FEBS Letters 454 (1999) 122–126 FEBS 22242

Amyloid-like aggregates of a plant protein: a case of a sweet-tasting protein, monellin

Takashi Konno[a,*], Kazuyoshi Murata[b], Kuniaki Nagayama[b]

[a] *Center for Brain Experiment, National Institute for Physiological Sciences, Okazaki 444-8585, Japan*
[b] *Department of Molecular Physiology, National Institute for Physiological Sciences, Okazaki 444-8585, Japan*

Received 28 May 1999

Abstract We report here a novel case of amyloid-like aggregation of a plant protein. A sweet-tasting protein, monellin, experiences an irreversible heat denaturation at pH 2.5 and 85°C. Addition of 100 mM NaCl couples this process with protein aggregation. The aggregates were structured as regular fibers with ~10 nm width and capable of binding to Congo red, similarly to well-known amyloid fibrils. The amyloid-like aggregation process was also successfully monitored with a calorimetric method. This work supports the universality of the amyloid-like aggregation, not restricted to some special categories of protein.

Key words: Monellin; Heat denaturation; Amyloid; Fibrous aggregation; Transmission electron microscopy; Calorimetry

1. Introduction.

For biophysicists who study conformational changes of protein, protein aggregation has been an artifact disturbing their thermodynamic analyses. However, this situation has largely been changed recently, since it is becoming evident that deposit of aggregates of denatured proteins or peptides to biological tissues is often correlated with pathology of important human diseases [1–3]. Structures of the amyloid-like aggregates of β-amyloid, huntingtin, immunoglobulin light chain, lysozyme, islet amyloid polypeptide, transthyretin, etc. [4–13], and mechanisms of their formation are now one of the most attractive subjects of biochemical and biophysical researches [14–16].

On the other hand, protein aggregation is a general phenomenon observed widely in proteins [14,17], and most of them do not exhibit amyloid-like fibrous aggregation but form an amorphous mass of denatured proteins. For systematic understanding of the mechanisms of amyloid-like aggregation, we should answer the question as to what are the common mechanisms that induce amorphous and fibrous aggregations while what makes a branch to the ordered fibrous aggregation. A further question should also be asked: what is the proper definition of an 'amyloid' fiber to make distinction between amyloid and other fibrous aggregates in a more general sense, which may also include pathologically important cases such as the fibrous aggregates of tau protein [18,19]. As a prerequisite to access to these questions, we should enumerate (ideally all) the possible physical processes that result in fibrous aggregates, among which we should adequately position the pathological cases. Previous investigations have suggested that amyloid fibril is formed via a partially unfolded state of protein [7,14,15], resulting in a cross-β-fiber structure, with β-strands perpendicular and β-sheets parallel to the fiber axis [10,20]. However, insights obtained only from some special cases often lead to misunderstanding or oversimplification. Recently it was found that non-pathogenic proteins SH3 domain [21] and acylphosphatase [22] also form amyloid-like aggregates, but such cases are still very rare. In this context, research to find out fibrous aggregation phenomena in a much wider range of protein species is of importance. This report intends to contribute to this point, and to support a view that amyloid-like fibrous aggregation is a universal phenomenon not restricted to some special categories of protein [21,22].

We are concerned here with a plant protein, monellin, isolated from the fruit of the tropical berry *Dioscoreophyllum cumminsii* [23,24]. It is a sweet-tasting protein composed of two subunits, one being 45 residues (A chain) and the other being 50 residues (B chain) [25]. X-ray and NMR methods have resolved the structure of monellin with four anti-parallel β-sheets and one α-helix [26,27]. The protein is very stable against acidification of solution, but undergoes heat and chemical denaturation rather easily [28–34]. The denaturation processes are often irreversible [28,29], and the tendency suggests that the system may be useful for studying protein aggregation phenomena. Our efforts were focused on searching for fibrous aggregation of this protein formed in the heat-denatured state. The purpose was partly motivated by the β-sheet propensity of monellin found in the native state because the amyloid formation is often related to or formed into β-structures [20]. We have succeeded in obtaining amyloid-like aggregates of monellin as expected, using sodium chloride as an additive. A common requirement for biophysical researches of protein aggregation and amyloid is applicability of many complementary measurements to gain sufficient resolution of the phenomenon. In this report, we employed circular dichroism (CD) spectroscopy, gel chromatography, a dye binding, calorimetry and transmission electron microscopy (TEM). Calorimetry especially was found to be very useful to follow the fibrous aggregation as a function of time and give direct estimates of the heat of the aggregation process.

*Corresponding author. Fax: (81) (564) 52-7913.
E-mail: konno@nips.ac.jp

Abbreviations: CD, circular dichroism; TEM, transmission electron microscopy; NMR, nuclear magnetic resonance; SAXS, solution X-ray scattering

PII: S0014-5793(99)00789-9

2. Materials and methods

2.1. Materials

Monellin was purchased from Sigma (USA) and was purified as described by Fan et al. [33]. Other chemicals were of reagent grade and were purchased from Nacalai Tesque (Kyoto, Japan). All the sample solutions contained 10 mM glycine (pH 2.5), pH being adjusted carefully with small amounts of HCl. The oncentration of monellin was determined using the extinction coefficient $\varepsilon_{277} = 1.46 \times 10^4$ M^{-1} cm^{-1} [34]. The concentration was 3 mg/ml monellin for all the experiments except for absorption measurements of Congo red. Heat treatment of the sample was done by incubating a sample tube in a dry incubator at 85°C. All the heat-treated samples were recovered at 25°C for 24 h.

2.2. CD measurements

CD spectra were measured with a Jasco J-720 WI spectropolarimeter (Jasco, Japan) using quartz cells with a light path of 0.1 or 1 mm. The temperature of the solutions was controlled by a JASCO temperature controller within ±0.1°C.

2.3. Size exclusion chromatography

Size exclusion chromatography was performed using the SMART system with an S-200 column (Pharmacia, Sweden). Equilibrium and elution buffers were composed of 10 mM glycine (pH 2.5) and 100 mM NaCl. The temperature of the column was kept at 25°C and the flow rate of the buffer was 60 μl/min. 15 μl of monellin solution was applied and the chromatogram was monitored by absorption at 280 nm.

2.4. Transmission electron microscopy

Electron micrographs of aggregates of monellin were taken with a JEM-1200EX electron microscope (JEOL, Japan) operated at 100 kV, at a magnification of ×40 000. The heat-treated monellin samples were applied to carbon grids and stained with 2% uranyl acetate. The images were recorded through a 2k×2k slow scan CCD camera (Gatan, USA) and saved on a PC computer.

2.5. Absorption spectra of Congo red

Monellin solutions (3 mg/ml, 10 mM glycine, 100 mM NaCl, pH 2.5) with and without heating were diluted 25 times with a dilution buffer (20 mM Na phosphate, 50 mM NaCl, pH 7.5) containing also Congo red. The final concentration of Congo red was 10 μM. Absorption spectra were taken with a U-3300 spectrophotometer (Hitachi, Japan).

2.6. Calorimetric measurements

The specific heat of monellin solutions was measured with a VP-DSC calorimeter (Microcal, USA) operated in the isothermal mode. 1 ml of sample was applied in the cell. The temperature of the cell was kept at 85°C and the data were collected every 10 s for 3 h.

3. Results and discussion

3.1. Heat denaturation of monellin

Detailed descriptions of the heat denaturation process of monellin in the reversible aspect will be given elsewhere (Konno, in preparation). We briefly summarize here them without showing data. Far- and near-ultraviolet CD spectroscopy have found that monellin at pH 2.5 in water loses its secondary and tertiary structures concurrently and cooperatively in a temperature range of 40–60°C. Addition of NaCl up to 100 mM did not affect the transition curves or the CD spectra in the native and the heat-denatured states, indicating that the salt induces no intermediate of denaturation and does not alter the structure of the heat-denatured state itself. ^{1}H nuclear magnetic resonance (NMR) spectra and solution X-ray scattering (SAXS) profiles of the heat-denatured monellin had patterns typical for highly denatured proteins with a flexible random-chain-like conformation. The folding rate of monellin from the heat-denatured state after the temperature jump depends strongly on the protein concentration, meaning that monellin in the heat-denatured state is dissociated into A and B chains.

3.2. Irreversible heat denaturation and aggregation of monellin

It has been claimed that the wild-type monellin at acidic pH is heat-denatured irreversibly [28]. However, if the heating time is short enough with a high concentration of protein and a long recovery time at ambient temperature, the heat denaturation as monitored by CD, ^{1}H NMR spectra and sweet taste was reversible almost completely. A heating time longer than 1 h was necessary to achieve significant irreversible changes of the CD spectrum in the condition without NaCl used here (pH 2.5, 3 mg/ml protein, heated at 85°C, recovery time 24 h). Fig. 1A shows changes of the CD spectra caused by incubation at pH 2.5 and 85°C for 3 h in 0 and 100 mM NaCl. Both spectra after the heat treatment have intermediate shapes as compared with those of the native (Fig. 1A, bold solid line) and the heat-denatured states (half-dotted line). Our present interest is in these irreversible denaturation processes. At a concentration of 3 mg/ml of monellin, protein gel or large aggregates were not observed even after heating for 3 h. The irreversible changes monitored by $[\theta]$ at 210 nm are shown in Fig. 1B. In the presence of 100 mM NaCl, the $[\theta]$ value started to increase quickly and saturated at around 3 h of heating time while the change in $[\theta]_{210nm}$ of the sample without NaCl came up more slowly. Increasing the heating time to longer than 3 h further altered the CD spectra in both

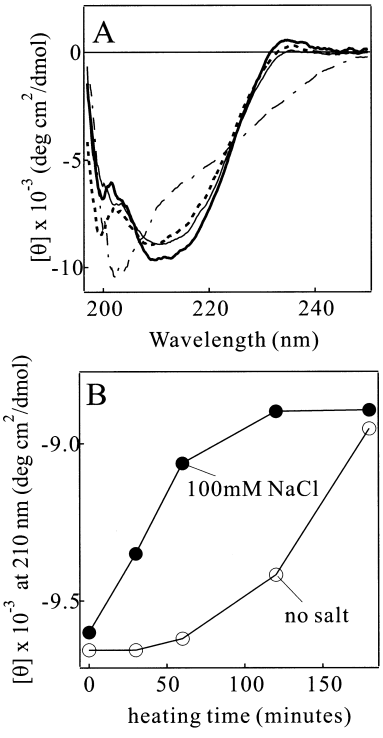

Fig. 1. A: Far-ultraviolet CD spectra of monellin (3 mg/ml) at pH 2.5 and 25°C. Bold solid line: no heating. Dotted line: heating at 85°C for 3 h without NaCl. Thin solid line: heating for 3 h in 100 mM NaCl. Half-dotted line: a spectrum of the heat-denatured state taken at 85°C. B: Changes in $[\theta]$ at 210 nm as a function of the heat incubation time.

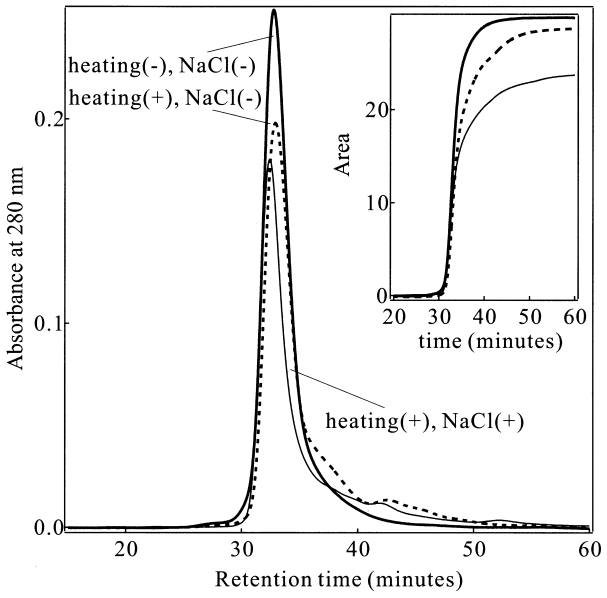

Fig. 2. S-200 size exclusion chromatograms. heating (−) or (+): no heating or heating for 3 h at 85°C. NaCl (−) or (+): 0 or 100 mM NaCl. Inset: Integration of the chromatograms. Each plot in the inset corresponds to that of the same line type in the main panel.

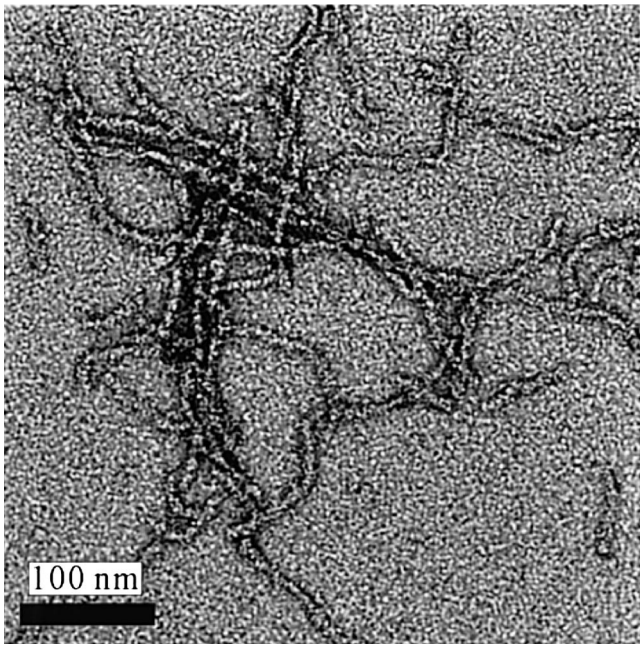

Fig. 3. A negatively stained TEM image of monellin after heating for 3 h at 85°C in 100 mM NaCl.

0 and 100 mM NaCl, although the events in this range are out of interest for the present.

To see whether the irreversible heat denaturation of monellin is coupled with protein aggregation, the samples before and after heating were applied to S-200 gel chromatography. The chromatogram of the sample without heating has a clear peak at a retention time of ~33 min (Fig. 2, bold solid line), and no change was observed even when the sample was in 100 mM NaCl if heating was not applied (data not shown). Using molecular weight standards, we identified that this main peak corresponds to ~10 kDa and is probably to be assigned to an isolated monellin molecule (molecular weight of monellin = 10 700 [24]). When the sample was heated for 3 h at 85°C, the area of the main peak decreased and smaller broad peaks appeared behind the main peak (Fig. 2). The reduction of the area of the main peak was more prominent for the sample with 100 mM NaCl than that without the salt (Fig. 2). The smaller peaks plausibly correspond to the A and B chains of monellin dissociated by denaturation or some hydrolyzed products, and indicate that some parts of the irreversibly denatured population did not form aggregates. Outside the range of 20–60 min, no peak was observed. The inset of Fig. 2 shows profiles that integrate the corresponding chromatograms in the main panel. The dotted line in the inset shows that the total amount of peptide components passing through the gel column was only marginally (~4% of total) reduced by the heat treatment in the absence of NaCl. On the other hand, 20% of the total could not pass through when the sample was heated in the presence of 100 mM NaCl, which indicates that aggregates larger than the pore size of the gel matrix were formed in this condition. It seems that the irreversible heat denaturation of monellin is accompanied by protein aggregation especially in the presence of NaCl. In fact, with a higher protein concentration (e.g. 10 mg/ml), we observed visible aggregates after heating but only in the presence of NaCl.

3.3. Amyloid-like aggregates of monellin

Morphological characterization of aggregates of monellin in 100 mM NaCl was performed by TEM. A negatively stained image of the samples after heating for more than 1 h showed many fibrous components (Fig. 3 for 3 h heating). However, this fibrous aggregate was not seen without adding NaCl or the heat treatment. This means that the fiber was formed by assembling the heat-denatured monellin molecules, and was specifically induced by NaCl. The fiber is solid and uniform with ~10 nm width, and this morphology is similar to that observed for amyloid fibrils [4–13]. We have also measured difference absorption spectra of Congo red in the monellin solution with 0 and 100 mM NaCl after heating for 3 h (the spectrum of the same sample without heating was subtracted). The spectra showed a clear peak at 532 nm especially with 100 mM NaCl (Fig. 4), indicating that the dye binds to the aggregates of monellin similarly to the case of amyloid fibrils [1].

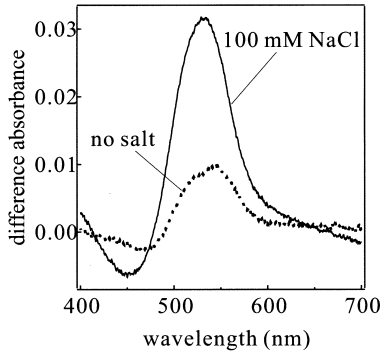

Fig. 4. Difference absorption spectra of Congo red in the monellin solutions with 0 and 100 mM NaCl after heating for 3 h (the spectrum of the same sample without heating was subtracted as a reference).

3.4. Calorimetric analysis of aggregation

Since the protein aggregation should bury a fraction of the hydrophobic surface area of the denatured protein into the protein matrix and then probably reduces the heat capacity of the solution, we expect to observe heat generation along with this process. Measurements of the heat by a calorimetric method will allow us to monitor the fibrous aggregation process quantitatively. In addition, the calorimetric method is the only way to obtain direct estimates of the thermodynamic parameters of the process, whose quantities must play essential roles in a detailed consideration of the mechanisms of protein-protein interaction [35]. However, this technique has not very often been applied to the amyloid formation process, probably because of technical reasons. We could overcome the difficulty rather easily because the aggregation process of monellin was well controlled by heat as well as an additive (NaCl). The rate of the process also falls in a range optimal for the measurements (completed within several hours). Moreover, monellin in the present condition does not form large aggregates or gel, which would disturb the calorimetric measurement very much.

We measured the specific heat necessary to keep the temperature (85°C) of the sample solutions as a function of incubation time. The trace for the sample without protein in Fig. 5 is almost constant and indicates that no reaction generated or absorbed heat. When no protein was added, the trace for the buffer with 100 mM NaCl was almost identical to that without the salt (data not shown). On the other hand, addition of monellin to the sample changed the shape of the trace, although this change was much less for the sample without NaCl (Fig. 5). In the presence of 100 mM NaCl, the trace was clearly inclining and showed that reactions generating heat proceeded as expected (Fig. 5, thin solid line). Since the unfolding rate of monellin at 85°C is fast (half decay of less than a few seconds), no unfolding process contributed to this trace of heat. The requirement of NaCl for the larger heat generation (Fig. 5) indicates that the heat generating reaction is mostly the fibrous aggregation. The inclining trace of the sample with NaCl was saturating at about 3 h of incubation time (Fig. 5, thin solid line). A slight positive slope of the trace for the sample without NaCl (dotted line in Fig. 4) may have originated from a slight amount of aggregation or some hydrolysis reaction.

To estimate the amount of the generated heat specific for the salt-induced aggregation, the data of [protein (+) NaCl (−)] in Fig. 5 was subtracted as a reference from that of [protein (+) NaCl (+)] and the resultant could be fitted well with an exponential function. The area between the two data traces gives estimates for the heat generated by the fibrous aggregation. Assuming that 15–20% of the total protein forms fibrous aggregates (inferred from the inset of Fig. 2) and after a minor correction of baseline, an estimate of the generated heat was 2.6–3.6 mcal/mg protein. This value is very close to that reported for amyloid formation of β25–35 peptide (∼2.8 mcal/mg peptide [36]). A decay time constant of the trace of heat, which is equivalent to the time constant of the fibrous aggregation process, was 47 min in the present condition. This time course followed by calorimetry is parallel to that of the change in CD spectra (filled symbols in Fig. 1B).

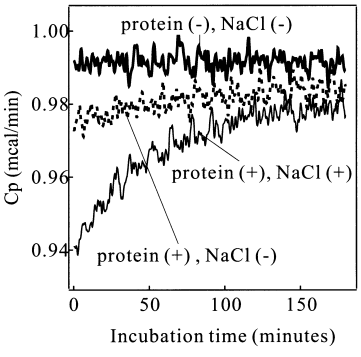

Fig. 5. Specific heat to keep the temperature of the sample at 85°C. protein (−) or (+): 0 or 3 mg/ml monellin added. NaCl (−) or (+): 0 or 100 mM NaCl.

3.5. Mechanism of amyloid-like aggregation of monellin

The fibrous aggregates of monellin presented here are amyloid-like with respect to the solid fibrous shape with ∼10 nm width, ability to bind to Congo red and heat generation equivalent to the case of β25–35 peptide. As far as we know, this is the first report of amyloid-like fibrous aggregation of a plant protein, and strongly supports the view that amyloid-like aggregation is a universal phenomenon observed in a very wide range of protein species [21,22], although the term 'amyloid' has not been well defined so far. The fiber formation of the present case could be controlled with NaCl. Considering the effective concentration range of NaCl (≤100 mM) at low pH, the most plausible mechanism of the salt-induced process is neutralization of positively charged residues on the protein chain. Reduction of electrostatic repulsive force among the denatured molecules accelerates to form assembly of the molecules. Molecular mechanisms that force the aggregate to be well-ordered into regular fibers are not known at present. It has often been argued that amyloid fibril is formed via a partially unfolded state of protein [7,15], but we did not observe any NaCl-induced accumulation of the intermediately denatured states (studied by CD, NMR and SAXS; data not shown). Differential roles of dissociated A and B chains of monellin in forming the fibers have not been clarified yet, either. Further studies should be targeted to the structure of the aggregates of monellin. A good aspect of the present case is that various physical techniques can be applied, among which the calorimetric method is very useful to monitor kinetics of the aggregation sensitively and quantitatively. We expect that understanding of amyloid-like aggregation will proceed by comparing the present case with the other cases.

Acknowledgements: We thank Y. Hirohata, K. Fujiyama and J. Nakamura (SiberHegner, Japan) for the calorimetric measurements. T.K. also thanks Dr. N. Tanaka and Dr. Y. Kamatari for their helpful discussions and suggestions throughout this work.

References

[1] Sipe, J.D. (1992) Annu. Rev. Biochem. 61, 947–975.
[2] Tan, S.Y. and Pepys, M.B. (1994) Histopathology 25, 403–414.
[3] Prusiner, S.B., Scott, M.R., DeArmond, S.J. and Cohen, F.E. (1998) Cell 93, 337–348.
[4] Hilbich, C., Kisters-Woike, B., Reed, J., Masters, C.L. and Beyreuther, K. (1991) J. Mol. Biol. 218, 149–163.
[5] Barrow, C.J., Yasuda, A., Kenny, T.M. and Zagorski, M.G. (1992) J. Mol. Biol. 225, 1075–1093.
[6] Terzi, E., Hoelzemann, G. and Seelig, J. (1995) J. Mol. Biol. 252, 633–642.

[7] Wetzel, R. (1996) Cell 86, 699–702.
[8] Lai, Z., Colon, W. and Kelly, J.W. (1996) Biochemistry 35, 6470–6482.
[9] Blake, C. and Serpell, L. (1996) Structure 4, 989–998.
[10] Sunde, M., Serpell, L.C., Bartlam, M., Fraser, P.E., Pepys, M.B. and Blake, C.C. (1997) J. Mol. Biol. 273, 729–739.
[11] Helms, L.R. and Wetzel, R. (1996) J. Mol. Biol. 257, 77–86.
[12] Booth, D.R., Sunde, M., Bellotti, V., Robinson, C.V., Hutchinson, W.L., Fraser, P.E., Hawkins, P.N., Dobson, C.M., Radford, S.E., Blake, C.C. and Pepys, M.B. (1997) Nature 385, 787–793.
[13] Kayed, R., Bernhagen, J., Greenfield, N., Sweimeh, K., Brunner, H., Voelter, W. and Kapuriniotu, A. (1999) J. Mol. Biol. 287, 781–796.
[14] Jaenick, R. and Seckler, R. (1997) Adv. Protein Chem. 50, 1–59.
[15] Kelly, J.W. (1998) Curr. Opin. Struct. Biol. 8, 101–106.
[16] Kelly, J.W. (1997) Structure 5, 595–600.
[17] Fink, A. (1995) Folding Design 3, R3–23.
[18] Grundke, I.I., Iqbal, K., Tung, Y.C., Quinlan, M., Wisniewski, H.M. and Binder, L.L. (1986) Proc. Natl. Acad. Sci. USA 83, 4913–4917.
[19] Arrasate, M., Perez, M., Armas-Portela, A. and Avila, J. (1999) FEBS Lett. 446, 199–202.
[20] Sunde, M. and Blake, C. (1997) Adv. Protein Chem. 50, 123–159.
[21] Guijarro, J.I., Sunde, M., Jones, J.A., Campbell, I. and Dobson, C.M. (1998) Proc. Natl. Acad. Sci. USA 95, 4224–4228.
[22] Chiti, F., Webster, P., Taddei, N., Clark, A., Stefani, M., Ramponi, G. and Dobson, C.M. (1999) Proc. Natl. Acad. Sci. USA 96, 3590–3594.
[23] Morris, J.A. and Cagan, R.H. (1972) Biochim. Biophys. Acta 261, 114–122.
[24] Morris, J.A. and Cagan, R.H. (1973) J. Biol. Chem. 248, 534–539.
[25] Kohmura, M., Nio, N. and Ariyoshi, Y. (1990) Agric. Biol. Chem. 54, 2219–2224.
[26] Somoza, J.R., Jiang, F., Tong, L., Kang, C.-H., Cho, J.M. and Kim, S.-H. (1993) J. Mol. Biol. 234, 390–404.
[27] Tomic, M.T., Somoza, J.R., Wemmer, D.E., Park, Y.W., Cho, J.M. and Kim, S.H. (1992) J Biomol. NMR 2, 557–572.
[28] Kim, S.-H., Kang, C.-H., Kim, R., Cho, J.M., Lee, Y.-B. and Lee, T.K. (1989) Protein Eng. 2, 571–575.
[29] Morris, J.A. and Cagan, R.H. (1975) Proc. Soc. Exp. Biol. Med. 150, 265–270.
[30] Jirgensons, B. (1976) Biochim. Biophys. Acta 446, 255–261.
[31] Brand, J. and Cagan, R.H. (1977) Biochim. Biophys. Acta 493, 178–187.
[32] Brand, J.G., Cagan, R.H. and Bayley, D.L. (1985) Proc. Soc. Exp. Biol. Med. 179, 76–82.
[33] Fan, P., Bracken, C. and Baum, J. (1993) Biochemistry 32, 1573–1582.
[34] Morris, J.A. and Cagan, R.H. (1980) Proc. Soc. Exp. Biol. Med. 164, 351–354.
[35] Weber, G. (1992) Protein Interactions, Chapman and Hall, New York.
[36] Terzi, E., Hoelzemann, G. and Seelig, J. (1994) Biochemistry 33, 1345–1350.