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Core mutants of the immunoglobulin binding domain of streptococcal protein G: stability and structural integrity

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Abstract A library of core mutants of the GB1 domain of streptococcal protein G was created, and the structure and stability of selected members was assessed by ^{1}H-^{15}N heteronuclear correlation NMR spectroscopy and fluorescence. All mutants comprised changes in β-sheet residues, with sidechains at positions 5 (Leu), 7 (Leu), 52 (Phe) and 54 (Val) forming the β-sheet side of the sheet-helix core interface. A solvent exposed position Ile-6 was chosen as a control. Randomization of bases at codon positions 1 and 3 with thymine at position 2 introduces five possible hydrophobic amino acids, namely Leu, Val, Ile, Phe, and Met. The distribution of encoded amino acids at all five positions is approximately as expected theoretically and indicates that no major bias was introduced towards particular residues. The overall structural integrity of several mutants, as assessed by NMR, ranges from very close to wild type to fully unfolded. Interestingly, the stability of the mutants is not strictly correlated with the number of changes or residue volume.

Key words: Protein G; B1 domain; Core mutant; Library; Protein design; Structure; Stability

1. Introduction

The most important factors governing the structure and stability of proteins are encoded in those amino acids which form the hydrophobic core. It is generally believed that a particular core is responsible for the class of chain fold and that the packing of residues within the core is crucial for the protein's thermodynamic stability [1]. A considerable amount of work has therefore been focused on investigating the various contributions of individual residues within protein cores on the structure and/or stability of particular proteins and the degree to which proteins are able to tolerate changes within their cores. An extensive body of mutational studies shows that proteins are remarkably tolerant to single site substitutions and it seems rare that a single site (and even up to three site) change completely obliterates the basic fold of a protein [2–7]. This could be interpreted to imply that the precise packing arrangement of side chains within protein cores is not of crucial importance. On the other hand, attempts at 'a priori' protein design seem to suggest that it is fairly easy to design proteins which have an approximately correct fold, but extremely hard to design a stable uniquely folded protein, indicating that correct sidechain packing is an essential component for native proteins [8]. This apparent contradiction seems puzzling and requires further investigation. It may well reflect the current limits of the two alternative experimental approaches. For instance, proteins recovered from random sequence libraries usually contain only a small number of different amino acids, thereby severely limiting all available possibilities that nature exploits by using permutations of all 20 amino acids [9–11]. Mutational studies generally look at single site or at most three site mutations in cores [12–14]. Given the fact that most protein cores consist of 10 or more residues, each subset will reflect only some of the possibilities. It therefore seems worthwhile to investigate the core of a small protein with the aim of examining as many core permutations as possible.

The B1 immunoglobulin binding domain of streptococcal protein G (GB1) can be described as a prototypic polypeptide for the purposes of studying protein folding and stability [15,16]. In particular, it is a small 56 residue domain which is highly thermostable and contains no disulfide bridges, prolines or prosthetic groups that could influence its structural organization and stability. Its native structure has been determined at high resolution by NMR [17] and crystallography [18,19], and the backbone dynamics of the folded state have been analyzed [20]. In addition, a structural and dynamic characterization of the urea denatured state has been carried out [21].

Due to its favorable properties (small size, high stability, high solubility, high resolution structure), GB1 has been employed as a model system for an increasing number of studies concerning basic questions in protein chemistry, such as β-sheet propensities of amino acids [22–25], mutagenesis via phage display [26], and as a scaffold for the design of a metal binding site [27]. Peptide fragments of GB1 have also been studied in isolation in water and trifluoroethanol, and it has been shown that a peptide comprising the β3-β4 turn exhibits a significant population of native conformer in water [28,29].

In the folded state, the GB1 domain is highly compact with 95% of the residues participating in regular secondary structure. The GB1 domain comprises a four stranded β-sheet arranged in a −1, +3x, −1 topology on top of which lies an α-helix (Fig. 1). The central part of the hydrophobic core of GB1 comprises residues Leu-5, Leu-7, Ala-26, Phe-30, Ala-34, Val-52 and Val-54 with residues Tyr-3, Gly-9, Tyr-33, and Val-39 at the boundaries.

In the present study, we have set out to generate a large number of mutants of GB1 comprising five sites, four of which are amino acids involved in the formation of the hydrophobic core. The core residues chosen are Leu-5, Leu-7, Phe-

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Abbreviations: GB1, the B1 immunoglobulin binding domain of streptococcal protein G; HSQC, heteronuclear single quantum coherence; NMR, nuclear magnetic resonance; GuHCl, guanidinium chloride

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52, and Val-54, all of which point upwards from the central two strands of the sheet and interact with the α-helix lying on top of the sheet (Fig. 1). The fifth position is Ile-6, which is positioned on the solvent exposed underside of the β-sheet and for which it is known that substitutions are tolerated, either in natural protein G domain variants (I6V in GB2) or by mutagenesis (I6A, I6G [24]).

2. Materials and methods

2.1. Mutagenesis

GB1 mutants at positions 5, 6, 7, 52 and 54 were generated by oligonucleotide directed methods using PCR amplification. Plasmid DNA for a synthetic GB1 variant (T2Q, I6G; the strain was kindly supplied by C.K. Smith and L. Regan) was used as template in PCR reactions using synthetic oligonucleotides comprising the 5′ and 3′ end of the gene in which the first and third nucleotide in the codon for amino acids 5, 6, 7, 52 and 54 was randomized in the synthesis and the second nucleotide was T only. In this manner, the following five hydrophobic amino acids Leu, Val, Ile, Phe and Met were encoded at these positions. Large quantities of PCR product were synthesized, digested with *Nde*I and *Bam*HI, purified and ligated into *Nde*I and *Bam*HI cut pET11a. Electrocompetent HMS174(DE3) cells were transformed with ~200 ng DNA. More than 5000 transformants were obtained, several hundred of which were picked and kept for further analysis.

2.2. Screening of mutant candidates

Initial screening was performed on miniprep DNA from individual candidate colonies. The presence of a single copy of the GB1 gene was assessed by digestion with *Hin*dIII and *Xba*I. 53 final candidates were subjected to automated DNA sequencing (Biotech Research Laboratories, Rockville, MD). Screening for protein expression was carried out in 2 ml cultures. After induction by 1 mM IPTG, cells were grown for 2.5 h, harvested by centrifugation, resuspended in Laemmli buffer, and aliquots run on 16% Tricine/SDS gels (Novex).

2.3. Sample preparation

GB1 (56 amino acids) was expressed and isolated as described previously [30]. Uniformly (>95%) ^{15}N labeled GB1 domain was prepared by growing the bacteria on minimal medium using $^{15}NH_4Cl$ as the sole nitrogen source. The samples for NMR contained approximately 0.5–1 mM protein, 50 mM sodium phosphate, pH 5.4. NMR spectra were run on crude extract samples.

2.4. NMR spectroscopy

^{1}H-^{15}N HSQC spectra [31,32] were recorded at 25°C on a Bruker AMX600 spectrometer equipped with a triple resonance z-shielded gradient probe. The spectra were recorded with $128^{*} \times 512^{*}$ complex points in the indirect (^{15}N) and acquisition (^{1}H) dimensions, respectively, and with total acquisition times of 64 ms in both dimensions.

2.5. Equilibrium unfolding

Samples for fluorescence contained heat released protein [17] which is ~95% pure as judged by SDS-PAGE. Tryptophan fluorescence was monitored at 25°C as a function of guanidinium chloride concentration. Excitation was at 295 nm and emission was integrated from 305 to 450 nm. Unfolding curves were analyzed by non-linear least squares according to a two state model [33].

3. Results and discussion

We chose positions for mutagenesis which are crucial for the formation of the hydrophobic core of GB1. Sidechains at positions 5 (Leu), 7 (Leu), 52 (Phe) and 54 (Val) have no solvent accessible surface area in the structure of GB1 [17] and form the β-sheet side of the sheet-helix core interface. The corresponding interacting sidechains on the α-helix are Ala-26, Phe-30 and Ala-34. A depiction of the detailed spacial arrangement of these residues within the GB1 structure is provided in Fig. 1. Position 6 (Ile) was chosen as a control, since it is known that several amino acid substitutions are tolerated at this position and it was hoped that any potential bias in the mutagenesis approach would be detected at this position. The template DNA encoded Leu at position 5, Gly at position 6, Leu at position 7, Phe at position 52 and Val at position 54. Since equal amounts of all four bases were used in the first and third codon positions, keeping T as the central one, 16 possible codons were introduced, coding for Leu, Val, Ile, Phe, and Met in a 6:4:3:2:1 ratio.

In our initial screen of miniprep DNAs, less than 5% of transformants contained either no insert or a wrong length insert. Candidates of that nature were omitted from further analysis. In total 53 mutant candidates were subjected to DNA sequencing. Four of those contained the template DNA sequence. The distribution of encoded amino acids at all five positions is summarized in Table 1. Comparison of the overall numbers indicates that for Leu and Phe the theoretically predicted number of codons was found by DNA sequencing. In the case of Val, more than the expected value was observed (80 experimental, 58 predicted), whereas for Ile and Met, slightly fewer than the expected numbers were observed (Ile: 30 observed, 43 predicted; Met: 7 observed, 14 predicted). In general, the differences between expected and observed codons are relatively small and indicate that no major bias in the mutagenesis procedure was introduced. Examining the different positions that were mutagenized, it appears that minor preferences for or against certain amino acids may be present. For example, Ile seems to be found less frequently than expected at position 5, accompanied by an concomitant increase in Leu. The small numbers, however, preclude any firm statements about avoidance or preference of particular amino acid codons at any of the five positions.

Eight clones exhibited a one base deletion at various positions, resulting in the introduction of a stop codon either at amino acid position 12 (five clones) or in other parts of the coding region. Three further clones contained other aberrations: one exhibited additional mutations in the ATG start and the second codon, the other two contained deletions in the T7 promoter. Subtracting aberrant as well as several duplicate protein sequences, we were finally left with 35 unique

Table 1
Distribution of amino acids at the various positions observed in the mutant library of GB1[a]

	Leu-5	Ile-6	Leu-7	Phe-52	Val-54	Σ
Leu	23 (17.2)	12 (17.2)	18 (17.2)	16 (17.2)	21 (17.2)	90 (86)
Val	14 (11.6)	20 (11.6)	15 (11.6)	17 (11.6)	14 (11.6)	80 (58)
Ile	2 (8.6)	7 (8.6)	6 (8.6)	6 (8.6)	8 (8.6)	29 (43)
Phe	4 (5.8)	4 (5.8)	6 (5.8)	8 (5.8)	2 (5.8)	24 (29)
Met	3 (2.8)	2 (2.8)	0 (2.8)	0 (2.8)	2 (2.8)	7 (14)

[a]Values given in parentheses are the theoretical expectation values for the particular amino acid at that position.

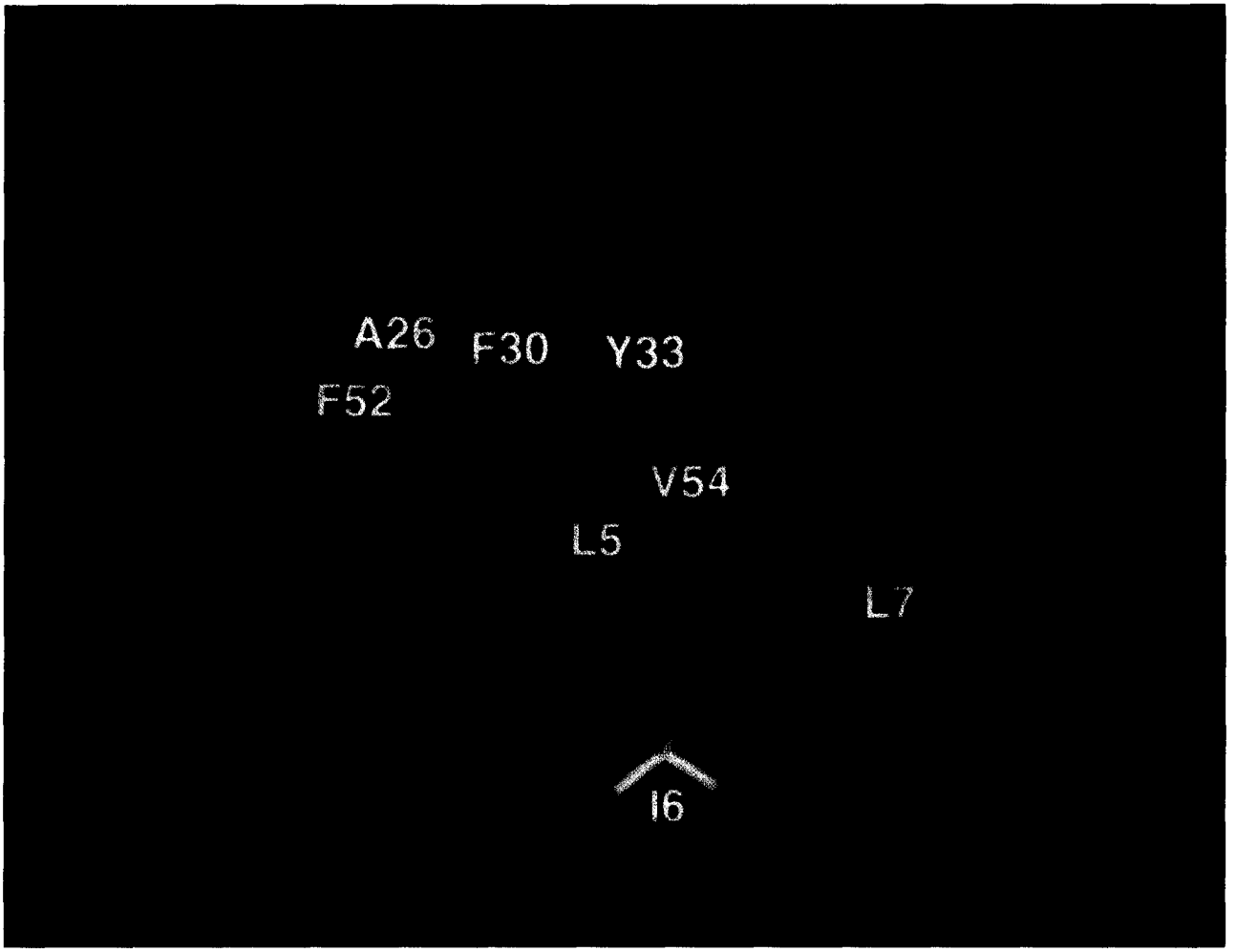

Fig. 1. Schematic ribbon diagram of the folded GB1 domain illustrating the four core residues (red) and the one external residue (yellow) on the β-sheet that were randomized in the present study. Also shown are interacting core residues projecting down from the helix (light blue).

mutant sequences of GB1. Upon screening for protein production, 23 of these showed good expression.

Several mutants were assessed with respect to their structural integrity using crude cell extracts of cells grown in minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source. The methodology employed has been described previously [30] and is based on the fact that ^1H-^{15}N HSQC spectra of small soluble ^{15}N labelled proteins can be obtained from crude *E. coli* extracts. The reasons for the success of the method are twofold; first, large proteins or multiprotein complexes (>50 kDa) will exhibit a slow rotational correlation time such that their resonances will be too broad to be observed. Second, since the signal to noise ratio is directly proportional to concentration, any overexpressed protein will be the predominant source of signal. Initially, three candidates were chosen at random after checking for protein expression and their ^1H-^{15}N HSQC spectra recorded. Two of those exhibited spectra very similar to folded GB1, whereas the third showed very few crosspeaks, resembling neither those of folded GB1 nor denatured GB1. SDS PAGE showed that approximately the same amount of GB1 mutant protein (~20% of total soluble protein) was present in this sample as in the other mutant protein samples. Thus, it is most likely that an aggregated form of this particular mutant is present. DNA sequencing revealed the following amino acids at the various positions for this mutant: Val-5, Met-6, Phe-7, Leu-52, Leu-54. This result demonstrates that high levels of protein expression cannot be used for selecting those mutants that represent stable, native-like folded proteins. This statement holds true for our entire set of mutants; indeed we observed good expression for some of the variants which exhibited NMR spectra of mostly unfolded protein at 25°C.

Examples of ^1H-^{15}N HSQC spectra of various mutant GB1s are shown in Fig. 2. Mutant #16 ($L_5L_6L_7F_{52}V_{54}$) contains the identical core residues to wild type and only Ile-6 is changed to Leu. Indeed the spectrum as well as other properties are extremely similar to wild type. Mutant #1 ($F_5F_6L_7F_{52}V_{54}$) and mutant #18 ($L_5V_6L_7V_{52}V_{54}$) are double mutants: both contain a change at the external position 6 plus a change in β-strand 1 for mutant #1 (L5F) and one in β-strand 4 for mutant #18 (F52V). The spectra for both these mutants exhibit the characteristically dispersed resonances of a folded GB1 variant. Mutant #7 ($L_5V_6V_7L_{52}V_{54}$) is a triple mutant with one change on β-strand 1(L7V) and one in β-strand 4 (F52L), as well as in the exposed 6 position. Again, a well dispersed spectrum characteristic of a folded protein is observed. Mutants #35 ($M_5I_6I_7L_{52}I_{54}$), #46 ($F_5L_6I_7V_{52}V_{54}$) and #47 ($V_5V_6F_7F_{52}L_{54}$) are quadruple mutants. In two of these (#46 and #47) one wild type core residue remains on β-strand 4, namely Val-54 in mutant #46 and Phe-52 in #47. In

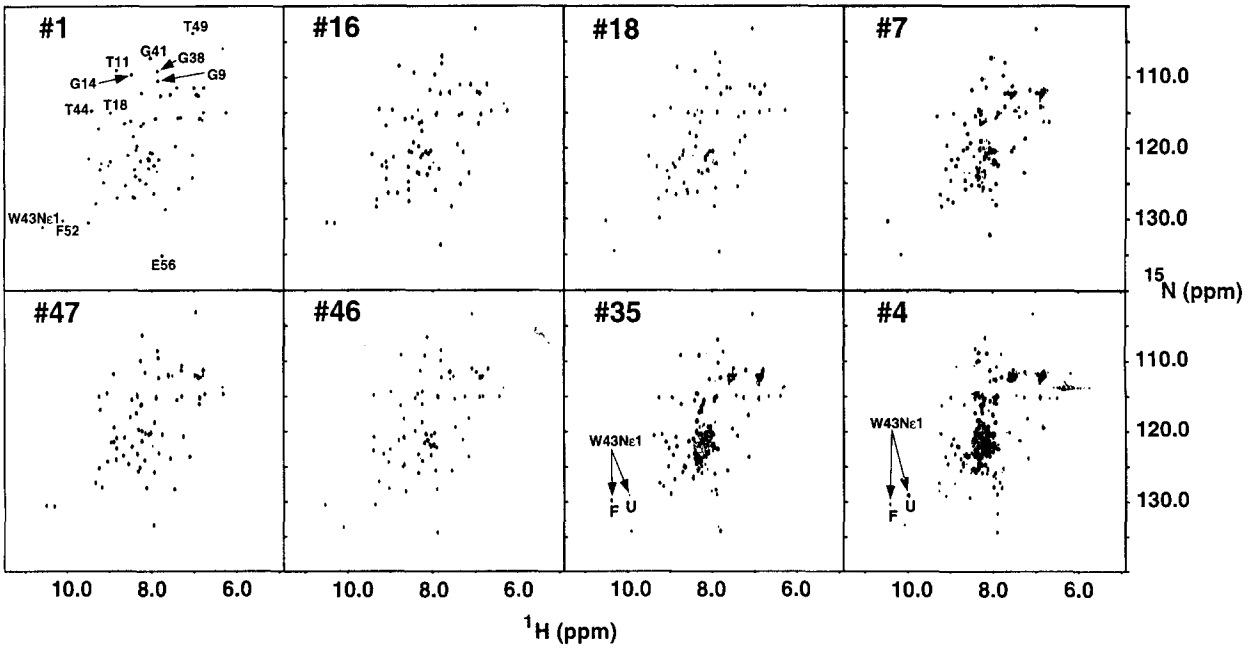

Fig. 2. ^{1}H-^{15}N HSQC spectra of GB1 mutants in 50 mM sodium phosphate buffer, pH 5.4 at 25°C.

contrast, in mutant #35 all core residues are changed and only the exposed position 6 remains an Ile. The ^{1}H-^{15}N HSQC spectra of mutants #46 and #47 are again characteristic of the native GB1 fold. In the case of mutant #35, however, smaller resonances are observed at low contour levels in addition to those resonances representing the native fold. These resonances exhibit chemical shifts very close to those obtained for unfolded GB1 [21], and thus most likely represent a small fraction of protein in the unfolded state. Integration of selected known resonances arising from the same residue in the folded versus unfolded form, allows one to estimate that the percentage of unfolded mutant #35 protein at 25°C is ~15%. Mutant #4 ($M_5V_6V_7V_{52}L_{54}$) is a quintuple mutant with changes in all the positions that were randomized. Its ^{1}H-^{15}N HSQC spectrum is characteristic of the unfolded state for this GB1 variant. However, at lower contour levels, small crosspeaks arising from the folded form of this mutant can be detected. Integration of the corresponding crosspeaks yields a percentage of folded mutant #4 at 25°C of ~20%.

The stability of a variety of mutants was assessed by equilibrium unfolding in guanidinium chloride monitoring the fluorescence of the single tryptophan at position 43. The resulting unfolding curves are shown in Fig. 3. The data for the different mutants were analyzed by non-least squares best fitting as described by Pace et al. [33] and the resulting free energies of unfolding are summarized in Table 2. Mutant #16 contains wild type hydrophobic core residues but has Ile-6 substituted by Leu. This substitution does affect the stability of this mutant to some degree. Indeed, we find that mutant #16 is ~1.23 kcal mol^{-1} less stable than our original wild type protein and two model studies aimed at exploring β-sheet propensities of different amino acids in exposed β-strand positions [22,24] report ΔG values for Leu versus Ile in the range of 0.49–0.88 kcal mol^{-1}. It should be noted, however, that all these protein variants are not strictly comparable to the present mutants since they all contain changes elsewhere in the amino acid sequence: in our case the original wild type sequence has a Thr at position 2 which in the present series is changed to Gln. This mutation could also destabilize the protein by up to 1 kcal mol^{-1}. It may be of interest to point out that the stability of the various mutants is not correlated with the number of amino acid changes; mutant #47 is a quadru-

Table 2
Thermodynamic parameters for unfolding of GB1 mutants as determined by GuHCl denaturation

Mutant[a]	5	6	7	52	54	Residue volume in core (Å^3)	$[GuHCl]_{mid}$ (M)	m^b (kcal mol^{-1} M^{-1})	ΔG_{H_2O} (kcal mol^{-1})
wild-type[c]	Leu	Ile	Leu	Phe	Val	417	3.14±0.02	1.79±0.08	5.61±0.25
#16	Leu	Leu	Leu	Phe	Val	417	2.43±0.01	1.83±0.06	4.45±0.13
#47	Val	Val	Phe	Phe	Leu	452	1.24±0.01	2.24±0.03	2.79±0.04
#18	Leu	Val	Leu	Val	Val	356	0.70±0.01	2.89±0.08	2.02±0.07
#7	Leu	Val	Val	Leu	Val	356	0.68±0.01	2.65±0.09	1.80±0.07
#46	Phe	Leu	Ile	Val	Val	392	0.48±0.02	2.42±0.11	1.17±0.09
#35	Met	Ile	Ile	Leu	Ile	413	0.45±0.04	2.16±0.22	0.96±0.18
#4[d]	Met	Val	Val	Val	Leu	359	–	–	~−0.8

[a]Position of residue in the amino acid sequence.
[b]$\Delta G = DG_{H_2O} - m[GuHCl]$ so that $m = \Delta G_{H_2O}/[GuHCl]_{mid}$. The value of m is a measure of the steepness of the unfolding curve.
[c]The wild type sequence contains a Thr at position 2, while the series of mutants generated in the present study was made in the T2Q background.
[d]ΔG_{H_2O} could not be determined from a GuHCl titration since the mutant is predominantly unfolded in H_2O; an approximate value was extracted from the ratio of folded to unfolded form observed in the ^{1}H-^{15}N HSQC spectrum.

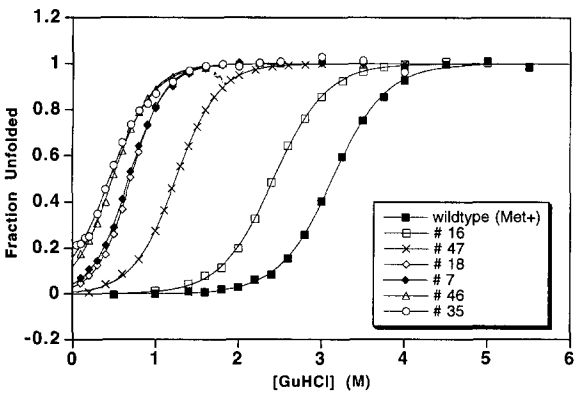

Fig. 3. Equilibrium unfolding of the GB1 domain in 50 mM sodium phosphate buffer, pH 5.4 and 25°C in guanidinium chloride as monitored by the fluorescence of the single tryptophan residue at position 43. (Fluorescence excitation was carried out at 295 nm and the emission was integrated from 305 to 450 nm.) The experimental points are presented as symbols, and the line represents the non-linear least squares best fit to a two-state model [33].

ple mutant in which only Phe-52 of the wild type sequence is retained, but ranks second in stability after mutant #16. This is in stark contrast to mutant #46 which is also a quadruple mutant with only Val-54 retained and which resides at the lower end of the stability scale. This clearly indicates that certain core positions seem to be more important for the overall stability of the protein than others. The least stable mutant in the present set is mutant #4 in which all five positions are altered compared to wild type. Clearly, no thermodynamic parameters can be obtained from the guanidinium chloride titration since the protein is almost completely unfolded at 25°C and pH 5.5. The ^{1}H-^{15}N HSQC spectrum exhibited two sets of resonances, one for the folded (~20%) and one for the unfolded form (~80%) and we used the relative amounts of these for an estimation of ΔG. Mutant #35 also exhibited a small amount (~15%) of unfolded protein in the ^{1}H-^{15}N HSQC spectrum, but it was nevertheless possible to obtain good guanidinium chloride unfolding data. We also do not observe a correlation between total residue volume of those residues altered in the core (see Table 2) and stability: indeed mutant #35 is closest to the wild type value amongst the current set, but clearly of much lower stability.

The set of mutant protein G core variants described in this papers lays the groundwork for an extensive study concerning core variability. It is demonstrated that ^{1}H-^{15}N HSQC spectroscopy on crude cell extracts (prepared by growing the bacteria on minimal medium with $^{15}NH_4Cl$ as the sole nitrogen source) in combination with fluorescence unfolding studies on purified heat released protein permits the fast and efficient characterization of the structure and stability of a large number of mutant GB1 proteins. It should also be pointed out that no selection procedure was employed in our mutational strategy used to generate the mutant library. Consequently, the mutants produced by this method are not limited to a set of candidates exhibiting a particular activity (e.g. IgG binding), thereby avoiding any potential bias towards functional proteins. The general approach employed here may also prove useful in structural assessment of designed binary code proteins.

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