changes associated with ATP and GroES binding. GroEL provides binding surfaces for substrate proteins on a ring of highly mobile domains. □

Received 20 May; accepted 24 August 1994.

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ACKNOWLEDGEMENTS. We thank J. Munn for EM support, R. Westlake and J. Bouquiere for computing support, R. J. Ellis and G. Laxer for discussion, the Birkbeck Photo Unit for photographic work and the Wellcome Trust and AFRC for project grants. A.R.C. is a Lister Institute Fellow.

Context is a major determinant of β-sheet propensity

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RESIDUES in β-sheets occur in two distinct tertiary contexts: central strands, bordered on both sides by other β-strands, and edge strands, bordered on only a single side by another β-strand[1]. The ΔΔG values for β-sheet formation measured at an edge β-strand of the IgG-binding domain of protein G(GB1) are quite different from those obtained previously[2,3] at a central position in the same protein. In particular, there is no correlation at the edge position with statistically determined β-sheet-forming preferences[4]. The differences between β-sheet propensities measured at central and edge β-strands, ΔΔΔG values, correlate with the values of water/octanol transfer free energies[5] and side-chain non-polar surface area for the amino acids[6]. These results strongly suggest that, unlike α-helix formation, β-sheet formation is determined in large part by tertiary context, even at solvent-accessible sites, and not by intrinsic secondary structure preferences.

The 20 naturally occurring amino acids were substituted at a solvent-exposed edge β-strand position, residue 44, by site-directed mutagenesis in a host molecule in which local interactions to the guest site had been minimized by replacing the nearest neighbours with alanine (see Fig. 1 legend). This edge β-strand is bordered on one side by another β-strand and on the other side by solvent (Fig. 1*a*). The stability of each protein (denoted AAA-44Xaa) was measured by thermal unfolding as monitored by circular dichroism (CD) at 218 nm (Fig. 1*b*). ΔΔG values for β-sheet formation, referenced to alanine, were obtained by assuming that changes in global stability result entirely from changes in the ability of the residue at the guest site to adopt a β-sheet conformation. In support of this assumption, molecules representative of the entire ΔΔG range were found to have $\Delta H_{\text{van't Hoff}}/\Delta H_{\text{cal}}$ ratios near unity, indicating that the two-state nature of the equilibrium unfolding transition observed for wild-type GB1 (ref. 7) remains intact (see Fig. 1 legend). The relative free energy differences for β-sheet formation at the edge position are listed in Table 1.

TABLE 1 Parameters of β-sheet formation

Amino acid	ΔΔG (kcal mol^{-1})	T_m (°C)	$K_a/K_a^{\text{AAA-44Thr}}$	ΔΔΔG (kcal mol^{-1})
Thr	0.83	60.2	1.0	−0.27
Ser	0.63	59.4	3.1	−0.07
Glu	0.31	57.0	1.5	0.30
Val	0.17	56.1	1.5	−0.65
Phe	0.16	56.1	1.1	−0.70
Tyr	0.11	55.9	1.1	−0.75
Cys	0.08	55.1	0.7	−0.44
Gln	0.04	54.7	1.9	−0.19
Ile	0.02	54.8	1.6	−0.98
Ala	0	54.7	2.9	0
His	−0.01	54.6	1.8	0.01
Met	−0.02	54.2	1.8	−0.74
Asp	−0.10	53.7	1.2	0.84
Trp	−0.17	53.3	3.0	−0.77
Asn	−0.24	52.6	1.0	−0.16
Leu	−0.24	52.8	1.0	−0.75
Lys	−0.40	51.4	0.9	−0.67
Arg	−0.43	51.2	1.0	−0.88
Gly	−0.85	47.6	1.4	0.35
Pro	< −4	<0	0	—

Listed are ΔΔG values for β-sheet formation at an edge position relative to alanine, thermal melting temperatures (T_m) for the AAA-44Xaa proteins, relative binding constants (K_a) to human Fc for the AAA-44Xaa proteins, and ΔΔΔG values for β-sheet formation, comparing $\Delta\Delta G_{\text{edge}} - \Delta\Delta G_{\text{centre}}$ values (see also ref. 2). The K_a for wild-type GB1 is 1.4×10^8 M^{-1} (ref. 30). The $K_a/K_a^{\text{AAA-44Thr}}$ value for wild-type GB1 is 7.1. ΔG values for unfolding at 321 K were calculated as described previously[2] using data obtained from CD thermal unfolding measurements and the Gibbs-Helmholz equation. A positive ΔΔG value indicates an increase in stability relative to alanine. Estimated errors in determination of T_m and ΔG are ±0.5 °C and ±0.06 kcal mol^{-1} respectively. Fc binding was measured as described previously[2] by adding variable amounts of competitor protein to a fixed quantity of protein G-alkaline phosphatase at 5 °C in 96-well plates. The ratio of affinity constants for the mutants was normalized to AAA-44Thr. GB1-Thr1 (see Fig. 1 legend) was included as an internal standard on each plate.

All the proteins tested, with the exception of unfolded AAA-44Pro, bind Fc with around sevenfold reduced affinity relative to wild-type GB1 (Table 1). There is some variation in binding constants but this is not correlated with protein stability. As residues 42–46 have been identified as participants in the Fc-binding interface[8,9] it seems likely that the observed affinity differences reflect direct effects on binding by substitutions at residue 44.

As a more detailed check on the conformation at the guest site of each molecule, the chemical shifts of the aromatic ring protons of Trp 43 were measured in each of the 20 variants. Trp 43 is expected to be sensitive to changes in structure as it immediately precedes the guest site and is part of the hydrophobic core of the molecule. The chemical shifts of the Trp 43 ring protons are similar in all the variants, with the exception

of unfolded AAA-44Pro, and are significantly different from the chemical shifts for free tryptophan (see Fig. 2 legend). Additionally, unambiguous cross-strand NOEs between the guest strand and its neighbouring strand can be found between upfield shifted $H^{\gamma 1}_{54}$ and $H^{\gamma 2}_{54}$ protons of Val 54 and the H^{δ}_{43}, $H^{\zeta 2}_{43}$, $H^{\zeta 3}_{43}$, $H^{\varepsilon 3}_{43}$, $H^{\eta 2}_{43}$, $H^{N\varepsilon}_{43}$ ring protons of Trp 43 in each folded protein.

Two molecules with substantially different stability—AAA-44Thr and AAA-44Ala—which also have different Fc-binding affinities, were characterized further by NMR. The ^{1}H–^{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 near the guest site that are present in wild-type GB1 (Fig. 2*c*). Taken together with the Fc-binding and Trp 43 chemical shift results, these data indicate that any structural changes between the variants are small and do not involve significant disruption of the backbone β-sheet structure.

The dramatic effect that context can have on β-sheet propensities is seen in the comparison of the thermodynamic preferences for β-sheet formation measured at the central and edge β-sheet positions (Fig. 3*a*). Although the overall magnitude of the scale for β-sheet formation at the edge position ($\sim$2 kcal mol^{-1}, excluding proline) is similar to that measured at the central β-sheet position[2,3], there is no apparent correlation with the statistically measured β-sheet frequencies of Chou and Fasman[4] (Fig. 3*b*). In addition, unlike the results obtained at the central position, for which the $\Delta\Delta G$ values are distributed fairly evenly over a wide range, more than half (13/20) of the $\Delta\Delta G$ values measured at the edge position fall within a small range ($\sim$0.4 kcal mol^{-1}).

The context dependence of β-sheet formation suggests that two components contribute to β-sheet propensity: (1) intrinsic ability to form a local extended β-strand structure; and (2) ability to interact with the surrounding tertiary β-sheet structure. The small range of β-sheet propensities measured at the edge position, together with the relative lack of preference for particular side chain rotamers in β-strand residues[10], suggests that (1) has only a minor role in determining β-sheet propensity. This situation contrasts with α-helix formation where a significantly biased side chain rotamer distribution[10] and a well-distributed range of α-helix propensity values are found[11-18].

The difference in β-sheet propensity between the edge and centre sites, designated as $\Delta\Delta\Delta G = \Delta\Delta G^{Xaa}_{edge} - \Delta\Delta G^{Xaa}_{centre}$, correlates with both the free energy of transfer for the amino acids from octanol to water[5] and with the non-polar accessible

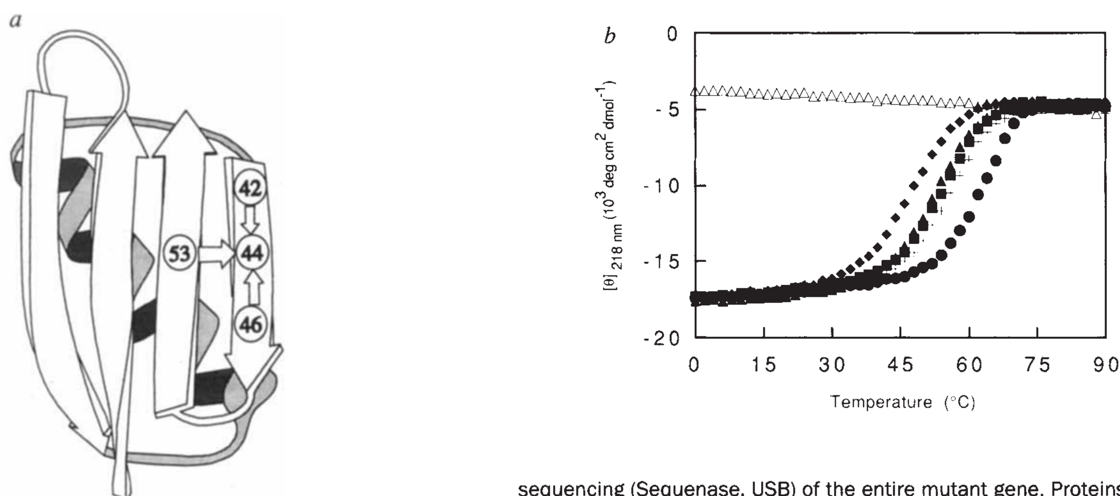

FIG. 1 *a*, Ribbon drawing[21] of GB1 based on the coordinate set 2GB1[22]. The positions of the guest site and the surrounding residues are indicated. The arrows represent the inter-residue contacts to the guest site made by the surrounding residues. *b*, Temperature dependence of the CD signal from representative GB1 variants: ●, AAA-44Thr; +, AAA-44Val; ■, AAA-44Ala; ▲, AAA-44Asn; ◆, AAA-44Gly; and △, AAA-44Pro, in 150 mM NaCl, 50 mM Na-acetate, pH 5.4.

METHODS. As described previously[2], major inter-residue contacts were identified[23] from the coordinate set 2GB1 and were defined as any atom pair having a centre-to-centre distance $\leqslant$150% of the sum of the van der Waals radii. Major contacts to residue 44 arise from residue 53 on the adjacent strand with fewer, more distant contacts being made by residues at positions $i+2$ and $i-2$ from the guest site. These residues were changed to alanine to create the host molecule E42A/D46A/T53A (denoted AAA). To test for possible effects from the $i+2$ and $i-2$ residues, which had been serine in our central position study[2], we also created the host molecule E42S/D46S/T53A (denoted SSA). Substitution at the guest position with threonine, an amino acid expected to be a very good β-sheet former, and glycine, an amino acid expected to be a very poor β-sheet former, yields identical $\Delta\Delta G$ values relative to alanine for β-sheet formation in both the AAA and SSA backgrounds. The host molecule bearing the smaller amino-acid substitutions, AAA, was chosen for further study. Recombinant GB1 mutants were expressed from a synthetic gene bearing the mutation Met 1→Thr (GB1-Thr1) described previously[2]. Mutations were generated by single-strand mutagenesis[24] and were verified by dideoxyribonucleotide sequencing (Sequenase, USB) of the entire mutant gene. Proteins were expressed in *Escherichia coli* (BL21 (DE3) pLysS) and were induced at an A_{600} of $\sim$0.5–0.8 with a final concentration of isopropylthiogalactoside of 0.4 mM for 2–4 h. All folded proteins were purified by affinity chromatography[7] with IgG 6 Fast Flow Sepharose (Pharmacia) followed by reverse phase high pressure liquid chromatography (HPLC) purification on a Vydac preparative C18 column using a linear H_2O-acetonitrile gradient in the presence of 0.1% TFA. The proteins are expressed as mixtures of N-terminally processed protein beginning with threonine at position 1 (56 residues) and non-processed protein (57 residues) beginning with methionine at position 0. The ratio of processed to unprocessed protein seems to depend on the stability of the molecule (data not shown). The HPLC purification step separates these two species and in all cases the 56-residue protein was used. For the unfolded molecule AAA-44Pro, the IgG affinity column step was replaced by G75 Sephadex chromatography in a buffer of 10 mM phosphate, 150 mM NaCl, pH 7.3. The identity of each HPLC purified protein was confirmed by laser desorption mass spectrometry (Finnigan Mat Lasermat). All measured molecular weights were within 3 atomic mass units of the expected mass. CD measurements were made and thermal unfolding curves were fitted as described previously[2]. ΔCp values for unfolding (data not shown) were similar ($\pm$15%) to those measured for the centre site AASS-Xaa molecules[2]. Protein concentration was determined by measuring absorbance of the unfolded protein[25]. Differential scanning calorimetry was performed with a Microcal MC-2 scanning calorimeter (Northampton, MA) as described previously[2]. AAA-44Thr, AAA-44Ser, AAA-44Gln, AAA-44Ala and AAA-44Gly were found to have $\Delta H_{van't\ Hoff}/\Delta H_{cal}$ ratios of 0.98, 0.94, 1.03, 0.98 and 0.99, respectively.

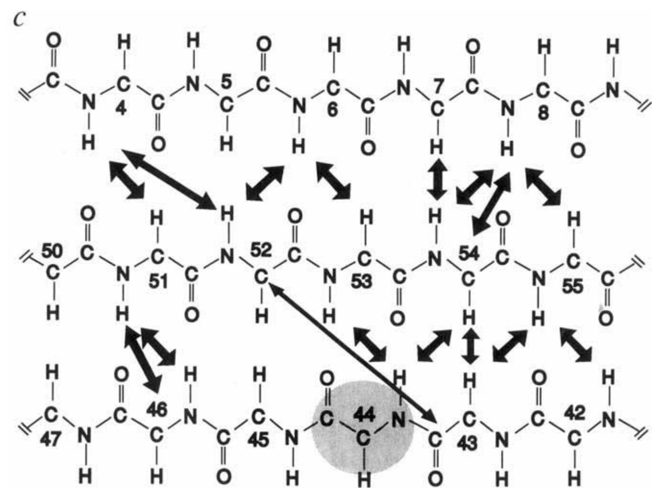

FIG. 2 ^{1}H-^{15}N correlation spectra of *a*, AAA-44Thr and *b*, AAA-44Ala proteins at 5 °C in 150 mM NaCl, pH 5.4 (10% D_2O). Labels indicate resonance assignments. *c*, Diagram of NOEs, present in both AAA-44Thr and AAA-44Ala, which are diagnostic for β-sheet structure at the guest site. Each arrow represents one or more NOEs. The position of the guest site is shaded grey. The range of chemical shifts (in p.p.m., see methods) for all the folded variants measured for the δH, εNH, ζ2H, η2H, ζ3H, protons of Trp 43 were respectively: 7.30–7.34, 10.32–10.38, 7.07–7.11, 6.45–6.50, 6.35–6.40. These chemical shifts are substantially different from the respective chemical shifts for free L-tryptophan: 7.01, 9.98, 7.24, 6.98, 6.90 and the respective chemical shifts for unfolded AAA-44Pro: 6.90, 10.00/9.96, 7.20, 6.91, not determined. (Two chemical shifts are seen for the εNH proton reflecting the influence of *cis* and *trans* isomers of Pro44[26]. The ζ3H resonance in AAA-44Pro could not be assigned owing to proximity to the diagonal.)

METHODS. ^{1}H-^{1}H DQF-COSY and NOESY spectra were collected at 5 °C, 150 mM NaCl, pH 5.4, 10% D_2O for all molecules. Spectral widths for ^{1}H-^{1}H experiments were 14.08 p.p.m. in both dimensions. *E. coli* harbouring the expression plasmid for AAA-44Thr or AAA-44Ala were grown in M9 media supplemented with $(^{15}NH_4)_2SO_4$ (99.7% ^{15}N, Isotec), as the sole nitrogen source to obtain uniformly (≥95%) ^{15}N-labelled protein[27]. Data were collected on a Bruker AMX 500 MHz NMR spectrometer. For heteronuclear experiments the spectral widths for the ^{1}H(F2) and ^{15}N(F1) dimensions were 14.08 p.p.m. and 31.98 p.p.m., respectively. Proton spectra were referenced to the carrier in both dimensions (4.76 p.p.m.). Heteronuclear spectra were referenced to the carrier in F1 (118.5 p.p.m.) and F2 (4.76 p.p.m.). Resonance assignments were made using standard methods[27,28] and were consistent with the assignments for wild-type GB1[22]. Observed cross strand NOEs include: H^N_4-H^α_{51}, H^N_6-H^N_{52}, H^N_6-H^α_{53}, H^α_7-H^N_{54}, H^N_8-H^N_{54}, H^N_8-H^α_{55}, H^N_{42}-H^N_{55}, H^α_{43}-H^α_{54}, H^N_{44}-H^N_{53}, H^N_{44}-H^α_{54}, H^N_{46}-H^N_{51}, H^β_{46}-H^N_{51}, H^N_{52}-H^N_4, H^δ_{52}-$H^{\zeta3}_{43}$, H^δ_{52}-$H^{\eta2}_{43}$, H^ε_{52}-$H^{\zeta3}_{43}$, H^ε_{52}-$H^{\eta2}_{43}$, H^β_{54}-$H^{\zeta3}_{43}$, H^β_{54}-H^δ_{43}, H^β_{54}-$H^{\zeta2}_{43}$, H^β_{54}-$H^{\eta2}_{43}$, H^β_{54}-H^α_{43}, H^β_{54}-$H^{\zeta3}_{43}$, $H^{\gamma1}_{54}$-H^δ_{43}, $H^{\gamma1}_{54}$-$H^{\zeta2}_{43}$, $H^{\gamma1}_{54}$-$H^{\zeta3}_{43}$, $H^{\gamma1}_{54}$-$H^{\varepsilon3}_{43}$, $H^{\gamma1}_{54}$-$H^{\eta2}_{43}$, $H^{\gamma1}_{54}$-$H^{N\varepsilon}_{43}$, $H^{\gamma2}_{54}$-H^δ_{43}, $H^{\gamma2}_{54}$-$H^{\zeta2}_{43}$, $H^{\gamma2}_{54}$-$H^{\zeta3}_{43}$, $H^{\gamma2}_{54}$-$H^{\varepsilon3}_{43}$, $H^{\gamma2}_{54}$-$H^{\eta2}_{43}$, $H^{\gamma2}_{54}$-$H^{N\varepsilon}_{43}$, H^N_{55}-H^α_{43}.

surface area of the side chain[6] (Fig. 3*c*, *d*). These correlations are striking as the amount of buried surface area differs substantially at centre and edge β-sheet positions[1]. Thus, these results suggest that interaction with the surrounding β-sheet structure (that is, component (2) above) is the dominant term for determining β-sheet propensity.

Our experiments indicate that β-sheet propensity is modulated strongly by tertiary context, even at solvent-exposed positions. This result provides an explanation for the differences in

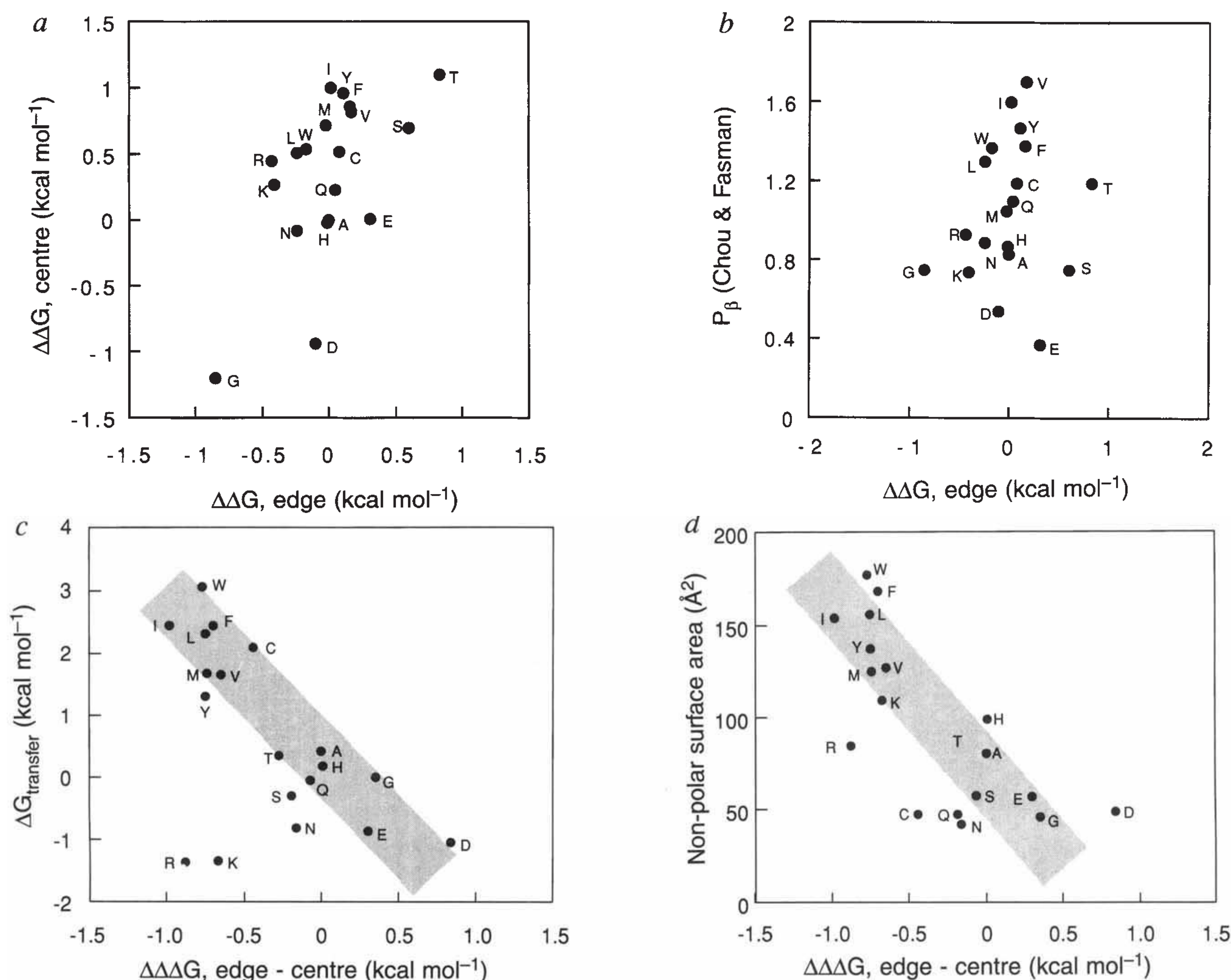

FIG. 3 *a*, Comparison of β-sheet forming propensities at central[2] and edge β-sheet positions. *b*, Comparison of the edge site β-sheet propensities with the β-sheet forming frequencies of Chou and Fasman[4]. The data are not correlated ($r=0.15$, $P>0.25$). *c*, Correlation of the difference in β-sheet forming propensity measured at edge and centre β-sheet positions, $\Delta\Delta\Delta G$, with ΔG of transfer for the amino acid side chains from octanol to water[5] ($r=0.57$, $P<0.005$; $r=0.80$, $P<0.0005$, excluding values of R and K, see methods) and *d*, side chain non-polar surface area[6] ($r=0.72$, $P<0.0005$). Amino acids are identified by the labels. Shaded areas are meant only to emphasize the overall trends in the data.

METHODS. Values for amino acid side chain non-polar surface areas are for 'set 1' from ref. 14. The values from 'set 2' show a similar trend. With respect to the correlation between $\Delta\Delta\Delta G$ and ΔG of transfer there are two clear outlying points, arginine and lysine. The apparent anomalous behaviour of these two residues probably reflects the large distance between the side chain charge and the backbone β-sheet structure. Correlation coefficients were calculated for the (x_i, y_i) pairs using the formula[29]:

$$r^2 = \left[\frac{\sum_{i=1}^{N} (x_i - \bar{x})(y_i - \bar{y})}{[\sum_{i=1}^{N} (x_i - \bar{x})^2 \sum_{i=1}^{N} (y_i - \bar{y})^2]^{1/2}} \right]^2$$

apparent β-sheet propensity measured in the zinc finger[19] and GB1 (refs 2, 3) model systems. More generally, our results emphasize that β-sheets are elements of both secondary and tertiary structure[20]. □

Received 13 June; accepted 2 August 1994.

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ACKNOWLEDGEMENTS. We thank Z.-Y. Peng for helpful advice and suggestions and L. M. Mayr, M. G. Oakley and P. A. Petillo critical reading of the manuscript. This work was supported by the Howard Hughes Medical Institute. D.L.M. is supported by a National Institutes of Health Biophysical Training Grant.