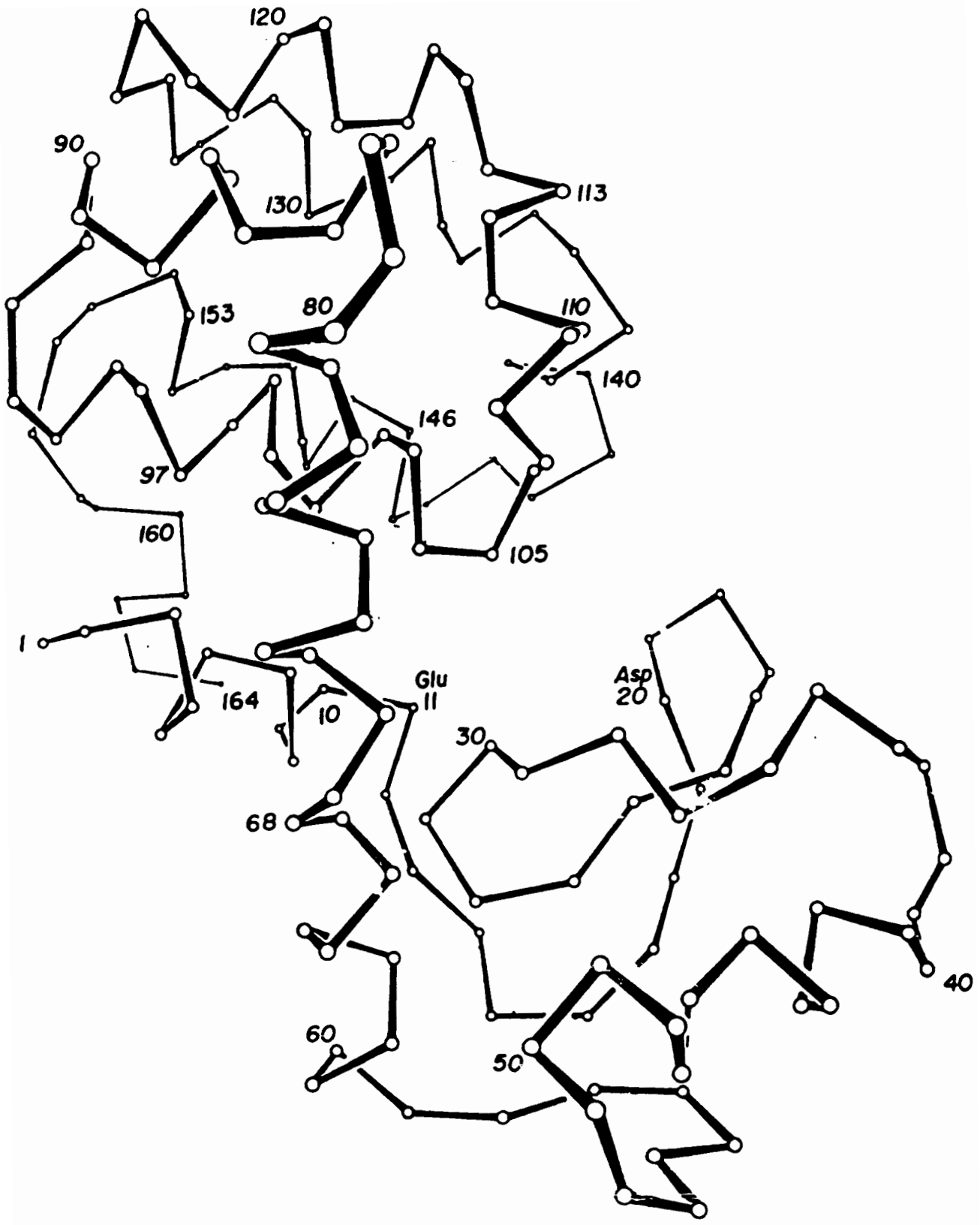
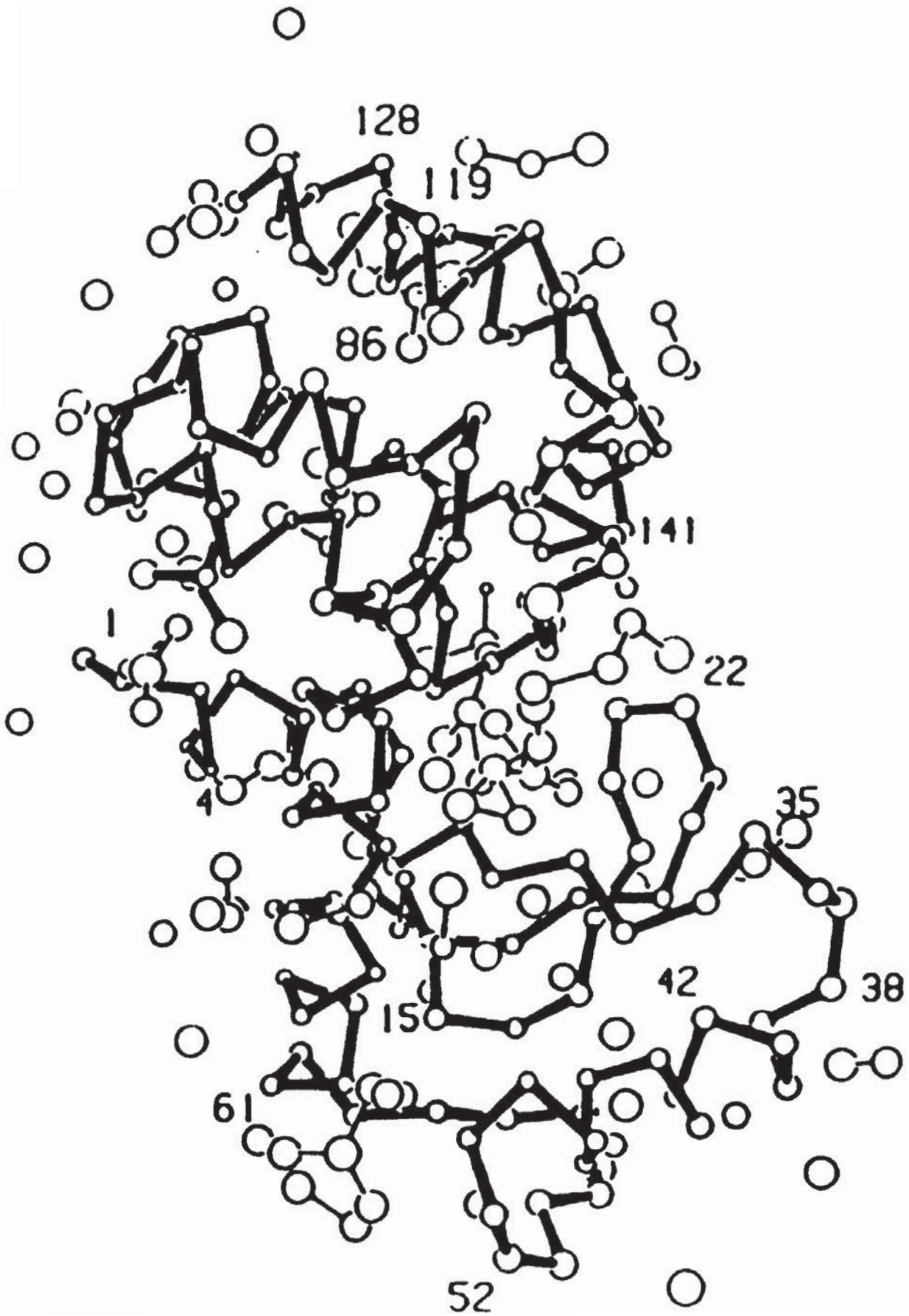


Backbone structure of T4 lysozyme


128
119
86
141
1
22
4
35
42
38
15
61
52

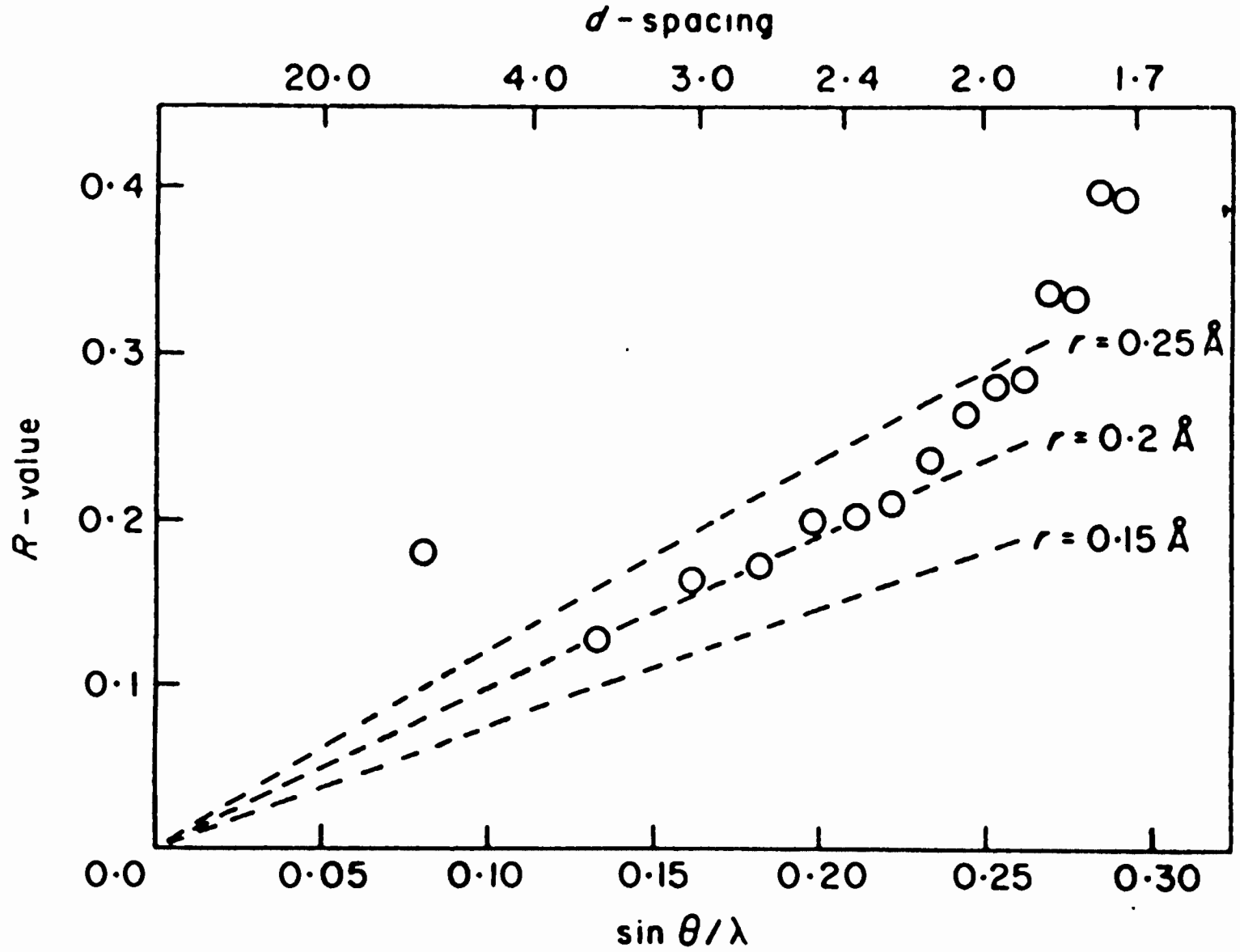


Figure 1. Luzzatti plot (Luzzatti, 1952) showing the observed crystallographic residual for all reflections and the expected profiles for co-ordinate errors of 0·15 Å, 0·20 Å and 0·25 Å.

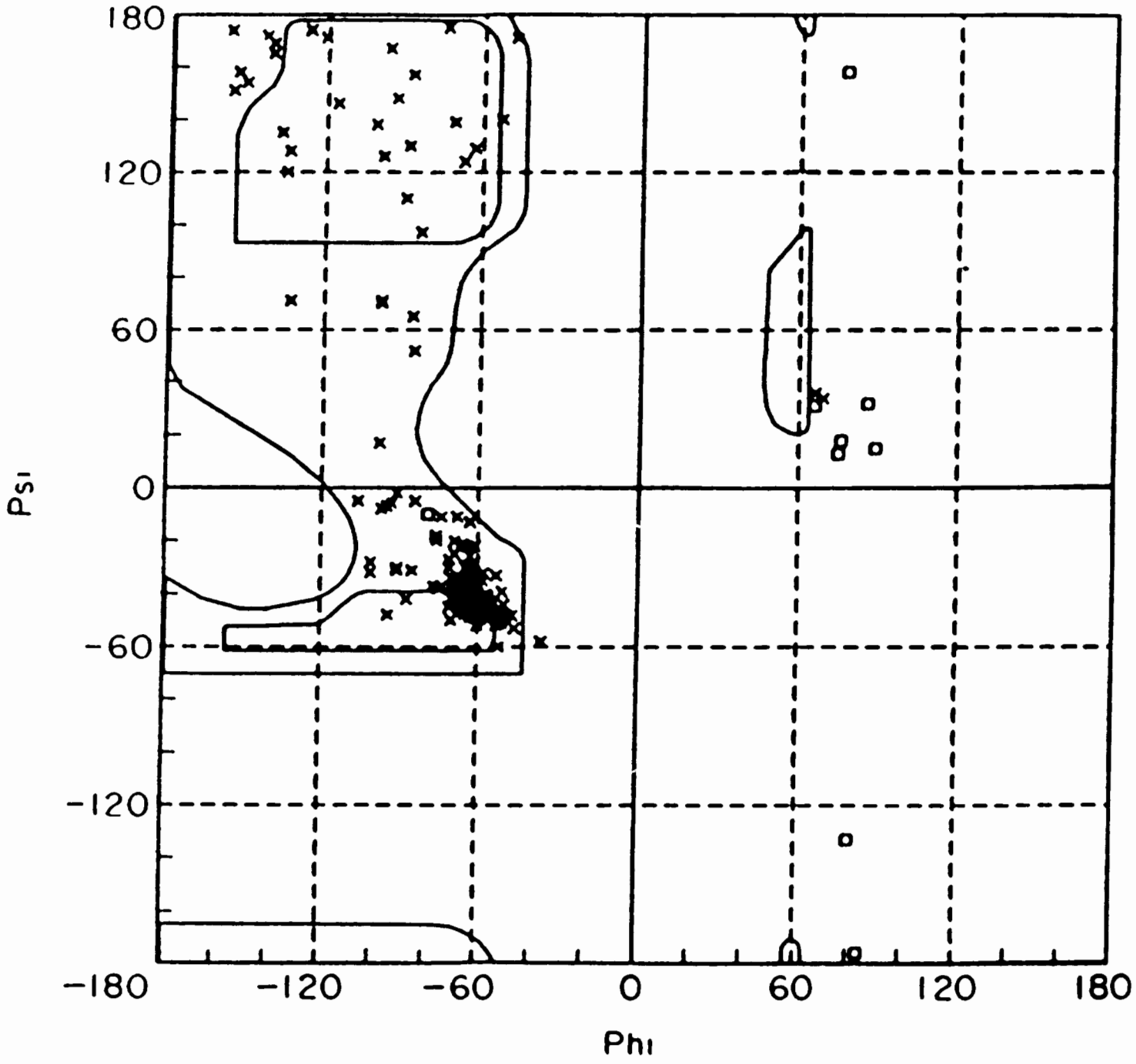


Figure 5. Ramachandran diagram for the lysozyme backbone. Glycine residues are indicated by squares and non-glycine by crosses.

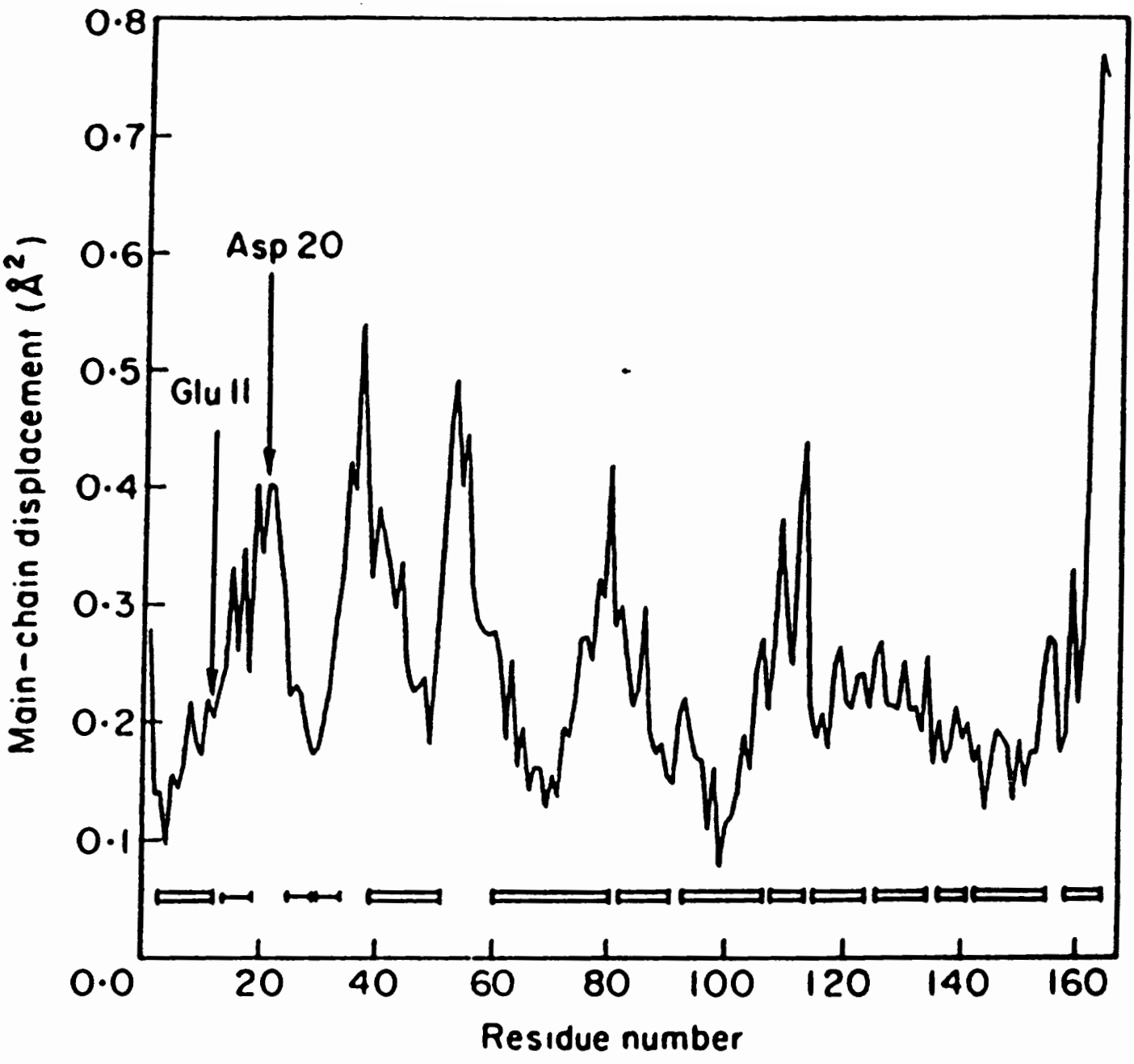
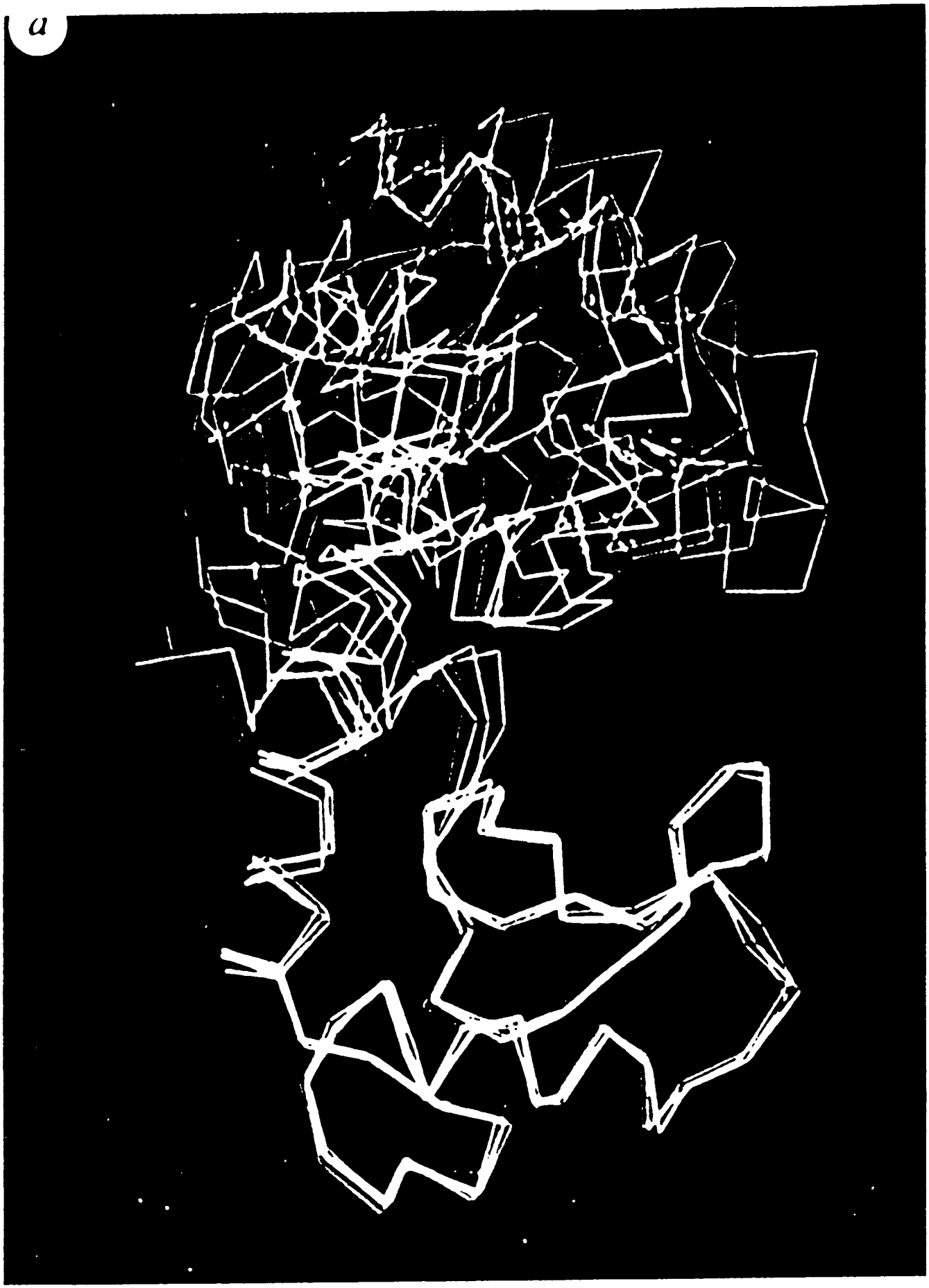
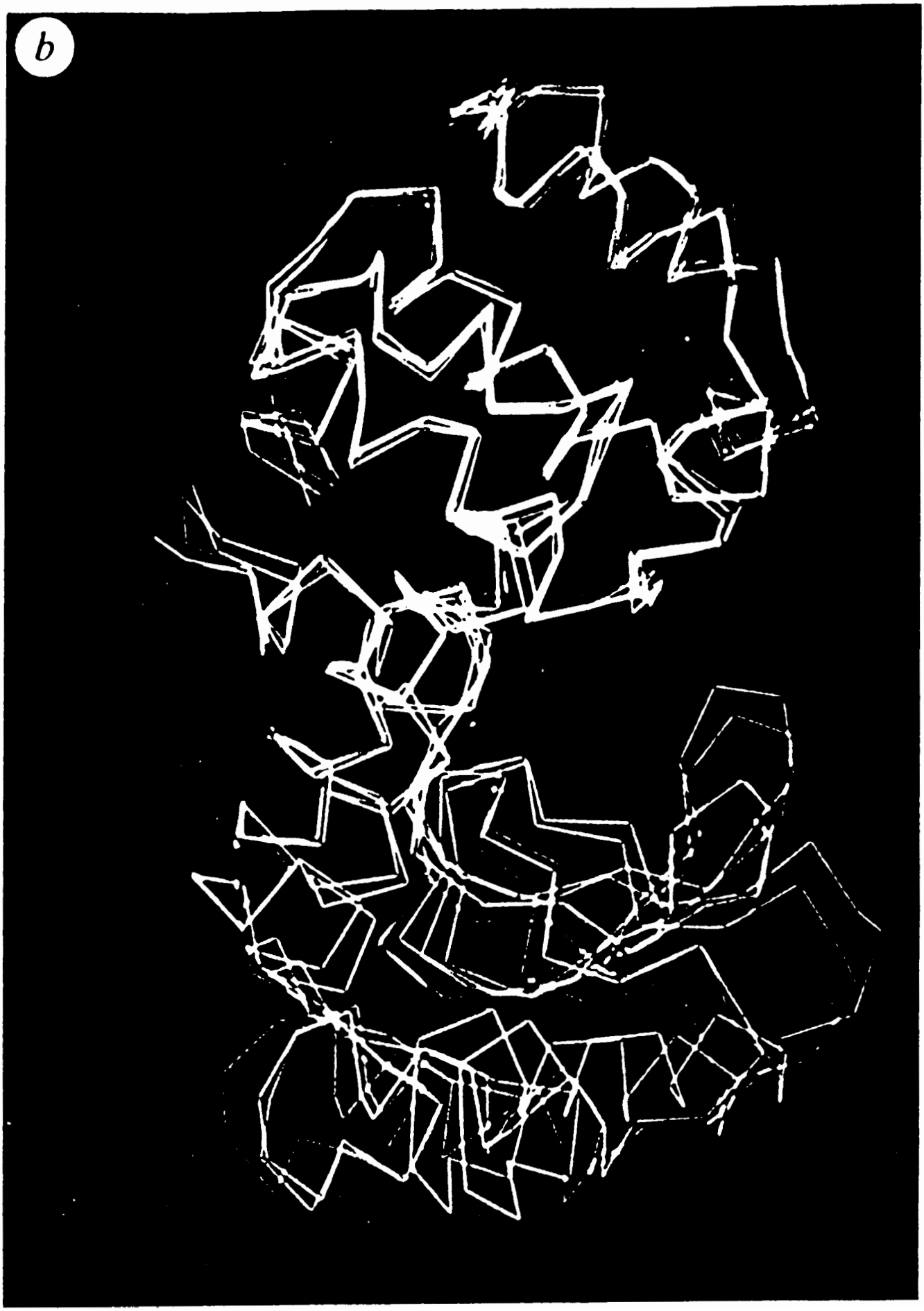


Figure 2. Apparent thermal motion of the phage lysozyme backbone plotted as a function of residue number. Locations of α-helices are indicated by double bars and β-sheet strands by single bars.

TABLE 1 Crystallographic data

Modification	Form I	Form II	
Crystals			
Space group	$P3_221$	$P2_12_12_1$	
Cell dimensions (a, b, c) (Å)	61.1, 61.1, 97.6	72.2, 73.8, 150.5	
Molecules per asymmetric unit	1	4	
Volume of crystals occupied by solvent (%)	56	54	
Data collection			
Diffractometer data	—	M6I	$HgCl_2$
Resolution (Å)	—	5.0	5.0
Reflections	—	3,526	3,616
R_{sym} (%)	—	6.6	8.4
Film data		M6I	
Resolution (Å)	1.8	2.1	
Reflections	13,645	28,684	
R_{merge} (%)	5.2	12.2	
Refinement			
Residual (%)	15.5	23.6	
No. solvent molecules	153	96	
r.m.s. deviations from ideal values			
Bonds (Å)	0.013	0.013	
Bond angles (degrees)	2.1	2.7	
Planarity (Å)	0.014	0.014	


a


b

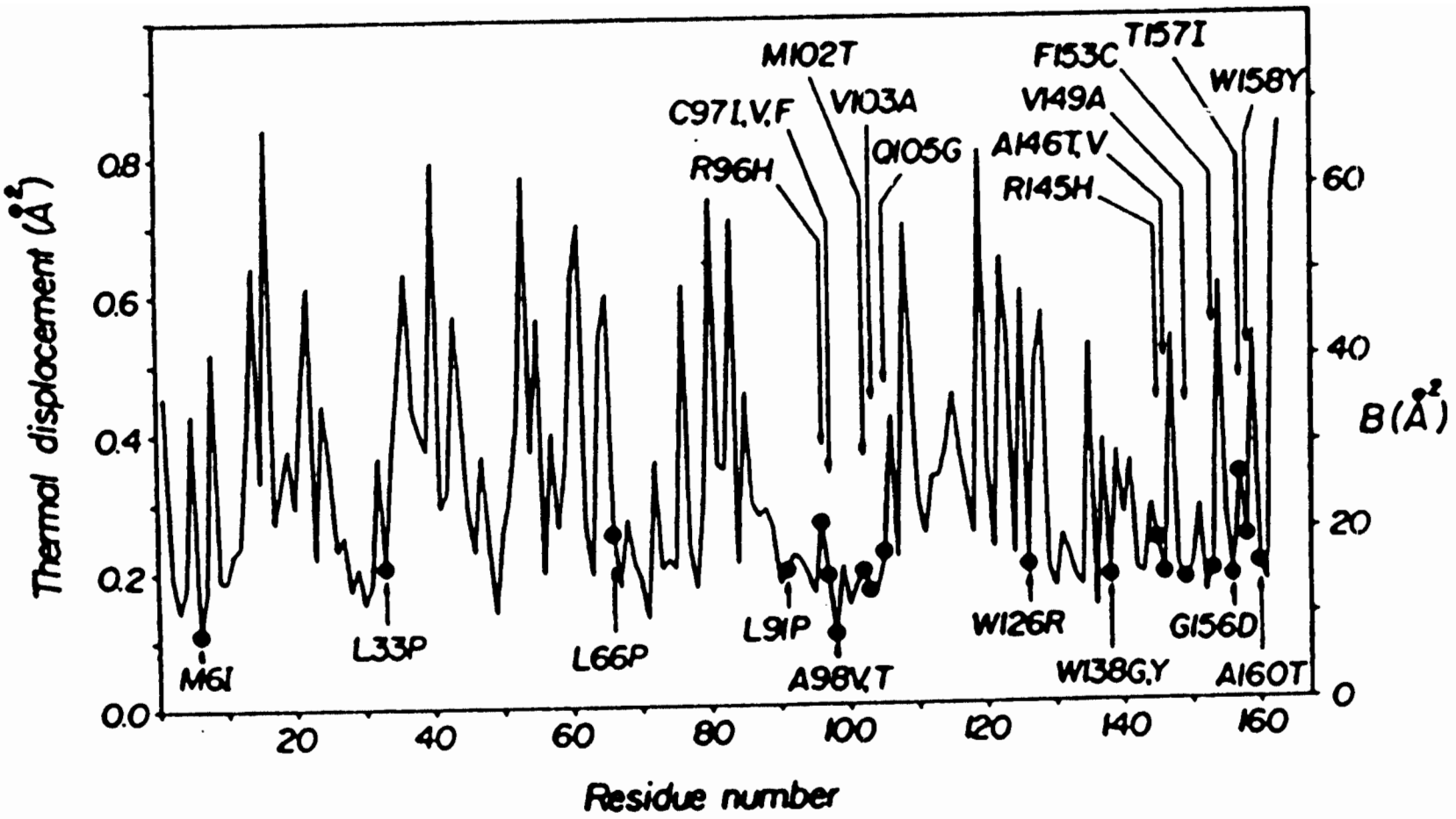


FIGURE 1: (a) Mean square thermal displacement of the side-chain atoms. The displacement for each residue was obtained by averaging the B values of the side-chain atoms (not including the α-carbon).

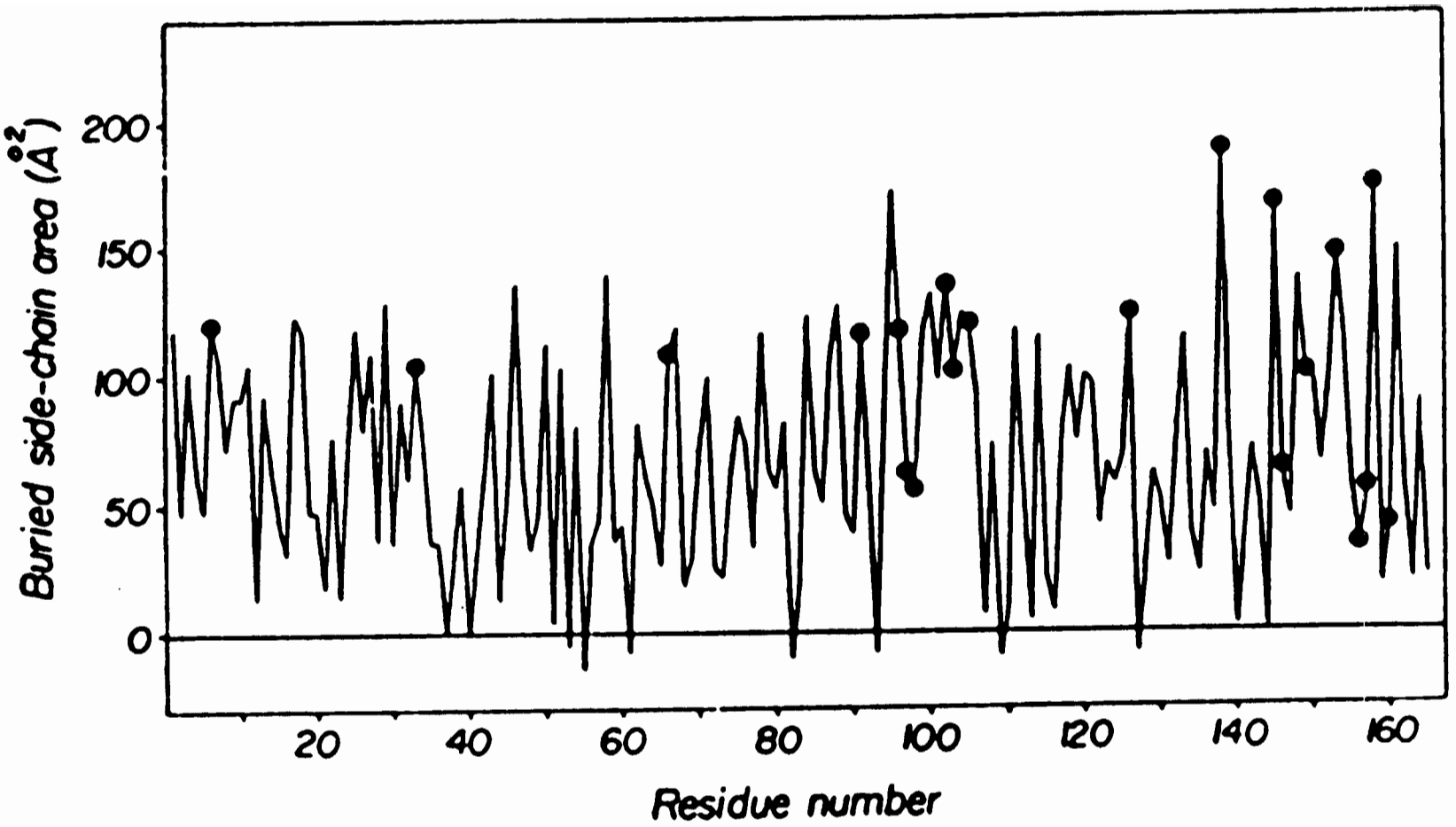


FIGURE 2: Buried surface area for each residue. The ordinate is $A_0 - A$, where A_0 is the side-chain surface area accessible to solvent in a model having the T4 lysozyme sequence in a fully extended conformation and A is the calculated side-chain solvent-accessible surface area in the X-ray crystal structure of phage T4 lysozyme. Dots indicate the ts mutations listed in Table I.

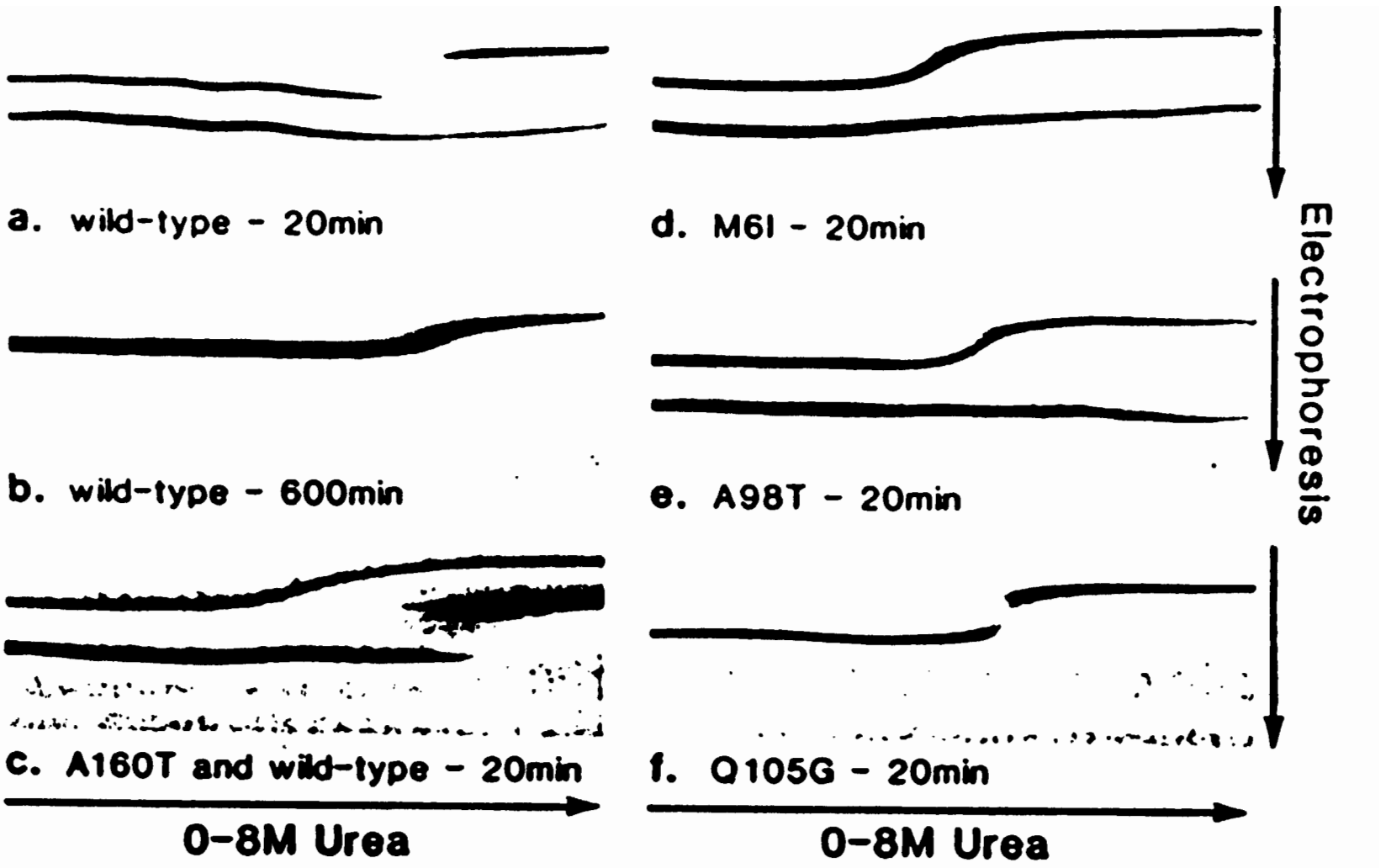
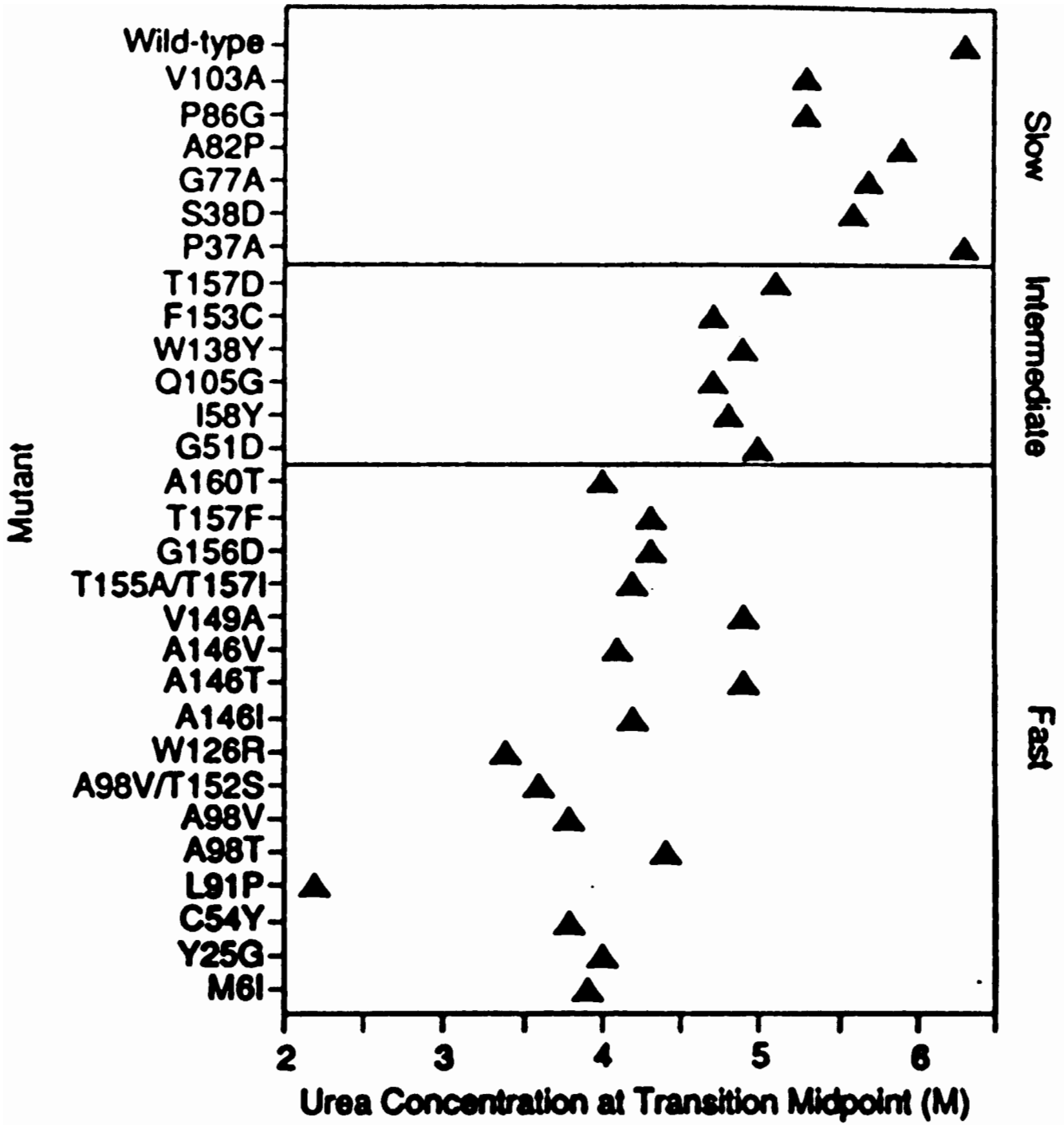


FIGURE 3: Urea gradient gel electrophoresis of wild-type and mutant forms of T4 lysozyme. The gels contained a continuous 0–8 M urea gradient with the urea concentration increasing from left to right.


Wild-type
V103A
P86G
A82P
G77A
S38D
P37A
T157D
F153C
W138Y
Q105G
I58Y
G51D
A160T
T157F
G156D
T155A/T157I
V149A
A146V
A146T
A146I
W126R
A98V/T152S
A98V
A98T
L91P
C54Y
Y25G
M6I
Mutant
Slow
Intermediate
Fast
2
3
4
5
6
Urea Concentration at Transition Midpoint (M)

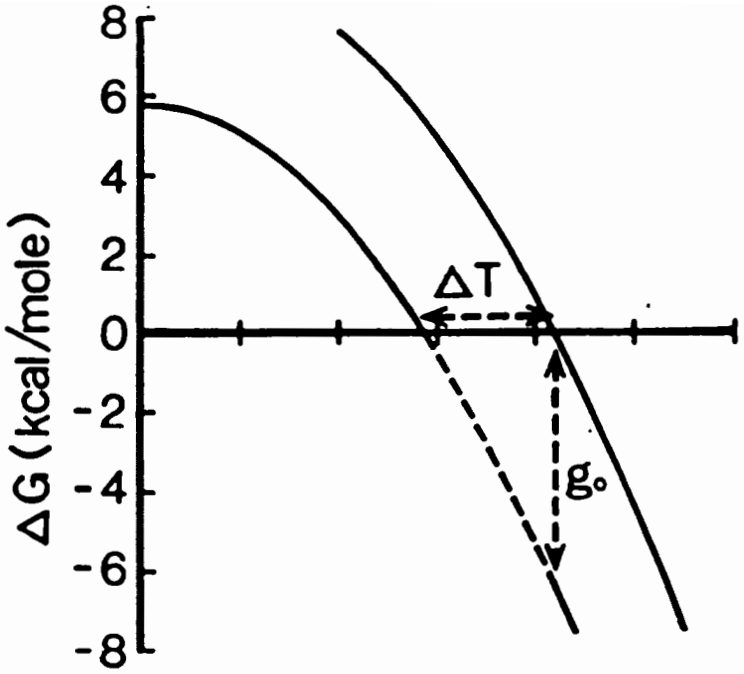


Fig. 8. The relationship between changes in melting temperature and changes in free energy.

It is possible to relate the change in melting temperature caused by a small perturbation to $g(T_g)$, the value of the perturbation free energy at the melting temperature of the protein in the reference state. This is demonstrated in Fig. 8. The slanting lines are the curves for $\Delta\bar{G}$ of the reference protein and $\Delta\bar{G}^*$ of the perturbed protein as they cross the abscissa at their melting temperatures T_g and T_g^*. As defined above, $g(T_g)$ is a vertical line connecting the two curves at T_g. Since $\partial\Delta\bar{G}/\partial T = -\Delta\bar{S}$, the slopes of the two curves at T_g and T_g^* are $-\Delta\bar{S}_g$ and $-\Delta\bar{S}_g^*$ respectively. If the stability curve for the perturbed protein can be approximated as a straight line, then from the figure,

$$\text{slope} = g(T_g)/(T_g^* - T_g) \quad \text{or} \quad \Delta T = g/\Delta\bar{S}_g^* \tag{18}$$

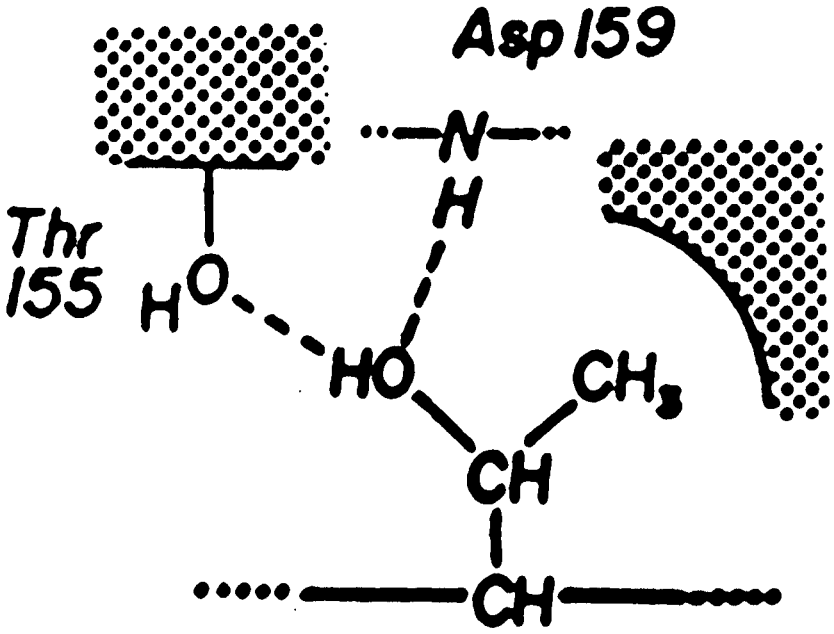
In the shorter notation on the right it is understood that g is evaluated at T_g and that ΔT is the difference between the melting temperatures of the mutant and reference proteins $(T_g^* - T_g)$. If we can further assume that the lines for $\Delta\bar{G}$ and $\Delta\bar{G}^*$ are parallel, then we can approximate $\Delta\bar{S}^*$ by $\Delta\bar{S}$ and write

$$\Delta T = g/\Delta\bar{S}_g = (gT_g)/\Delta\bar{H}_g \tag{19}$$

or in its most useful form

$$g = \Delta T\left(\Delta\bar{H}_g/T_g\right) \quad \text{or} \quad g = \Delta\bar{S}_g\Delta T \tag{20}$$

The utility of this formula is that if T_g and $\Delta\bar{H}_g$ are known for the protein in the reference state, then an estimate of g, the stabilization or destabilization of the perturbed state, can be obtained from a measurement of ΔT, which is the simplest measurement that one can make.


Asp 159
Thr 155
Thr 157
(Wild type)

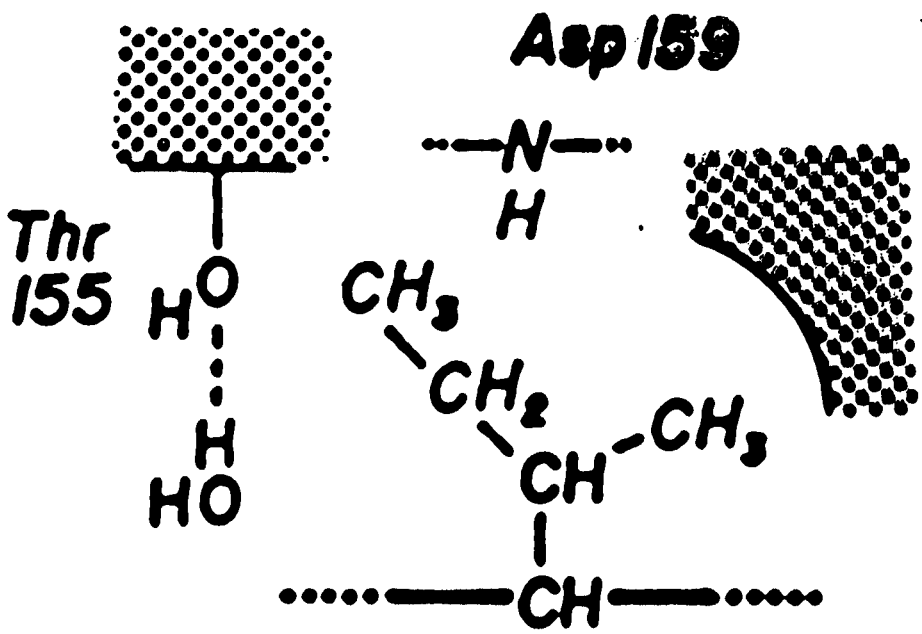

Asp 159
Thr 155
Ile 157
(Ts mutant)

Table 2 Thermodynamic stability of lysozymes with different amino acids at position 157

Amino acid at position 157	ΔT_m(°C)	$\Delta\Delta G$ (kcal mol^{-1})
Thr (wild type)	—	—
Asp	−1.7	0.45
Ser	−2.5	0.66
Asp	−4.2	1.1
Gly	−4.2	1.1
Cys	−4.9	1.3
Leu	−5.0	1.3
Arg	−5.1	1.3
Ala	−5.4	1.4
Glu	−5.8	1.5
Val	−6.0	1.6
His	−7.9	2.1
Phe	−9.2	2.4
Ile (ts mutant)	−11.0	2.9

Table 1 Thermal stability of lysozymes with different amino acids at position 3

Amino acid at position 3	ΔT_m (°C)		$\Delta\Delta G$ (kcal mol^{-1})		$-\Delta G_{tr}$ (kcal mol^{-1})
	*p*H 2.0	*p*H 6.5	*p*H 2.0	*p*H 6.5	
Trp	−16.4	−8.0	−3.6	−2.8	3.00
Ile	0.0	0.0	−0.0	0.0	2.97
Tyr	−9.5	−5.9	−2.7	−2.3	2.87
Phe	−4.0	−3.0	−1.0	−1.1	2.65
Leu	3.0	0.9	0.9	0.4	2.42
Val	−2.1	−1.2	−0.6	−0.4	1.69
Met	−0.9	−2.3	−0.3	−0.9	1.30
Cys (S-H)	−1.9	−3.7	−0.4	−1.2	1.00
Cys (S-S)	4.8	3.3	1.0	1.2	1.00
Ala	−3.8	−1.8	−1.1	−0.7	0.73
Thr	−6.1	−6.0	−1.7	−2.3	0.44
Ser	−7.0	−4.6	−1.9	−1.7	0.04
Gly	−7.2	−5.8	−1.8	−2.1	0.00
Glu	−4.1	−5.7	−1.1	−2.0	0.55 (−1.63)
Asp	−6.5	−8.5	−1.8	−3.2	0.54 (−2.63)

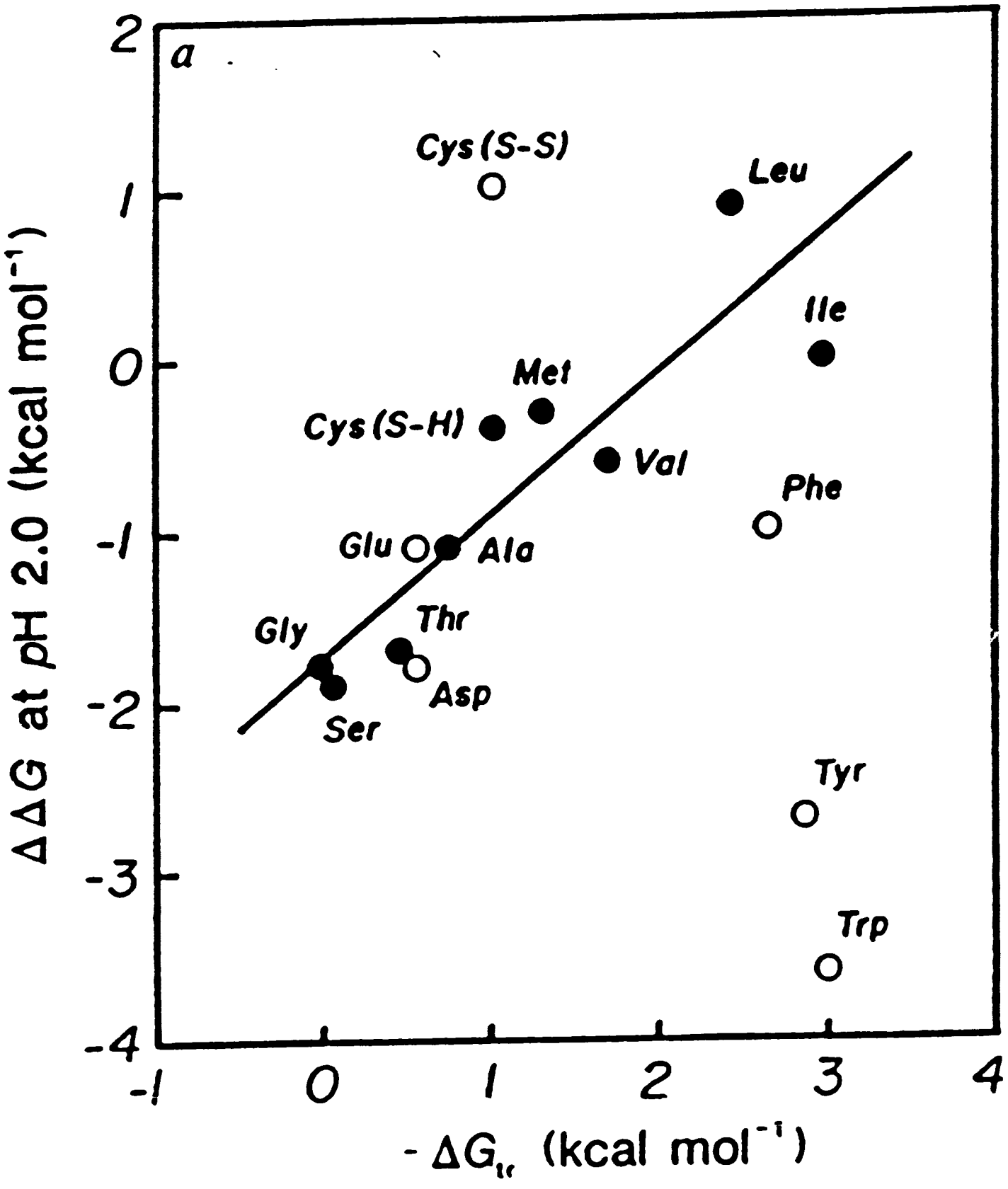

a
Cys (S-S)
Leu
Ile
Met
Cys (S-H)
Val
Phe
Glu
Ala
Gly
Thr
Asp
Ser
Tyr
Trp
2
1
0
-1
-2
-3
-4
-1
0
1
2
3
4
ΔΔG at pH 2.0 (kcal mol⁻¹)
- ΔG_tr (kcal mol⁻¹)

Table III: Changes in Free Energy and Enthalpy of Denaturation, at $t_{1/2}$ for the Wild-Type Protein, Produced by the Various Mutations[a]

protein	pH 2.0, 38.8 °C		pH 2.5, 46.2 °C		pH 3.0, 53.6 °C		pH 2.00, 42 °C
	$\Delta\Delta G°_d$	$\Delta\Delta H_d$	$\Delta\Delta G°_d$	$\Delta\Delta H_d$	$\Delta\Delta G°_d$	$\Delta\Delta H_d$	$\Delta\Delta G°_d$[b]
T157A	1.1	−2.1	0.9	−3.4	0.5	−4.6	1.4
T157E	1.5	9.7	1.3	11.9	0.8	14.1	1.1
T157I	2.1	−2.2	1.9	−1.1	1.5	0.0	2.9
T157L	1.7	7.2	1.7	9.2	1.7	11.1	1.3
T157N	1.0	−3.7	1.1	−5.8	1.2	−7.8	0.45
T157R	1.1	−3.5	0.6	−1.8	−0.3	−0.1	1.3
T157V	1.7	1.3	1.6	0.9	1.2	0.4	1.6

[a] $\Delta\Delta G°_d$ and $\Delta\Delta H_d$ (kcal mol^{-1}). Estimated uncertainties: $\Delta\Delta G°_d$, ±0.4 kcal mol^{-1}; $\Delta\Delta H_d$, ±4 kcal mol^{-1} (average value). [b] Alber et al., 1987.

Table II: Stability of Mutant Lysozymes[a]

	pH	T_m (°C)	ΔT_m (°C)	ΔH (kcal/mol)	$\Delta\Delta G$ at T_m of the mutant (kcal/mol)
WT*	3.0	51.6		117	
M102K		16.6	−35.0	12.4	−8.9
L133D		36.7	−14.9	47.9	−4.9
WT*	4.4	64.6		138	
L133D		49.6	−15.0	59.1	−5.5
WT*	5.3	65.3		135	
M102K		45.0	−20.3	53	−6.9
WT*	6.5	61.9		121	
L133D		44.0	−17.9	50.8	−5.7
WT*	10.4	47.9		79.5	
M102K		37.8	−10.1	77.4	−2.2
L133D		28.9	−19.0	47.1	−3.7

[a] T_m is the melting temperature of the mutant lysozyme, and ΔT_m is the difference between mutant and wild type. ΔH is the enthalpy of unfolding at T_m, and $\Delta\Delta G$ is the difference between the free energy of unfolding of the mutant and that of WT*. $\Delta\Delta G$ values at each pH value were determined at the T_m of the mutant by means of a van't Hoff plot of the WT* data

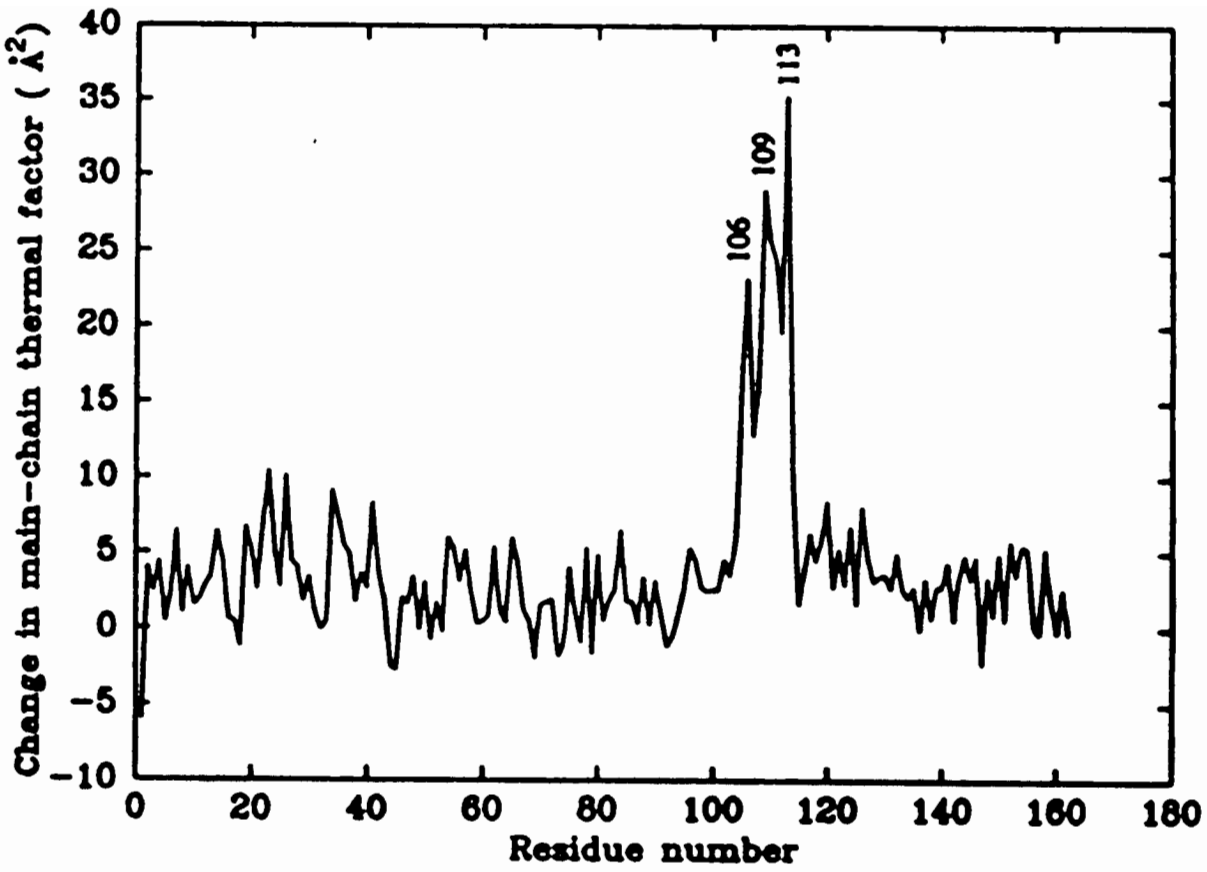


FIGURE 6: Plot showing the difference between the backbone mobility of mutant M102K and wild-type lysozyme. For each residue in the respective structures the individual crystallographic thermal factors for N, CA, C, and O were averaged to give a mean value. The plot shows the differences between these values for M102K and wild-type lysozymes.

Table 1. Thermodynamic parameters for wild-type and mutant lysozymes

Protein	T_m, °C	ΔT, °C	ΔH, kcal/mol	ΔS, cal/deg·mol	$\Delta\Delta H$, kcal/mol	$\Delta\Delta S$, cal/deg·mol	$\Delta\Delta G$, kcal/mol	$-T_m\Delta S_{Z,Y}$, kcal/mol
				Denaturation at pH 2.0				
Wild type	41.9 ± 0.4	—	89 ± 5	282 ± 16	—	—	—	—
G77A	40.5 ± 0.7	−1.4 ± 0.8	85 ± 4	270 ± 13	−1 ± 6	−3 ± 21	−0.4	0.8
A82P	42.7 ± 0.1	0.8 ± 0.4	90 ± 5	283 ± 16	−1 ± 7	−2 ± 23	0.3	1.3
				Denaturation at pH 6.5				
Wild type	64.7 ± 0.5	—	129 ± 9	381 ± 27	—	—	—	—
G77A	65.6 ± 0.2	0.9 ± 0.5	125 ± 9	368 ± 27	−6 ± 13	−18 ± 38	0.4	0.8
A82P	66.8 ± 0.2	2.1 ± 0.5	126 ± 9	371 ± 26	−7 ± 13	−22 ± 37	0.8	1.4

The thermodynamic parameters were derived from van't Hoff analyses of reversible thermal denaturations of the wild-type and mutant proteins. Equilibrium constants were obtained from the fraction of native protein present under a given set of conditions of sequence, temperature, and pH. T_m is the temperature of denaturation, and ΔT is the difference in melting temperature. ΔH is the enthalpy of unfolding, and $\Delta\Delta H$ is the difference in unfolding enthalpy of mutant and wild-type proteins measured at the melting temperature of the wild-type protein. ΔS is the entropy of unfolding, and $\Delta\Delta S$ is the difference in unfolding entropy of mutant and wild-type proteins. The difference between the free energy of unfolding of mutant and wild-type proteins, $\Delta\Delta G$, is the observed free energy of stabilization and can be compared with $-T_m\Delta S_{Z,Y}$, which is the backbone entropic stabilization estimated from Eq. 3. The temperature variation of the enthalpy and entropy of denaturation for these T4 lysozymes, ΔC_p, was determined to be 2.0 ± 0.2 kcal/deg·mol. Additional details will be presented elsewhere.

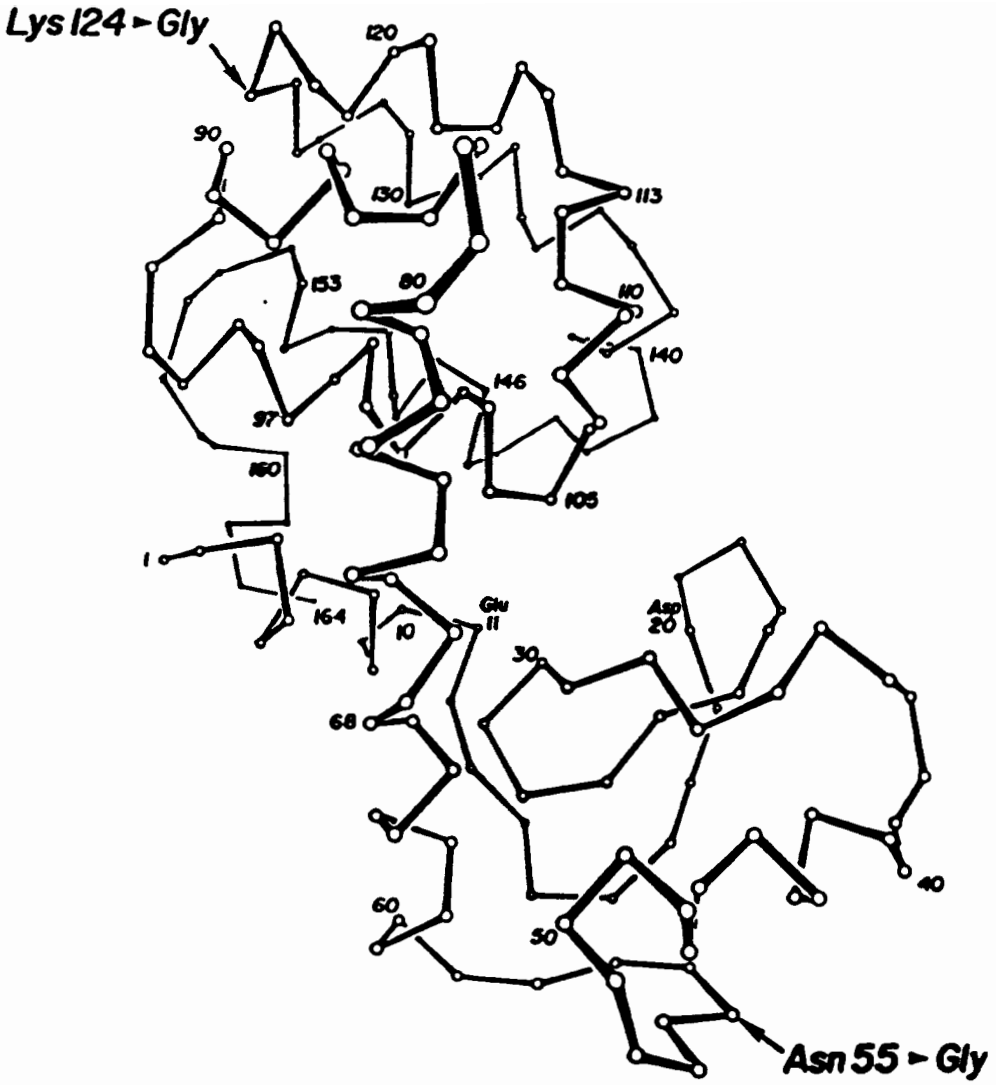


Figure 1. Backbone of T4 lysozyme showing the locations of the 2 left-handed helical residues that were replaced by glycine.

Table 1
Thermodynamic stability of mutant lysozymes

Protein	pH 2·0			pH 6·5		
	t_m (°C)	t_m (°C)	$\Delta\Delta G$ (kcal/mol)	t_m (°C)	t_m (°C)	$\Delta\Delta G$ (kcal/mol)
WT	41·9	—	—	64·7	...	...
N55G	40·2	−1·7	−0·5	63·1	−1·6	−0·6
K124G	41·9	0·0	0·0	64·5	−0·2	−0·1

t_m is the melting temperature (±0·5 deg.C), Δt_m the change in the melting temperature relative to wild-type lysozyme, and $\Delta\Delta G$ is the change in the free energy of stabilization of the mutant structure relative to wild-type, estimated from Δt_m by the relationship given by Becktel & Schellman (1987). Wild-type data are from Matthews *et al.* (1987).

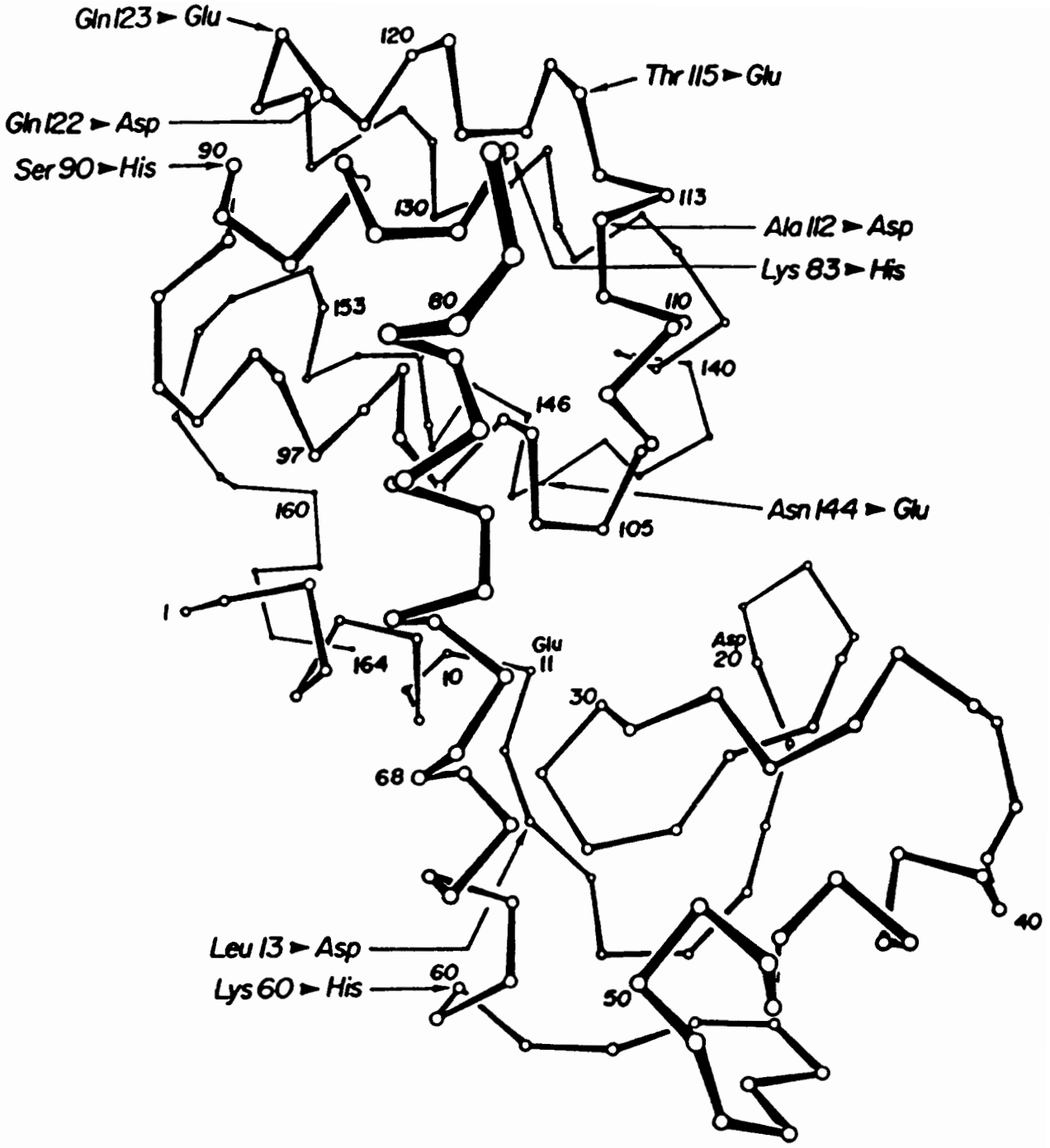


FIGURE 1: Backbone of T4 lysozyme showing the mutations used both to introduce putative salt bridges. The control mutations, used to delete the interacting partner, are not shown.

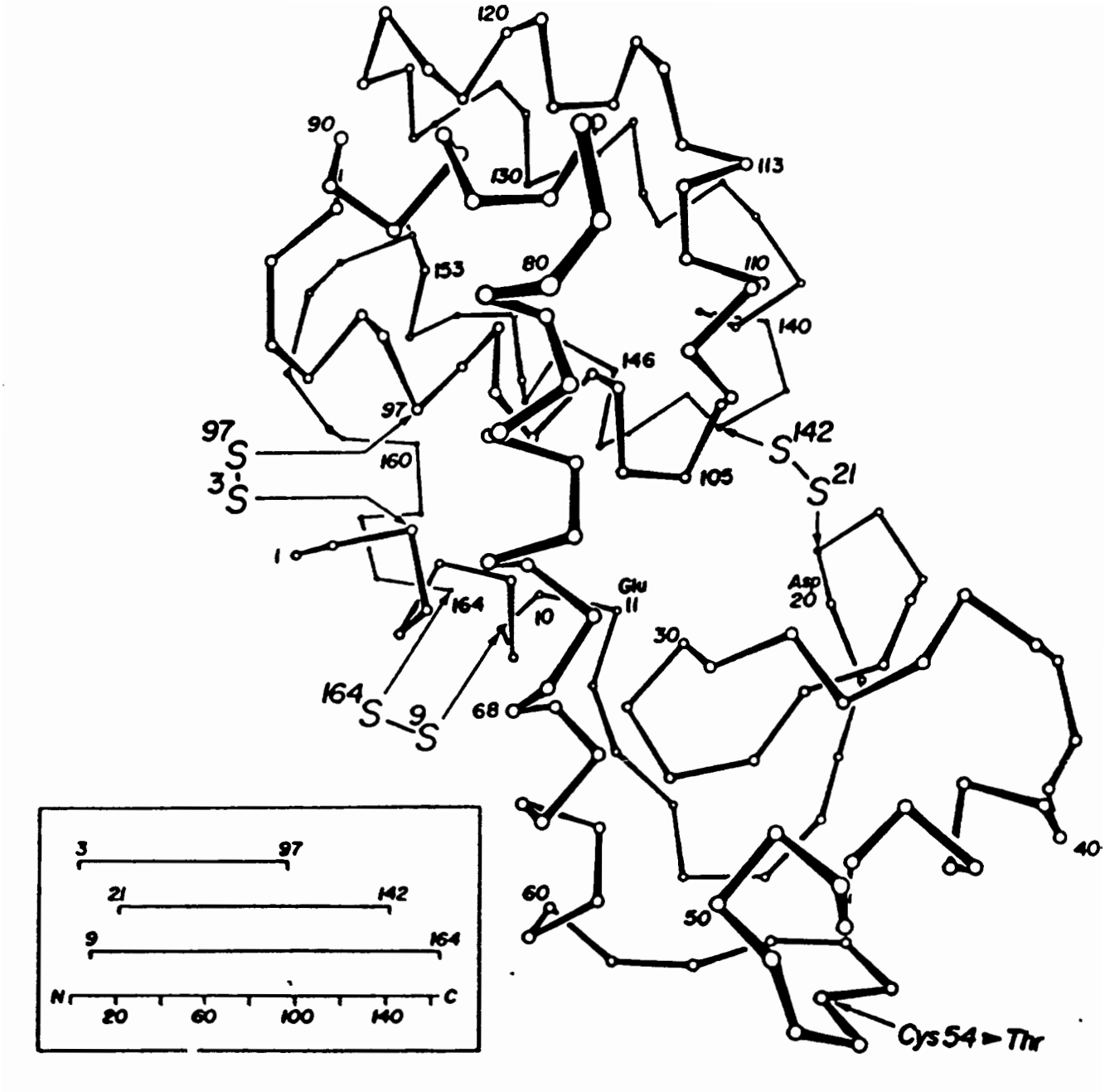
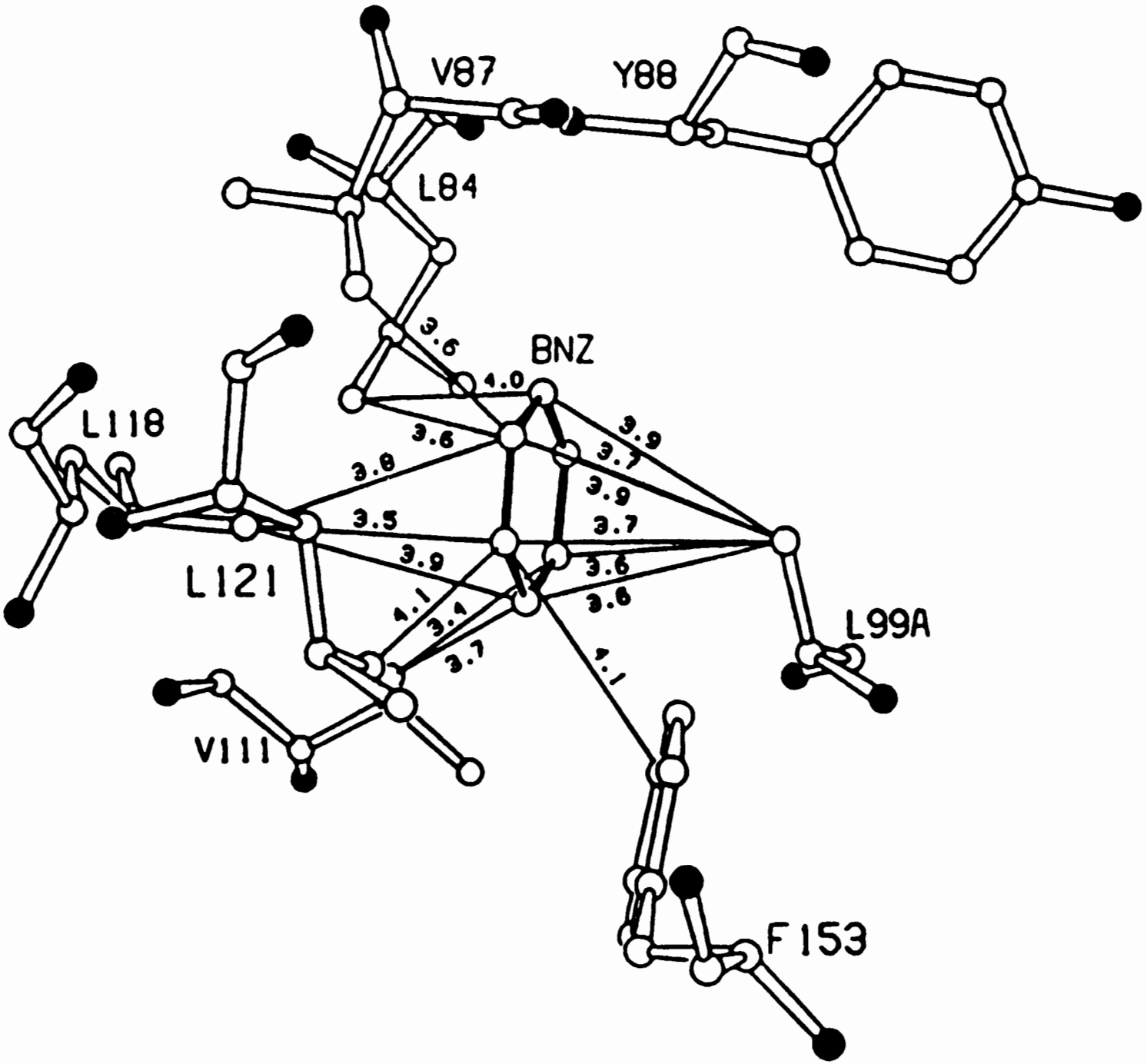


FIG. 1 Backbone of T4 lysozyme showing the locations of the three engineered disulphide bridges. The insert illustrates the loops formed by these bridges.

TABLE 1 Effect of benzene on the stability of mutant lysozymes

Protein	Concentration of benzene (mM)	pH	T_m (°C)	ΔT_m (°C)	ΔH (kcal mol^{-1})	$\Delta\Delta G$ (kcal mol^{-1})
WT*	0.0	3.01	51.6	—	119.0	—
	10.4	3.01	51.5	−0.1	113.3	0.0
L99A	0.0	3.01	36.2	—	82.4	—
	2.6	3.01	40.7	4.5	97.2	1.4
	5.6	3.01	41.6	5.4	100.6	1.7
	7.5	3.01	42.2	6.0	98.8	1.9
F153A	0.0	3.01	39.5	—	72.5	—
	3.0	3.01	39.5	0.0	71.6	0.0
	7.2	3.01	39.4	−0.1	70.6	0.0
L99A/F153A	0.0	4.46	41.7	—	45.3	—
	10.4	4.46	44.7	3.0	63.5	0.6

WT* is a pseudo wild-type lysozyme in which the two cysteines present in normal wild-type lysozyme were replaced with threonine and alanine (C54T/C97A)[21]. All mutants were constructed in this background. T_m is the melting temperature of the protein; ΔT_m is the change in melting temperature of the same protein in the presence of benzene. ΔH is the enthalpy of unfolding at T_m. $\Delta\Delta G$ is the change in the free energy of unfolding of the protein caused by the presence of benzene, estimated from the relationship[22] $\Delta\Delta G = \Delta S \cdot \Delta T_m$, where ΔS is the entropy of unfolding of the benzene-protein complex. A positive value of $\Delta\Delta G$ indicates an increase in stability. The estimated error in T_m is ±0.2 °C and in ΔH is ±4 kcal mol^{-1}. Thermal unfolding[23] was done in 25 mM KCl, 20 mM KPO_4 in the presence of varying amounts of benzene. Experiments for the double mutant were at pH 4.46 as this protein is partially unfolded at pH 3.0. Because of this change in pH, the T_m of the double mutant cannot be directly compared with the other proteins. Folding was >95% reversible in all cases.


V87
Y88
L84
3.6
BNZ
4.0
L118
3.6
3.9
3.7
3.8
3.9
3.5
3.7
3.9
3.6
L121
3.6
4.1
3.4
3.7
4.1
L99A
V111
F153

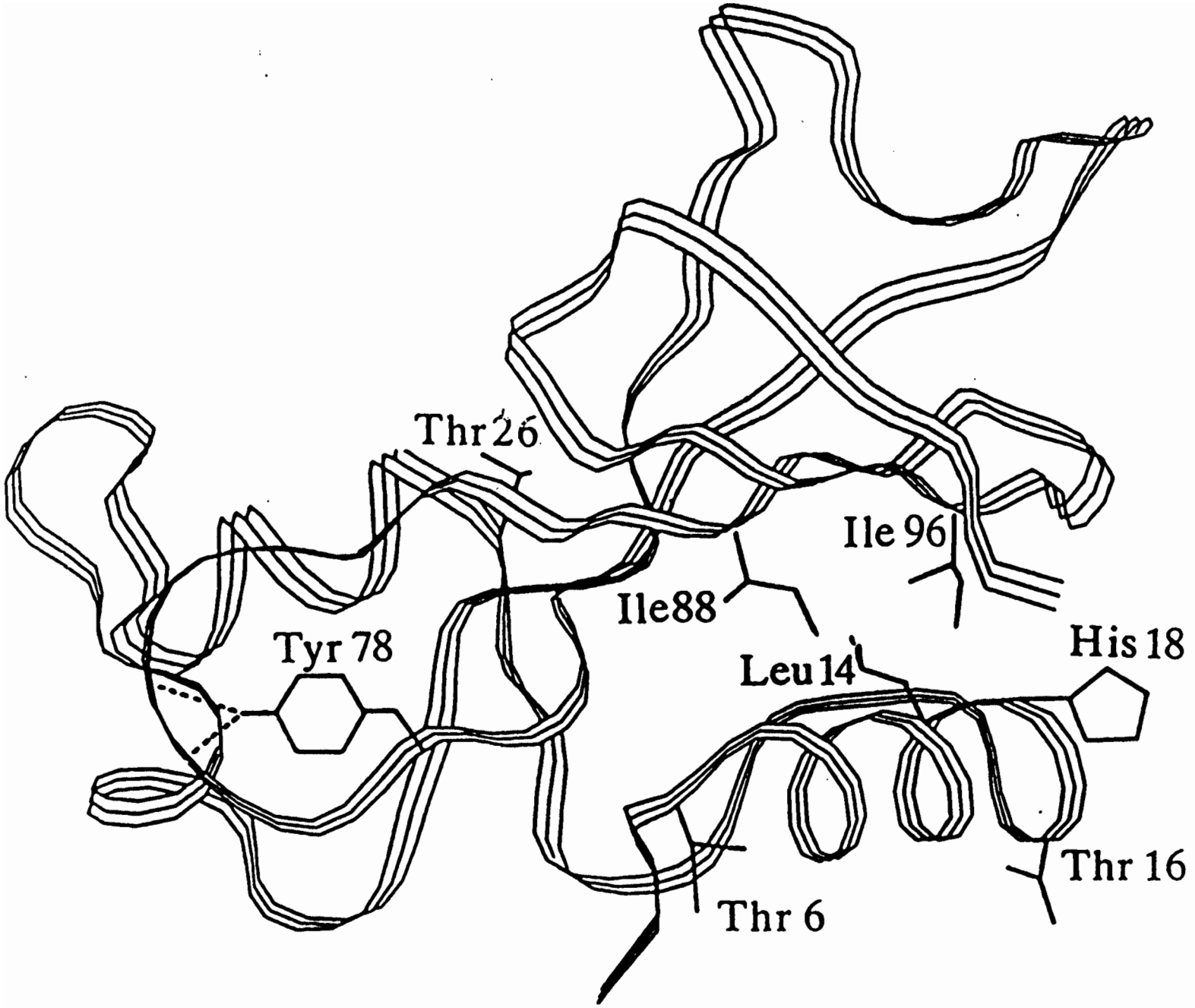


Figure 1. Sketch of barnase, showing the initial residues mutated in this study.

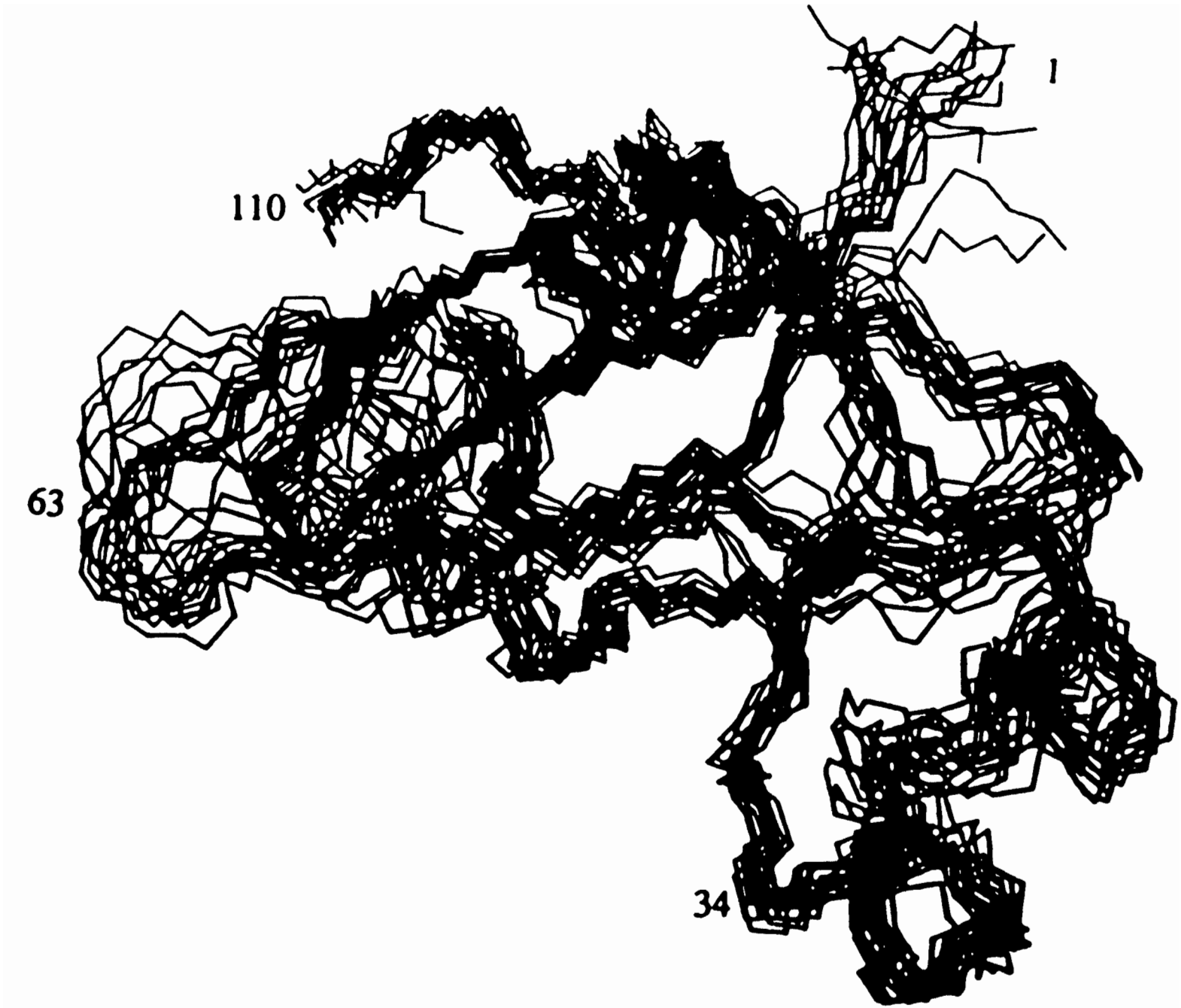


FIGURE 2: Superposition of the backbone atoms of the 20 solution structures of barnase.

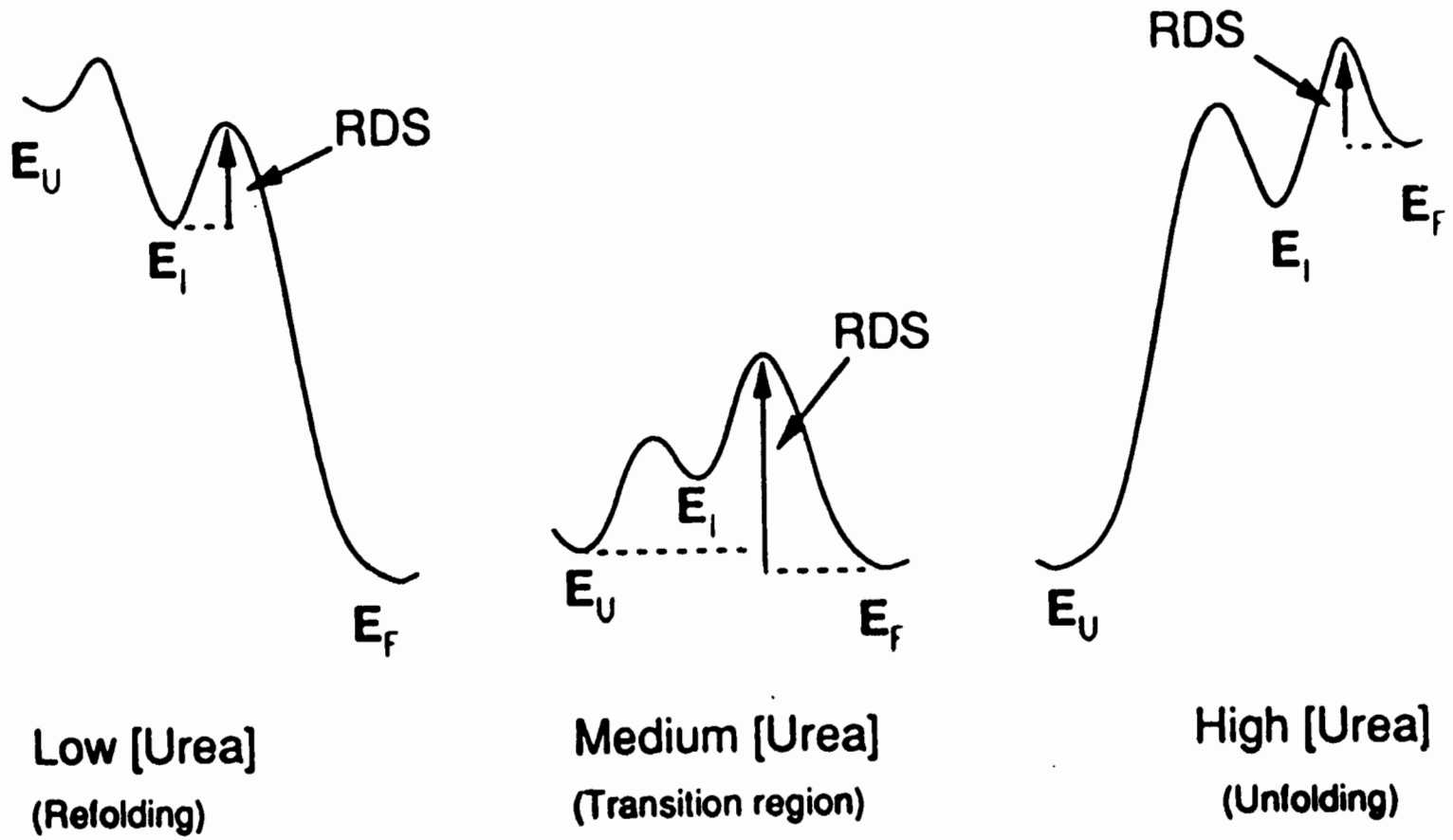


Figure 2. Minimal free energy profiles that describe the unfolding and refolding of barnase at low (< 2 M), medium (4–5 M) and high (> 6 M) [urea].

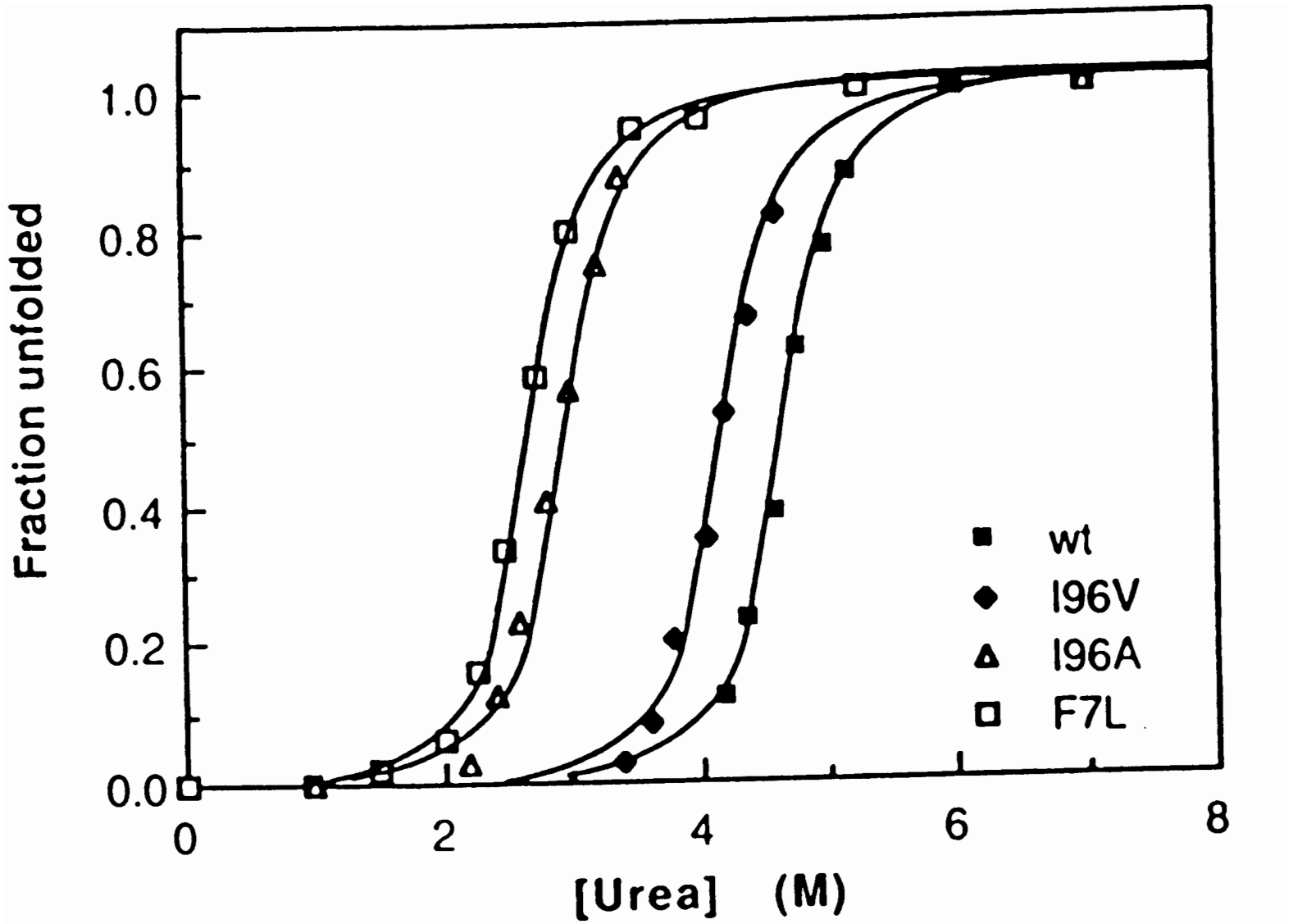


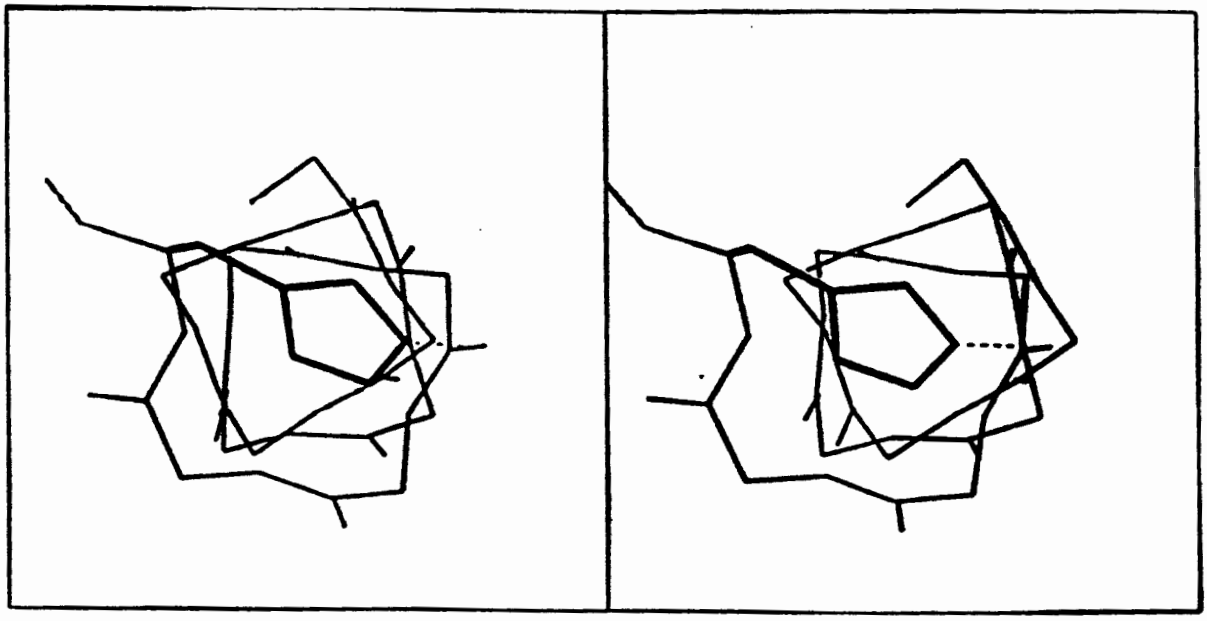
Fig. 1 Urea denaturation of recombinant barnase and engineered mutants. Denaturation was observed spectroscopically using difference spectroscopy at 286 and 270 nm.

Mutant	$\Delta\Delta G_D^*$ (in urea)	$\Delta\Delta G_D^{H_2O}$† (at 0 M urea) (kcal mol^{-1})	ΔG_{solv}‡
Ile → Val96	1.1	1.2	−0.12
Ile → Ala96	4.0	4.0	−0.15
Phe → Leu7	4.6	4.1	2.24

* $\Delta G_{D(wt)} - \Delta G_{D(mut)}$ calculated by direct comparison at the same concentrations of urea (wild type and Ile → Val96) or interpolation to an intermediate concentration of urea (Ile → Val96 and Ile → Ala96 at 3.5 M urea, Ile → Ala96 and Phe → Leu7 at 2.8 M urea, see Fig. 2 and text). The similarities in the slopes of the denaturation curves renders the interpolation insensitive to the choice of [urea] within ±1 M.

† $\Delta G^{H_2O}_{D(wt)} - \Delta G^{H_2O}_{D(mut)}$ from extrapolation to [urea] = 0 M (equation (1)). Note that there is expected to be a small discrepancy between this and the previous column because of the different stabilities of the exposed side chains in the denatured state when exposed to water and urea. For complete exposure of the side chain, this is negligible for Ile → Val, +0.15 kcal mol^{-1} for Ile → Ala and is +0.18 kcal mol^{-1} for Phe → Leu at 4 M urea (the larger side chains being more stable in urea)[25].

‡ Data from ref. 23 for the change in free energy of solvation of side chain ($G_{solv(mut)} - G_{solv(wt)}$).



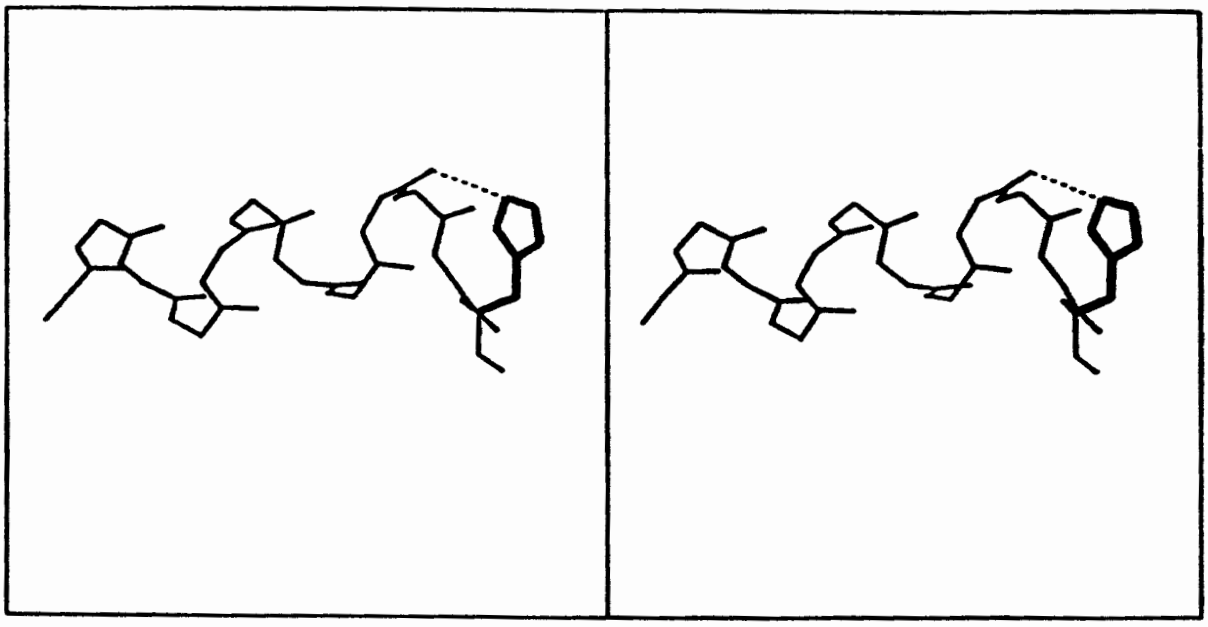


TABLE 2 Change in free energy of unfolding ($\Delta\Delta G_U$) on mutation of N-caps (Thr 6 and Thr 26)*

Mutation	$\Delta\Delta G_U$ (kcal mol^{-1}) Position 6	Position 26
Thr → Ser	0.22	0.56
Thr → Val		2.31
Thr → Ala	2.53	2.11
Thr → Gly	1.34	1.58
Thr → Asn	1.27	1.29
Thr → Asp	−0.11	
Thr → Gln	1.87	1.72
Thr → Glu	0.27	0.05
Asp → Asn	1.38	
Glu → Gln	1.60	1.67
Asp → Glu	0.38	
Asn → Gln	0.60	0.43
Ser → Ala	2.31	1.55
Ser → Gly	1.12	1.02
Gly → Ala	1.19	0.53

* $\Delta\Delta G_U$ is the free energy of unfolding of wild-type enzyme minus that of mutant. The free energies of unfolding of wild-type protein and mutants were analysed by urea denaturation monitored by fluorescence[5-7]

TABLE 3 Comparison of statistical and energetic surveys of N-cap stabilities

Preferred residue at N-cap	Stabilization energy relative to Thr (kcal mol^{-1})*	Frequency at N-cap†	Relative value‡
Asp	−0.1	27	2.1
Thr	0.0	21	1.6
Glu	0.2	5	0.4
Ser	0.4	34	2.3
Asn	1.3	34	3.5
Gly	1.5	33	1.8
Gln	1.8	3	0.4
Ala	2.3	10	0.5
Val	2.3	1	0.1

* Average value at positions 6 and 26.

† Frequency found in survey in ref. 2.

‡ Frequency at N-cap normalized for frequency of occurrence in proteins in general[2].

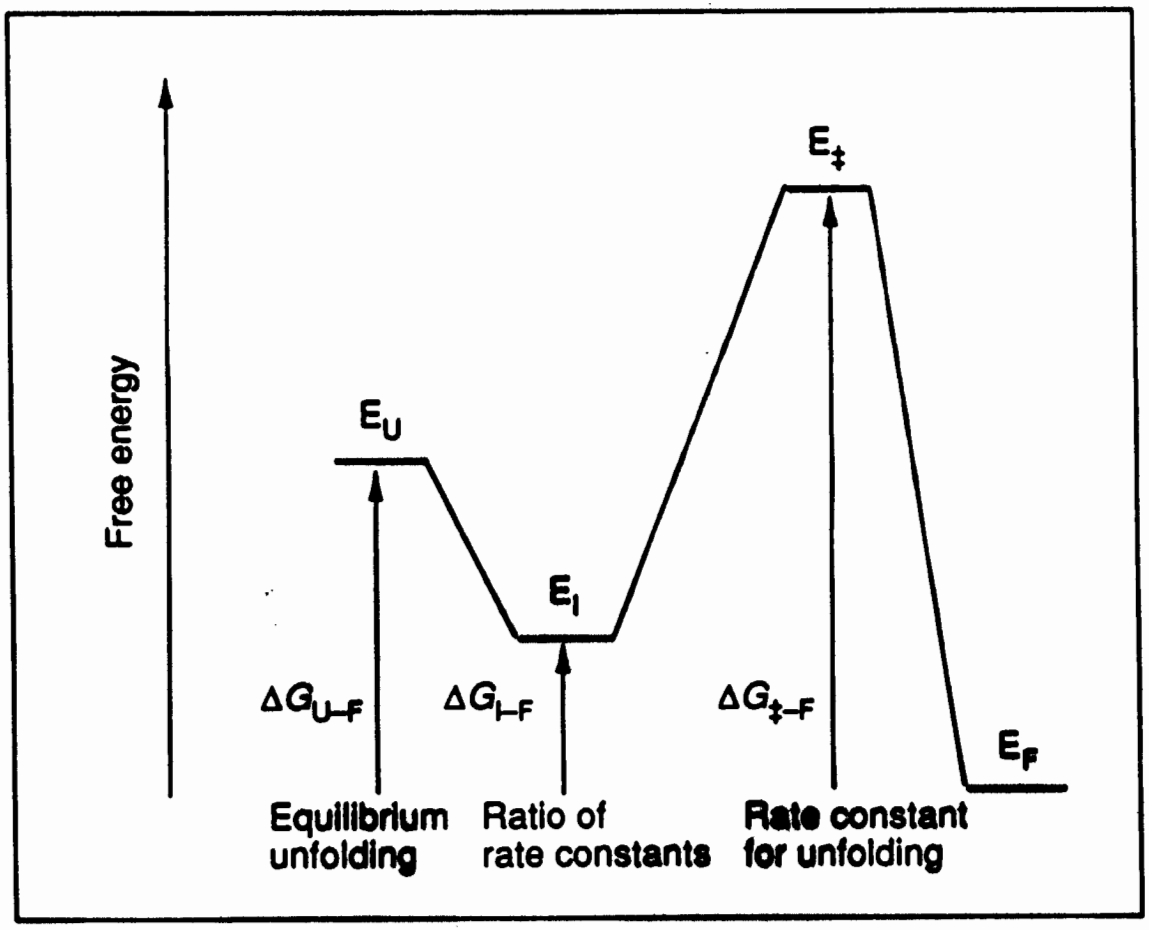


Fig. 4. Free energy profile of barnase folding showing how the energies of the different states can be determined.

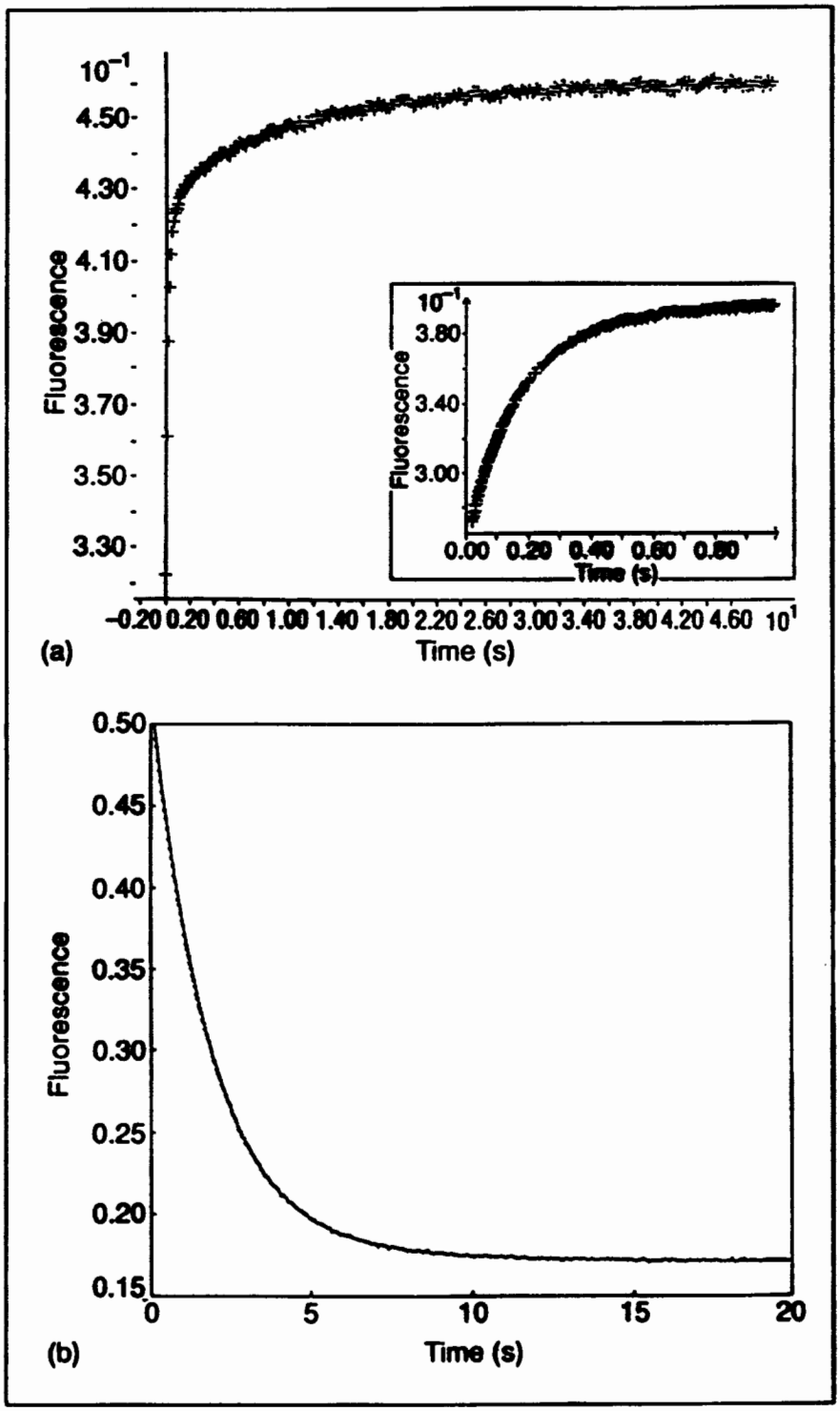


Fig. 3. Kinetic (a) folding and (b) unfolding curves of barnase. Reproduced from (41) and (44) with kind permission from Academic Press and Macmillan Publishers.

Table 1 Destabilization energies and ϕ-values for the mutations of barnase.

Mutation	$\Delta\Delta G_{U-F}$	ϕ_I	ϕ_{ts}
Ile→Val 4	0.60	−0.1	−0.1
Ile→Ala 4	1.35	−0.1	−0.1
Val→Ala 4	0.75	−0.1	0.0
Asn→Ala 5	1.85	0.0	0.0
Thr→Gly 6	1.24	0.3	0.3
Thr→Ala 6	2.15	0.3	0.3
Asp→Ala 8	0.89	0.7	0.7
Val→Thr 10	2.48	0.2	0.4
Val→Ala 10	3.39	0.2	0.3
Tyr→Ala 13	3.34	0.4	0.5
Tyr→Ala 13/17	4.18	0.3	0.4
Leu→Ala 14	4.32	0.5	0.6
Gln→Ile 15	−1.06	0.6	0.7
Thr→Ser 16	1.68	0.8	0.9
Thr→Ser 16/Tyr→Ala 17	2.15	0.5	0.5
Tyr→Ala 17	2.03	0.4	0.5
His→Gln 18	1.58	0.9	0.9
Asn→Ala 23	2.25	−0.1	−0.2
Ile→Val 25	1.12	−0.3	−0.3
Ile→Ala 25	3.52	−0.5	−0.5
Val→Ala 25	2.40	−0.6	−0.6
Thr→Ala 26	1.94	0.0	−0.1
Glu→Gly 29	1.76	−0.2	−0.2
Leu→Gln 33	1.31	−0.1	−0.1
Val→Thr 36	1.15	−0.1	0.0
Val→Ala 36	1.30	−0.3	−0.1
Asn→Asp 41	2.51	0.0	0.0
Val→Thr 45	2.44	−0.1	−0.1
Val→Ala 45	1.75	−0.4	−0.2
Ile→Val 51	1.80	−0.2	−0.2
Asp→Asn 54	2.42	−0.2	−0.2
Asp→Ala 54	2.97	−0.2	−0.2
Ile→Thr 55	0.89	0.2	0.4
Ile→Ala 55	1.15	0.5	0.5
Val→Thr 55	0.62	0.0	0.3
Val→Ala 55	0.88	0.6	0.6
Asn→Ala 58	2.17	0.9	0.9
Lys→Arg 62	0.43	0.7	0.9
Ile→Val 76	0.82	−0.2	−0.1
Ile→Ala 76	1.89	0.2	0.4
Val→Ala 76	1.07	0.4	0.8
Asn→Ala 77	1.65	−0.2	−0.2
Tyr→Phe 78	1.35	0.1	0.1
Asn→Ala 84	2.02	0.2	0.1
Ile→Val 88	1.34	0.6	0.9
Ile→Ala 88	4.01	0.6	1.0
Val→Ala 88	2.67	0.6	1.0
Leu→Val 89	0.30	0.4	0.1
Leu→Thr 89	2.82	0.5	1.0
Val→Thr 89	2.52	0.5	1.0
Ser→Ala 91	1.93	0.6	0.9
Ser→Ala 92	2.79	0.6	1.0
Ile→Val 96	0.88	0.6	0.5
Ile→Ala 96	3.17	0.7	0.9
Val→Ala 96	2.29	0.7	1.0
Thr→Val 99	2.67	0.8	0.2
Thr→Val 105	2.24	0.3	0.5
Ile→Val 109	0.76	0.1	0.1
Ile→Ala 109	2.07	0.4	0.6
Val→Ala 109	1.31	0.5	0.9

$$\phi_I = \frac{\Delta\Delta G_{U-I}}{\Delta\Delta G_{U-F}}$$

$$\phi_{ts} = \frac{\Delta\Delta G_{U-ts}}{\Delta\Delta G_{U-F}}$$

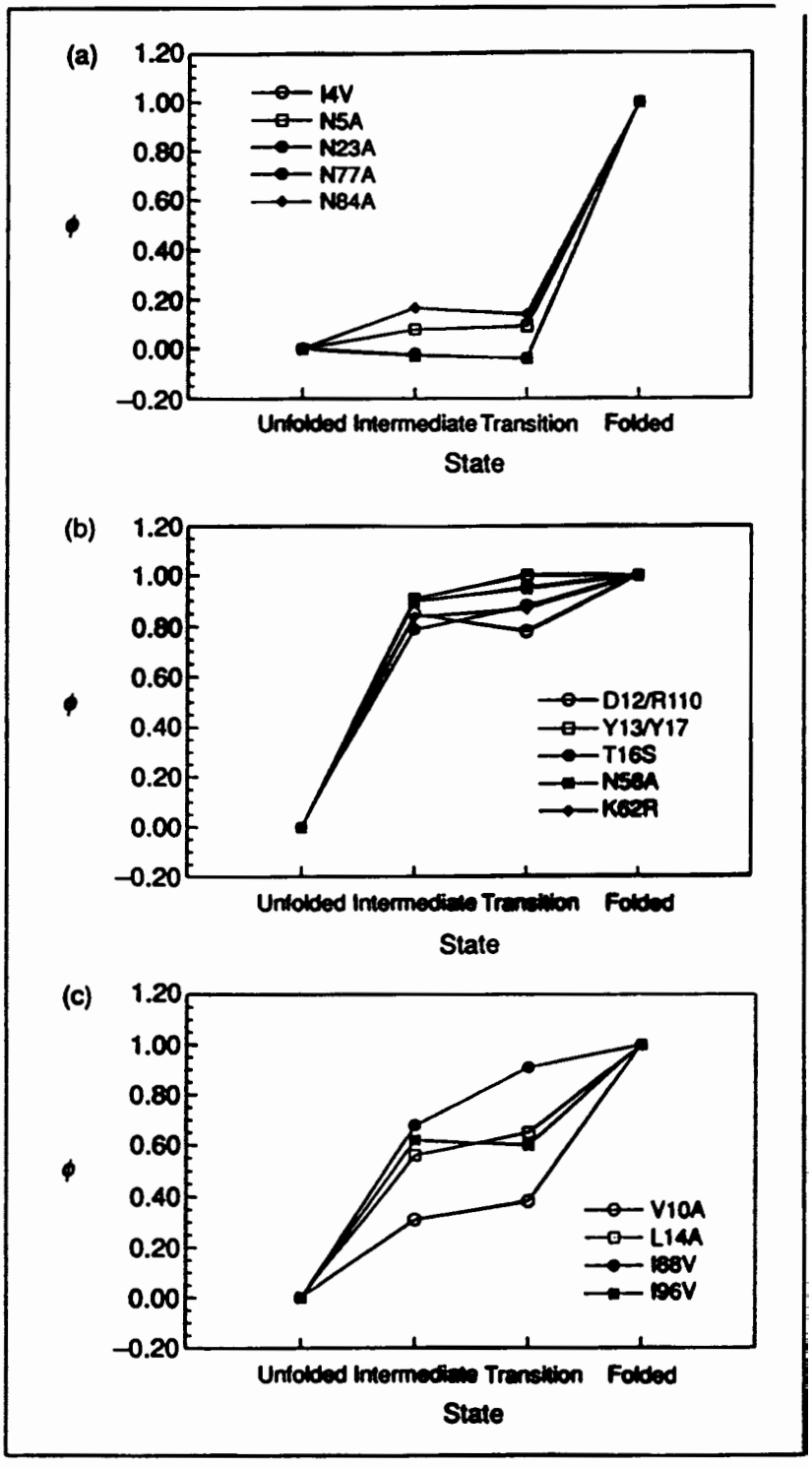


Fig. 5. ϕ-plots for the patterns of formation of different interactions in barnase. (a) ϕ-plots for several mutations affecting surface loops and tertiary interactions (Ile → Val 4, Asn → Ala 5, Asn → Ala 23, Asn → Ala 77, Asn → Ala 84). (b) ϕ-plots for mutations affecting an α-helix (Asp → Ala 12 / Arg → Ala 110, Tyr → Ala 13 / Tyr → Ala 17, Thr → Ser 16) and a β-turn and a specific loop (Asn → Ala 58, Lys → Arg 62). (c) ϕ-plots for mutations affecting hydrophobic $core_1$ (Val → Ala 10, Leu → Ala 14, Ile → Ala 88, Ile → Ala 96).

Changes in the free energies of unfolding of wild-type barnase, the single mutants B(Tyr→Ala13), B(Tyr→Ala17), B(Tyr→Phe17), and B(Tyr→Phe13) and the double mutants B(Tyr→Ala13, Tyr→Ala17) and B(Tyr→Phe17, Tyr→Phe13) determined by reversible denaturation with urea

Mutant	$\Delta G_u^{H_2O}$ (kcal/mol)†	m‡	$[urea]_{50\%}$ (M)§	$\Delta\Delta G_u^{[urea]_{50\%}}$ (kcal/mol)‖	$\Delta\Delta G_u^o$ (kcal/mol)¶
Wild-type	10·15	2·22	4·57	...	-
Ala13-Tyr17	6·46	2·26	2·86	3·71	3·64
Tyr13-Ala17	7·94	2·25	3·53	2·26	2·21
Ala13-Ala17	5·39	2·22	2·43	4·64	4·61
Phe13-Tyr17	9·52	2·17	4·38	0·41	0·37
Tyr13-Phe17	9·57	2·16	4·43	0·30	0·30
Phe13-Phe17	8·92	2·08	4·29	0·61	0·60

Denaturation was performed as described in Experimental Procedures.

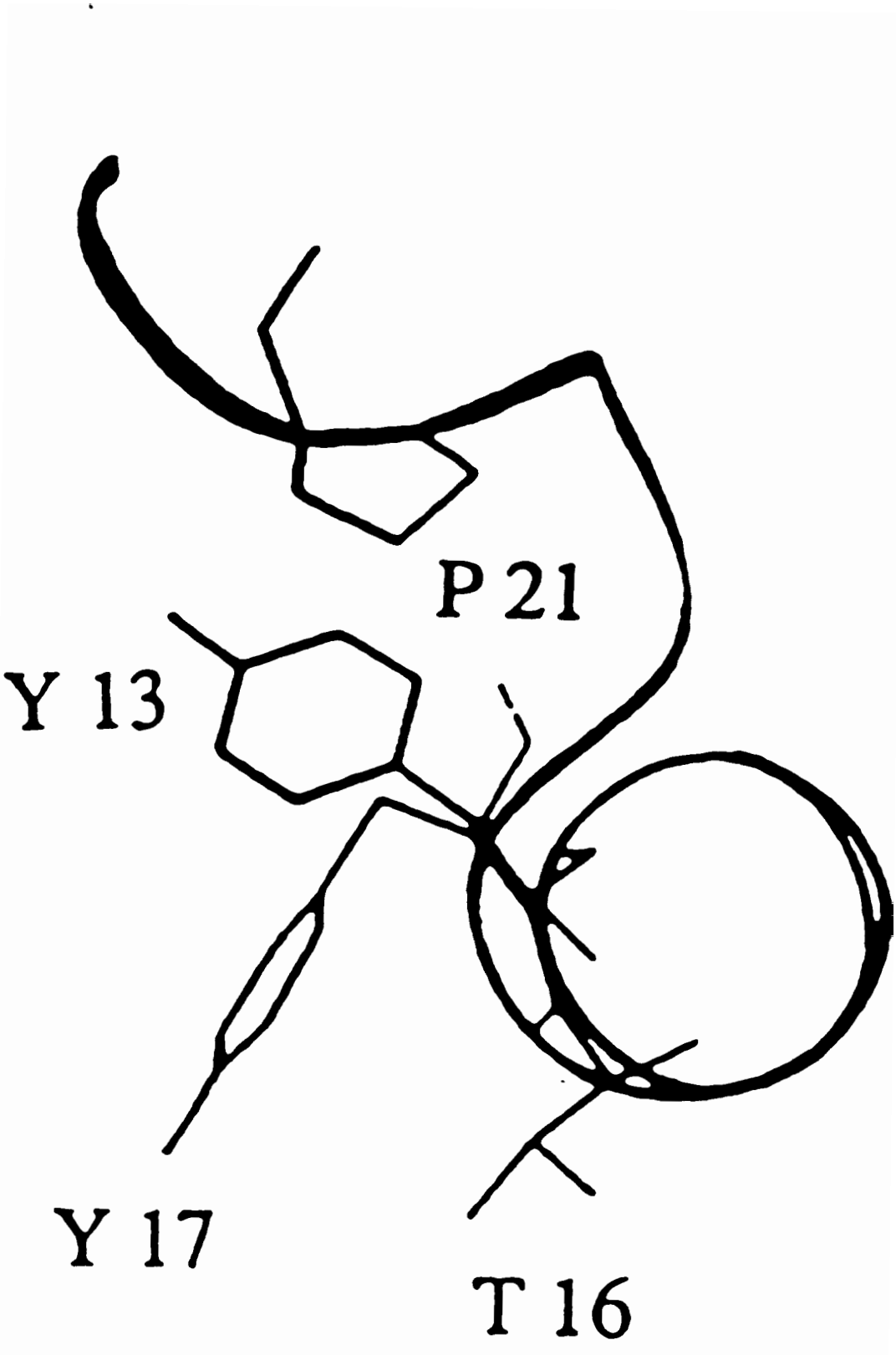
† $\Delta G_u^{H_2O}$ was determined by denaturation of the proteins with urea and extrapolation of the data to zero denaturation.

‡ m is the slope of the linear denaturation plot, $-d\Delta G_u/[urea]$.

§ $[urea]_{50\%}$ is the concentration of urea at which 50% of the protein is unfolded.

‖ $\Delta\Delta G_u^{[urea]_{50\%}}$ was determined by subtracting $\Delta G_u^{[urea]_{50\%}}$ for each mutant from the wild-type, and multiplying the difference by the average slope $\langle m \rangle (= 2{\cdot}17)$.

¶ $\Delta\Delta G_u^o$ was calculated very simply and accurately because the denaturation curves of all the proteins overlap in a scaled manner, as indicated in Experimental Procedures.



view of barnase 1st α-helix

orientation of Asp8 and Asp12 with respect to Arg110

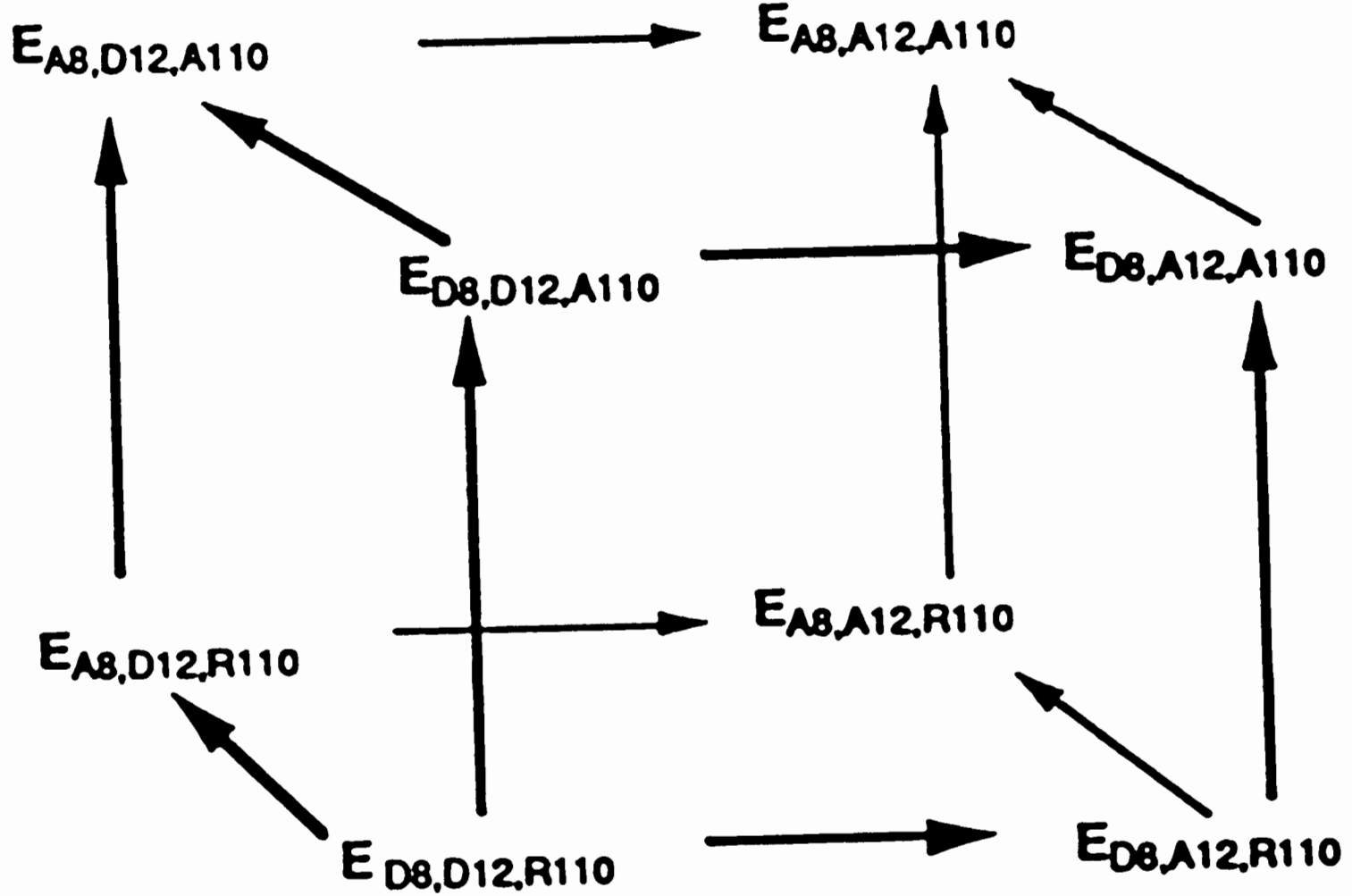


Figure 3. Triple mutant box constructed to determine the strength and co-operativity of the salt bridges between Arg110 with Asp8 and Asp12, respectively. Here, E stands for barnase and the single letter notation for amino acids is used.

Free energies of unfolding of wild-type barnase and three single alanine mutants, three double alanine mutants and one triple alanine mutant at positions 8, 12 and 110 in the enzyme, as determined by reversible urea denaturation

Enzyme	$\Delta G_u^{H_2O}$ (kcal mol^{-1})†	m (kcal mol^{-1} M^{-1})	$[Urea]_{50\%}$ (M)	$\Delta\Delta G_u^{H_2O}$ (kcal mol^{-1})‡
Asp8, Asp12, Arg110	9·93	2·02	4·58	0·00
Ala8, Asp12, Arg110	8·94	2·15	4·12	−0·99
Asp8, Ala12, Arg110	9·59	2·12	4·42	−0·34
Asp8, Asp12, Ala110	9·47	2·26	4·37	−0·46
Ala8, Ala12, Arg110	9·04	1·98	4·16	−0·89
Ala8, Asp12, Ala110	9·46	1·96	4·36	−0·47
Asp8, Ala12, Ala110	10·38	2·14	4·78	+0·45
Ala8, Ala12, Ala110	10·04	2·16	4·63	+0·11

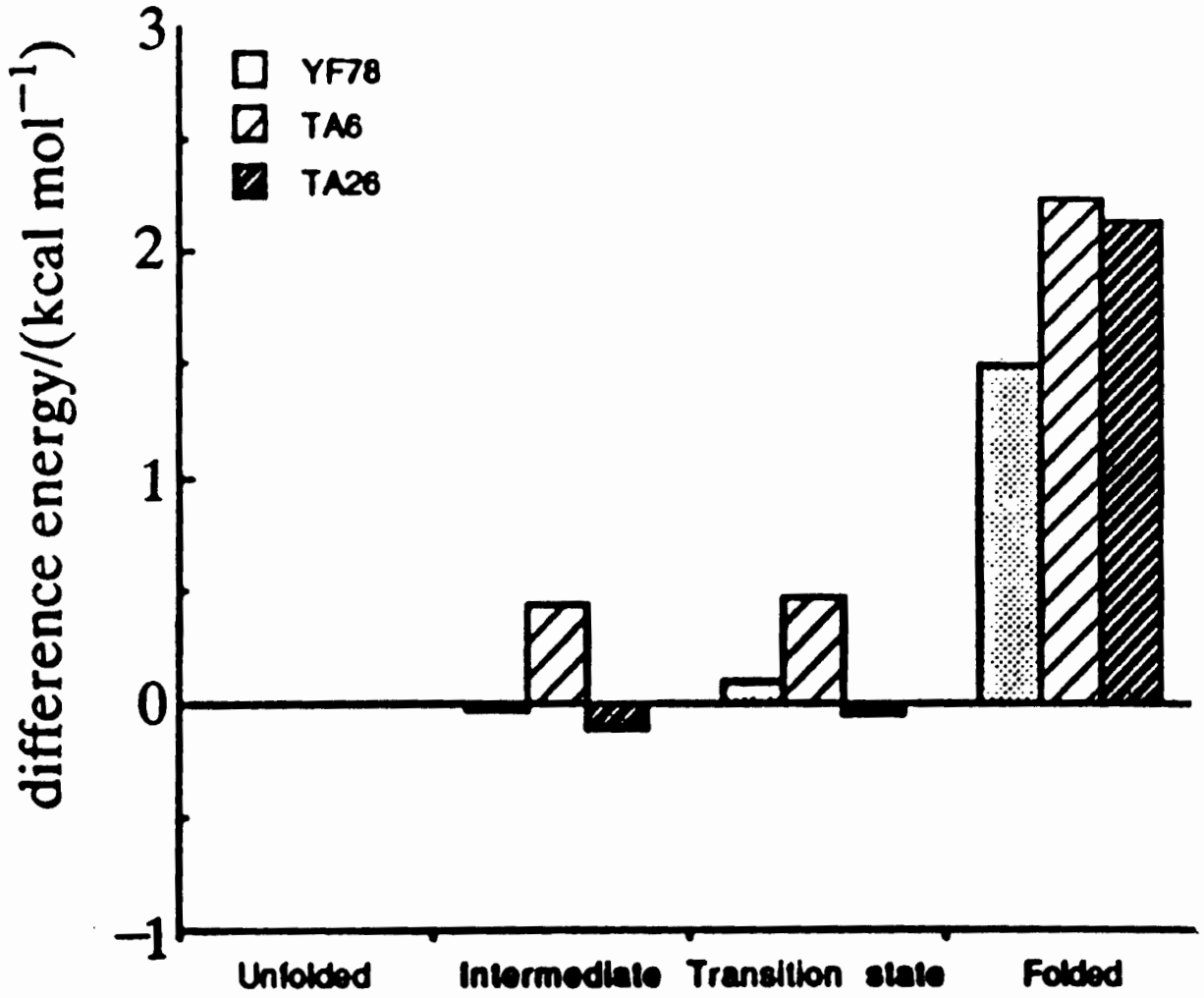
Denaturation experiments were done in 50 mM-Mes (pH 6·3) at 25°C as described in Materials and Methods.

† $\Delta G_u^{H_2O}$ was calculated by multiplying the $[urea]_{50\%}$ concentration by 2·17, which is the average value of m, the slope of the linear denaturation plot (see Materials and Methods).

‡ Values of $\Delta\Delta G_u^{H_2O}$ are relative to wild-type enzyme. The error in $[urea]_{50\%}$ is ±0·01 M, which is equivalent to ±0·022 kcal mol^{-1}. Any discrepancies are due to rounding errors.

Salt dependence of the coupling energies for the interactions in the triad

Interaction	0 M-NaCl	0·2 M-NaCl	0·5 M-NaCl
(Asp8, Arg110) in wild-type enzyme	−0·98	−0·52	−0·35
(Asp12, Arg110) in wild-type enzyme	−1·25	−0·79	−0·68
(Asp8, Asp12) in wild-type enzyme	−0·44	−0·60	−0·37
(Asp8, Arg110) in the Asp12→Ala mutant	−0·21	−0·06	+0·05
(Asp12, Arg110) in the Asp8→Ala mutant	−0·48	−0·33	−0·28
(Asp8, Asp12) in the Arg110→Ala mutant	+0·33	−0·14	+0·03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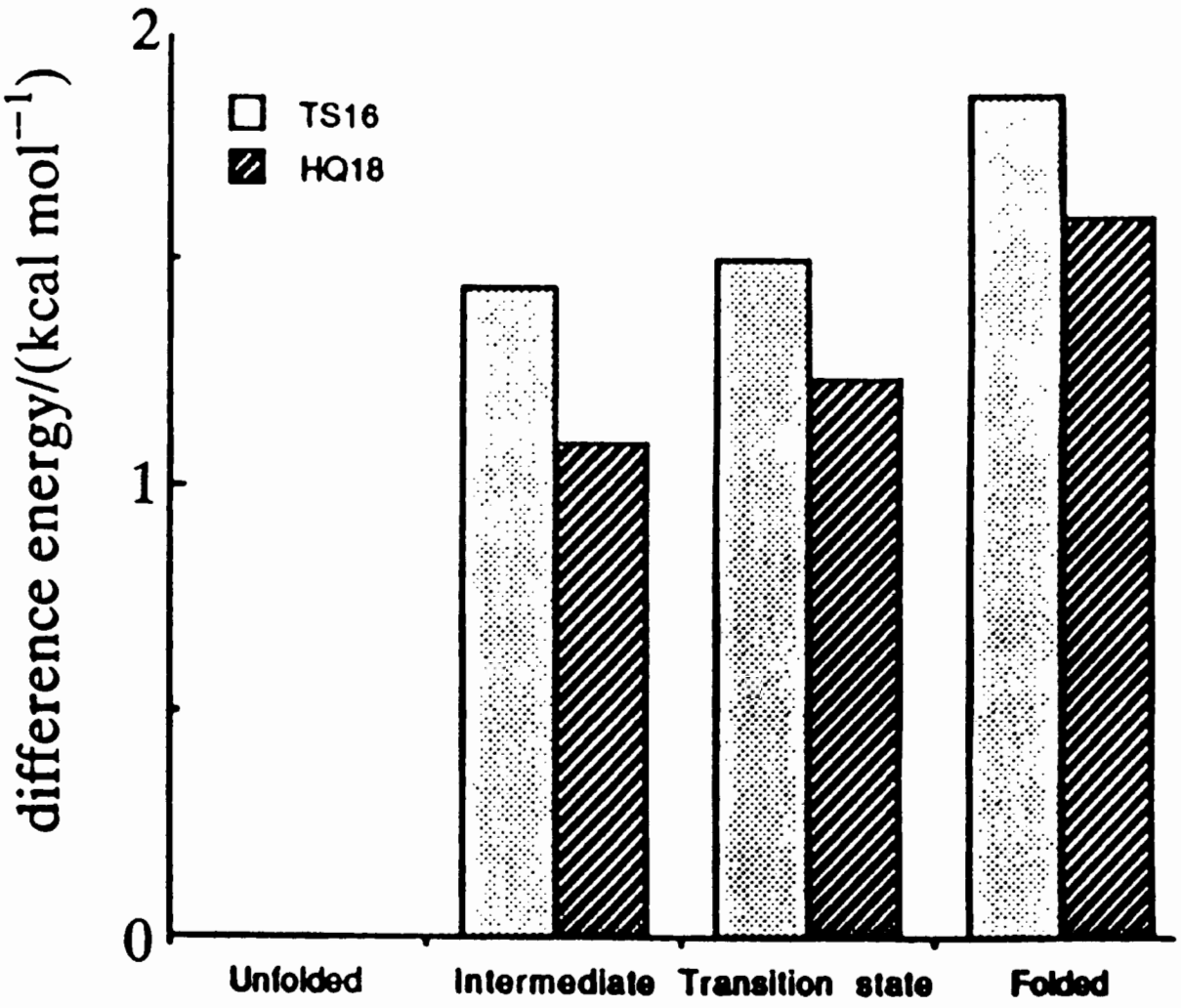
1
0
−1
YF78
TA6
TA26
Unfolded
Intermediate
Transition state
Folded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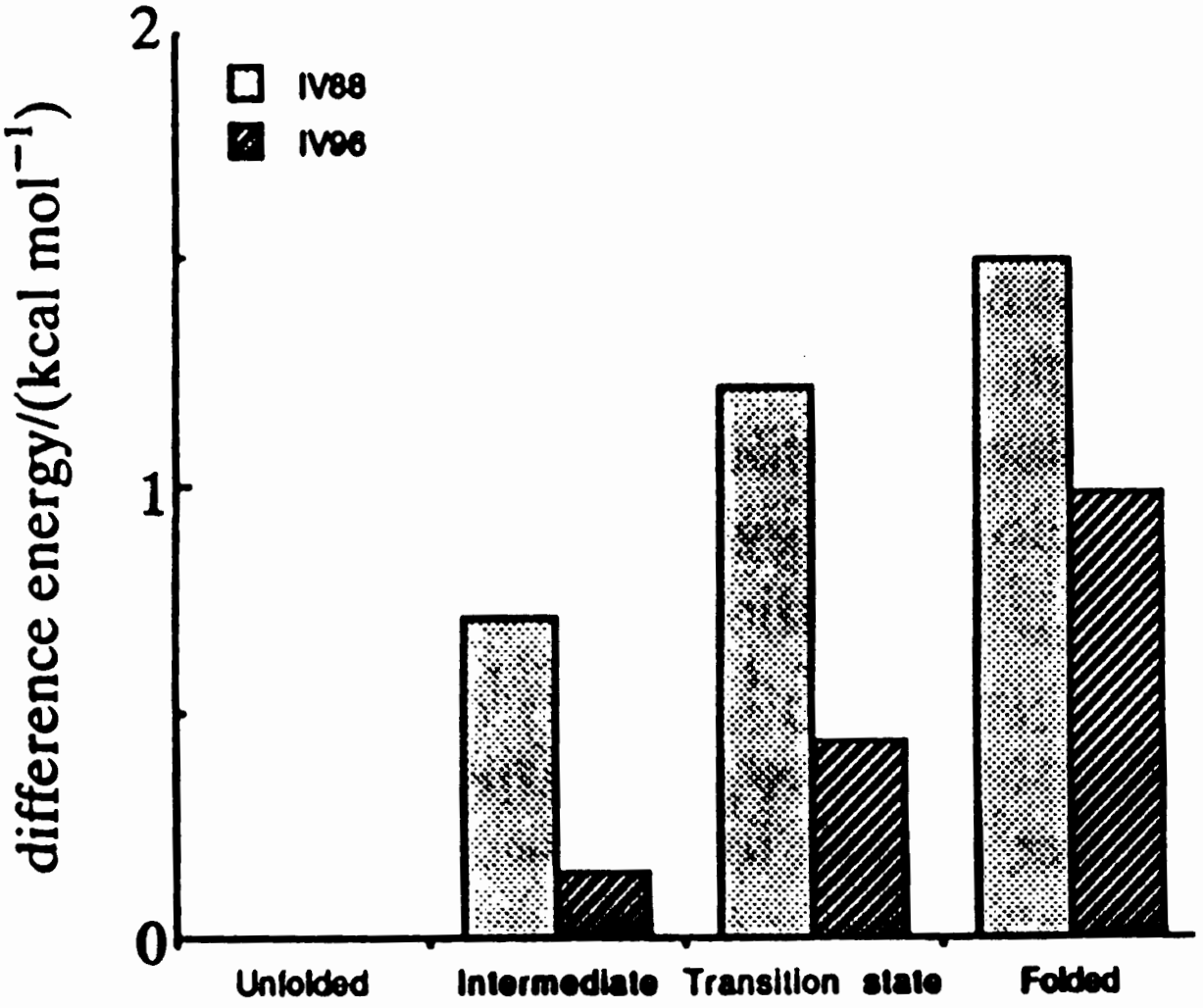
1
0
difference energy/(kcal mol^{-1})
TS16
HQ18
Unfolded
Intermediate
Transition state
Folded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0
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IV96
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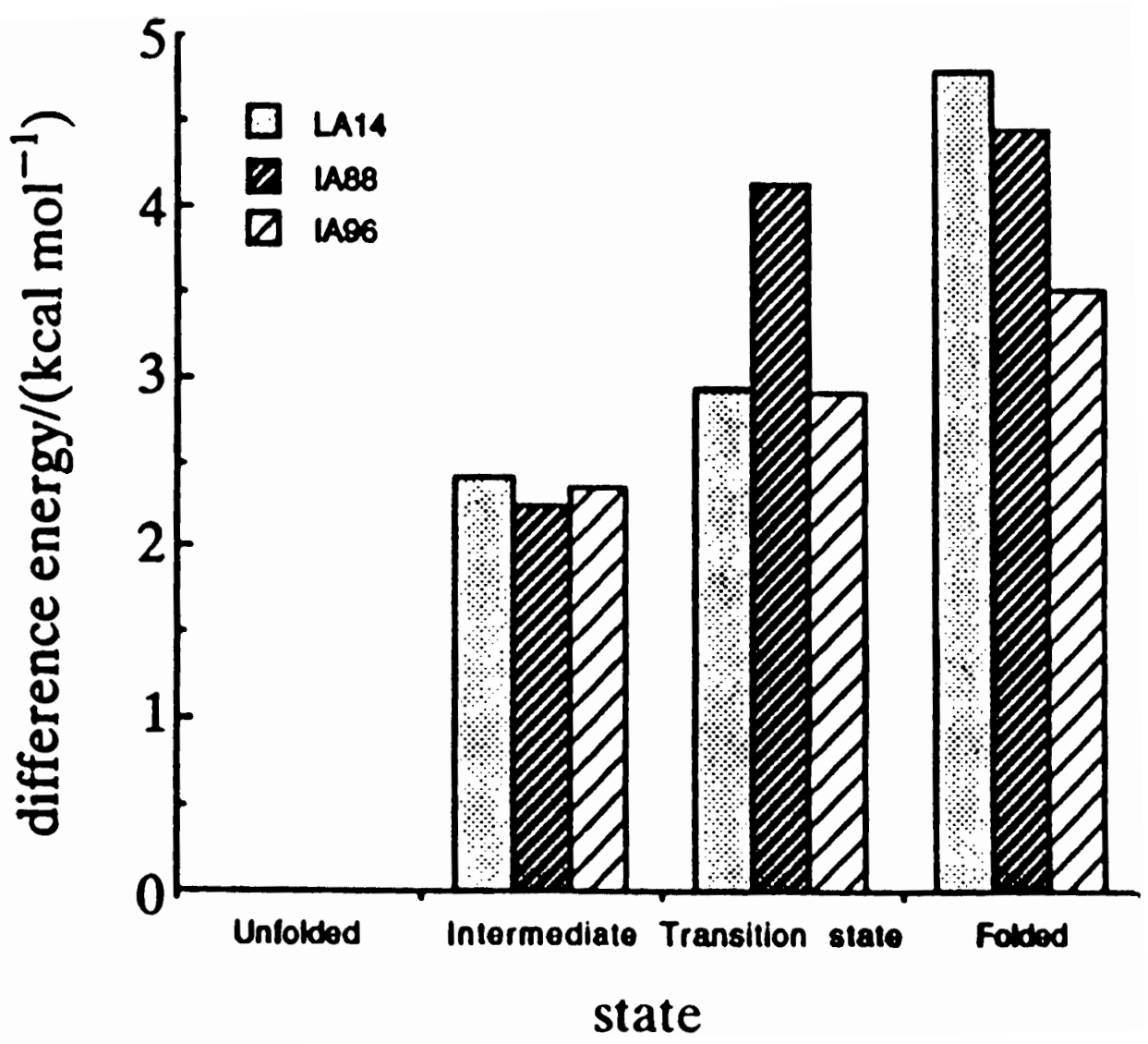
Unfolded
Intermediate
Transition state
Folded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3
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IA88
IA96
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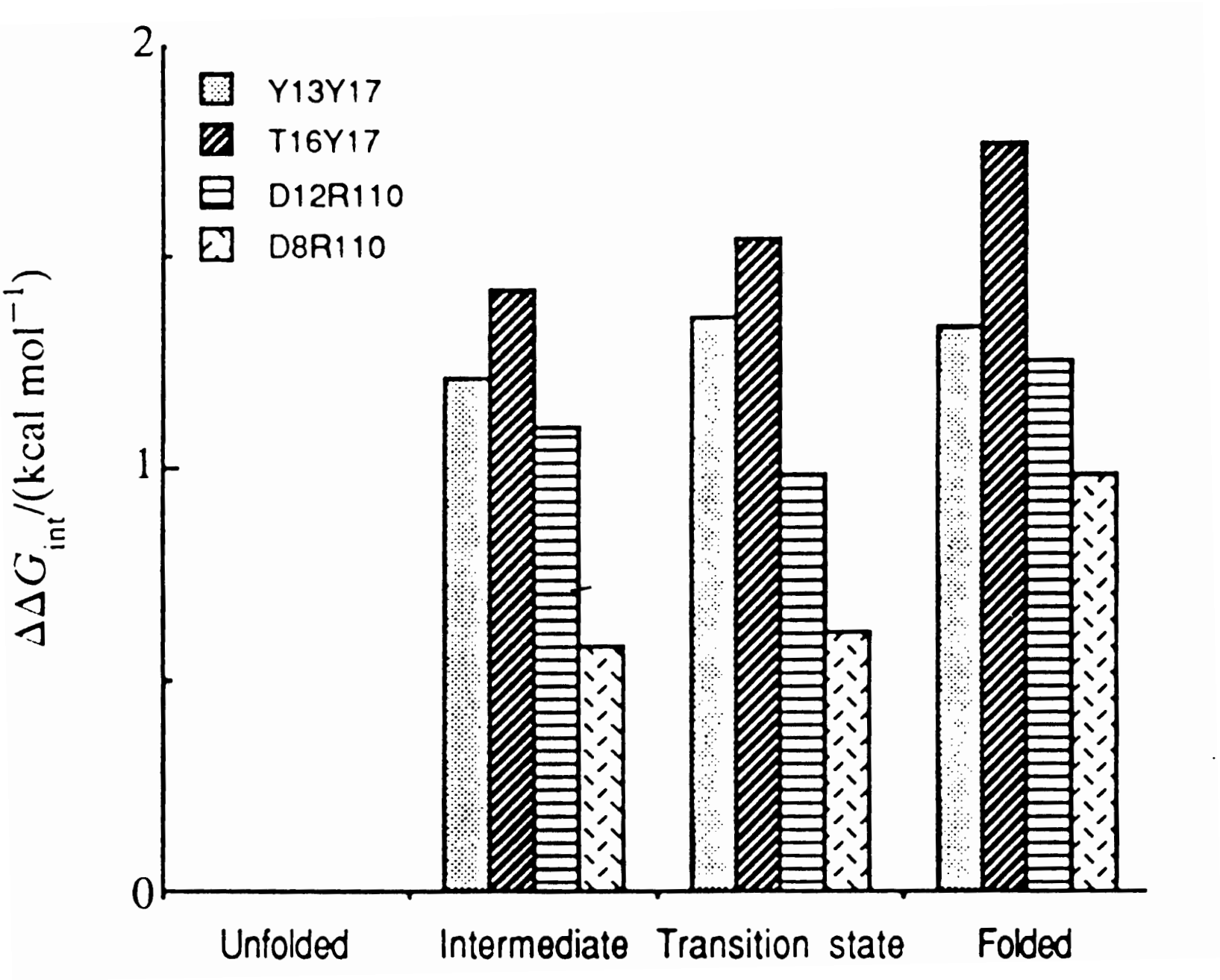
state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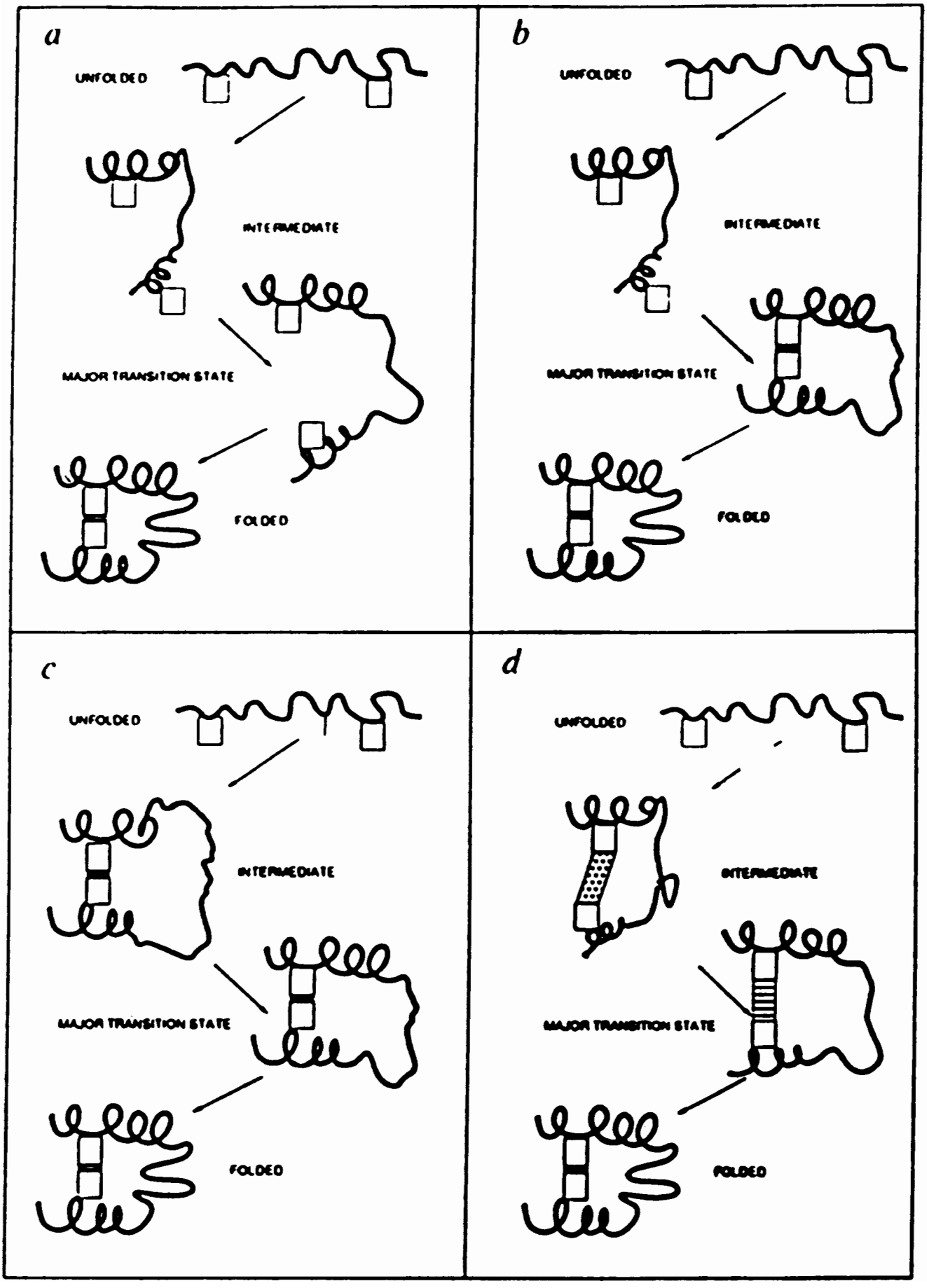
T16Y17
D12R110
D8R110
$\Delta\Delta G_{int}$/(kcal mol^{-1})
Unfolded
Intermediate
Transition state
Folded


a
UNFOLDED
INTERMEDIATE
MAJOR TRANSITION STATE
FOLDED
b
UNFOLDED
INTERMEDIATE
MAJOR TRANSITION STATE
FOLDED
c
UNFOLDED
INTERMEDIATE
MAJOR TRANSITION STATE
FOLDED
d
UNFOLDED
INTERMEDIATE
MAJOR TRANSITION STATE
FOLDED