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Complement assembly of two fragments of the streptococcal protein G B1 domain in aqueous solution

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Abstract **We examined the complementation of various pairs of fragments derived from the streptococcal protein G B1 domain by NMR and CD. Most were not associated; however, one pair of fragments (1–40) and (41–56) interacted sufficiently enough to regenerate a stable 1:1 complex, $K_d = 9 \times 10^{-6}$ M. A 2D-NMR analysis showed that the structure of the complex resembled that of native domain. Here we discuss the complementation from the viewpoint of the folding pathway of the protein.**

Key words: Protein G; Protein folding; Complementation; Nuclear magnetic resonance; Circular dichroism

1. Introduction

The mechanism of protein folding remains a challenge for biochemists. Understanding this mechanism would help to predict three-dimensional structures of proteins from their amino acid sequence or to design and synthesize proteins with desired functions. Protein folding has been studied by several investigators, and their significant efforts have made some generalized aspects [1–5]. These investigators consider that proteins fold by means of a specific pathway with defined intermediates rather than according to a random search for conformational space. More recent studies of protein folding have primarily focused upon the structure of intermediates along the folding pathway. The conformation of the some protein fragments as models of intermediates in the early stage of folding pathway have been investigated: ribonuclease A [6], bovine pancreatic trypsin inhibitor [7], cytochrome *c* [8], myoglobin [9] and tendamistat [10]. The reported intermediates commonly possess undefinable structures in aqueous solution under physiological conditions which are stabilized into defined secondary structures at low temperatures and/or in low dielectric solvents such as trifluoroethanol or dimethylsulfoxide. It is also considered that the early folding intermediate proceeds to assembly with another segment to form a tertiary structure, which was confirmed by the study of the complementation of peptide fragments: barnase [11–13], adenylate kinase [14] *trp* repressor [15], cytochrome *c* [16] and chymotrypsin inhibitor-2 [17].

Protein G is a immunoglobulin G binding protein located on the cell wall of group G streptococci containing the immunoglobulin G binding domains, B1, B2 and B3 that are nearly identical [18–20]. Although these domains comprise only 56 amino acid residues without disulfide bridges, they have high heat denaturation points. The thermodynamic features of the protein G B1 domain have been shown by Alexander et al. [21,22]. The domain is extraordinarily heat stable (denaturation temperature 87.5°C). The NMR structure in water of the Protein G B1 domain determined by Gronenborn et al. [23] possesses a monomeric and unique folding topology, which was also confirmed by X-ray crystallography by Gallagher et al. [24] (Fig. 1). Since this domain is one of the smallest proteins lacking a disulfide bridge that cooperatively folds like the other globular proteins, it seemed to be a suitable model for investigating the folding and unfolding pathway of proteins. Furthermore, this domain may be useful as a the target of theoretical analysis using a more powerful computing system in the future.

Here, we demonstrated the complementation of various fragments in the protein G B1 domain (PGB1), which were examined by circular dichroism (CD) and proton nuclear magnetic resonance (^{1}H NMR) in an aqueous solution.

2. Experimental

2.1. Sample preparation

PGB1 and its fragments shown in Fig. 2 were synthesized by Fmoc strategy on a HMP resin support using the Fast Moc method on an ABI 430A solid phase peptide synthesizer combined with manual synthesis. The synthetic peptides were purified by preparative reverse phase HPLC and their homogeneity was confirmed by analytical reverse phase HPLC. The amino acid components were analyzed using a Hitachi L-8500 amino acid analyzer, the sequences with a Shimadzu PPSQ-10 amino acid sequencer and the molecular mass with a JEOL JMS-HX110HF double-focusing mass spectrometer. The sample concentration of PGB1 and its fragments was determined using the molar extinction coefficients for tryptophan and tyrosine in model compounds [26].

2.2. Circular dichroism

Circular dichroism (CD) spectra were measured from 260 to 195 nm using a Jasco J-600 spectropolarimeter controlled with a NEC PC-9801VM computer, which was calibrated with ammonium *d*-10-camphor sulfonate [27]. The temperature of the samples was manipulated using a Haake F-3 temperature control unit. Cells with a path length of 0.02, 0.1, 1 and 2 cm were used depending on sample concentration. The spectra were obtained as the average of 4 scans, and are represented as molar ellipticity [q]. Lyophilized powdered peptides were dissolved in 50 mM phosphate buffer at pH 5.5, and the pH values were adjusted with 1 N HCl or NaOH. Unspecified solvent dichroic ellipticity was subtracted from the spectra by computer manipulation.

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Abbreviations: CD; circular dichroism; $d_{\alpha N}(i,j)$, intramolecular distance between the protons C$^\alpha$H and NH on the residues i and j; DQF-COSY, two-dimensional double quantum-filtered coherence transfer spectroscopy; HOHAHA, two-dimensional homo-nuclear Hartmann-Hahn spectroscopy; K_d, dissociation constant; NOESY, two-dimensional nuclear Overhauser and exchange spectroscopy; PGB1, protein G B1 domain; ROESY, two-dimensional rotating frame nuclear Overhauser and exchange spectroscopy; [x+y], equimolar mixture of fragments x and y; NMR, nuclear magnetic resonance.

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2.3. NMR spectroscopy

We examined complementation between all pairs of fragments in samples were prepared by dissolving 500 nmol of lyophilized peptides in 0.5 ml of 99.996% D_2O, containing 50 mM phosphate buffer at pH 7.0. For 2D-NMR measurement, an equimolar mixture of N40 and C16 [N40+C16] (3.6 mM) was dissolved in 0.5 ml of 99.996% D_2O or 90% H_2O/10% D_2O containing 50 mM phosphate buffer at pH 5.5. NMR experiments were performed using Bruker AM500, AMX500 or AMX600 spectrometers at 298 K with ^1H resonance frequencies of 500.13 and 600.13 MHz, respectively. The reference for all spectra was sodium 3-trimethylsilylpropionate-d_4 at zero ppm. All 2D spectra were recorded in the pure absorption mode according to the time-proportional phase incrementation method (Redfield and Kuntz, 1975): (1) DQF-COSY [28], (2) HOHAHA [29] with DIPSI-2 sequence [30] for spin-locking periods of 45 and 87 ms, (3) ROESY [31], and (4) NOESY [32]. NOESY and ROESY spectra were recorded at mixing times of 100–400 and 75–300 ms, respectively. Spin-locking fields for the HOHAHA and ROESY experiments were set at 9 and 2 kHz, respectively. Solvent suppression for NOESY in 90% H_2O/10% D_2O was achieved using the 'jump-and-return' sequence ($90°y$, τ, $90°-y$) [33], with the final single observation pulse being replaced, for HOHAHA using a three-pulse sequence composed of a flip-back $90°-y$, τ', jump-and-return sequence. After the DIPSI-2 spin-locking sequence, τ' was the same value for the spin-locking time.

3. Results

Fig. 3 shows the aliphatic and α proton regions of the 1D spectra of 0.5 mM fragments and mixtures derived from PGB1 domain. Only fragments N40 and C16 greatly changed in the overall spectral region when the fragments were mixed in D_2O. That is, up-fielded signals (up to −1.5 ppm) appeared in the aliphatic region and significant changes were observed in the aromatic and α proton region of the spectrum. The changed spectral pattern of [N40+C16] resembled that of the native protein, PGB1. The other pairs of fragments [N10+C46], [N20+C36], [N30+C26], [N20+C16], [N30+C16], [H20+C16] and [N20+H20] did not show any spectral change and they are considered to be a simple summation of the spectra generated by the original fragments.

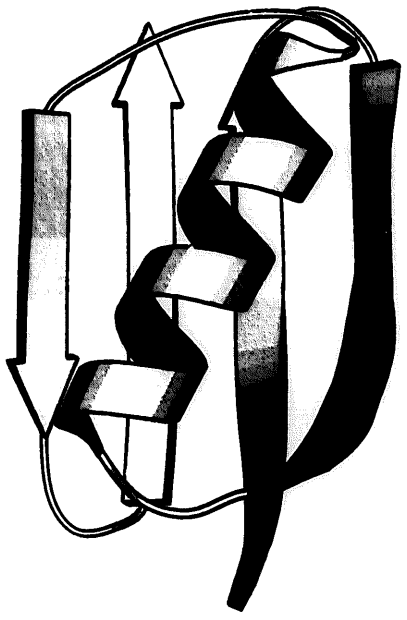

Fig. 1. Ribbon diagram of the protein G B1 domain, produced by the program MOLSCRIPT [25] with coordinate information determined by Gallagher et al. [24] (Brookhaven Protein Data Bank accession number 1PGA).

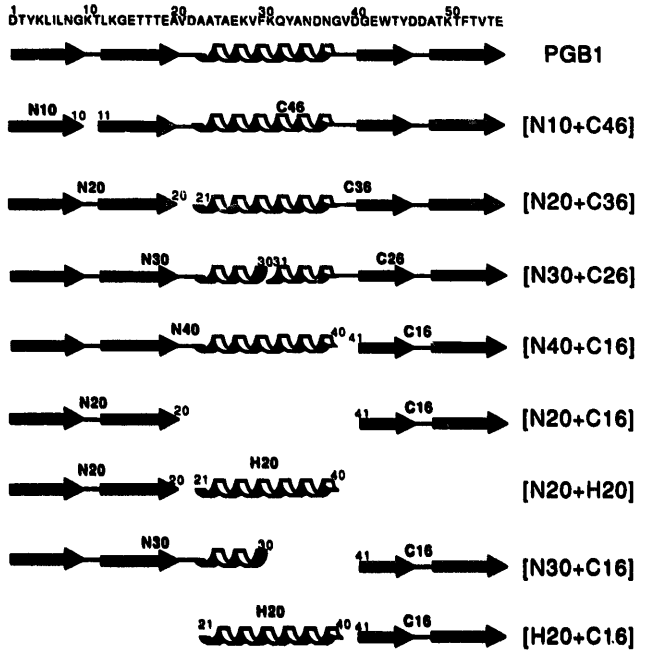

Fig. 2. Schematic representation of PGB1 and the pairs of fragments studied. The amino acid sequence of PGB1 is shown above the illustration of PGB1. The secondary structure is represented by the ribbon model as shown in Fig. 1.

To investigate the conformational features of the mixture, we measured the far-UV CD spectra of [N40+C16], PBG1, N40 and C16 (Fig. 4). The CD spectrum of [N40+C16] significantly changed. It was analogous to that of native PGB1 and far from the summation of the N40 and C16 spectra. The two fragments were bound in an equimolar manner and the dissociation constant was evaluated by the concentration dependence of the CD spectrum at 222 nm ($K_d = 9.0 \times 10^{-6}$ M).

The conformational features of [N40+C16] were further analyzed by 2D NMR and compared with that of PGB1. The resonances of the mixture and PGB1 in 90% H_2O/10% D_2O were assigned by the conventional methodology of 2D NMR [34]. Namely, the types of amino acid residues were identified via through-bond connectivities, by DQF-COSY and HOHAHA, followed by sequential assignment using $d_{\alpha N}(i,i+1)$, $d_{\beta N}(i,i+1)$ and $d_{NN}(i,i+1)$ in the NOESY experiments. For example, the $d_{\alpha N}(i,i+1)$ for the segment Gly^{41}-Glu^{56} are shown in Fig. 5. In the NOESY spectra of [N40+C16], several sets of proton resonances exhibited positive cross peaks which were due to neither NOE nor scalar magnetization transfer. These cross peaks also appeared in the ROESY spectrum as positive peaks and their intensity depended upon the mixing ratio of fragments N40 to C16, indicating that the N40 and C16 fragments inter-converted slowly on a NMR time scale between the free and bound states. Therefore, all proton resonances of [N40+C16] and PGB1 were completely assigned and that of free N40 and C16 were also assigned by the positive peaks that arose in the ROESY spectra.

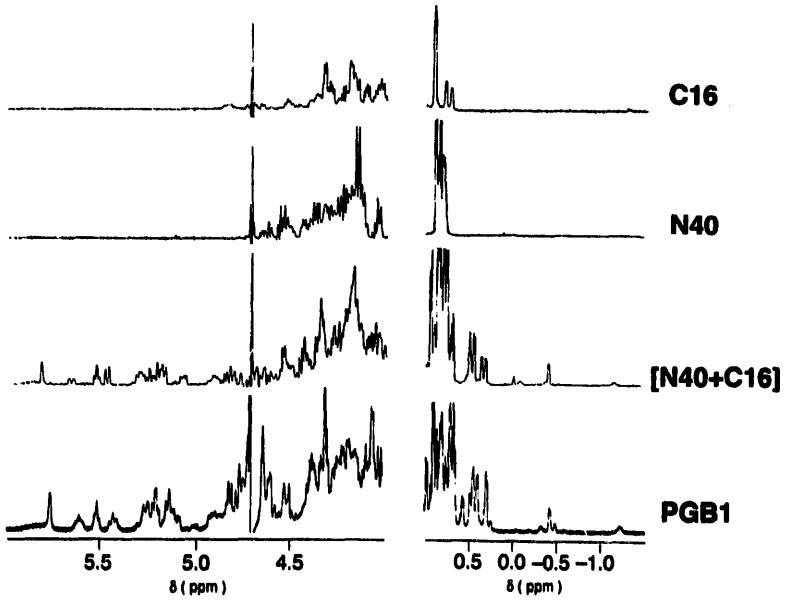

Fig. 3. Amide and aliphatic region of 1D ^{1}H NMR of N40 (top), C16 (second from top), [N40+C16] (second from bottom) and PGB1 (bottom). The respective sample concentrations were 0.5, 0.5, 3.6 and 2.7 mM. All spectra were measured at 298 K in 99.996% D_2O, 50 mM phosphate buffer, pH 5.5.

4. Discussion

We examined reassociating fragments which were split at the boundary between the secondary structures in PGB1, using seven pairs of synthetic fragments. The spectral changes in 1D NMR can be easily monitored as largely up-field, down-field or broadened signals when a pair of equimolar fragments is associated at a high concentration (above 0.5 mM). The signals in the aliphatic region of [N40+C16], assigned to Val^{54} CγH$_3$ (−0.41 ppm), Val^{54} CβH (−0.12 ppm) and Leu^5 CβH (−1.15 ppm) by 2D-NMR, were up-fielded probably due to the effect of the current created by the aromatic rings of Trp^{43} or Phe^{30}, indicating that these protons are involved in a hydrophobic core formed in complexed N40 and C16. The signals of Val^{54} CγH$_3$, Val^{54} CβH and Leu^5 CβH in native PGB1 were similarly shifted up-field to −0.42, −0.38 and −1.28 ppm. Aliphatic signals in the region of −1.8~0.5 ppm did not appear in free N40 and C16. Down-field signals in the α proton region, which indicates the formation of ordered β-sheet, are also important for monitoring conformational changes. Though the down-field shift of alpha proton signals in both free N40 and C16 was not observed between 5.0~6.0 ppm, many signals appeared in the region when the fragments were mixed (Fig. 3). Since there was no concentration dependence by all free fragments within an extensive range of concentration (30 μM~3 mM), the changes can be ascribed to the heterogeneous association of N40 and C16. On the other hand, there were no significant changes in 1D NMR spectra in the other mixtures, [N10+C46], [N20+C36], [N30+C26], [N20+C16], [N20+H20] and [H20+C16].

Free N40 and C16 may be regarded as highly disordered structure from the CD spectral patterns of the peptides. However, NMR indicated otherwise for C16 (discussed later). The spectral features of [N40+C16] were analogous to those of PGB1, suggesting that PGB1 was regenerated in [N40+C16].

The fingerprint α-amide region of the 2D NMR spectrum is quite sensitive to structural changes in proteins. The patterns of sequential assignment also depend on the structure of proteins. Those of PGB1 were nearly identical with that of [N40+C16] (partially represented in Fig. 5). The difference in the chemical shifts between PGB1 and [N40+C16] were en-

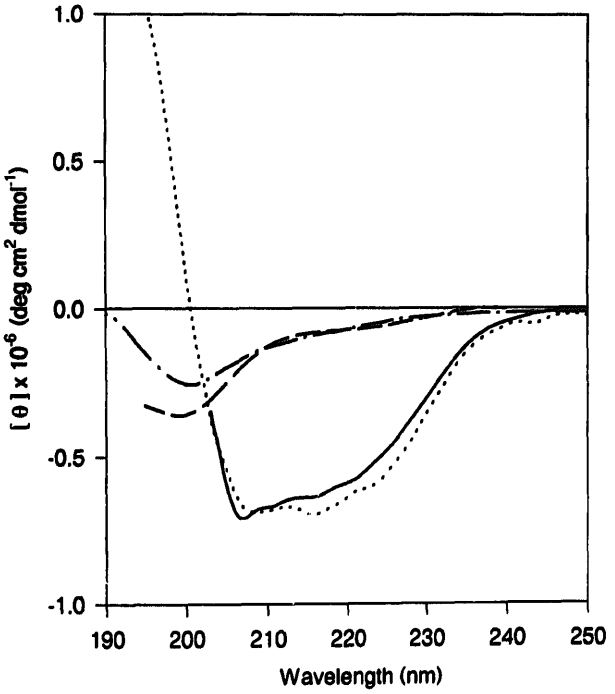

Fig. 4. CD spectra of N40 (– – – –), C16 (–·–·–), [N40+C16] (——) and PGB1 (·····). The respective sample concentrations were 50, 50, 502 and 136 μM. The condition of measurement except for the sample concentration were the same as those described in Fig. 2.

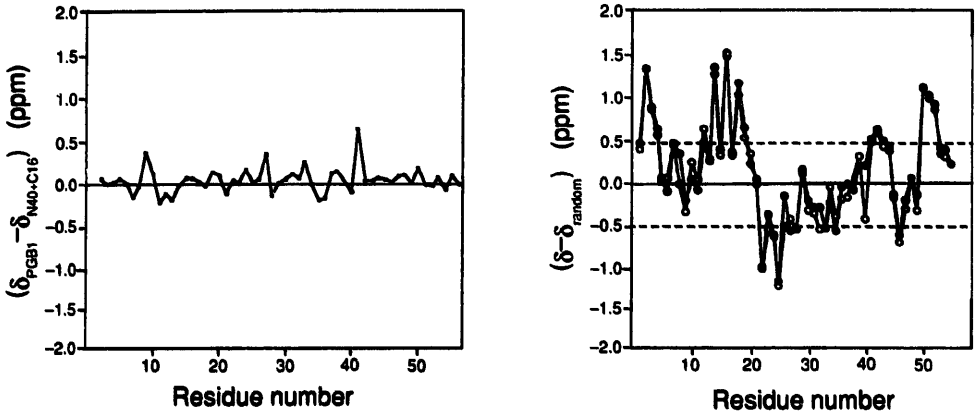

Fig. 5. Amide-α regions of the NOESY spectra of PGB1 (top) and [N40+C16] (bottom) measured at a mixing time of 150 ms, 298 K in 90% H_2O/10% D_2O, 50 mM phosphate buffer, pH 5.5. The sample concentrations are same as those described in the legend to Fig. 3. The sequential connectivities are partially indicated by solid lines labeled with the residue number of the amide proton resonance.

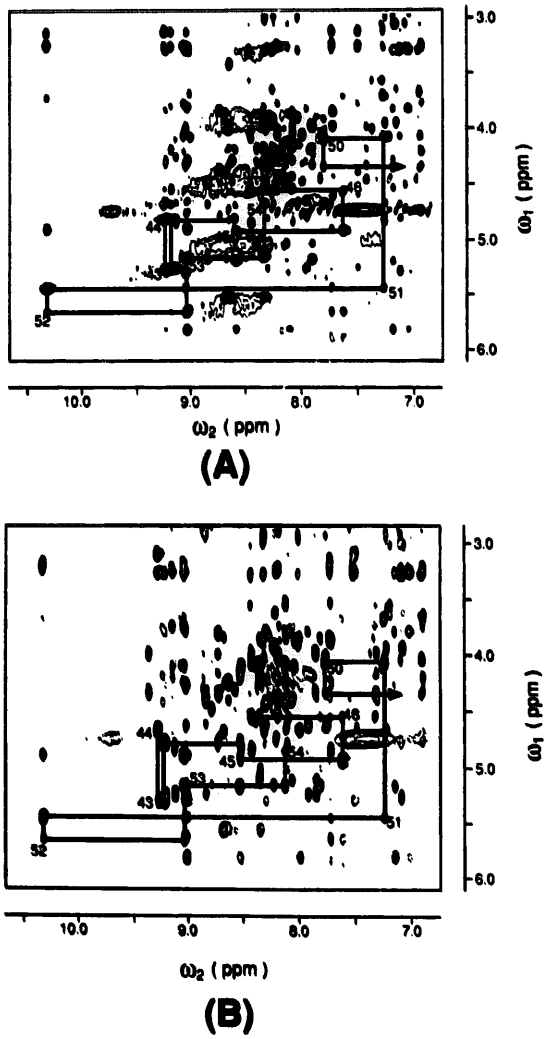

Fig. 6. Magnitude of changes in chemical shift of α proton resonances in the spectrum of PGB1 relative to [N40+C16] (left), and conformation-dependent chemical shifts of alpha protons of PGB1 (●) and [N40+C16] (○) determined by 2D-NMR (right) under the same conditions described in the legend to Fig. 3.

tirely within ± 0.5 ppm (Fig. 6), showing that the structure of complex of the fragments N40 and C16 is similar to that of native PGB1, and also suggesting the formation of a 1:1 complex in aqueous solution. This equivalent binding was also confirmed by the proton resonance of free N40 or C16 in the mixed system, which were assigned as positive peaks in the ROESY spectra. The magnitude of these peaks was minimal at the mixing ratio of 1:1. Generally, conformational shifts of alpha proton signals provide information about the secondary structure. The deviation of chemical shifts of α proton signals with respect to the random reference values [35], shown in Fig. 6, indicated that [N40+C16] possess the same pattern of defined secondary structures as PGB1.

We concluded that only one pair of fragments, (1–40) and (41–56), associates to form the native structure of PGB1. We showed by NMR that the synthetic fragments N20, N30 and N40, seem to assume a highly disordered structures in water, and that C16, C36 and C46 which contain the C16 sequence, have a non-random coil structure [36]. These results seem to conflict with these of the CD spectra in this study. However, we consider that C16 does not have a complete random structure but rather a nascent ordered structure without the features of a secondary structure in the CD spectra. We also suppose that C16 initiates the folding of PGB1. Now we are promoting the study of complementation using N40 and C16 analogs and also investigating the fine structure, structural energetics, and folding kinetics of regenerated PGB1, which will be helpful in understanding the folding pathway of PGB1.

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