Structure changes of natively disordered Humanin in the presence of lipid

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Abstract

While neuroprotective activities of Humanin peptides have been clearly demonstrated, the functional mechanism has not been fully understood. Humanin and a majority of Humanin analogs showed a disordered structure at low peptide concentrations and aggregation at higher concentrations in aqueous solution at pH 7.0. Here we have examined the structure in lipid environments, i.e., in the presence of liposome by circular dichroism. Humanin underwent a large structure change into a typical β-sheet structure at

neutral pH in the presence liposome made of a negatively charged 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG), but not an electrically neutral 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC). As Humanin possesses a positive charge at neutral pH, the observed structure changes with DOPG suggest electrostatic binding of the peptide with the lipid. No effect of NaCl on the Humanin structure was observed in neutral solution and in the presence of DOPC liposome. Increasing temperature resulted in changes in the structure due to aggregation. On the other hand, the effects of temperature on the Humanin structure showed that it has a relatively stable structure in the presence of DOPG liposome independent of the presence of NaCl.

Introduction

One of the biggest challenges in drug discovery and development is to find effective treatments for neuronal diseases, including Alzheimer's disease (AD). Currently, there are a very limited number of treatments available for the Alzheimer's disease [1, 2]. Humanin (HN), a 24 amino acid peptide (MAPRGFSCLLLLTSEIDLPVKRRA), was discovered as a potential therapeutic agent against AD-related diseases [3-10]. However, the neuroprotecive mechanism of HN has been little understood [11]. Several HN binding proteins, including cell-surface receptors, have been shown to be involved in HN actions [12-17]. The structure that is involved in binding to cellular proteins is also unclear [11]. Various structures have been proposed, e.g., disordered at low peptide concentration in aqueous buffer solutions, or immunoglobulin-like fold in the presence of trifluoroethanol (TFE) or sodium dodecylsulfate (SDS)[18-24]. These reagents are often used to mimic

membrane environments, as such a short peptide as HN may require structure changes before binding to the target proteins, e.g., cell surface receptors [25]. Here we have examined the secondary structure of HN in the presence of liposome, by circular dichroism (CD), which more closely mimics the cell surface membrane. The effects of NaCl and temperature on the HN structure in solution and in the presence of liposome were also investigated

Materials and Methods

Chemicals. HN was obtained from Peptide Institute, Inc. (Osaka, Japan). The purity was confirmed by the vendor using amino acid and mass spectrometry analysis. Two lipids, 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoglycerol sodium salt (DOPG), were obtained from NOF Corp. (Tokyo, Japan). Sodium dihydrogenphosphate dehydrate were from Nacalai Tesque Inc. (Kyoto, Japan). NaCl was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Formation of unilamellar liposomes with DOPC and DOPG. Unilamellar liposome with DOPC and DOPG were prepared by the lipid extrusion protocol as described by previous reports [26, 27]. Briefly, the lipids dissolved in chloroform were placed in a round glass vial and subjected to evaporation in a vacuum desiccator over 12 hours to remove the solvent. The dry lipid films thus obtained were hydrated with 20 mM sodium phosphate buffer, pH 7.0 and vortexed for several seconds. The resulting suspension was extruded through a polycarbonate filter (pore size of 200 nm) using a Mini Extruder

(Avanti Polar Lipids, Inc., Alabaster, AL), leading to an apparently transparent liposome preparation with uniform size. All the process was carried out at room temperature.

Circular Dichroism measurements. Far-UV CD measurements were carried out at various temperatures using a Jasco J-720 spectropolarimeter (Tokyo, Japan). HN was first dissolved in distilled water for a final concentration of 0.2 mg/ml. The peptide concentration was spectrophotometrically determined using the molar extinction coefficient of 195 at 257.5 nm. The aqueous peptide solution was then mixed with an equal volume of various solutions of interest, in which the CD spectra were to be determined. The samples thus prepared were incubated at indicated temperature for a sufficient time to reach equilibrium structure: no time dependence was observed. When time course was measured, CD measurements were immediately initiated upon arrival at 37°C.

Results and Discussion

1. In 5 mM sodium phosphate buffer.

We have shown before that HN peptides aggregate less in low phosphate concentration, i.e., 5 mM [23]. CD measurements were repeated on 0.1 mg/ml HN in 5 mM phosphate buffer, pH 7.0. Fig.1 shows the far-UV CD spectra of HN at 0.1 mg/ml at 25°C as a function of phosphate buffer concentration. The results were similar to the previous data that the secondary structure of HN is essentially disordered in water and 5 or 10 mM

phosphate buffer. There was a slight upper shift in intensity around 200 nm in the presence of phosphate buffer, but without changes in spectral shape. Note that the mean residue ellipticity was also similar to the previous report, although the peptide concentration was determined using phenylalanine absorbance here compared to the weight-based measurements in the previous report.

The observed disordered structure has raised a question about the functional structure of HN [11]. Many peptide ligands, in particular those for G-protein coupled receptors, have been shown to undergo conformational changes at the cell surface membranes [25]. We have here tested such a possibility using liposomes. Fig.2 shows the far-UV CD spectra of 0.1 mg/ml HN in 5 mM phosphate buffer, pH 7.0, in the presence of 0.17 mM DOPG or DOPC. The spectrum in DOPC was nearly identical to the spectrum in 5 mM phosphate alone, indicating no apparent effects of DOPC on the HN structure. This result means either that HN did not interact with this liposome or that the DOPC-bound HN underwent no structural changes. The latter possibility may be less likely, as a large structure transition was observed with DOPG (Fig.2). Namely, HN underwent a drastic structure change in the presence of DOPG, indicating that HN bound to the liposome, leading to a large change in the secondary structure. The structure was typical of β-sheet, as observed in antibody fold [18, 19, 28].

2. In 10 mM sodium phosphate buffer.

A more physiological phosphate concentration of 10 mM was then used to confirm the above results. Fig.3 shows the far-UV CD spectra of 0.1 mg/ml HN at 25°C in various solution conditions. The spectra were essentially identical in 10 mM phosphate with and without 0.15 M NaCl, corresponding to a disordered structure. DOPC had no apparent effects on HN structure independent of the presence of 0.15 M NaCl, similar to the result in 5 mM phosphate buffer. Thus, HN underwent no structure changes at 10 mM phosphate buffer concentration, by the addition of NaCl and in the presence of DOPC. Conversely, a drastic change was observed again with 0.17 mM DOPG. The structural features with a negative peak at ~219 nm and a positive peak at ~200 nm were distinctive (Fig.3), corresponding primarily to the immunoglobulin-like β-sheet structure [18, 19, 28]. The structure is reminiscent of that observed in the intermediate concentrations of TFE and SDS [24]. At higher concentration of these reagents, the HN structure further changed to other structures containing both disordered and β-sheet structures [24]. It thus appears that the observed intermediate structure in TFE or SDS might correspond to the structure that HN acquires upon binding to the DOPG liposome. The observed differences in the HN structure between DOPC and DOPG suggest that the structure and the function of HN are affected by the surface property of lipid membrane and hence cell types, although no such study has been done to our knowledge. DOPC is electrically neutral, while DOPG is negatively charged. Therefore, the structure alteration of HN with DOPG suggests that the electrostatic interactions contribute to the binding of HN to this liposome. Binding and consequent structure changes do not appear to occur for electrically neutral DOPC liposome.

Fig.3 also shows the effect of 0.15 M NaCl on the HN structure induced by DOPG liposome. Although the spectral shape was largely retained, the magnitude was reduced by about half of the intensity in the absence of salt. However, this far-UV CD spectrum should be viewed with caution. This sample, i.e., a combination of 0.17 mM DOPG, 0.1 mg/ml HN and 0.15 M NaCl resulted in turbidity development immediately after sample preparation. One of the explanations for the observed turbidity is due to disruption of the liposome arising from the addition of 0.15 M NaCl and high external osmolality (no NaCl is present inside the liposome). However, this is unlikely as DOPC developed no turbidity by the addition of 0.15 M NaCl. It is thus possible that the HN bound to DOPG liposome indeed underwent structure changes by the addition 0.15 M NaCl. Another possibility cannot be ruled out that aggregation of liposome-bound HN occurred due to higher ionic strength and such aggregation has distorted the CD spectrum.

Temperature effects

The effects of temperature on HN structure were examined at 5, 10, 25 and 37°C. Fig.4 shows the spectra of 0.1 mg/ml HN in 10 mM phosphate buffer, pH 7.0. The spectra were essentially identical at 5°C and 10°C. The spectrum began to change at 25°C, as seen in decrease at ~220 nm and slight increase at ~200 nm. The spectrum significantly changed at 37°C and the upward shift continued with incubation time. The results were similar in the presence of 0.17 mM DOPC. As shown in Fig.5, the spectra were identical at 5°C and 10°C and slightly changed at 25°C in the presence of DOPC. Further change occurred at 37°C. The structural changes at 37°C in the presence and absence of DOPC had a similar

time course, as shown in Fig.6, occurring slowly with incubation time and leveling off around 30 min. That is, these results imply that HN in fact did not bind to the DOPC liposome and were present in solution phase. A same conclusion was obtained in the presence of 0.15 M NaCl (data not shown). Namely, HN underwent spectral changes with temperature in both aqueous solution and DOPC liposome containing 0.15 M NaCl, just as seen in the absence of NaCl. Thus, HN does not appear to bind to the DOPC liposome regardless of the ionic strength. The observed changes with temperature in the presence and absence of liposome or in the presence and absence of NaCl are most likely due to aggregation as previously proposed [19, 23]. Such aggregation at 37°C has resulted in time dependent changes in CD signals. The small changes observed at 25°C is also most likely due to aggregation, although no change with time was observed perhaps due to slow kinetics of aggregation.

On the contrary, the DOPG-bound HN was highly stable with change in temperature. The CD spectra were essentially independent of the incubation temperature, i.e., the immunoglubulin-like fold of HN that occurs upon binding to the DOPG liposome was retained at physiological temperature (Fig.7). The same was true in the presence of 0.15 M NaCl. Fig.8 shows little changes in CD spectrum with temperature. As described in Fig.3, however, the addition of 0.15 M NaCl might cause changes in the secondary structure of DOPG-bound HN. Thus, although some unknown changes might occur in HN structure by the addition of 0.15 M NaCl to the DOPG liposome system, whatever the structure that was attained on the liposome, it is also stable with temperature.

Biological relevance

It appeared clear that HN undergoes structure change upon binding to negatively charged DOPG liposome. It is the first report that HN forms a stable structure on lipid membranes. It has been speculated that many short peptides are structurally disordered in solution, but undergo structure transition upon binding to lipid membrane and the membrane-bound structure is responsible for binding to their cognate receptors [25]. Cell surface receptors have been identified for HN to transmit the signal and hence lead to neuroprotection [11-16]. First question is whether the observed immunoglubulin-like structure is responsible for receptor binding. The observed structure of HN in the intermediate TFE or SDS concentrations also supports this notion that HN can assume such a stable structure [24]. Second question is whether such a short peptide as HN can form a stable structure of β-sheet. Naturally β-sheet structure is formed when two or more β-strand associate with each other. If it occurs with the HN chain, the N-terminus of HN should turn back onto the C-terminal region to form anti-parallel packing of the peptide. Even if such a turn occurs with a potential strain posed on this short peptide, a question is raised whether there is enough length to fold back to form a stable β-sheet in the liposome-bound state. Another possibility is that two or more HN peptides associate on the liposome to form a stable β-sheet. It has been suggested that HN forms a functional dimer in solution [8], which was later ascribed to its tendency to aggregate [11]. We subjected the HN sequence, MAPRGFSCLLLLTSEIDLPVKRRA, to various secondary structure predication programs, none of which indicated strong turn potentials: note that a strong potential for β-sheet structures was clearly predicted in both N- and C-terminal regions.

One possible sequence for a turn was found near the N-terminus. However, even if this sequence makes a turn, there is not enough length N-terminal to this turn and hence to make intra-chain anti-parallel β-sheet structure. Therefore, the observed β-sheet structure is more likely accounted for by self-association of HN when bound to the liposome.

Summary

The structure change of HN is schematically illustrated in Fig.9. HN is monomeric at low peptide concentration and structurally disordered in solution or in the presence of DOPC (shown inside the box). As the ionic strength, temperature or peptide concentration is increased, it aggregates as shown by a box arrow. The peptide forms a β-sheet structure in the presence of DOPG or at the intermediate concentrations of TFE and SDS and is assumed to become a functional dimer. This assumption has to be experimentally confirmed. At higher SDS and TFE concentrations, the HN peptide will remain monomeric, which also needs to be demonstrated.

References

[1] S.A. Lipton, Curr. Alzheimer Res, 2 (2005) 155-165.

[2] S.A. Lipton, Curr. Drug Targets, 8 (2007) 621-632.

[3] Y. Hashimoto, T. Niikura, H. Tajima, Y. Yasukawa, H. Sudo, Y. Ito, Y. Kita, M. Kawasumi, K. Kouyama, M. Doyu, G. Sobue, T. Koide, S. Tsuji, J. Lang, K. Kurokawa, I. Nishimoto, Proc. Natl. Acad. Sci. USA, 98 (2001) 6336-6341.

[4] Y. Hashimoto, Y. Ito, T. Niikura, Z. Shao, M. Hata, F. Oyama, I. Nishimoto, Biochem. Biophys. Res. Commun, 283 (2001) 460-468.

[5] Y. Hashimoto, T. Niikura, Y. Ito, H. Sudo, M. Hata, E. Arakawa, Y. Abe, Y. Kita, I. Nishimoto, J. Neurosci, 21 (2001) 9235-9245.

[6] H. Tajima, T. Niikura, Y. Hashimoto, Y. Ito, Y. Kita, K. Terashita, K. Yamazaki, K. Koto, S. Aiso, I. Nishimoto, Neuro. Lett, 324 (2002) 227-231.

[7] T. Niikura, H. Tajima, Y. Kita, Curr. Neuropharmacol, 4 (2006) 139-147.

[8] K. Terashita, Y. Hashimoto, T. Niikura, H. Tajima, Y. Yamagishi, M. Ishizaka, M. Kawasumi, T. Chiba, K. Kanekura, M. Yamada, M. Nawa, Y. Kita, S. Aiso, I. Nishimoto, J. Neurochem, 85 (2003) 1521-1538.

[9] Y. Yamagishi, Y. Hashimoto, T. Niikura, I. Nishimoto, Peptides, 24 (2003) 585-595.

[10] Y. Hashimoto, K. Terashita, T. Niikura, Y. Yamagishi, M. Ishizaka, M. Kanekura, T. Chiba, M. Yamada, Y. Kita, S. Aiso, M. Matsuoka, I. Nishimoto, Eur. J. Neurosci, 19 (2004) 2356-2364.

[11] T. Arakawa, Y. Kita, T. Niikura, Curr. Med. Chem, 15 (2008) 2086-2098.

[12] G. Ying, P. Iribarren, Y. Zhou, W. Gong, N. Zhang, Z.X. Yu, Y. Le, Y. Cui, J.M. Wang, J. Immunol, 172 (2004) 7078-7085.

[13] B. Guo, D. Zhai, E. Cabezas, K. Welsh, S. Nouraini, A.C. Satterthwait, J.C. Reed Nature, 423 (2003) 456-461.

[14] D. Zhai, F. Luciano, X. Zhu, B. Guo, A.C. Satterthwait, J.C. Reed, J. Biol. Chem, 280 (2005) 15815-15824.

[15] T. Niikura, Y. Hashimoto, H. Tajima, M. Ishizaka, Y. Yamagishi, M. Kawasumi, M. Nawa, K. Terashita, S. Aiso, I. Nishimoto, Eur. J. Neurosci, 17 (2003) 1150-1158.

[16] M. Ikonen, B. Lu, Y. Hashimoto, L. Ma, K.W. Lee, T. Niikura, I. Nishimoto, P. Cohen, Proc. Natl. Acad. Sci. USA, 100 (2003) 13042-13047.

[17] M. Harada, Y. Habata, M. Hosoya, K. Nishi, R. Fujii, M. Kobayashi, S. Hinuma, Biochem. Biophys. Res. Commun, 324 (2004) 255-261.

[18] F. Arisaka, T. Niikura, T. Arakawa, Y. Kita, Int. J. Biol. Macromol, 43 (2008) 88-93

[19] T. Arakawa, T. Niikura, H. Tajima, Y. Kita, J. Pept. Sci, 12 (2006) 639-642.

[20] D. Benaki, C. Zikos, A. Evangelou, M. Vlassi, E. Mikros, M. Pelecanou, Biochem. Biophys. Res. Commun, 329 (2005) 152-160.

[21] D. Benaki, C. Zikos, A. Evangelou, M. Vlassi, E. Mikros, M. Pelecanou M. Biochem. Biophys. Res. Commun, 349 (2006) 634-642.

[22] A. Rojo-Dominguez, G. Ramirez-Galicia, G. Havel, L.H. Gutierrez-Gonzalez, Protein Pept. Lett, 14 (2007) 618-624.

[23] F. Arisaka, T. Arakawa, T. Niikura, Y. Kita, Protein Pept. Lett, 16 (2009) 132-137.

[24] Y. Kita, T. Niikura, F. Arisaka, T. Arakawa, Protein Pept. Lett, 15 (2008) 510-515.

[25] R. Sankararamakrishnan R. Biosci. Rep, 26 (2006) 131-158.

[26] N.K. Subbarao, R.I. MacDonald, K. Takeshita, R.C. MacDonald, Biochim. Biophys. Actam 1063 (1991) 147-154.

[27] R.C. MacDonald, R.I. MacDonald, B.P. Menco, K. Takeshita, N.K. Subbarao, L.R. Hu, Biochim. Biophys. Acta, 1061 (1991) 297-303.

[28] D. Ejima, K. Tsumoto, H. Fukada, R. Yumioka, K. Nagase, T. Arakawa, J.S. Philo, Proteins, 66 (2007) 954-962.

Figure 1

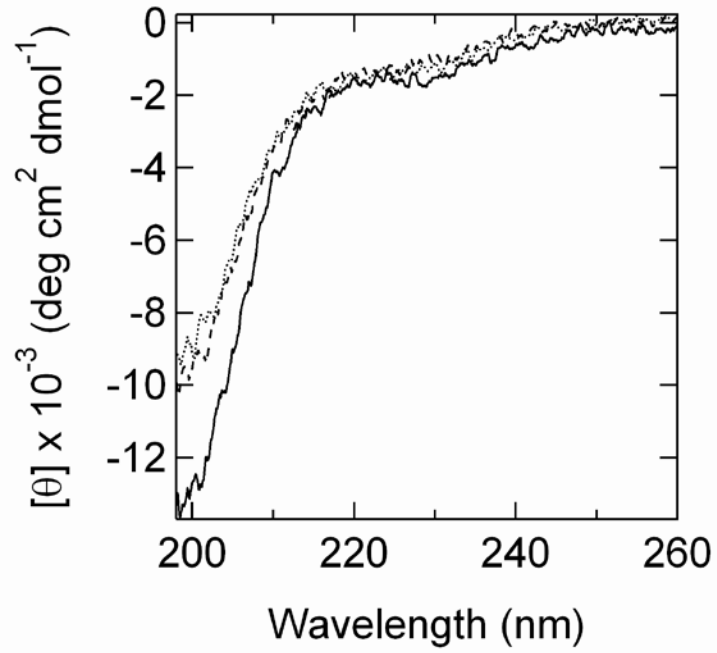

Figure 1. Far UV CD spectra of 0.1 mg/ml HN at 25 ˚C in aqueous solution. Solid line, in water; dotted line, in 5 mM phosphate buffer, pH 7.0; broken line, in 10 mM sodium phosphate buffer, pH 7.0.

Figure 2

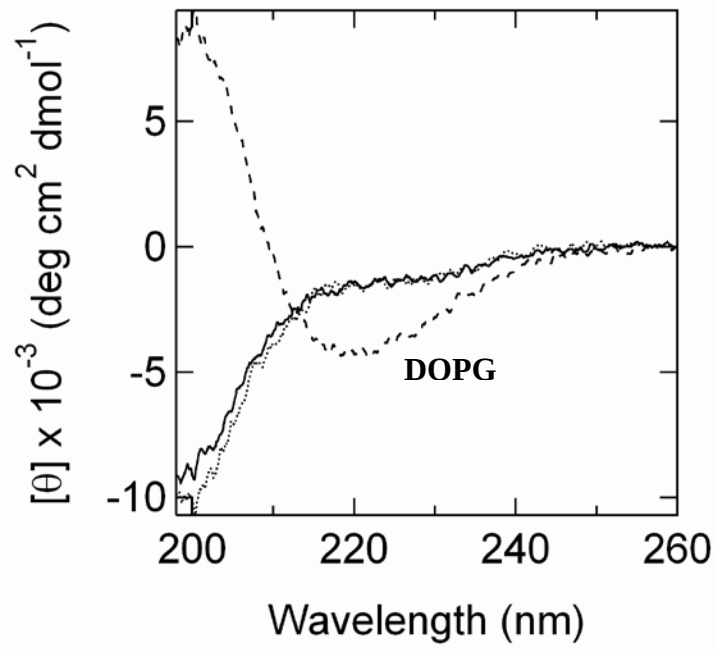

Figure 2. Far UV CD spectra of 0.1 mg/ml HN at 25 ˚C in the presence or absence of 0.17 mM DOPC or DOPG in 5 mM of sodium phosphate buffer, pH7.0. Solid line, 5 mM phosphate buffer; dotted line, DOPC; broken line, DOPG.

Figure 3

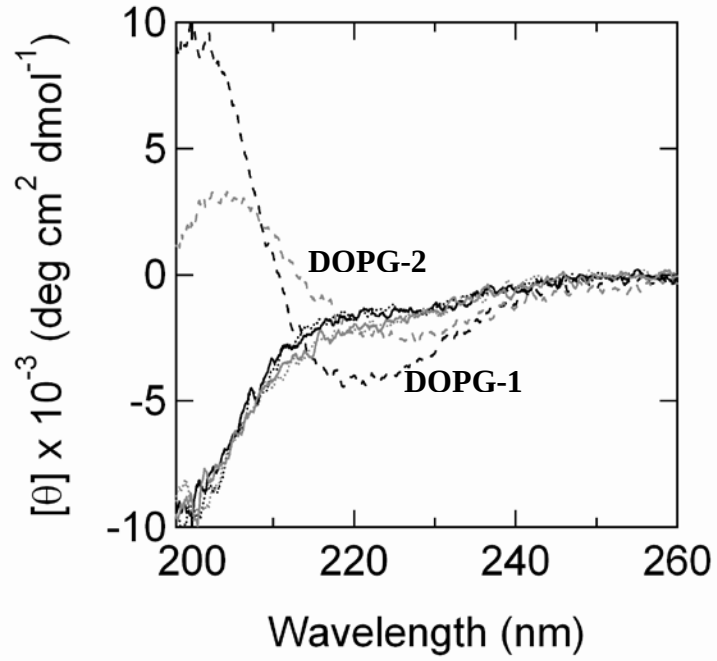

Figure 3. Far UV CD spectra of 0.1 mg/ml HN at 25 ˚C in the presence or absence of 0.17 mM DOPC or DOPG in 10 mM of sodium phosphate buffer, pH7.0. Black lines and grey lines were taken in the absence and presence of 0.15 M NaCl, respectively. Solid line, no lipids; dotted line, DOPC; broken line, DOPG.

DOPG-1, no NaCl. DOPG-2, in 0.15 M NaCl.

Figure 4

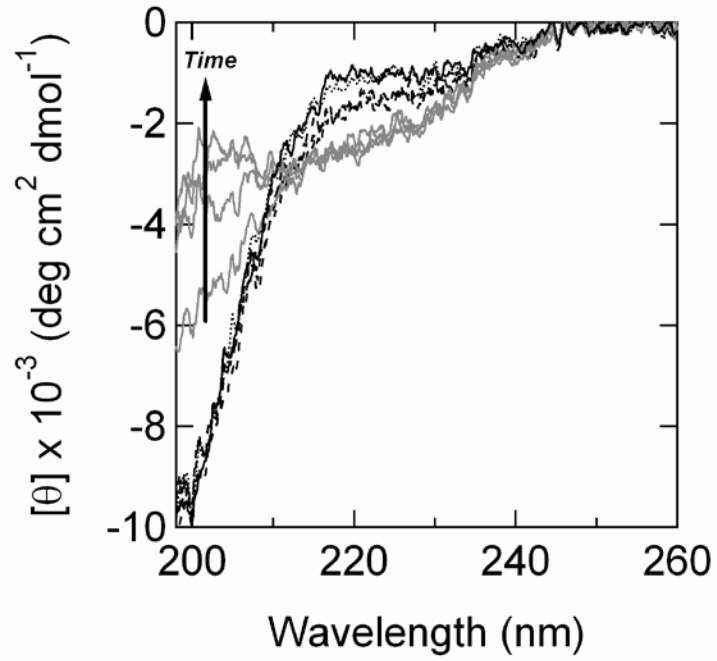

Figure 4. Far UV CD spectra at different temperatures of 0.1 mg/ml HN in 10 mM of sodium phosphate buffer, pH 7.0. Solid line, 5 ˚C; dotted line, 10 ˚C; broken line, 25 ˚C; gray lines, 37 ˚C.

Arrow indicates increasing incubation time (see Fig.6 for time course).

Figure 5

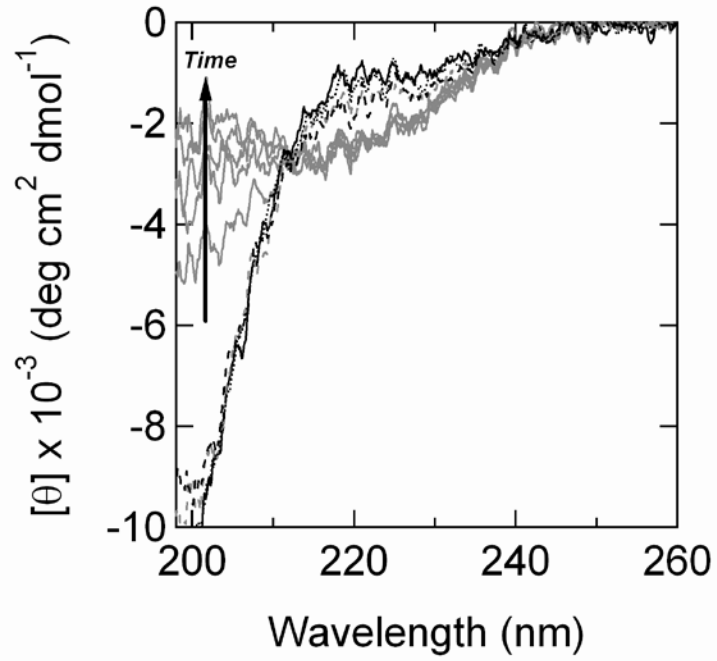

Figure 5. Far UV CD spectra at difference temperatures of 0.1 mg/ml HN in the presence of 0.17 mM DOPC in 10 mM of sodium phosphate buffer, pH 7.0. Solid line, 5 ˚C; dotted line, 10 ˚C; broken line, 25 ˚C; gray lines, 37 ˚C.

Arrow indicates increasing incubation time (see Fig.6 for time course).

Figure 6

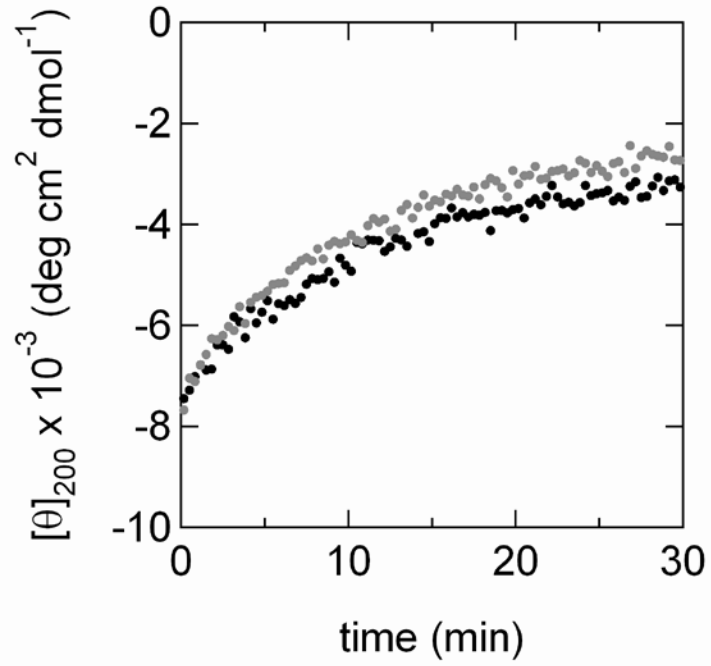

Figure 6. Time course of structural change of 0.1 mg/ml HN in the absence and presence of 0.17 mM DOPC in 10 mM of sodium phosphate buffer, pH7.0. Black and gray symbols were taken in the presence and absence of DOPC.

CD signal at 200 nm was monitored as a function of temperature.

Figure 7

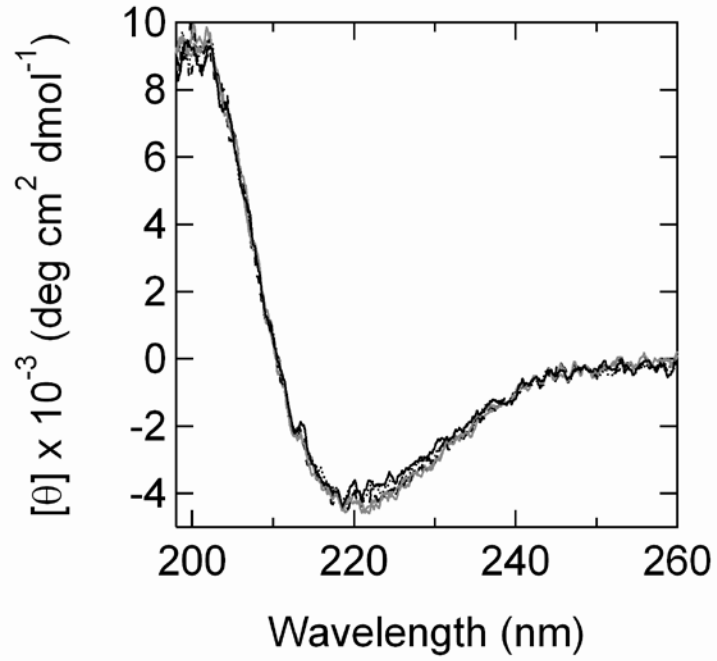

Figure 7. Far UV CD spectra at different temperatures of 0.1 mg/ml HN in the presence 0.17 mM DOPG in 10 mM of sodium phosphate buffer, pH 7.0. Solid line, 5 ˚C; dotted line, 10 ˚C; broken line, 25 ˚C; gray line, 37 ˚C.

Figure 8

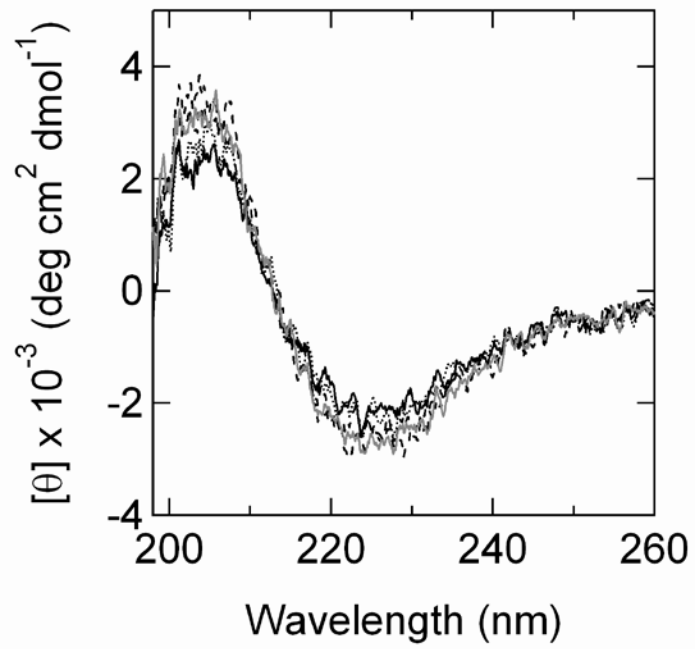

Figure 8. Far UV CD spectra at different temperatures of 0.1 mg/ml HN in the presence of 0.17 mM DOPG with 0.15 M NaCl in 10 mM of sodium phosphate buffer, pH 7.0. Solid line, 5 ˚C; dotted line, 10 ˚C; broken line, 25 ˚C; gray line, 37 ˚C.

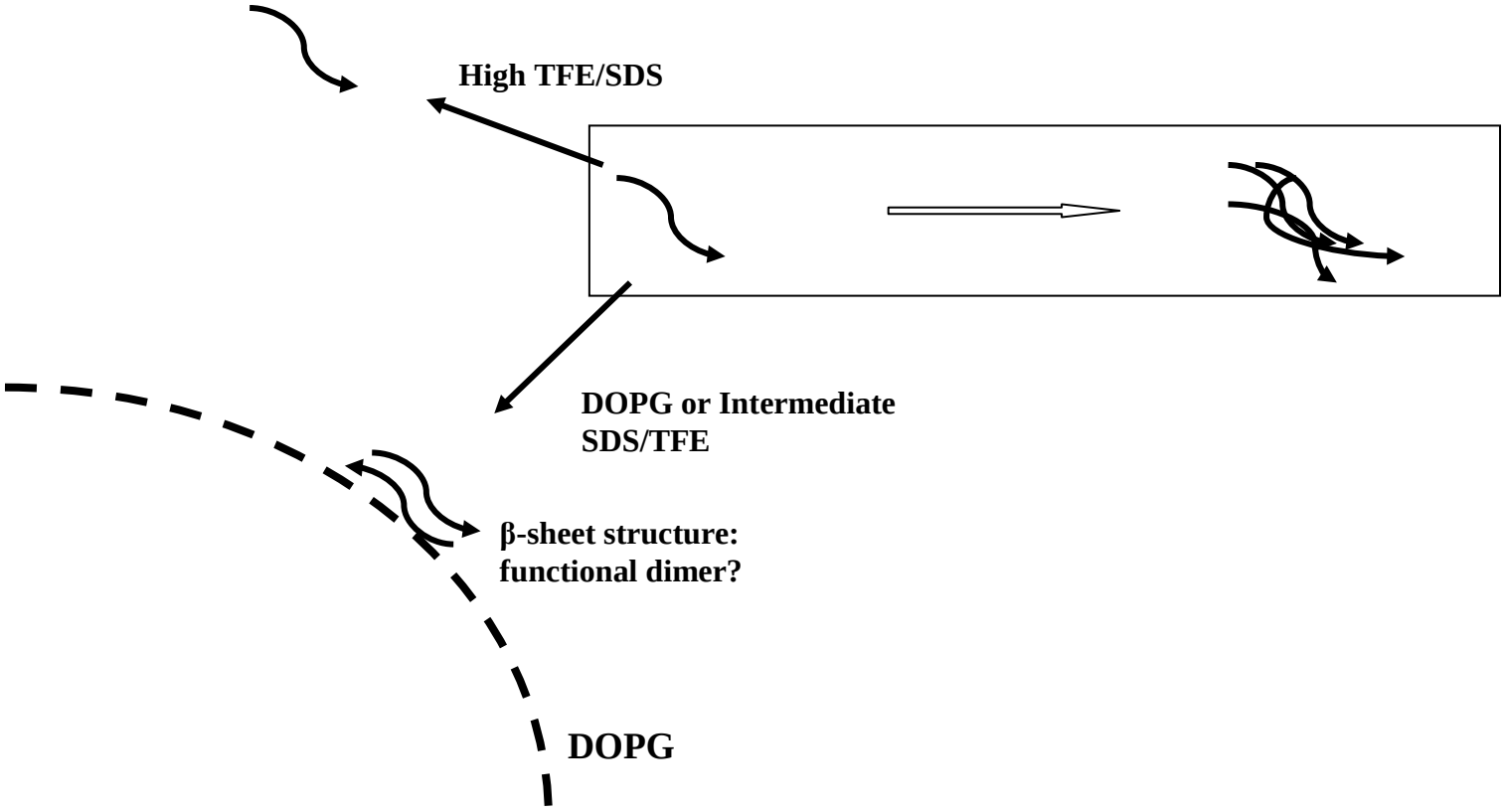

Fig.9 Schematic illustration of structure changes of HN

Box indicates HN in either solution or in the presence of DOPC.

Box arrow indicates increasing temperature, ionic strength or peptide concentration.