Measurement of the β-sheet-forming propensities of amino acids

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SEVERAL model systems have been used to evaluate the α-helical propensities of different amino acids[1–7]. In contrast, experimental quantitation of β-sheet preferences has been addressed in only one model system, a zinc-finger peptide[8]. Here we measure the relative propensity for β-sheet formation of the twenty naturally occurring amino acids in a variant of the small, monomeric, β-sheet-rich, IgG-binding domain from protein G. Amino-acid substitutions were made at a guest site on the solvent-exposed surface of the β-sheet. Several criteria were used to establish that the mutations did not cause significant structural changes: binding to the Fc domain of IgG, calorimetric unfolding and NMR spectroscopy. Characterization of the thermal stabilities of these proteins leads to a thermodynamic scale for β-sheet propensities that spans a range of ~2 kcal mol^{-1} for the naturally occurring amino acids, excluding proline. The magnitude of the differences suggests that β-sheet preferences can be important determinants of protein stability.

The immunoglobin-binding domain B1 from protein G (denoted GB1) is a small, soluble, monomeric, highly stable protein comprising a four-stranded β-sheet and a single α-helix (Fig. 1*a*). GB1 has been shown to undergo a two-state, reversible, thermal unfolding transition[9,10]. These properties make GB1 an attractive model system for studying β-sheet propensities. A 'guest' site, into which all twenty natural amino acids could be substituted, was chosen at a solvent-exposed position (residue 53) in a central β-strand of the protein. This position was chosen to avoid potential effects from the edges or ends of the sheet (Fig. 1*a*).

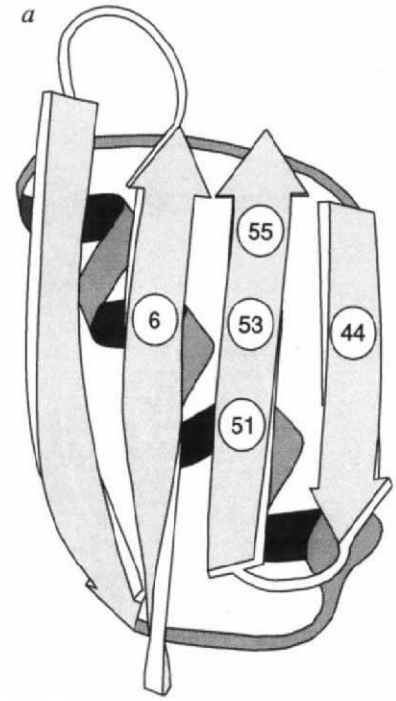
As we wanted to measure the intrinsic propensities for β-sheet formation, we constructed potential host molecules that minimized local interactions to the guest site. Three potential host systems were constructed in which the guest position was surrounded by small neutral amino acids. The majority of inter-residue contacts to the guest site arise from the two residues on each of the immediately adjacent β-strands (Fig. 1*a*). These residues, Ile 6 and Thr 44, were replaced by alanine. The residues $i-2$ and $i+2$ from the guest site (residues 51 and 55) make fewer, more distant contacts and were replaced simultaneously with alanine, serine or threonine.

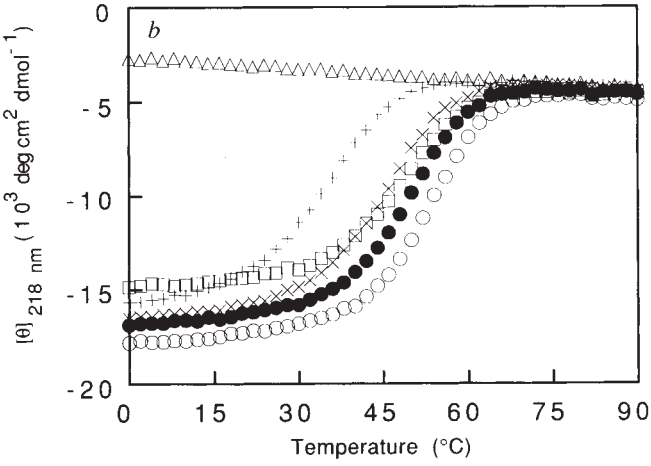
Glycine, an amino acid expected to have a low β-sheet propensity[8,11], was substituted at the guest site in order to evaluate the potential host systems. In the system containing alanine subsitutions at the $i-2$ and $i+2$ positions, with glycine at the guest position, the circular dichroism (CD) thermal unfolding curve lacks a clearly defined folded baseline (unpublished results). A well-defined folded baseline, however, is obtained with the glycine mutant in the systems containing serine or threonine substitutions at the $i-2$ and $i+2$ positions. The host containing serine, the smaller side-chain substitution, at the $i-2$ and $i+2$ positions (I6A/T44A/T51S/T55S; denoted AASS) was chosen for further study.

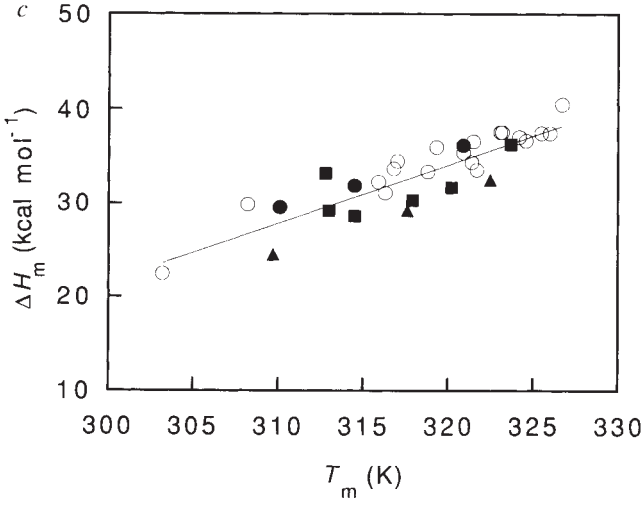
The twenty naturally occurring amino acids were substituted for residue 53 in the AASS background by site-directed mutagenesis. The stability of each protein (designated AASS-53Xaa) was measured by thermal unfolding as monitored by CD (Fig. 1*b*). $\Delta\Delta G$ values for β-sheet formation were obtained by assuming that changes in global stability result entirely from changes

FIG. 1 *a*, Ribbon drawing of GB1 (based on a diagram produced with the program RIBBON[17] using the coordinate set 2GB1[10]). The positions of the guest site and the surrounding residues are indicated. The arrows represent the inter-residue side-chain–side-chain contacts to the guest site made by the surrounding residues. In the native structure of GB1, the number of contacts to residue 53 from residues 6, 44, 51 and 55 is twelve, seven, three and two respectively. In the AASS background the number would be reduced to three, two, one and two respectively, assuming that the backbone structure is unperturbed from that of GB1. *b*, Temperature dependence of the CD signal from representative GB1 mutants. AASS-53Thr (○), AASS-53Ser (●), AASS-53Gln (X), AASS-53Asp (+), AASS-53Trp (□), AASS-53Pro (△). The folded baselines of all molecules fall within the bounds of the range shown here, with the exception of AASS-53Gly, which has a $[\theta]_{218nm}$ value of −13,100 deg cm^2 dmol^{-1} at 0 °C. The differences in folded baseline $[\theta]_{218nm}$ values probably result largely from different aromatic contributions to the CD signal. Residue 53 is situated above W43, which is in the hydrophobic core of the protein. Molecules containing the mutation W43F show changes in both the folded and unfolded $[\theta]_{218nm}$ baseline values of 45 and 35% respectively (data not shown), indicating that W43 is making a substantial contribution to the CD signal at 218 nm (see also text and refs 13, 14). *c*, Plot of ΔH_m versus temperature for the AASS mutants. Open circles indicate T_m and ΔH_m values from the AASS-53Xaa mutants. Filled symbols indicate T_m and ΔH_m for AASS-53Thr (●), AASS-53Phe (▲) and AASS-53Val (■), at different pH values. The slope of the line is ΔC_p (624 cal mol^{-1} deg^{-1}).

METHODS. Calculations of nearest-neighbour side-chain contacts were made using a program written by T. G. Oas[18]. Significant contacts were identified from the coordinate set 2GB1 and were defined as any atom pair having a centre-to-centre distance less than or equal to 150% of the sum of the van der Waals radii. A synthetic gene for the GB1 sequence[19] was constructed and cloned into a T7 expression plasmid[20], pAED4 (ref. 21), using standard cloning procedures[22]. All proteins were purified by affinity purification[9] on IgG 6 Fast-Flow Sepharose (Pharmacia, 17-0969-01) followed by reverse-phase high-pressure liquid chromotography (HPLC) purification on Vydac preparative C18 column using a linear H_2O-acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. This purification scheme yielded two species of GB1, a 56-residue protein containing an N-terminal methionine and a 55-residue protein in which the N-terminal methionine had been removed. The 55-residue species was substantially less stable (by ~1.7 kcal mol^{-1}) than the 56-residue species. The mutation Met1→Thr was made to produce a 56-residue, N-terminally processed protein that began with threonine at position 1. This protein had the same stability as the 56-residue GB1. All mutant genes were made in the GB1 background containing threonine at position 1 by site-directed mutagenesis[23]. Proteins were purified by affinity and HPLC chromatography as described, except for AASS-53Pro, for which the IgG affinity-column step was replaced by G75 chromatography in a buffer of 10 mM phosphate, 150 mM NaCl, pH 7.3. Mutant genes were sequenced completely (Sequenase, USB) and the identity of each HPLC-purifed protein was confirmed by laser desorption mass spectrometry (Finnegan Mat Lasermat). All relative molecular masses were within 3 units of the expected M_r. CD measurements were made at a concentration of 10 μM using a 1-cm pathlength cell with an Aviv model 62DS circular dichroism spectrometer equipped with a thermoelectric temperature controller. Sample buffer contained 50 mM CH_3COO Na, 150 mM NaCl, pH 5.4. The melting curves were fitted (Kaleidagraph; Abelbeck Software) to the equation $\theta = \theta_u + (\theta_f - \theta_u)/(1 + e((-\Delta H_m/R)(1/T - 1/T_m) + (\Delta C_p/R)[((T_m/T) - 1) + \ln(T/T_m)]))$, using a fixed ΔC_p of 624 cal mol^{-1} deg^{-1}, where θ is the measured CD signal, θ_f and θ_u are linear functions representing the folded and unfolded baselines respectively, T_m is the melting temperature, and ΔH_m is the enthalpy of unfolding at T_m (ref. 24). The two-state unfolding model was tested for AASS-53Thr and AASS-53Ala by measuring the enthalpy of unfolding by differential scanning calorimetry. The results are $\Delta H_{cal} =$ 39.9 kcal mol^{-1} and 32.8 kcal mol^{-1}, $\Delta H_{cal}/\Delta H_{van't\ Hoff} = 0.99$ and 0.97 for AASS-53Thr and AASS-53Ala respectively. Differential scanning calorimetry experiments were performed using a Microcal MC-2 scanning calorimeter (Northampton, MA). Calorimetry samples contained ~3 mg ml^{-1} protein in 50 mM sodium acetate, 150 mM NaCl, pH 5.4, and were dialysed extensively against this buffer before the experiment. Samples were degassed under vacuum before loading into the sample chamber and were heated from 4 to 95 °C using a scanning rate of one deg per min. The pH dependence of T_m was measured in a buffer of 1 mM each of phosphate, acetate, citrate and borate in 196 mM NaCl. Protein concentration was determined for all samples from the absorbance of the unfolded protein[25].







in the ability of the residue at the guest site to adopt a β-sheet conformation. ΔC_p for unfolding, a necessary parameter for calculating the temperature dependence of the free energy of unfolding, was determined by combining data from the pH dependence of stability for AASS-53Thr, AASS-53Phe and AASS-53Val with the stability data obtained at pH 5.4 for each of the guest site mutants (Fig. 1*c*). The resultant value ($\Delta C_p = 624 \pm 62$ cal mol^{-1} deg^{-1}) is in excellent agreement with the previously reported value for wild-type GB1 (621 ± 71 cal mol^{-1} deg^{-1})[9] and these data illustrate that these mutations have little effect on ΔC_p, as might be expected for mutations at solvent-exposed positions[12]. The relative free energy differences for β-sheet formation are listed in Table 1.

There are differences among the mutant proteins in the magnitude of the CD signal at 218 nm (Fig. 1*b*). However, all the

TABLE 1 $\Delta\Delta G$ values for β-sheet formation relative to alanine, thermal melting temperatures (T_m), and relative binding constants to human Fc for the AASS-53Xaa proteins normalized to the binding of AASS-53Thr

Amino acid	$\Delta\Delta G$ (kcal mol^{-1})	T_m(°C)	$K_a/K_a^{AASS\text{-}53Thr}$
Thr	1.1	53.7	1.0
Ile	1.0	53.0	1.0
Tyr	0.96	52.5	1.0
Phe	0.86	51.6	1.0
Val	0.82	51.2	1.1
Met	0.72	50.2	1.0
Ser	0.70	50.1	1.1
Trp	0.54	48.7	1.1
Cys	0.52	48.5	1.1
Leu	0.51	48.4	1.0
Arg	0.45	47.9	1.0
Lys	0.27	46.3	1.1
Gln	0.23	45.8	1.2
Glu	0.01	44.0	1.1
Ala	0.00	43.8	1.1
His	−0.02	43.3	1.0
Asn	−0.08	42.9	1.0
Asp	−0.94	35.2	1.1
Gly	−1.2	30.2	1.0
Pro	<−3	<0	0

The K_a for wild-type GB1 is 1.4×10^8 M^{-1} (ref. 15). The $K_a/K_a^{AASS\text{-}53Thr}$ value for wild-type GB1 is 1.1. ΔG values for unfolding of the AASS-Xaa proteins were calculated using the Gibbs–Helmholtz equation $\Delta G(T) = \Delta H_m\ (1 - T/T_m) - \Delta C_p[T_m - T) + T\ln(T/T_m)]$, assuming $\Delta C_p = 624$ cal mol^{-1} deg^{-1} for all molecules (Fig. 1c). $\Delta\Delta G$ values were calculated at 321 K, the mean of the T_ms excluding the glycine mutant, to minimize extrapolations in the calculation of $\Delta G(T)$. A positive value of $\Delta\Delta G$ indicates an increase in stability. The estimated errors in determination of T_m and ΔG are ±0.5 °C and ±0.06 kcal mol^{-1} respectively. Fc was made by proteolytic digestion of human IgG (Sigma I-4506) with papain and was purified from other digestion products by affinity (protein G)[16] and gel-filtration (G75) chromatography in a buffer of 10 mM phosphate, 150 mM NaCl, pH 7.3, followed by FPLC purification using a linear gradient of NaCl (0.05–1 M) in a buffer of 50 mM sodium acetate, pH 4.75, on Mono-S resin (Pharmacia). The purified material was >98% pure as judged by SDS polyacrylamide gel electrophoresis. The binding assay was carried out using standard methods[16] on 96-well microtitre-plates (Costar), coated for 2 h with a 100 μg ml^{-1} solution of Fc and then blocked for 2 h with 3% bovine serum albumin (BSA). The assay was carried out at 5 °C using a fixed quantity of protein G–alkaline phosphatase (100 μg ml^{-1}) (Pierce 31399X) and a variable amount of competitor protein (0.6–3.5 mM) in the presence of 1.5% BSA in a buffer of 50 mM sodium acetate, 150 mM NaCl, pH 5.4. Samples were allowed to equilibrate for 39–48 h. Control experiments indicated that equilbrium was reached after ~25 h. Wells were developed with a fresh solution of 1 mg ml^{-1} *p*-nitrophenol phosphate (Pierce 34045X) and the absorbance at 410 nm was read using a microplate reader (Dynatech). The ratio of affinity constants of the competitor molecule with protein G was determined using the equation $(Y \times Y)/(Y-1) = -(K_a^{protein\ G}/K_a^{competitor})$[competitor], where Y is the fraction of protein G–alkaline phosphatase bound. The ratio of affinity constants for the mutants was normalized to AASS-53Thr, which was run as an internal standard on each plate.

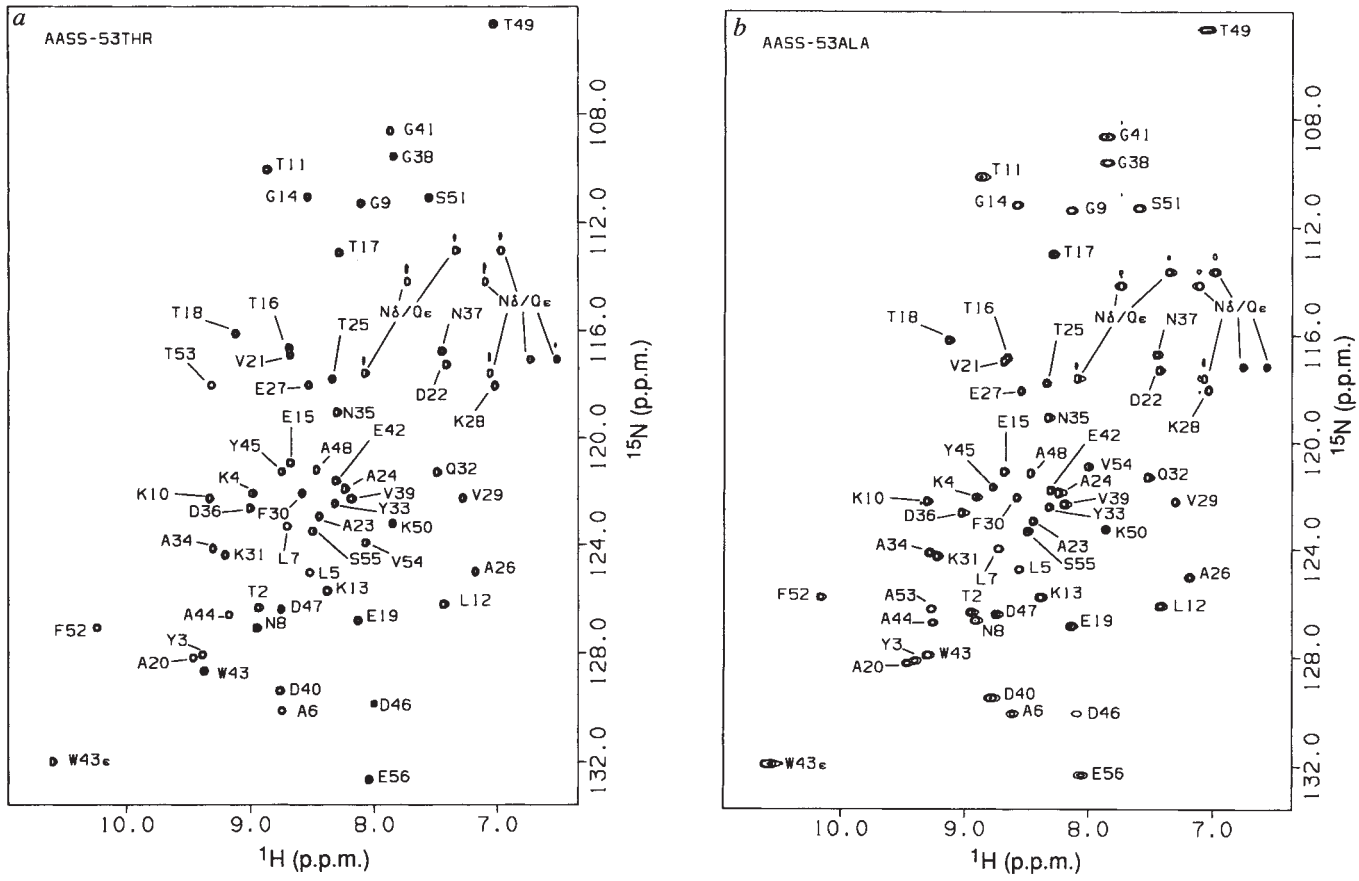
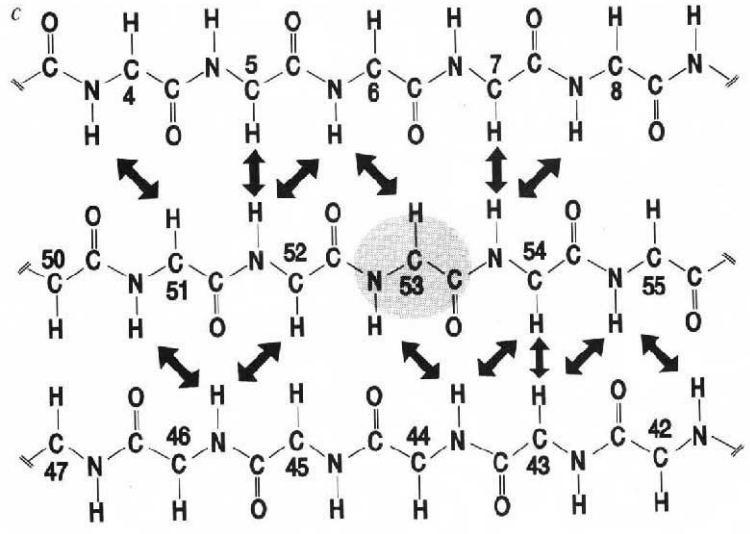


FIG. 2 ^1H–^{15}N correlation spectra[26] of *a*, AASS-53Thr and *b*, AASS-53Ala proteins at 5 °C in 150 mM NaCl, pH 5.4 (10% D_2O). Labels indicate resonance assignments. The side-chain amide resonances from the one Gln and three Asn residues are unassigned and are labelled as *Nδ/Qε*. *c*, Diagram of observed NOEs present in both AASS-53Thr and AASS-53Ala diagnostic for *β*-sheet structure at the guest site. Each arrow represents one or more NOEs. The position of the guest site is shaded grey. Side-chain positions are omitted for clarity. Observed NOEs include: H^N_{51}–H^N_{46}, H^α_{51}–H^N_4, H^N_{52}–H^α_5, H^N_{52}–H^N_6, H^N_{52}–H^N_4, H^α_{52}–H^N_{46}, H^N_{52}–H^β_5, H^N_{53}–H^N_{44} (not seen for AASS-53Ala because of overlap), H^α_{53}–H^N_6, H^N_{54}–H^α_7, H^N_{54}–H^N_8, H^α_{54}–H^α_{43}, H^α_{54}–H^N_{44}, H^N_{55}–H^N_{42}, H^N_{55}–H^α_{43}, $H^{\gamma 2}_{54}$–H^δ_{43}, $H^{\gamma 2}_{54}$–H^ε_{43}, $H^{\gamma 2}_{54}$–$H^{\zeta 2}_{43}$, $H^{\gamma 2}_{54}$–$H^{\zeta 3}_{43}$, $H^{\gamma 2}_{54}$–H^η_{43}, $H^{\gamma 2}_{54}$–$H^{N\varepsilon}_{43}$, $H^{\gamma 1}_{54}$–H^ε_{43}, $H^{\gamma 1}_{54}$–H^δ_{43}, $H^{\gamma 1}_{54}$–$H^{\zeta 2}_{43}$, H^β_{54}–H^δ_{43}, H^β_{54}–$H^{\zeta 2}_{43}$, H^β_{54}–$H^{\zeta 3}_{43}$, H^β_5–H^η_{43} and H^β_5–$H^{\zeta 3}_{43}$.
METHODS. *Escherichia coli* harbouring the expression plasmid for AASS-54Thr or AASS-53Ala were grown in M9 medium supplemented with $(^{15}NH_4)_2SO_4$ (99.7% ^{15}N; Isotec, Ohio) to obtain uniformly (⩾95%) ^{15}N–labelled protein. Data were collected on a Bruker AMX 500 MHz NMR spectrometer. Spectral widths in the $^1H(F_2)$ and $^{15}N(F_1)$ dimensions were 14.08 p.p.m. and 31.98 p.p.m., respectively. Spectra were referenced to the carrrier in F_1 (118.5 p.p.m.) and trimethylsilylpropionate (TMSP) in F_2 corrected for the pH dependence of TMSP[27]. Resonance assignments were made by standard methods[28,29] and were consistent with the assignments for wild-type GB1 (ref. 10).



proteins bind Fc with the same affinity as wild-type GB1, with the exception of the unfolded mutant AASS-53Pro (Table 1). This suggests that none of the folded molecules has undergone substantial changes in overall conformation. In light of this result, it is likely that differences in the baseline values of the molar ellipticity at 218 mm ($[\theta]_{218nm}$) arise from contributions to the CD signal from aromatic side chains (compare refs 13, 14) at this wavelength (see also Fig. 1 legend).

Two molecules representing each end of the stability scale, AASS-53Thr and AASS-53Ala, were chosen for further characterization. For both molecules, ΔH_{cal} was found to be equal to $\Delta H_{van't\ Hoff}$ (Fig. 1 legend), suggesting that the two-state nature of the unfolding reaction for the wild-type GB1 protein[9] is preserved in the variants studied here.

AASS-53Thr and AASS-53Ala were also investigated by NMR. The ^1H-^{15}N correlation spectra of these two molecules are very similar except for resonances from residues in the immediate vicinity of the guest site (Fig. 2*a*, *b*). The NMR spectra of both proteins also contain cross-strand nuclear Overhauser effect (NOE) patterns which are present in wild-type GB1 and are

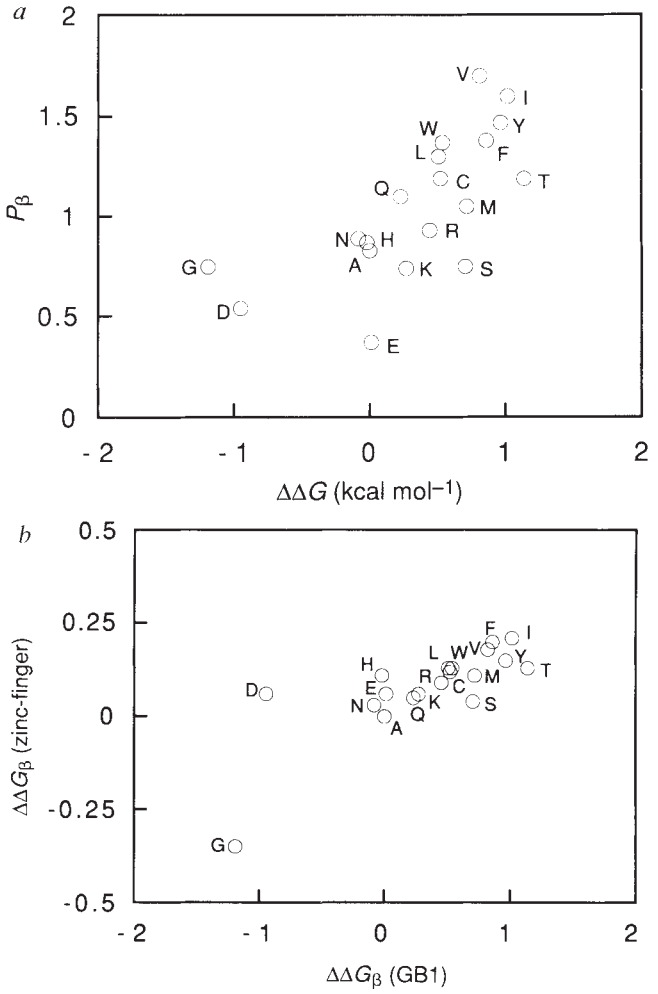


FIG. 3 *a*, Correlation of the ΔΔG values for β-sheet formation measured in the AASS system (Table 1) with the β-sheet-forming propensities (P_β) of Chou and Fasman[11]. *b*, Correlation of the ΔΔG values for β-sheet formation obtained here and in the zinc-finger system[8]. Note the difference in scale between the two systems. The value for proline is omitted.

indicative of β-sheet structure at the guest site (Fig. 2*c*). In particular, these data suggest that any structural changes between the mutants are small and do not involve significant disruption of the backbone β-sheet structure, such as fraying of residues 53–56.

Our results indicate that there are significant differences in β-sheet-forming propensities among the naturally occurring amino acids. The propensity scale suggests that β-branching of the side chain favours β-sheet formation, and the rank order shows a modest correlation with β-sheet preferences measured by statistical methods[11] and with the rank order measured in the zinc-finger system[8] (Fig. 3). It is striking, however, that the range of ΔΔG values for the β-sheet formation obtained here is an order of magnitude larger than the values obtained with the zinc-finger host (2.05 kcal mol^{-1} versus 0.21 kcal mol^{-1} full scale, excluding proline and glycine).

Finally, the magnitude of β-sheet preferences obtained here is comparable to those measured for α-helices[1,7], suggesting that β-sheet propensities can be as important as α-helix propensities in determining protein stability. The thermodynamic preferences for β-sheet formation reported here should be useful for deciphering the rules involved in protein stability, protein folding and protein design. □

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