20% being the 4S-protein. At pH ≤ 6.0, the protein produce long virus-like helical aggregates called repolymerized protein [6].

But TMV CP also turned out to be a good model for studies of heating-induced unordered aggregation [7, 8]. The process of irreversible TMV CP aggregation is highly reproducible and its rate can be easily manipulated by changing solution ionic strength, the protein concentration, and temperature [7, 8]. Figure 1 shows the TMV CP subunit structure in the virion as deduced from X-ray fiber diffraction data with 0.29 nm resolution [9]. It can be seen that the TMV CP molecule consists of a classical four α-helix bundle, located in the virion at a small distance from the virion long axis, and of less regular structure including so-called "hydrophobic girdle" [10], containing all Trp and Tyr residues and located at a large distance, closer to the virion surface (Fig. 1). This hydrophobic girdle serves as one of the main centers of axial and lateral intersubunit interactions in the TMV CP

Abbreviations: TMV) tobacco mosaic virus; CP) coat protein; PB) phosphate buffer.

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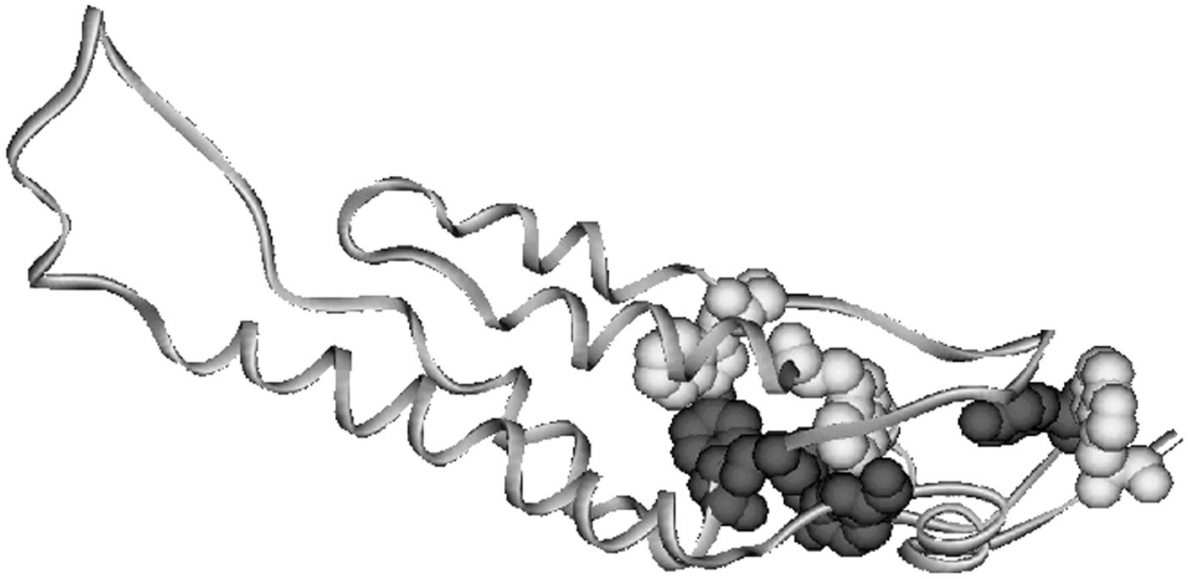

Fig. 1. Side-view of the structure of TMV CP subunit in the virion. The virion axis is on the left. Tyrosine and tryptophan residues are highlighted (dark-gray and light-gray, respectively). The figure was generated from the X-ray diffraction data [9] using the WebLab ViewerLite© program.

ordered aggregates ("polymers") and the virus particles [6, 9].

Finding of efficient aggregation inhibitors and their subsequent use for treatment of "amyloid" illness is a declared aim of all protein aggregation studies. While searching for TMV CP heating-induced aggregation inhibitors, we found that the aggregation can be prevented by rather low SDS concentrations. These SDS concentrations were found to induce disordering of the large-distance part (including hydrophobic girdle) of the TMV CP subunit in parallel with strong stabilization of the small-distance part (the four α-helix bundle) of the molecule.

MATERIALS AND METHODS

Purification of viruses and CP preparation. Wild-type (strain U1) TMV was obtained as described elsewhere [11] and its coat protein was isolated by the acetic acid method [12]. The CP preparations were stored at concentrations of 4 to 5 mg/ml in 5 mM Na/Na phosphate buffer (PB), pH 8.0, at 4 or –20°C. The protein concentration was measured by UV spectroscopy using the extinction coefficient $E_{280}^{0.1\%}$ of 1.30 [13].

UV spectroscopy. The absorption spectra and the kinetics of CP macroscopic aggregation at constant temperature (52°C) were recorded in 0.5- or 1-cm cuvettes using a Specord UV-VIS spectrophotometer (Carl Zeiss, Germany). The protein concentration in kinetic experiments was varied from 0.05 to 0.3 mg/ml. A cuvette filled with 50 mM Na/Na PB, pH 7.0, and SDS (Sigma, USA) of desired concentration was placed at 52°C for 10 min; thereafter, an aliquot (0.015-0.1 ml) of a stock CP solution preheated at 35°C was added and thoroughly stirred with the buffer for 15 sec (measurement "dead time"). The final volume of the mixture was 1 ml in 0.5-cm cuvettes or 2 ml in 1-cm cuvettes. The kinetics of turbidity changes was monitored at 313 nm. The initial rate of aggregation (v_{in}) was calculated in absorbance units per minute from the initial linear portion of the kinetic curve.

Fluorescence measurements. Spectral fluorescence intensity measurements were taken using a Hitachi MPF-4 spectrofluorimeter (Japan) in 1-cm cuvettes at room temperature or at 52°C. Fluorescence from 0.2 mg/ml TMV CP in 10 or 100 mM Na/Na PB, pH 7.0, was excited at 280 nm; the emission spectra were recorded between 300 and 400 nm. At room temperature, SDS of desired concentration was added to the buffer solution 10 min before or 10 min after the protein sample addition. These changes in the reagent addition order did not lead to any changes in experimental results. At 52°C, the detergent was, naturally, added first, and spectra were recorded as soon as possible after the protein addition. Measurement dead time was 15 sec. Fluorescence spectrum measurement took about 1 min.

CD spectroscopy. The CD spectra of the TMV protein were recorded between 198 and 250 nm using a modified Mark V dichrograph (Jobin-Ivon, France) as described previously [7, 8]. In the step-wise heating ("melting") experiments, the TMV CP samples (0.2 mg/ml) in 10 or 50 mM Na/Na PB, pH 7.0, were

heated for 15 min in about 10°C steps in a 0.1-cm temperature-controlled cuvette. SDS concentration was 0.005 or 0.013%.

RESULTS AND DISCUSSION

The TMV CP heating-induced amorphous aggregation, in contrast to that of most other proteins, occurs without any lag-period ([7, 8], see also Fig. 2). Probably for this reason all agents tested by us known to inhibit protein aggregation (tetracycline, dithiothreitol, EDTA, small chaperones and so on) turned out to be ineffective in the case of the TMV CP. SDS was found to be almost the only agent which prevented the TMV CP heating-induced aggregation.

The process of the TMV CP thermally induced amorphous aggregation can be most easily monitored by a large increase in turbidity ("absorption" at $\lambda > 305$ nm) in the course of heating at, say, 52°C [7]. Throughout this work, we employed Na/Na phosphate buffer, instead of the K/K buffer used in our previous works [7, 8]. Replacement of K/K PB by Na/Na PB by itself produced absolutely no changes in any parameters of the TMV CP heating-induced aggregation. As can be seen in Fig. 2 (curves *1* and *2*) heating at 52°C in 50 mM Na/Na PB, pH 7.0, of 0.2 mg/ml (11.5 μM) TMV CP resulted in a fast increase in turbidity ("absorption" at 313 nm) identical to that observed in 50 mM K/K PB.

Addition of different (low) SDS concentrations resulted (Fig. 2, curves *3* to *7*) in a gradual decrease in the initial rate of aggregation. Complete inhibition of the heating-induced TMV CP aggregation under these conditions was observed at SDS concentration of 172.5 μM (0.005%), which corresponds to the molar SDS/CP ratio of 15 : 1, i.e., about one SDS molecule per 10 amino acid residues. In similar experiments with different TMV CP concentrations it was found that the observed SDS effect is determined not by the absolute SDS concentration, but by the molar SDS/protein ratio (Fig. 3). It should be kept in mind that, as shown in the previous work [7], the TMV CP initial rate of aggregation is proportional to the square of the protein concentration, i.e., this rate increases fourfold on increase in TMV CP concentration from, say, 0.1 to 0.2 mg/ml. Due to this, in Fig. 3 the *relative* initial rates of aggregation inhibition by SDS ($1 - v_{in}^{SDS}/v_{in}^{0}$) are presented, where v_{in}^{SDS} and v_{in}^{0} are the initial rates of aggregation in the identical experiments with and without SDS. The observed linear dependence of ($1 - v_{in}^{SDS}/v_{in}^{0}$) on the SDS/CP ratio argues in favor of a suggestion that the aggregation inhibition is caused by some kind of direct interaction of SDS molecules with the TMV CP subunits.

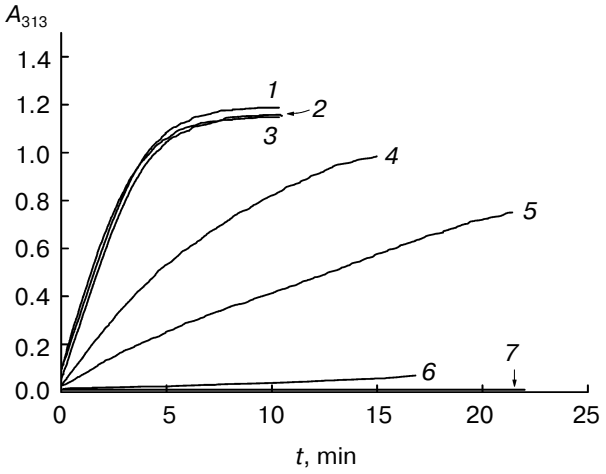

Fig. 2. Effect of SDS on TMV CP amorphous aggregation at 52°C, as assessed from "absorption" at 313 nm of 0.2 mg/ml (11.5 μM) TMV CP in 50 mM K/K PB, pH 7.0 (curve *1*), or Na/Na PB, pH 7.0 (curves *2*-*7*). Curves *1* and *2*, without SDS; curves *3* to *7*, with 11.5, 57.5, 80.5, 115, and 172.5 μM SDS, respectively (SDS/CP molar ratios are 1 : 1, 5 : 1, 7 : 1, 10 : 1, and 15 : 1).

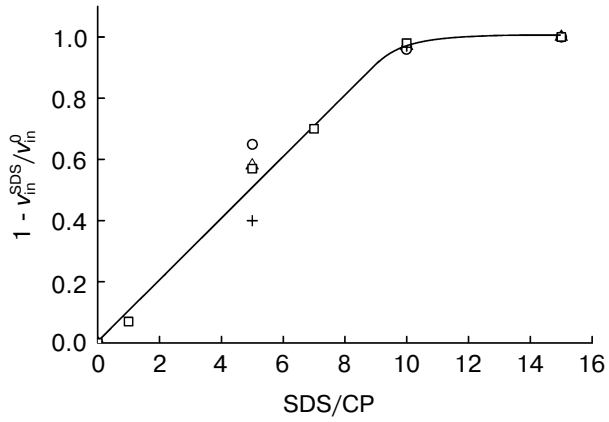

Fig. 3. Effect of the molar SDS/CP ratios on the protein aggregation inhibition in 50 mM Na/Na PB, pH 7.0. v_{in}^{SDS} and v_{in}^{0} are initial rates of aggregation in a pair of identical experiments with and without SDS. The TMV CP concentrations: 0.05 (Δ), 0.1 (○), 0.2 (□), and 0.3 (+) mg/ml. The correlation coefficient for the linear part of the curve is 0.991.

The TMV CP is a very "good" protein for intrinsic fluorescence studies. It is characterized by rather high tryptophan fluorescence intensity and the classical for globular protein maximum position (320 nm). On thermal denaturation, the TMV CP fluorescence maximum is shifted to 340 nm and the fluorescence intensity at 320 nm drops about fivefold [8, 14]. Previously we observed that the TMV CP heating-induced aggregation is preceded by a very fast drop in fluorescence intensity and suggested that it is disordering of a small large-distance part of the subunit (including Tyr and Trp residues (see Fig. 1)), which transforms the molecule into an amorphous aggregation-prone state [8]. In the experi-

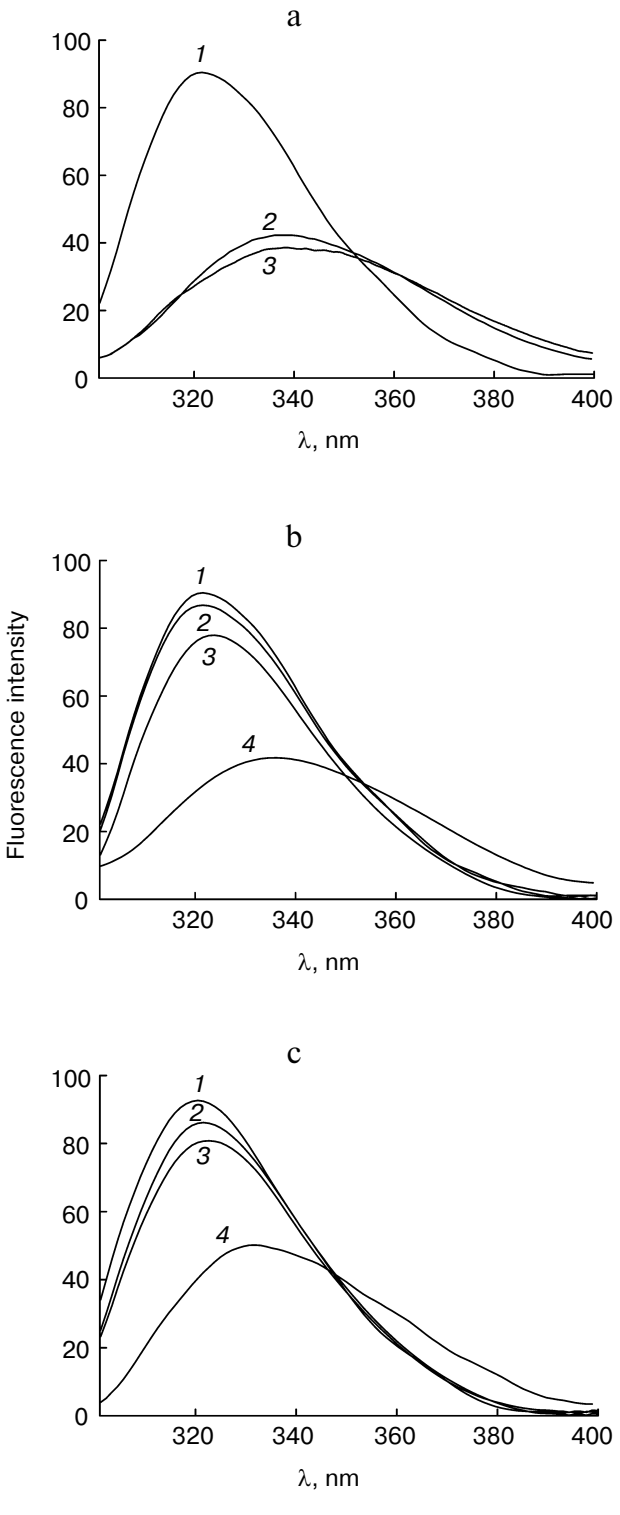

Fig. 4. Effects of SDS on fluorescence spectra of TMV CP (0.2 mg/ml) at 52°C (a) and at room temperature (b, c). a: *1*) Unheated TMV CP in 10 mM Na/Na PB, pH 7.0; *2*, *3*) TMV CP immediately after heating to 52°C without SDS (*2*) and with 172.5 μM SDS (15 : 1) (*3*). b) Fluorescence spectra at room temperature of TMV CP with different SDS/CP molar ratios in 10 mM Na/Na PB, pH 7.0: *2*) 10 : 1; *3*) 15 : 1; *4*) 40 : 1; *1*) without SDS; c) the same as in Fig. 4b, but in 100 mM Na/Na PB, pH 7.0.

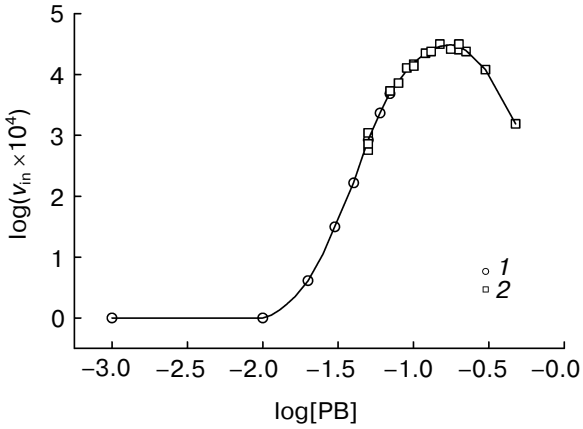

Fig. 5. Log–log ionic strength dependence of the initial rate of TMV CP amorphous aggregation at pH 7.0. Protein concentrations: 0.133 (*1*) and 0.067 mg/ml (*2*); 0.067-mg/ml initial rate values were multiplied by 4 (see text). Measurements were performed at 52°C.

ments on fluorescence-monitored TMV CP heating at 52°C (in 10 mM PB, pH 7.0), it was found that SDS (at the ratio 15 : 1) did not prevent this disordering (Fig. 4a). More than that, SDS was found to decrease the TMV CP fluorescence even at room temperature and complete loss of the native fluorescence signal was observed at the SDS/protein ratio of 40 : 1. This effect was independent of PB molarity in the 10 to 100 mM range (Figs. 4b and 4c). Presumably, this indicates a hydrophobic nature of SDS–girdle interactions. A similar picture was observed when we followed the SDS-induced hydrophobic girdle disordering at 25°C by near UV ("aromatic") CD spectra in the 250-300 nm region (data not shown).

As shown in our previous study [7], the rate of the TMV CP heating-induced aggregation is extremely sensitive to the phosphate buffer molarity (Fig. 5). At the same time, the TMV CP partial denaturation temperature demonstrated almost no dependence on the buffer molarity [7]. Thus, it seems as if temperature- or SDS-induced disordering of the TMV CP large-distance segment structure is not determined by electrostatic effects, while the heating-induced unordered aggregation can occur only after neutralization of strong repulsive electrostatic interactions [8].

These results suggest extremely low stability of the "external" part of the TMV CP subunits when they are not incorporated into the virions or the ordered helical aggregates. For some enzymes it has been shown that their activity is not inactivated even in 1% SDS [15, 16]. Extremely low thermal stability of free TMV CP in contrast to that of TMV virions had been demonstrated in classical works of Jockusch in the 1960s [17]. As shown by Jockusch, free TMV CP loses the ability to form ordered assemblies already after heating to temperatures as low as 40°C. This means that *inter*subunit interactions in the

TMV virions (and in the helical repolymerized TMV CP) are much stronger, than *intra*subunit interactions in the protein molecule. The present results suggest that it is the "girdle" region that determines the very low thermal stability of free TMV CP functional activity [17]. At the same time, a part of the TMV CP four α-helical bundle posses very high thermal stability [7], and this stability is further increased by SDS (see below).

According to the far UV (200-250 nm) CD data [7], in the absence of SDS at about 42°C the TMV CP is transformed from the native form ($[\Theta]_{208} = -16{,}300$ deg·cm^2· dmol^{-1}) to a partially disordered structure with $[\Theta]_{208} = -10{,}000$ deg·cm^2·dmol^{-1}, which retains stability up to 90°C. This $[\Theta]_{208}$ value corresponds to preservation of about half of the initial 50% α-helix content of the protein [18]. The transition observed was almost independent of PB molarity in the 10 to 50 mM range [7].

In 10 mM PB, pH 7.0, SDS (15 : 1) induced no changes in $[\Theta]_{208}$ value of the TMV CP at room temperature, and only minor reduction of the CD signal was observed even after heating up to 90°C (Fig. 6a). Thus, in the presence of SDS no transition to the "−10,000-form" is observed. Minimal (negative) $[\Theta]_{208}$ value (attained at 90°C) equaled −15,000 deg·cm^2·dmol^{-1}, and after cooling to room temperature $[\Theta]_{208}$ was even slightly higher than that for the native protein (Fig. 6a). Besides, SDS also reproducibly increased (negative) CD signal in 220-240 nm region possibly through its effect on the region of TMV CP aromatic acid residues (see above). The same picture was registered in 10 mM PB with 0.46 mM SDS (40 : 1).

To study possible mechanisms of the low SDS concentration stabilizing action on the TMV CP four α-helix bundle, we tried to perform the protein "CD-melting" at higher solution ionic strengths. But it turned out that in 50 mM PB, 172.5 μM SDS did not completely prevent, but only delay (in temperature) TMV CP aggregation. At 70°C, the large-scale aggregation accompanied by well-known distortions of the CD spectra within 200-250 nm region took place (Fig. 6b), making estimation of the protein secondary structure impossible [7, 19, 20]. But below this temperature about 20% decrease in CD signal intensity at 208 to 220 nm was observed suggesting a decrease in α-helix content of the TMV CP. Ionic strength of 0.12 M (corresponding to 50 mM PB) usually is not sufficient for complete disruption of electrostatic interactions in proteins, but further increase in PB molarity resulted in further acceleration of the TMV CP aggregation [7].

Thus, an interaction of a small number of SDS molecules with the TMV CP four α-helix bundle strongly stabilizes its structure against thermal unfolding. As judged by the far-UV CD within 200-250 nm region, the residual (25%) α-helical structure of internal region of TMV CP subunit seems to be highly stable. It is not disrupted by heating up to 90°C in 10 mM PB, pH 8.0 [7], and in 172.5 μM SDS the fraction of stable α-helices increased to almost 100%. In the pioneering work of Schubert and Krafczyk of 1969 [21], the TMV CP was shown not to lose its 208 nm CD negative maximum even in 66% acetic acid. And only by heating to 80°C at pH 2.8 we succeeded in reversible shifting of this maximum to 202 nm with some loss in intensity (our unpublished results).

SDS-induced stabilization of α-helical structure has been described for several proteins [15, 16, 20, 22-24]. But this stabilization is observed at much higher (millimolar) SDS concentrations and is usually explained by micelle formation. Our results do not allow definite conclusions on mechanisms of the SDS-induced stabilization of the TMV CP four α-helix bundle. But most probably this stabilization is determined by strong electrostatic interactions between the detergent molecule anionic "heads" and positively-charged residues of the bundle especially nearby positioned Arg112-Arg113 or Arg90-Arg92.

Thus, SDS produces three effects on the TMV CP molecules: it prevents heating-induced aggregation, destabilizes the large-distance region, and stabilizes the small-distance α-helical bundle of the subunits. All the three effects are induced by rather small amounts of SDS—15 molecules per the CP subunit or about one detergent molecule per 10 amino acid residues.

SDS is widely considered as a model of cellular lipids (see, for instance, [20]) and it is well known that virus infection may lead to drastic changes in lipid metabolism and membrane structure in plant cells [25, 26]. The TMV produces enormous amounts of the CP in infected cells, but, in the case of non-mutant virus, this protein never forms large amorphous aggregates. Interaction with host lipids may be one of the mechanisms of the TMV CP protection from unordered aggregation *in vivo*.

In our previous work [8] we suggested that disordering of only the large-distance segment of the TMV CP subunit is sufficient to initiate its thermally-induced macroscopic aggregation. The interaction of SDS with the large-distance region (the hydrophobic girdle) residues even at room temperature disrupts its native structure but inhibits thermal aggregation. Probably, this inhibition is determined by binding of hydrophobic tail of SDS molecule(s) to surface-exposed hydrophobic residues of the large-distance region of TMV CP subunits, making impossible its interactions with the identical regions of other TMV CP molecules. Such interactions were supposed to be a driving force of heating-induced amorphous TMV CP aggregation [8].

Thus, it seems that the TMV CP heating-induced amorphous aggregation is driven by non-native *inter*subunit interactions of those regions of the molecules which in the native state participate in very strong *inter*subunit interactions leading to formation of ordered aggregates (helical "polymers" and virions). This agrees not too well with the predictions of the Goldberg–Wetzel model [4, 5]. But the capacity to produce highly stable ordered

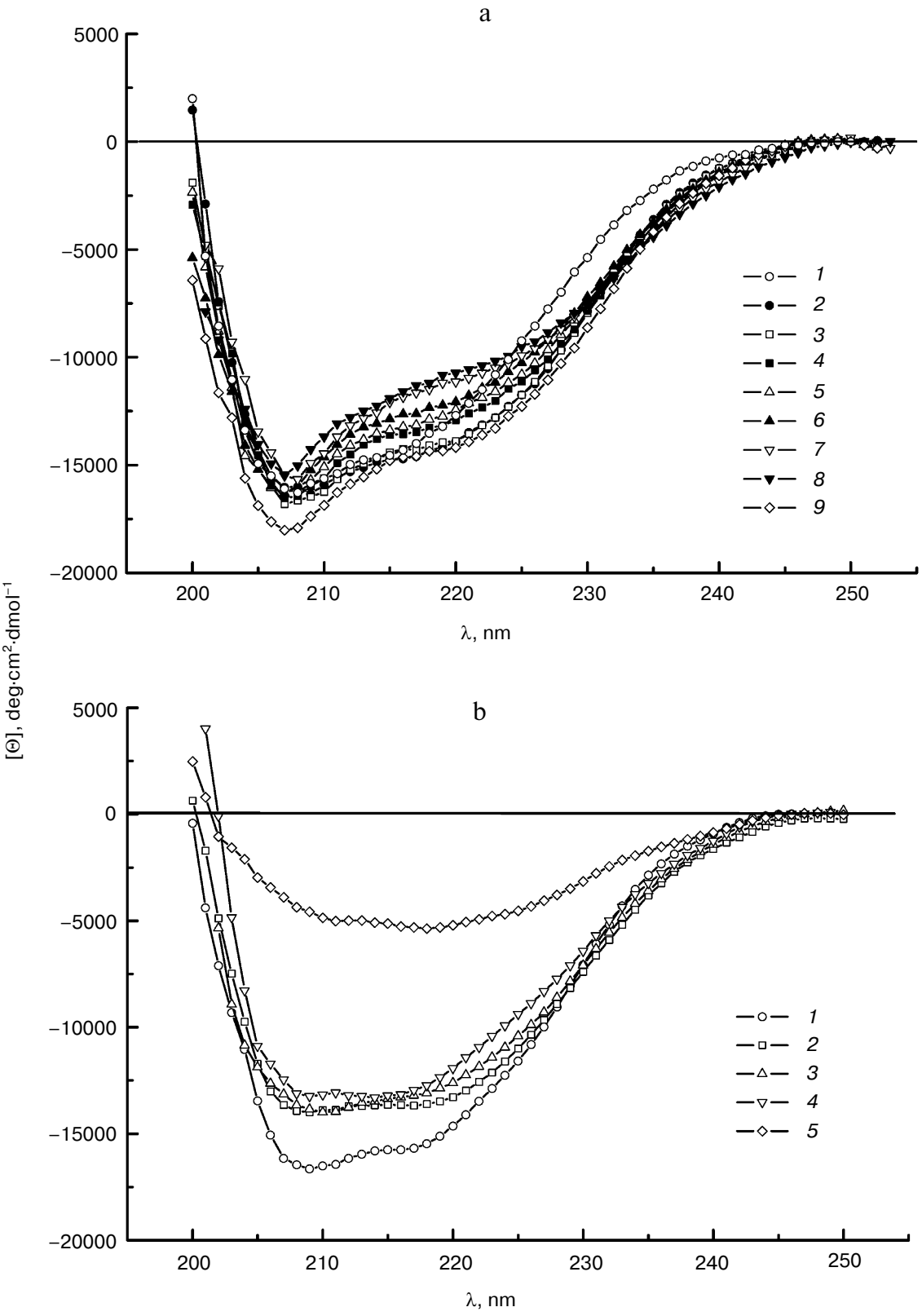

Fig. 6. Effect of temperature on CD spectra of 0.2 mg/ml TMV CP in 10 mM (a) or 50 mM (b) PB, pH 7.0, with SDS. a) TMV CP without SDS at 25°C (*1*); with 172.5 μM SDS (15 : 1) at 25 (*2*), 35 (*3*), 45 (*4*), 55 (*5*), 65 (*6*), 75 (*7*), and 90°C (*8*); CP with SDS cooled to 25°C (*9*). b) 25 (*1*), 35 (*2*), 50 (*3*), 60 (*4*), and 70°C (*5*).

aggregates is the most prominent feature of the TMV CP, and the Goldberg–Wetzel model still can be true for a vast majority of proteins which in their native state do not display such strong propensity for intermolecular interactions.

The work was supported by the Russian Foundation for Basic Research (grant No. 02-04-48842).

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