

STUDIES ON PROTEIN STABILITY WITH T4 LYSOZYME

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I. INTRODUCTION

The combination of directed mutagenesis with high-resolution structure analysis has made it possible to address systematically some of the fundamental questions of protein folding and stability. This chapter reviews recent results in this area based on such studies of the lysozyme of bacteriophage T4.

II. TOLERANCE TO AMINO ACID SUBSTITUTION

Table I summarizes essentially all the mutants of T4 lysozyme that have been characterized to date in our laboratory. A quick perusal of Table I shows that the majority of the substitutions have a very modest effect on the stability of the protein. Also, in most cases the activity of the mutants is comparable with that of the wild type.

An even more extensive set of variants was generated by Poteete and co-workers (Rennell *et al.*, 1991), who used the suppression of *amber* mutants to substitute, in turn, 13 different amino acids at 163 of the 164 residue sites in T4 lysozyme. Of the resulting 2015 single-amino acid substitutions, only 173 were seriously deleterious. These occur at the 53 sites marked in Fig. 1. As can be seen, essentially every one of these amino acids has low mobility in the native structure, strongly supportive of the view (Alber *et al.*, 1987a) that the stability of the protein is dominated by its rigid parts and that flexible, solvent-exposed parts contribute little. More than half of the residues in T4 lysozyme tolerate all of the substitutions examined by Poteete and co-workers (Rennell *et al.*, 1991). It has also been verified that residues on the surface of T4 lysozyme can

TABLE I
Mutant T4 Lysozymes^a

Mutant ^b	PDB ID ^c	Activity ^d (%)	Thermodynamic data ^e			Crystallographic data ^f			Ref. ^h
			ΔT_m (°C)	$\Delta\Delta G$ (kcal/mol)	pH	Space group	Resolution (Å)	Comment ^g	
WT	3LZM	100	(67.2)	—	5.3	$P3_221$	1.7	Wild type	1, 2
WT	4LZM	—	(40.6)	—	2.0	—	—	—	—
WT	5LZM	—	—	—	—	$P3_221$	1.8	Wild type, medium salt	2
WT	6LZM	—	—	—	—	$P3_221$	1.8	Wild type, low salt	2
WT* (i.e., C54T/C97A)	1L63	103	(65.8)	—	5.3	$P3_221$	1.8	Pseudo wild type	3-5
WT*	—	—	(39.15)	—	2.0	—	—	—	—
I3A	—	~WT	-1.8	-0.7	6.5	No crystals	—	Hydrophobic replacement	6, 7, 19
I3C (S-H)	—	90	-3.7	-1.2	6.5	No crystals	—	Hydrophobic replacement	6-8
I3C (S-S)	—	96	3.3	1.2	6.5	No crystals	—	S-S bridge to Cys-97	6-8
I3C/I9C/T21C/C54T/ T142C/L164C red.	—	43	-8.5	—	2.0	N/A	—	Triple S-S bridge	9
I3C/I9C/T21C/C54T/ T142C/L164C ox.	—	0	23.4	—	2.0	No crystals	—	Triple S-S bridge	9
I3D	—	~WT	-8.5	-3.2	6.5	No crystals	—	Hydrophobic replacement	6, 7
I3E	—	~WT	-5.7	-2.0	6.5	No crystals	—	Hydrophobic replacement	6, 7
I3F	—	~WT	-3.0	-1.1	6.5	No crystals	—	Hydrophobic replacement	6, 7, 19
I3G	—	~WT	-5.8	-2.1	6.5	No crystals	—	Hydrophobic replacement	6, 7, 19
I3L	—	~WT	0.9	0.4	6.5	No crystals	—	Hydrophobic replacement	6, 7
I3M	—	~WT	-2.3	-0.9	6.5	No crystals	—	Hydrophobic replacement	6, 7
I3P	1L96	~WT	-7.3	-2.8	3.01	$P3_221$	2.0	Proline replacement	10
I3P	1L97	~WT	—	—	—	$P2_12_12$	2.0	Nonisomorphous hinge bending	10
I3S	—	~WT	-4.6	-1.7	6.5	No crystals	—	Hydrophobic replacement	6, 7
I3T	—	~WT	-6.0	-2.3	6.5	No crystals	—	Hydrophobic replacement	6, 7
I3V	1L17	~WT	-1.2	-0.4	6.5	$P3_221$	1.7	Hydrophobic replacement	6, 7, 19

I3W	—	~WT	-8.0	-2.8	6.5	No crystals	—	Hydrophobic replacement	6, 7
I3Y	1L18	~WT	-5.9	-2.3	6.5	$P3_2I$	1.7	Hydrophobic replacement	6, 7
M6I	—	100	-3.4	-1.4	6.0	$P3_2I$	1.8	Isomorphous form of M6I	11
M6I	150L	100	-3.4	-1.4	6.0	$P2_1I2_1$	2.1	Nonisomorphous hinge-bending mutant	11
I9C/L164C/WT* red.	—	99	-6.5	-1.5	2.0	—	—	Stabilizing S-S bridge	8
I9C/L164C/WT* ox.	1L35	106	6.4	1.5	2.0	$P3_2I$	1.8	Stabilizing S-S bridge	4
R14K/WT*	—	106	-0.08	-0.03	5.4	—	—	Random st mutant	43
K16E	1L42, 43	142	1.1	0.5	5.3	$P3_2I$	1.8	Cumulative charge-change	12
K16E/R119E	—	88	0.9	0.4	5.3	N/A	—	Cumulative	12
K16E/R119E/K135E/K147E	—	40	-2.5	-1.0	5.3	N/A	—	charge-change	12
K16E/K135E	—	132	-1.6	-0.7	5.3	N/A	—	charge-change	12
K16E/K135E/K147E	—	93	-4.0	-1.7	5.3	N/A	—	Cumulative charge-change	12
K16E/R154E	—	175	-1.9	-0.8	5.3	N/A	—	Cumulative charge-change	12
Y18D/T26Q/WT*	—	13	-4.0	—	5.3I	$P3_2I$	1.8	charge-change	13
Y18H/T26Q/WT*	—	4.7	-3.4	—	5.3I	$P3_2I$	1.8	Second-site revertant	13
T21C/T142C/WT* red.	—	68	-2.7	-0.7	2.0	$P3_2I$	1.8	Active site S-S bridge	3, 8, 14
T21C/T142C/WT* ox.	—	0	11	2.8	2.0	$P3_2I$	1.7	Active site S-S bridge	3, 8, 14
E22K/WT*	—	40	1.37	0.57	5.4	—	—	Random st mutant	43
T26E/WT*	—	0	—	—	—	$P2_1I2_1$	1.9	Covalent adduct	51
T26Q/WT*	—	1.9	-0.9	—	5.3I	No crystals	—	Low activity, random selection	13
T26S/WT*	131L	75	1.35	0.57	5.4	$P3_2I$	1.7	Random st mutant	43, 57
P37A	—	—	-0.8	—	2.0	—	—	Remove proline	19
S38D	1L19	320	1.6	0.6	6.7	$P3_2I$	1.7	Helix-dipole interaction	15, 16
S38D/N144D	—	430	3.7	1.4	5.0	N/A	—	Two helix-dipole interactions	15

(continued)

TABLE I (Continued)

Mutant ^b	PDB ID ^c	Activity ^d (%)	Thermodynamic data ^e			Crystallographic data ^f			Comment ^g	Ref. ^h
			ΔT_m (°C)	$\Delta\Delta G$ (kcal/mol)	pH	Space group	Resolution (Å)			
S38N/WT*	1L61	—	-0.1	0.0	6.7	P3 ₂ 21	1.8	Helix-dipole interaction		16
L39A/WT*	—	~WT	-2.5	-0.9	5.45	N/A	—	Insertion control		52
L39P	—	—	-5.5	—	2.0	—	—	Add proline		19
N40A/WT*	—	~WT	1.2	0.43	3.0	Isom.	—	Poly(alanine) helix 40-49		17
N40-[A]/WT*	102L	~WT	-7.8	-2.8	5.45	P3 ₂ 21	1.74	Insertion		52, 56
N40-[AA]/WT*	—	~WT	-2.9	-1.1	5.45	No crystals	—	Insertion		52, 56
N40-[AAA]/WT*	—	~WT	-4.7	-1.7	5.45	No crystals	—	Insertion		52, 56
N40-[AA]/K48-[LP]/WT*	—	~WT	-9.8	-3.3	5.45	N/A	—	Insertion control		52
N40-[AAAA]/WT*	—	~WT	-6.4	-2.2	5.45	No crystals	—	Insertion		52
A40-42/44-45/47-49/WT*	1L64	~WT	3.09	1.06	3.0	Isom.	—	Poly(alanine) helix 40-49		17
A40-49/WT*	1L64	~WT	-8.47	-2.11	3.0	P3 ₂ 21	1.9	Poly(alanine) helix 40-49		17
N40-[ES]/WT*	—	~WT	-1.6	-0.7	5.45	N/A	—	Insertion "recovery"		52, 56
N40-[SLD]/WT*	103L	~WT	-1.9	-0.7	5.45	P3 ₂ 21	1.9	Insertion "recovery"		52, 56
N40-[SLD]/L46A/WT*	—	~WT	-8.1	-2.8	5.45	P3 ₂ 21	—	Insertion control		52
N40D/WT*	—	124	1.14	0.44	5.4	—	—	Random st mutant		43
N40L-[A]/WT*	—	~WT	-3.8	-1.3	5.45	N/A	—	Insertion		52, 56
N40L/K43A/S44-[A]/WT*	—	~WT	-5.4	-1.8	5.45	N/A	—	Insertion control		52
A41D/WT*	—	105	0.71	0.29	5.4	—	—	Random st mutant		43
A41S/WT*	120L	~WT	-1.77	-0.6	3.0	P3 ₂ 21	1.8	Add hydroxyl		54
A41V	—	—	1.33	0.45	2.8	P3 ₂ 21	1.7	Entropic stabilization		18
A41V/V131A	—	—	1.81	0.6	2.8	N/A	—	Entropic stabilization		18
A42K/WT*	—	~WT	-11.0	-3.7	5.45	N/A	—	Insertion control		52
A42S/WT*	—	~WT*	-7.49	-2.3	3.0	P3 ₂ 21	1.75	Add hydroxyl		54
K43A/WT*	1L66	~WT	-2.95	-0.96	3.0	P3 ₂ 21	1.7	Poly(alanine) helix 40-49		17
S44A/WT*	1L68	~WT	1.2	0.44	3.0	P3 ₂ 21	1.7	Poly(alanine) helix 40-49		17
S44-[A]/WT*	—	~WT	-11.7	-3.8	5.45	No crystals	—	Insertion		52, 56

S44-[AA]/WT*	104L	~WT	-9.9	-3.4	5.45	R32	2.8	Insertion	52, 56
S44-[AAA]/WT*	205L	~WT	-6.0	-2.2	5.45	P3 ₂ 21	2.1	Insertion	52, 56
S44-[AAA]/L46A/WT*	—	~WT	-11.2	-3.7	5.45	P3 ₂ 21	—	Insertion control	52
S44-[AAAA]/WT*	—	~WT	-7.3	-2.5	5.45	No crystals	—	Insertion	52
S44A-[AA]/WT*	—	~WT	-4.4	-1.7	5.45	R32	2.6	Insertion	52, 56
S44C/WT*	—	~WT	-0.35	-0.11	3.0	—	—	Helix propensity analysis	49, 50
S44D/WT*	—	~WT	-0.32	-0.11	3.0	—	—	Helix propensity analysis	49, 50
S44E/WT*	1HYD	~WT	0.00	0.00	3.0	P3 ₂ 21	1.7	Helix propensity analysis	49, 50
S44E/WT*	217L	~WT	0.00	0.00	3.0	P2	1.9	Helix propensity analysis	49, 50
S44F/WT*	137L	~WT	0.18	0.06	3.0	P2	1.85	Helix propensity analysis	49, 50
S44G/WT*	107L	—	-1.55	-0.53	3.0	P3 ₂ 21	1.8	Helix propensity analysis	49, 50
S44H/WT*	—	~WT	0.12	0.04	3.0	—	—	Helix propensity analysis	49, 50
S44I/WT*	108L	~WT	0.91	0.31	3.0	P3 ₂ 21	1.8	Helix propensity analysis	49, 50
S44K/WT*	109L	~WT	0.57	0.20	3.0	P3 ₂ 21	1.85	Helix propensity analysis	49, 50
S44L/WT*	110L	~WT	1.09	0.39	3.0	P3 ₂ 21	1.7	Helix propensity analysis	49, 50
S44M/WT*	—	~WT	0.92	0.33	3.0	—	—	Helix propensity analysis	49, 50
S44N/WT*	111L	~WT	-0.40	-0.14	3.0	P3 ₂ 21	1.8	Helix propensity analysis	49, 50
S44P/WT*	112L	—	-10.3	-3.03	3.0	P3 ₂ 21	1.8	Helix propensity analysis	49, 50
S44Q/WT*	—	~WT	0.75	0.27	3.0	—	—	Helix propensity analysis	49, 50
S44R/WT*	113L	~WT	0.68	0.24	3.0	P3 ₂ 21	1.8	Helix propensity analysis	49, 50
S44T/WT*	114L	~WT	0.03	0.01	3.0	P3 ₂ 21	1.8	Helix propensity analysis	49, 50
S44V/WT*	115L	~WT	0.29	0.10	3.0	P3 ₂ 21	1.8	Helix propensity analysis	49, 50
S44W/WT*	116L	~WT	0.15	0.05	3.0	P1	2.05	Helix propensity analysis	49, 50
S44Y/WT*	—	~WT	0.54	0.19	3.0	—	—	Helix propensity analysis	49, 50
E45A/WT*	—	~WT	1.47	0.55	3.0	Isom.	—	Poly(alanine) helix 40-49	17
E45A/K48A/WT*	—	~WT	1.04	0.38	3.0	Isom.	—	Poly(alanine) helix 40-49	17
L46A/WT*	1L67	~WT	-8.39	-2.62	3.0	P3 ₂ 21	1.9	Poly(alanine) helix 40-49	17, 20
D47A/WT*	1L65	~WT	-0.81	-0.28	3.0	P3 ₂ 21	1.7	Poly(alanine) helix 40-49	17
K48A/WT*	—	~WT	-0.93	-0.32	3.0	Isom.	—	Poly(alanine) helix 40-49	17
K48-[A]/WT*	—	~WT	-11.2	-3.7	5.45	No crystals	—	Insertion	52, 56
K48-[AA]/WT*	—	~WT	-10.3	-3.5	5.45	No crystals	—	Insertion	52, 56
K48-[AAA]/WT*	—	~WT	-13.7	-4.2	5.45	No crystals	—	Insertion	52, 56

(continued)

TABLE I (Continued)

Mutant ^b	PDB ID ^c	Activity ^d (%)	Thermodynamic data ^e			Crystallographic data ^f			Ref. ^g
			ΔT_m (°C)	$\Delta\Delta G$ (kcal/mol)	pH	Space group	Resolution (Å)	Comment ^h	
K48-[AAAA]/WT*	—	~WT	-12.5	-4.0	5.45	No crystals	—	Insertion	52
K48-[HP]/WT*	201L	~WT	-7.0	-2.4	5.45	P2 ₁	2.0	Insertion "recovery"	52, 56
K48-[LP]/WT*	—	~WT	-7.3	-2.5	5.45	N/A	—	Insertion "recovery"	52
A49S/WT*	221L	~WT	-1.53	-0.5	3.0	P3 ₂ 2 ₁	1.7	Add hydroxyl	54
C54T/C97A	—	—	—	—	—	—	—	See WT*	—
N55G	1L21	110	-1.6	-0.6	6.5	P3 ₂ 2 ₁	1.9	"Left-handed helical" residue	21
T59A/WT*	1LYJ	~WT	-4.0	-1.5	6.5	P3 ₂ 2 ₁	1.8	Replace helix cap, Thr-59	22
T59D/WT*	1LYI	~WT	-3.1	-1.2	6.5	P3 ₂ 2 ₁	2.0	Replace helix cap, Thr-59	22
T59G/WT*	1LYH	~WT	-4.1	-1.6	6.5	P3 ₂ 2 ₁	1.7	Replace helix cap, Thr-59	22
T59N/WT*	1LYG	~WT	-2.8	-1.1	6.5	P3 ₂ 2 ₁	1.8	Replace helix cap, Thr-59	22
T59S/WT*	1LYF	~WT	-0.4	-0.2	6.5	P3 ₂ 2 ₁	1.8	Replace helix cap, Thr-59	22
T59V/WT*	1LYE	~WT	-4.0	-1.5	6.5	P3 ₂ 2 ₁	1.8	Replace helix cap, Thr-59	22
K60H	—	—	-0.4	-0.2	6.5	N/A	—	Salt bridge control	23
K60H/L13D/WT*	—	115	-7.1	-2.8	6.5	No crystals	—	Salt bridge	23
K60P	1L56	—	-0.1	0.0	6.5	P3 ₂ 2 ₁	1.8	Entropic stabilization	24
N68C/A93C/WT*	139L	0	—	—	—	P3 ₂ 2 ₁	1.7	Cross-linked dimer	60
Q69P	—	88	-7.6	-2.9	6.5	ND	—	Proline disruption	25
V71P	—	—	—	—	—	—	—	Proline disruption	25
D72P	1L76	57	-7.1	-2.7	6.5	P3 ₂ 2 ₁	1.9	Proline disruption	25
A73S/WT*	122L	~WT	-1.27	-0.4	3.0	P3 ₂ 2 ₁	1.8	Add hydroxyl	54
A74P	—	66	-12.1	-4.6	6.5	ND	—	Proline disruption	25
V75T/WT*	127L	~WT	-3.70	-1.3	3.0	P3 ₂ 2 ₁	1.85	Substitute hydroxyl	54
G77A	1L23	~WT	0.9	0.4	6.5	P3 ₂ 2 ₁	1.7	Entropic stabilization	26
R80K/WT*	—	44	-0.43	-0.17	5.4	—	—	Random st mutant	43

R80K/R119H/WT*	—	104	-1.20	-0.47	5.4	—	Random st mutant	43
A82P	1L24	~WT	2.1	0.8	6.5	P3 ₂ 21	Entropic stabilization	26, 19
A82S/WT*	123L	~WT	-0.99	-0.3	3.0	P3 ₂ 21	Add hydroxyl	54
K83H	—	—	-1.0	-0.4	6.5	N/A	Salt bridge control	23
K83H/A112D/WT*	1L41	110	-3.9	-1.5	6.5	P3 ₂ 21	Salt bridge	23
K83M/T115E	—	—	0.1	0.04	6.5	N/A	Salt bridge	23
P86A	1L25	50	-2.0	—	2.0	N/A	Proline within helix	27, 19
P86C	1L26	70	-1.0	—	2.0	P3 ₂ 21	Proline within helix	27
P86D	1L27	110	0	—	2.0	P3 ₂ 21	Proline within helix	27
P86G	1L28	70	-2.0	—	2.0	P3 ₂ 21	Proline within helix	27
P86H	1L29	70	-2.0	—	2.0	P3 ₂ 21	Proline within helix	27
P86I	—	70	-2.0	—	2.0	N/A	Proline within helix	27
P86L	1L30	50	-2.0	—	2.0	P3 ₂ 21	Proline within helix	27
P86R	1L31	20	-2.0	—	2.0	P3 ₂ 21	Proline within helix	27
P86S	1L32	70	-1.0	—	2.0	P3 ₂ 21	Proline within helix	27
P86T	—	80	-1.0	—	2.0	N/A	Proline within helix	27
V87T/WT*	128L	~WT	-4.55	-1.6	3.0	P3 ₂ 21	Substitute hydroxyl	54
S90C/Q122C/WT* red.	—	102	-5.8	-1.4	2.0	—	Destabilizing S-S bridge	8
S90C/Q122C/WT* ox.	—	58	-0.5	-0.1	2.0	No crystals	Destabilizing S-S bridge	8
S90H	—	—	-2.9	-1.1	6.5	N/A	Salt bridge control	23
S90H/Q122D/WT*	—	100	-5.7	-2.2	6.5	P222 ₁	Salt bridge	23, 28
D92N/WT*	1L55	—	-3.7	-1.4	6.7	P3 ₂ 21	Helix-dipole interaction	16
A93C/WT*	138L	~WT	—	—	—	P3 ₂ 21	Dimer control	60
A93P	—	—	0.2	0.1	6.5	No crystals	Entropic stabilization	24
A93S/WT*	224L	~WT	-0.52	-0.2	3.0	P3 ₂ 21	Add hydroxyl	54
A93T/WT*	128L	105	0.13	0.06	5.4	P3 ₂ 21	Random st mutant	43, 57
R96H	1L34	100	-8.7	-3.2	3.0	P3 ₂ 21	Random ts mutant	29-31
A98S/WT*	125L	~WT	-7.47	-2.5	3.0	P3 ₂ 21	Add hydroxyl	54
A98V	1L48	80	-14.8	-4.9	3.0	P3 ₂ 21	Helix-packing ts mutant	32
A98V/V149C/T152S	1L50	75	-12.1	-4.4	3.0	P3 ₂ 21	Helix-packing analysis	32
A98V/V149I/T152S	1L51	—	-12.0	-4.4	3.0	P3 ₂ 21	Helix-packing analysis	32
A98V/T152S	1L49	72	-13.9	-4.8	3.0	P3 ₂ 21	Helix-packing analysis	32

(continued)

TABLE 1 (Continued)

Mutant ^b	PDB ID ^c	Thermodynamic data ^d			Crystallographic data ^f			Comment ^g	Ref. ^h
		Activity ^d (%)	ΔT_m (°C)	$\Delta\Delta G$ (kcal/mol)	pH	Space group	Resolution (Å)		
L99A/WT*	1L90	—	-15.65	-5.0	3.01	$P3_221$	1.75	Cavity-creating mutant	20
L99A/WT*(+Bz)	1L83	—	—	—	—	$P3_221$	1.7	Cavity plus benzene	33
L99A/F153A/WT*	1L89	—	-41.8	-8.3	3.01	$P3_221$	1.9	Cavity creating	20
L99A/F153A/WT*(+Bz)	1L84	—	—	—	—	$P3_221$	1.9	Cavity plus benzene	33
L99F/WT*	1L91	—	-1.1	-0.4	3.01	$P3_221$	1.8	Hydrophobic replacement	5
L99F/M102L/WT*	—	104	-2.34	-0.71	3.01	Isom.	—	Alternative core packing	34
L99F/M102L/V1111/WT*	1L80	47	-4.02	-1.15	3.01	$P3_221$	1.8	Alternative core packing	34
L99F/M102L/V1111/ F153L/WT*	1L81	87	-1.82	-0.54	3.01	$P3_221$	2.1	Alternative core packing	34
L99F/M102L/F153L/WT*	1L82	81	-0.68	-0.21	3.01	$P3_221$	2.0	Alternative core packing	34
L99F/V1111/WT*	1L79	85	-2.7	-0.82	3.01	$P3_221$	1.9	Alternative core packing	34
L99F/F153L/WT*	—	66	0.09	0.03	3.01	Isom.	—	Alternative core packing	34
L99I/WT*	1L92	—	-4.0	-1.4	3.01	$P3_221$	1.7	Hydrophobic replacement	5
L99M/WT*	1L93	—	-2.0	-0.7	3.01	$P3_221$	1.8	Hydrophobic replacement	5
L99V/WT*	1L94	—	-6.6	-2.3	3.01	$P3_221$	1.8	Hydrophobic replacement	5
M102K/WT*	1L54	35	-20.3	-6.9	5.3	$P3_221$	1.9	Buried lysine	35
M102L/WT*	1L77	77	-2.54	-0.74	3.01	$P3_221$	2.05	Alternative core packing	34
M102L/V111F/WT*	—	42	-5.49	-1.51	3.01	Isom.	—	Alternative core packing	34
M102V	—	60	-12.7	—	3.0	$P3_221$	2.1	Random ts mutant	36-38
Q105A	1L00	33	-1.6	-0.6	5.8	$P3_221$	1.9	Perturb Trp-138	58
Q105E	1L98	19	-3.0	-1.1	5.8	$P3_221$	1.8	Perturb Trp-138	58
Q105G	1L99	18	-3.9	-1.5	5.8	$P3_221$	1.95	Perturb Trp-138	58
T109D/WT*	1L62	—	1.5	0.6	6.7	$P3_221$	1.7	Helix-dipole interaction	16
T109N/WT*	1L59	—	0.3	0.1	6.7	$P3_221$	1.75	Helix-dipole interaction	16
V111F/WT*	—	86	-4.77	-1.43	3.01	Isom.	—	Alternative core packing	34

V111F/F153L/WT*	—	55	-3.52	-1.09	3.01	Isom.	—	Alternative core packing	34
V111I/WT*	2L78	87	-2.32	-0.69	3.01	P3 ₂ I	2.0	Alternative core packing	34
G113A	1L60	—	0.8	0.3	6.5	P3 ₂ I	1.65	Entropic stabilization	24
G113E/WT*	—	165	0.79	0.30	5.4	—	—	Random st mutant	43
T115E/WT*	1L37	370	0.7	0.3	6.5	P3 ₂ I	1.85	Helix-dipole/salt bridge	16, 23
N116D	1L57	—	1.6	0.6	5.7	P3 ₂ I	1.85	Helix-dipole interaction	16
N116D/R119M	—	—	1.6	0.6	5.7	N/A	—	Helix-dipole control	16
S117F/WT*	—	10	2.8	1.1	3.0	P3 ₂ I	2.0	Stable repacking mutant	53
L118A/WT*	—	—	-12.2	-3.5	3.01	P3 ₂ I	1.9	Cavity-creating mutant	20
R119E	1L44	169	-0.1	-0.04	5.3	P3 ₂ I	1.7	Cumulative charge change	35
R119E/K135E	—	72	-2.6	-1.1	5.3	N/A	—	Cumulative charge change	35
R119E/K135E/K147E	—	68	-3.8	-1.6	5.3	N/A	—	Cumulative charge change	35
R119H/WT*	—	105	-0.74	-0.29	5.4	—	—	Random st mutant	43
R119M	—	—	0.3	0.1	5.7	N/A	—	Helix dipole control	16
L121A/WT*	—	—	-9.2	2.7	3.01	P3 ₂ I	2.0	Cavity-creating mutant	20
L121A/A129M/V149I/WT*	141L	~WT	—	-1.4	3.0	P3 ₂ I	2.0	Core repacking	55
L121A/A129M/F153L/WT*	140L	~WT	—	-1.1	3.0	P3 ₂ I	2.1	Core repacking	55
L121A/A129V/L133A/ F153L/WT*	142L	~WT	—	-3.5	3.0	P3 ₂ I	2.0	Core repacking	55
L121A/A129V/L133M/ F153L/WT*	143L	~WT	—	-2.3	3.0	P3 ₂ I	2.0	Core repacking	55
L121I/A129L/L133M/ F153W/WT*	144L	~WT	—	-1.3	3.0	P3 ₂ I	2.1	Core repacking	55
L121I/A129W/L133M/WT*	145L	~WT	—	-1.4	3.0	P3 ₂ I	2.0	Core repacking	55
L121M/A129L/L133M/ V149I/F153W/WT*	146L	~WT	—	-1.3	3.0	P3 ₂ I	1.85	Core repacking	55
L121M/L133V/F153L/WT*	147L	~WT	—	-2.5	3.0	P3 ₂ I	2.1	Core repacking	55
Q123/WT*	1L38	280	1.2	0.4	6.5	P3 ₂ I	1.8	Salt bridge	23
K124G	1L22	140	-0.2	-0.1	6.5	P3 ₂ I	1.7	"Left-handed helical" residue	21
W126Y	—	—	-2.7	—	2.0	No crystals	—	Amber suppressor	59
W126Y/W138Y/W158Y	—	—	-7.2	—	2.0	No crystals	—	Amber suppressor	59

continued)

TABLE I (Continued)

Mutant ^b	PDB ID ^c	Thermodynamic data ^a			Crystallographic data ^f			Ref. ^h
		Activity ^d (%)	ΔT_m (°C)	$\Delta\Delta G$ (kcal/mol)	pH	Space group	Resolution (Å)	
D127A/E128A	1L72	—	0.8	0.2	2.0	$P3_221$	1.9	Poly(alanine) helix 126–134 39
D127C/R154C/WT* red.	—	79	-5.4	-1.3	2.0	—	—	Destabilizing S–S bridge 8
D127C/R154C/WT* ox.	—	52	-2.4	-0.5	2.0	No crystals	—	Destabilizing S–S bridge 8
A127–132	1L73	—	3.8	1.0	2.0	$P3_221$	2.05	Poly(alanine) helix 126–134 39
A127–134	1L75	—	-9.4	-2.3	2.0	$P3_221$	1.9	Poly(alanine) helix 126–134 39
E128A	—	70	0.6	0.16	2.0	Isom.	—	Poly(alanine) helix 126–134 40
E128A/V131A	1L71	70	1.5	0.41	2.0	$P3_221$	1.9	Poly(alanine) helix 126–134 39, 40
E128A/V131A/N132A	1L36	70	3.4	0.94	2.0	$P3_221$	1.7	Poly(alanine) helix 126–134 39, 40
E128K	—	4	-5.3	—	3.0	$P3_221$	2.4	Low activity 37, 41
A128–134	1L74	—	-10.3	-2.6	2.0	$P3_221$	1.7	Poly(alanine) helix 126–134 39
A129V	—	—	-1.9	-0.7	2.85	$P3_221$	1.9	Cavity filling 42
A130S/WT*	1I8L	~WT	-2.89	-1.0	3.0	$P3_221$	1.8	Add hydroxyl 54
V131A	1L33	—	0.66	0.26	3.0	$P3_221$	1.7	Helix propensity analysis 18, 49, 50
V131A/N132A	1L70	—	2.3	0.62	2.0	$P3_221$	1.9	Poly(alanine) helix 126–134 39, 40
V131D	1DYA	~WT	0.22	0.08	3.0	$P3_221$	1.9	Helix propensity analysis 49, 50
V131E	1DYG	~WT	0.52	0.20	3.0	$P3_221$	2.1	Helix propensity analysis 49, 50
V131G	1DYB	~WT	-1.80	-0.68	3.0	$P3_221$	1.75	Helix propensity analysis 49, 50

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V131I	IDYC	~WT	0.41	0.16	3.0	P3 ₂ I	2.1	Helix propensity analysis	49, 50
V131L	IDYD	~WT	0.23	0.09	3.0	P3 ₂ I	1.9	Helix propensity analysis	49, 50
V131M	IDYF	~WT	0.32	0.12	3.0	P3 ₂ I	1.9	Helix propensity analysis	49, 50
V131S	IDYE	~WT	-0.12	-0.05	3.0	P3 ₂ I	1.8	Helix propensity analysis	49, 50
V131T	—	—	-0.33	-0.12	3.0	P3 ₂ I	1.7	Helix propensity analysis	18
L133A	IL69	—	-10.55	-3.6	3.01	P3 ₂ I	1.9	Cavity-creating, poly(alanine)	20, 39
L133D/WT*	—	4	-17.9	-5.7	6.5	No crystals	—	Buried aspartate	35
L133F	—	—	-0.8	-0.3	2.85	P3 ₂ I	1.9	Cavity filling	42
A134S/WT*	IL19L	~WT	-0.44	-0.1	3.0	P3 ₂ I	1.65	Add hydroxyl	54
K135E	IL45	104	-2.3	-1.0	5.3	P3 ₂ I	1.7	Cumulative charge change	12
K135E/K147E	—	128	-3.7	-1.5	5.3	N/A	—	Cumulative charge change	12
P143A	IL58	—	—	—	—	—	—	Proline replacement	19
N144D	IL20	160	1.4	0.5	6.9	P3 ₂ I	1.9	Helix-dipole interaction	15, 16
N144E/WT*	IL39, 40	115	1.5	0.5	6.5	P3 ₂ I	1.85	Salt bridge	23
N144E/K147M	—	—	1.2	0.4	6.5	N/A	—	Salt bridge	23
N144H/WT*	—	—	0.7	0.3	6.7	No crystals	—	Helix-dipole control	16
A146F	—	—	—	—	—	P4 ₁ I ₂	3.0	New crystal form	44
A146T	—	55	-9.4	—	3.0	P3 ₂ I	2.1	Random ts mutant	36-38
K147E	IL46	120	-1.6	-0.7	5.3	P3 ₂ I	1.7	Cumulative charge change	12
V149C	IL53	67	-5.1	-2.2	3.0	P3 ₂ I	1.85	Helix-packing analysis	32
V149T/WT*	I26L	~WT	-10.08	-2.8	3.0	P3 ₂ I	1.8	Substitute hydroxyl	54
T151S/WT*	I30L	86	0.93	0.39	5.4	P3 ₂ I	1.7	Random st mutant	43, 57
T152S	IL52	94	-6.6	-2.6	3.0	P3 ₂ I	1.7	Helix-packing analysis	32
F153A/WT*	IL85	—	-12.3	-3.5	3.01	P3 ₂ I	2.0	Cavity creating	20
F153A/WT*(+Bz)	—	—	—	—	—	P3 ₂ I	2.7	Cavity plus benzene	33
F153I/WT*	IL86	—	-1.5	-0.5	3.01	P3 ₂ I	1.8	Hydrophobic replacement	5
F153L/WT*	IL87	—	0.6	0.2	3.01	P3 ₂ I	1.8	Hydrophobic replacement	5
F153M/WT*	IL88	—	-2.4	-0.8	3.01	P3 ₂ I	1.85	Hydrophobic replacement	5
F153V/WT*	IL95	—	-6.1	-1.8	3.01	P3 ₂ I	2.0	Hydrophobic replacement	5
R154E	IL47	90	-2.6	-1.1	5.3	P3 ₂ I	1.7	Cumulative charge change	12
T155A/T157I	IL01	—	—	—	—	P3 ₂ I	1.7	H bond, incidental	45

(continued)

TABLE I (Continued)

Mutant ^b	PDB ID ^c	Activity ^d (%)	Thermodynamic data ^e		Crystallographic data ^f			Ref. ^h
			ΔT_m (°C)	$\Delta\Delta G$ (kcal/mol)	pH	Space group	Resolution (Å)	
G156D	1L16	50	-6.1	-2.3	6.5	$P3_221$	1.7	Random ts mutant 46
T157A	1L02	~WT	-5.4	-1.4	2.0	$P3_221$	1.7	H-bond analysis 45
T157C	1L03	~WT	-4.9	-1.3	2.0	$P3_221$	1.7	H-bond analysis 45
T157D	1L04, 5	~WT	-4.2	-1.1	2.0	$P3_221$	1.7	H-bond analysis 45
T157E	1L06	~WT	-5.8	-1.5	2.0	$P3_221$	1.7	H-bond analysis 45
T157F	1L07	~WT	-9.2	-2.4	2.0	$P3_221$	1.7	H-bond analysis 45
T157G	1L08	~WT	-4.2	-1.1	2.0	$P3_221$	1.7	H-bond analysis 45
T157H	1L09	~WT	-7.9	-2.1	2.0	$P3_221$	1.7	H-bond analysis 45
T157I	1L10	90	-11.0	-3.1	2.0	$P3_221$	1.7	Ts mutant 47
T157L	1L11	~WT	-5.0	-1.3	2.0	$P3_221$	1.7	H-bond analysis 45
T157N	1L12	~WT	-1.7	-0.45	2.0	$P3_221$	1.7	H-bond analysis 45
T157R	1L13	~WT	-5.1	-1.3	2.0	$P3_221$	1.7	H-bond analysis 45
T157S	1L14	~WT	-2.5	-0.66	2.0	$P3_221$	1.7	H-bond analysis 45
T157V	1L15	~WT	-6.0	-1.6	2.0	$P3_221$	1.7	H-bond analysis 45
N163D/WT*	—	193	-0.50	-0.21	5.4	—	—	Random st mutant 43

^a Mutant lysozymes for which the protein has been purified and, at least to some degree, characterized.

^b Any mutant that includes the designation WT* was constructed in the cysteine-free pseudo-wild-type background, which includes the two replacements C54T and C97A.

^c PDB ID is the identification used by the Brookhaven Protein Data Bank.

^d Activity indicated as ~WT means that the mutant lysozyme appeared to have activity roughly the same as wild type, but activity was not measured explicitly.

^e Thermodynamic data are for a representative value of pH, as quoted. Numbers given in parentheses for WT and WT* indicate the melting temperatures of these two reference proteins. ΔT_m is the change in melting temperature of the mutant protein relative to WT or WT*, whichever is the appropriate reference protein. $\Delta\Delta G$ is the change in the free energy of unfolding of the mutant protein relative to WT or WT*, a positive value corresponding to a mutant that is more stable than wild type.

^f If crystallization was attempted but was unsuccessful this is indicated by No crystals. If crystals were obtained and appeared to be isomorphous with wild type but were not actually characterized, the space group is designated Isom. In some cases no attempt (N/A) was made to obtain crystals.

^g Explains why the mutant was made, or other pertinent information. "Random st mutant" and "Ts mutant" are used to identify variants that were identified using random screens to select, respectively, thermostable (st) and temperature-sensitive (ts) mutants.

- ^h Key to references: (1) Weaver, L. H., and Matthews, B. W. (1987). *J. Mol. Biol.* **193**, 189-199; (2) Bell, J. A., Wilson, K. P., Zhang, X.-J., Faber, H. R., Nicholson, H., and Matthews, B. W. (1991). *Protein Struct. Funct. Genet.* **10**, 10-21; (3) Matsumura, M., and Matthews, B. W. (1989). *Science* **243**, 792-794; (4) Pjura, P. E., Matsumura, M., Wozniak, J. A., and Matthews, B. W. (1990). *Biochemistry* **29**, 2592-2598; (5) Eriksson, A. E., Baase, W. A., Wozniak, J. A., and Matthews, B. W. (1992). *J. Mol. Biol.* **229**, 747-769; (6) Matsumura, M., Becktel, W. J., and Matthews, B. W. (1988). *Nature (London)* **334**, 406-410; (7) Matsumura, M., Wozniak, J. A., Dao-pin, S., and Matthews, B. W. (1989). *J. Biol. Chem.* **264**, 16059-16066; (8) Matsumura, M., Becktel, W. J., Levitt, M., and Matthews, B. W. (1989). *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6562-6566; (9) Matsumura, M., Signor, G., and Matthews, B. W. (1989). *Nature (London)* **342**, 291-293; (10) Dixon, M. L., Shewchuk, L., and Matthews, B. W. (1992). *J. Mol. Biol.* **227**, 917-933; (11) Faber, H. R., and Matthews, B. W. (1990). *Nature (London)* **348**, 263-266; (12) Dao-pin, S., Soderlind, E., Baase, W. A., Wozniak, J. A., Sauer, U., and Matthews, B. W. (1991). *J. Mol. Biol.* **221**, 873-887; (13) Potete, A. R., Dao-pin, S., Nicholson, H., and Matthews, B. W. (1991). *Biochemistry* **30**, 1425-1432; (14) Jacobson, R., Matsumura, M., Faber, H. R., and Matthews, B. W. (1992). *Protein Sci.* **1**, 46-57; (15) Nicholson, H., Becktel, W. J., and Matthews, B. W. (1988). *Nature (London)* **336**, 651-656; (16) Nicholson, H., Anderson, D. E., Dao-pin, S., and Matthews, B. W. (1991). *Biochemistry* **30**, 9816-9828; (17) Heinz, D. W., Baase, W. A., and Matthews, B. W. (1992). *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3751-3755; (18) Dao-pin, S., Baase, W. A., and Matthews, B. W. (1990). *Protein Struct. Funct. Genet.* **7**, 198-204; (19) Chen, B.-L., Baase, W. A., Nicholson, H., and Schellman, J. A. (1992). *Biochemistry* **31**, 1464-1476; (20) Eriksson, A. E., Baase, W. A., Zhang, X.-J., Heinz, D. W., Blaber, M., Baldwin, E. P., and Matthews, B. W. (1991). *Science* **255**, 178-183; (21) Nicholson, H., Soderlind, E., Tronrud, D. E., and Matthews, B. W. (1989). *J. Mol. Biol.* **210**, 181-193; (22) Bell, J. A., Becktel, W. J., Sauer, U., Baase, W. A., and Matthews, B. W. (1992). *Biochemistry* **31**, 3590-3596; (23) Dao-pin, S., Sauer, U., Nicholson, H., and Matthews, B. W. (1991). *Biochemistry* **30**, 7142-7153; (24) Nicholson, H., Tronrud, D. E., Becktel, W. A., and Matthews, B. W. (1992). *Biopolymers* **32**, 1431-1441; (25) Sauer, U. H., Dao-pin, S., and Matthews, B. W. (1992). *J. Biol. Chem.* **267**, 2393-2399; (26) Matthews, B. W., Nicholson, H., and Becktel, W. J. (1987). *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6663-6667; (27) Alber, T., Bell, J. A., Dao-pin, S., Nicholson, H., Wozniak, J. A., Cook, S., and Matthews, B. W. (1988). *Science* **239**, 631-635; (28) Wozniak, J., and Matthews, B. W., (1992), unpublished; (29) Grutter, M. G., Hawkes, R. B., and Matthews, B. W. (1979). *Nature (London)* **277**, 667-669; (30) Weaver, L. H., Gray, T. M., Grutter, M. G., Anderson, D. E., Wozniak, J. A., Dahlquist, F. W., and Matthews, B. W. (1989). *Biochemistry* **28**, 3793-3797; (31) Kitamura, S., and Sturtevant, J. M. (1989). *Biochemistry* **28**, 3788-3792; (32) Dao-pin, S., Alber, T., Baase, W. A., Wozniak, J. A., and Matthews, B. W. (1991). *J. Mol. Biol.* **221**, 647-667; (33) Eriksson, A. E., Baase, W. A., Wozniak, J. A., and Matthews, B. W. (1991). *Nature (London)* **355**, 371-373; (34) Hurley, J. H., Baase, W. A., and Matthews, B. W. (1992). *J. Mol. Biol.* **224**, 1143-1159; (35) Dao-pin, S., Anderson, D. E., Baase, W. A., Dahlquist, F. W., and Matthews, B. W. (1991). *Biochemistry* **30**, 11521-11529; (36) Grutter, M. G., Weaver, L. H., Gray, T. M., and Matthews, B. W. (1983). In "Bacteriophage T4 (C. K., Mathews, E. M., Kutter, G., Mosig, and P. M. Berget, eds.), pp. 356-360. American Society for Microbiology, Washington, D.C.; (37) Hawkes, R., Grutter, M. G., and Schellman, J. A. (1984). *J. Mol. Biol.* **175**, 195-212; (38) Alber, T., Grutter, M. G., Gray, T. M., Wozniak, J. A., Weaver, L. H., Chen, B.-L., Baker, E. N., and Matthews, B. W. (1986).

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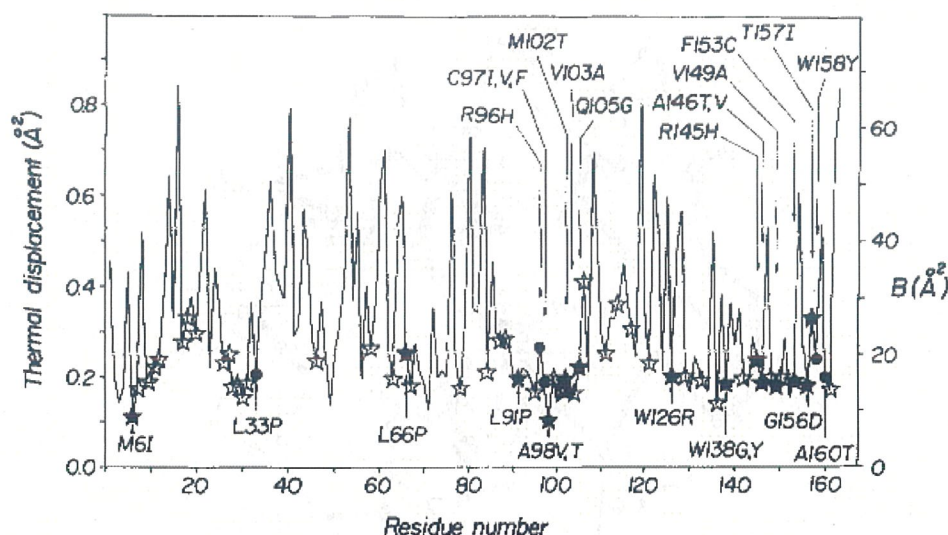


FIG. 1. Locations of substantially destabilizing mutations in T4 lysozyme. The vertical axis shows the average "thermal motion" of each side chain in the crystal structure of wild-type T4 lysozyme. The solid circles indicate the locations of temperature-sensitive mutants of T4 lysozyme, tabulated by Alber *et al.* (1987a), and identified on the figure. Destabilizing substitutions are observed only in the more rigid parts of the structure. The open stars show sites at which substitution with one or more of the amino acids Gly, Ala, Leu, Gln, Cys, Ser, Tyr, Phe, His, Pro, Glu, Arg, or Lys gives a negative suppression pattern at 37°C or 25°C (Rennell *et al.*, 1991). Reproduced, with permission, from the *Annual Review of Biochemistry*, (Matthews, 1993) Volume 62, © 1993, by Annual Reviews, Inc.

routinely be replaced with alanine with very little change in stability or structure (Zhang *et al.*, 1991, 1992; Heinz *et al.*, 1992). This tolerance to change (Matthews, 1987) occurs in part because conformational flexibility often allows the protein to minimize the effects of a potentially deleterious substitution by locally readjusting to give an alternative structure that is energetically comparable to that of the wild type (Alber *et al.*, 1987b; Gibbs *et al.*, 1990).

III. TOLERANCE TO AMINO ACID INSERTION

Although it might be expected that amino acid insertions would be much more destabilizing than substitutions, due to disruption of the polypeptide backbone, this need not always be the case. T4 lysozyme has been shown to tolerate insertion of up to four alanines at the beginning, the middle, and the end of the α helix that includes residues 39–50. This α helix is amphipathic, with its hydrophobic side contributing to the hydrophobic core of the protein and the largely hydrophilic side exposed to solvent. The loop regions at each end of the helix are also relatively exposed to solvent (Fig. 2).

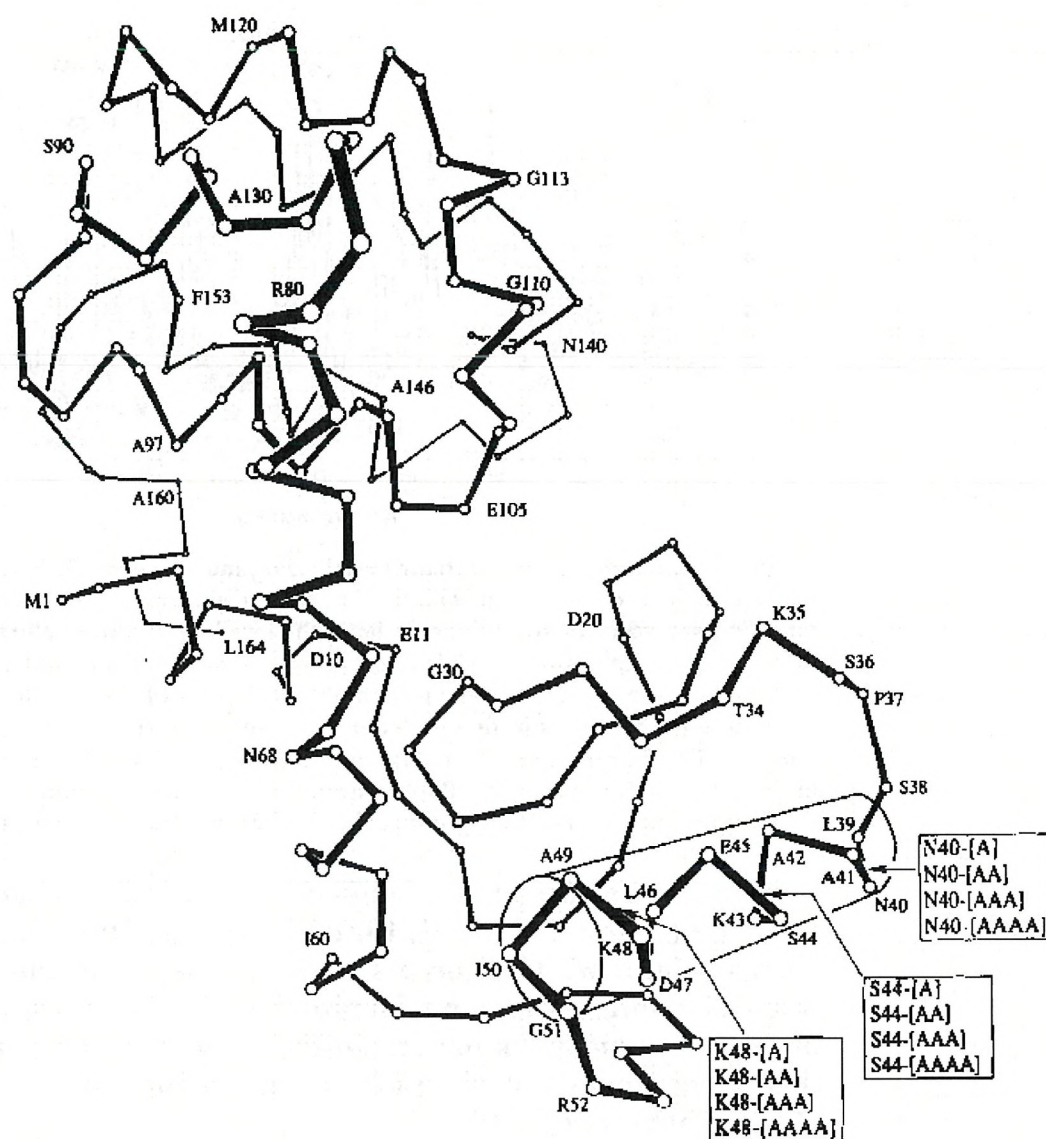


FIG. 2. α -Carbon backbone of T4 lysozyme showing the initial alanine and poly(alanine) amino acid insertions made in α helix 39–50. Reprinted from Heinz *et al.* (1994), with permission.

In a number of cases the insertion mutants could be crystallized and their structures determined. In other cases high-resolution nuclear magnetic resonance (NMR) could be used to determine the structural response of the protein to the insertion. The results are summarized in Figs. 3 and 4 (Heinz *et al.*, 1993, 1994).

There are two distinct ways in which the protein responded to the amino acid insertions: (1) The inserted amino acids were incorporated into the helix by replacing preceding wild-type amino acids and causing

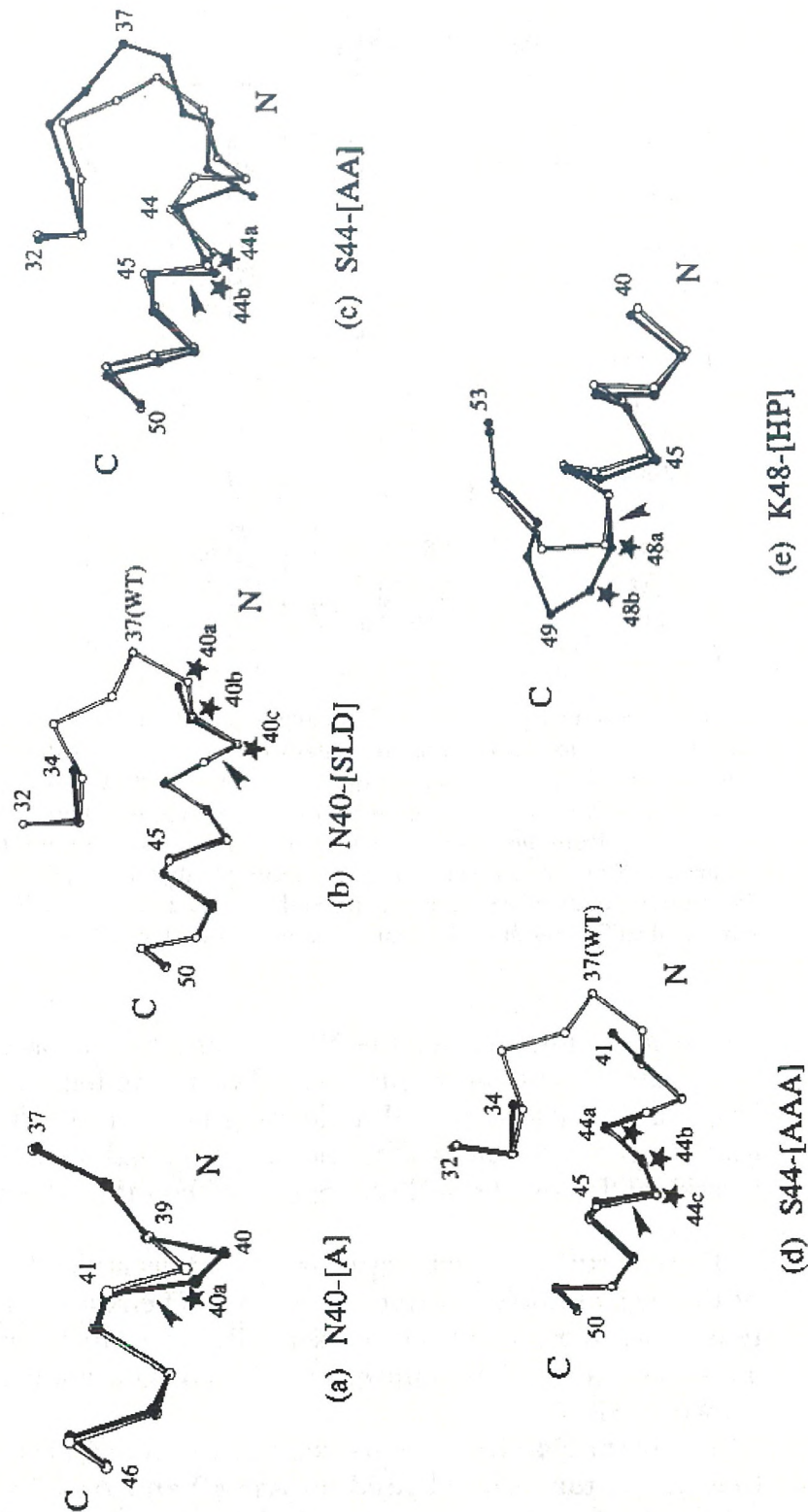
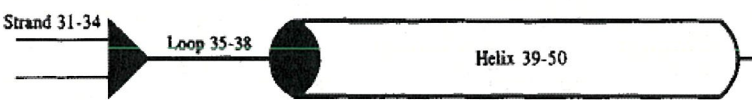


FIG. 3. Superposition of C α positions from the crystal structures of five insertion mutants (solid bonds) on the wild type (open bonds). Arrowheads show the sites of the insertion. Inserted amino acids are labeled using the suffix a, b, or c and are marked with stars. Letters N and C indicate the N and C termini of the α helix. Disordered residues 35-40 in the structures of N40-[SLD] and S44-[AAA] are omitted. Reprinted with permission from *Nature* (Heinz *et al.*, 1993). Copyright (1993), Macmillan Magazines Limited.



	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	$\Delta\Delta G$ (kcal/mol)
Wild type:	L	T	K	S	P	S	L	N	A	A	K	S	E	L	D	K	A	I	G	
Mutant:																				
N40-[A]	"	"	"	"	"	"	N	A	"	"	"	"	"	"	"	"	"	"	"	-2.8
N40L-[A]	"	"	"	S	P	S	L	A	"	"	"	"	"	"	"	"	"	"	"	-1.3
N40-[AA]	"	"	"	S	P	S	L	A	A	"	"	"	"	"	"	"	"	"	"	-1.1
N40-[AAA]	"	"	"	(SPSLN)			A	A	A	"	"	"	"	"	"	"	"	"	"	-1.7
N40-[SLD]	"	"	"	(SPSLN)			S	L	D	"	"	"	"	"	"	"	"	"	"	-0.8
S44-[A]	"	"	"	"	"	"	"	"	"	"	S	A	"	"	"	"	"	"	"	-3.8
N40L/K43A/ S44-[A]	"	"	"	S	P	S	L	A	A	A	S	A	"	"	"	"	"	"	"	-1.8
S44-[AA]	"	"	"	S	P	S	L	N	A	A	K	A	A	"	"	"	"	"	"	-3.4
S44A-[AA]	"	"	"	S	P	S	L	N	A	A	K	A	A	"	"	"	"	"	"	-1.7
S44-[AAA]	"	"	"	(SPSLN)			A	A	K	S	A	A	A	"	"	"	"	"	"	-2.2
K48-[HP]	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	HPA	"	"	-2.4

FIG. 4. Summary of the structural alignment of 11 insertion mutants with residues 33–51 of WT* (top row) based on crystal structure and NMR data. Inserted amino acids are underlined. Anchor residues (see text) are in vertical boxes. Positions identical in mutants and WT* are denoted by double primes ("). Residues "looped out" are shown slightly offset from the respective row. Residues translocated by one, two, or three positions toward the N terminus due to a shift in register are denoted <, <<, or <<<, respectively. Disordered residues are shown in parentheses. The column at the right gives the free energy of unfolding of each mutant relative to wild type. Based on Heinz *et al.* (1994).

a shift in register toward the N terminus. As a consequence, wild-type (WT) amino acids were translocated from the helix into the preceding loop. (2) Insertions caused a "looping out" within the α helix. In this case the perturbation was confined to a minimal region in the immediate vicinity of the insertion. No change in the length of the helix was detected in either case.

The structural response appears to be determined by the maintenance of the hydrophobic interface between the helix and the rest of the protein. This interface remains essentially intact in all variant structures. This behavior can be appreciated by consideration of the mutations shown in Fig. 3.

In mutant N40-[A], the insertion of the single alanine residue in the first helical turn caused residues Asn-40 and Ala-40a to loop out into solvent, leading to a distortion of the helix (Fig. 3a). [N40A would indicate

a mutant lysozyme in which Asn-40 is substituted by alanine. N40-[A] indicates a mutant in which the amino acid(s) in brackets, in this case a single alanine, is inserted following Asn-40.] The positions of the neighboring amino acids, Leu-39 and Ala-41, were practically unchanged. In this case the polypeptide backbone did not translocate toward the amino terminus because it would have resulted in the clearly unfavorable placement of the polar side chain of Asn-40 in the largely buried hydrophobic site previously occupied by Leu-39.

The insertion N40-[SLD] caused a shift in register such that residues Ser-40a, Leu-40b, and Asp-40c occupied the former WT* positions of Ser-38, Leu-39, and Asn-40 (Fig. 3b). This caused a displacement of these residues into the preceding loop (35–38) and an associated expansion of the loop. This loop, which was already mobile in the isomorphous WT* structure, became even more flexible in the mutant, to such a degree that amino acids 35 to 40 could not be seen in the electron density map. In this case, translocation was favored over looping out because Ser-38 and Asn-40 in the wild-type structure are solvent exposed, and therefore very tolerant of change. Position 39 in the wild type is partly buried and might be sensitive to substitution, but translocation in the mutant leaves a leucine at this site, which is well tolerated.

The refined structure of mutant S44[AAA] (Fig. 3d) showed a striking resemblance to N40-[SLD] (see above). The insertion of three alanines, in this case in the middle of the helix, again caused a shift in register by three amino acids toward the N terminus. The three inserted alanines were located in the positions previously occupied by Ala-42, Lys-43, and Ser-44 (Figs. 3 and 4). This in turn leads to the further “substitutions” or “replacements” Ala-41 → Ser, Asn-40 → Lys, and Leu-39 → Ala within the remaining residues of the N-terminal half of the helix. Thus, the translocation affected almost two complete helical turns. As in N40-[SLD], the preceding loop, 35–38, was lengthened by three amino acids and was disordered. Within the translocated region, however, only one of the two residues contributing to the hydrophobic interface between the helix and the rest of the protein (Leu-39) was changed to Ala. This substitution is destabilizing (Table I), but is still tolerated. The second interface residue, Ala-42, was substituted by the inserted Ala-44a.

S44-[AA] showed a very similar response to the insertion as observed for S44-[AAA]. The insertion was again incorporated into the α helix via a register shift that led to the translocation of residues 39–44 by two positions so that the dislocation was ultimately transferred into the preceding loop (Fig. 3c). The polar amino acid Ser-44 now occupied the former position of the completely buried Ala-42. The O γ atom of Ser 44 formed a hydrogen bond with the main-chain carbonyl oxygen of

Asn-40, an $i, i - 4$ interaction often seen for serines in α helices. Overall, this translocation was even more destabilizing than in S44-[AAA] (Table I), but was still preferred to looping out.

Finally, the insertion of His-48a and Pro-48b in the last turn of the helix caused the formation of a well-defined type I β turn that comprised residues His-48a, Pro-48b, Ala-49, and Ile-50. The preceding residue, His-48a, is located in a crevice on the surface of the molecule and the imidazole ring hydrogen bonds with the main-chain amide of Ala-49 and with a solvent molecule. The side chain of Ile-50 still occupied the same region as in WT*, although with slightly changed side-chain torsion angles. Translocation would have resulted in the replacement of the completely buried Ile-50 by the inserted proline. The size mismatch of the proline relative to the isoleucine side chain, accompanied by the disruption by the proline of the α helix, would clearly be destabilizing, and was avoided by looping out.

The results exemplify the plasticity and the adaptability of the protein structure that allows the incorporation of additional amino acids into a secondary structure element without large structural perturbations, as long as vital internal interactions are preserved.

The results also agree with inferences based on sequence/structure comparisons of members of the globin family (Lesk and Chothia, 1980; Bashford *et al.*, 1987). In the case of the globins the interfaces between secondary structure elements were always preserved, indicating their importance for folding and stability. An increase in the length of the helix was not observed for any of the insertion mutants. This suggests that the delineation of the beginning and the end of this α helix is determined not only by the sequence of amino acids that constitute the helix, but also by the interactions between these residues and the remainder of the protein (Heinz *et al.*, 1993, 1994).

IV. HYDROPHOBIC STABILIZATION

It is generally agreed that the hydrophobic effect is the major factor in stabilizing the folded structure of globular proteins. Until recently it has also been generally agreed that the strength of the hydrophobic effect, i.e., the energy of stabilization provided by the transfer of hydrocarbon surfaces from solvent to the interior of a protein, is about 25–30 cal/mol/Å² (Chothia, 1974; Richards, 1977). Some studies using site-directed mutagenesis and protein denaturation have, however, suggested that the strength of the hydrophobic effect might be much larger (Kellis *et al.*, 1989; Shortle *et al.*, 1990; Sandberg and Terwilliger, 1991; Pace, 1992).

To address this question Eriksson *et al.* (1992) created a number of "cavity-creating" leucine-to-alanine replacements in T4 lysozyme and determined their crystal structures. In some mutants the protein structure hardly changed at all. In other cases the protein relaxed substantially (shifts up to ~ 1 Å) and atoms moved into part of the space vacated by the leucine side chain. In each case, however, a cavity remained in the mutant protein. An approximate linear relationship was noted between the size of the cavity created by a given replacement and the loss of protein stability (Fig. 5). Figure 5 suggests that the decrease in protein stability associated with a Leu $\rightarrow$ Ala replacement consists of a constant

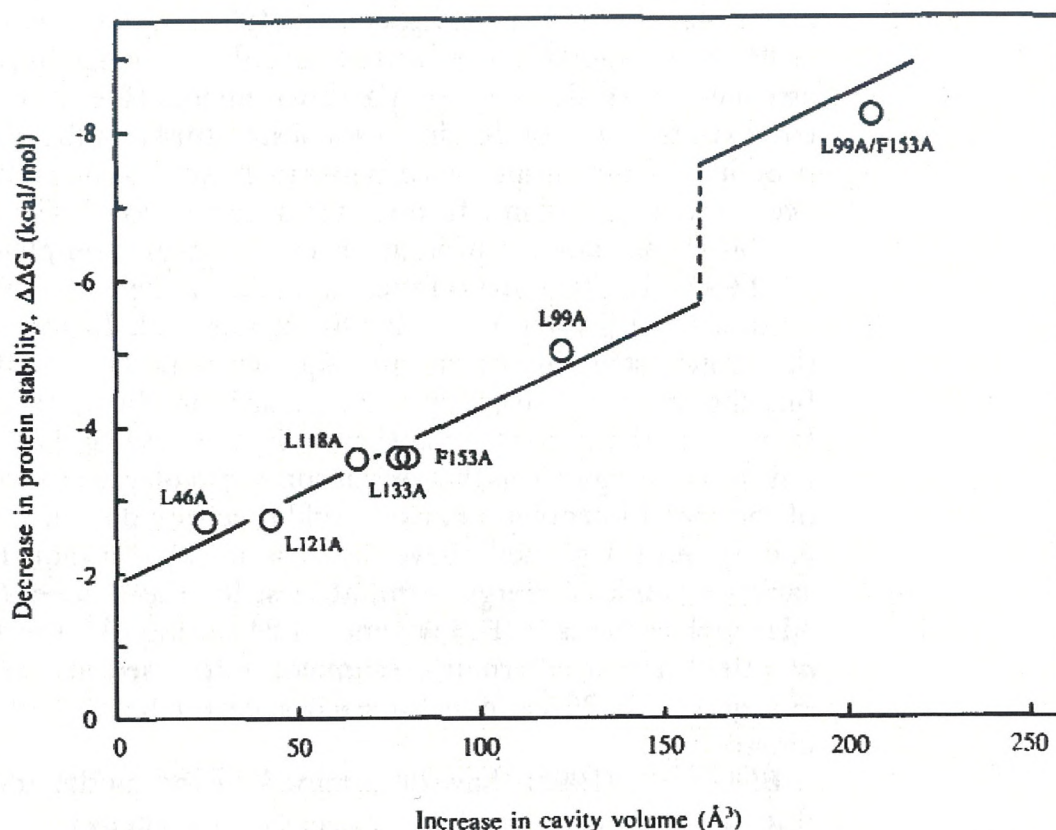


FIG. 5. Change in the free energy of unfolding ($\Delta\Delta G$) of a series of Leu $\rightarrow$ Ala and one Phe $\rightarrow$ Ala mutant lysozymes relative to wild type plotted as a function of the cavity volume created by the amino acid substitution(s). In the vicinity of the double mutant L99A/F153A an additional -1.9 kcal/mol is added to $\Delta\Delta G$ to reflect the fact that in this case both a leucine and a phenylalanine (which is here regarded as equivalent to a second leucine) have been replaced with alanine. Reprinted, with permission, from Eriksson, A. E., Baase, W. A., Zhang, X.-J., Heinz, D. W., Blaber, M., Baldwin, E. P., and B. W. Matthews. Response of a protein structure to cavity-creating mutations and its relation to the hydrophobic effect. *Science* **255**, 178–183 (1992), © AAAS.

energy term of approximately 1.9 kcal/mol plus an energy component that depends on the size of the cavity created by the substitution. The constant term of 1.9 kcal/mol is equal to the difference in hydrophobicity of leucine and alanine estimated by transfer from water to octanol and corresponds to about 25 cal/mol/Å².

Figure 5 suggests that the change in energy associated with the replacement of a buried leucine with an alanine consists of two parts. The first part is a constant and is presumed to depend only on the identities of the two amino acids being compared, in this instance leucine (or phenylalanine) and alanine. Physically this energy term can be considered as the difference in energy required to desolvate (i.e., transfer from solvent to protein interior) a leucine relative to an alanine. As noted above, Chothia (1974) and Richards (1977) have estimated this energy as 25–30 cal/mol/Å². The second part of the change in protein stability associated with the Leu → Ala replacements (Fig. 2) depends on the context within the three-dimensional structure and the way in which the protein structure adjusts in response to the substitution. One can imagine two extreme situations. In one case a Leu → Ala replacement is made at a buried site and the protein structure remains completely unchanged (cf. L99A). In this situation the size of the created cavity is large and the mutant (Ala) protein is maximally destabilized. In the other extreme, the protein structure relaxes in response to the Leu → Ala substitution, fills the space occupied by the leucine side chain, and so avoids the formation of any cavity whatsoever. In this case, and in the absence of any other energy terms that might come into play, the decrease in energy of the mutant protein relative to wild type would reduce to the constant energy term described above, i.e., about 1.9 kcal/mol. In general, the cavity-dependent energy term, at least for the Leu → Ala (Or Phe → Ala) replacements in T4 lysozyme, is 24 cal/mol/Å³ [see also Eriksson *et al.* (1992) for an alternative estimate]. Expressed in terms of area, the energy cost is 20 cal/mol for each square angstrom of cavity surface created.

Blaber *et al.* (1993b) have determined the thermodynamic cost of introducing a polar group within the core of a protein by constructing a series of nine Ala → Ser and three Val → Thr substitutions in T4 lysozyme. The sites were all within α helices, but ranged from fully solvent exposed to totally buried. The range of destabilization incurred by the Ala → Ser substitutions was found to be very similar to that for the Val → Thr replacements. For the solvent-exposed and partly exposed sites the destabilization was modest (≤0.5 kcal/mol). For the completely buried sites the destabilization was larger, but variable (~1–3 kcal/mol). Crystal structure determinations showed that the Ala → Ser mutant structures were, in

general, very similar to their wild-type counterparts, even though the replacements introduce a hydroxyl group. This is in part because the introduced serines are all within α helices and at congested sites can avoid steric clashes with surrounding atoms by making a hydrogen bond to a backbone carbonyl oxygen in the preceding turn of the helix. The three substituted threonine side chains essentially superimpose on their valine counterparts, but display somewhat larger conformational adjustments. The results illustrate how a protein structure will adapt in different ways to avoid the presence of an unsatisfied hydrogen bond donor or acceptor. In the most extreme case, Val-149 $\rightarrow$ Thr, which is also the most destabilizing variant ($\Delta\Delta G = -2.8$ kcal/mol), a water molecule is incorporated in the mutant structure where it forms several hydrogen bonds including one to Thr-149. The results are consistent with the view that many hydrogen bonds within proteins contribute only marginally to stability, but that noncharged polar groups that lack a hydrogen-bonding partner are very destabilizing ($\Delta\Delta G \geq 3$ kcal/mol).

V. CORE PACKING IN T4 LYSOZYME

Side chains in proteins interiors or "cores" are tightly packed (Richards, 1974) and usually adopt single well-defined low-energy conformations (Chandrasekaran and Ramachandran, 1970; Janin *et al.*, 1978).

However, genetic experiments by Sauer and co-workers demonstrated that λ repressor tolerated multiple substitutions within the interior (Lim and Sauer, 1989; Lim *et al.*, 1992). Many variants with different combinations of interior hydrophobic side chains were functional, and a significant fraction were only moderately destabilized (Lim *et al.*, 1992). This result poses two questions: (1) how are the different side chains arranged in the interior, and (2) how has the rest of the protein adjusted? To address these questions, T4 lysozyme variants with altered hydrophobic cores were obtained and their three-dimensional structures determined (Baldwin *et al.*, 1993).

The largest contiguous hydrophobic core in T4 lysozyme is within the carboxy-terminal domain (residues 81–164). Five adjacent buried residues in this region were targeted for substitution: Leu-121, Ala-129, Leu-133, Val-149, and Phe-153 (Fig. 2). High-resolution structures and folding energies of eight variants containing three to five substitutions were determined (Table I).

All variants were less stable than the wild-type protein (-1.1 to -3.5 kcal/mol), but not by more than that expected for a single interior substitution (Dao-pin *et al.*, 1991; Eriksson *et al.*, 1992, 1993) (Table I). Thus, as observed for λ repressor (Lim and Sauer, 1989; Lim *et al.*,

1992), the structure and activity of T4 lysozyme are tolerant to a variety of combinations of hydrophobic residues in the core.

Although the distal atoms of some of the replacement side chains changed their positions up to 1–2 Å, their torsion angles were within 20° of the wild type, with the exception of position 153 (Fig. 6). In other words, the mutant side chains usually retained the same rotational conformation as present in wild type. Three of the variants that had Phe or Trp at position 153 (II, III, and IV) had essentially wild-type torsion angles ($\chi_1 = -81^\circ$ to -91° , $\chi_2 = 108^\circ$ to 129°). In a variant V, which had the largest structural perturbations, there was a Phe at position 153 with $\chi_1 = -143^\circ$ and $\chi_2 = 140^\circ$. The other four variants (I, VI, VII and VIII) had Leu at position 153, and all four had similar conformations ($\chi_1 = -160^\circ$ to -173° , $\chi_2 = 45^\circ$ to 80°). Differences in side-chain rotational conformations occurred in only 13 out of 59 possible torsion angles or in 7 out of 33 possible side chains. Change in conformation of even a single side chain may be difficult to accommodate because it may require conformational changes in surrounding side chains.

Root-mean-square (RMS) shifts in backbone atoms for the eight variants ranged from 0.17 to 0.63 Å (Fig. 7a). Helices moved up to 0.8 Å (Fig. 7a) by a combination of rotations and translations, with individual α -carbon atoms moving up to 1.7 Å. The largest backbone adjustments occurred in the variants having the largest increase in core volume and corresponded to an overall expansion of the COOH-terminal domain.

Thus, instead of the backbone remaining fixed and the side chains adopting new rotamer angles, the protein accommodated the alternative core-packing residues by adjusting the backbone and, in most instances, leaving the side-chain rotamer angles essentially the same as in the wild-type structure.

Figure 7b shows the shifts in the backbone observed within the carboxy-terminal domain in a series of T4 lysozyme variants that crystallize in forms different from wild type. These are mutant structures but the substitution(s) are in the amino-terminal domain. Therefore the structural changes seen in Fig. 7b are presumably not due to the mutation, but rather to the different crystal-packing interactions that occur in these novel crystal forms. It is striking that the shifts in the backbone observed for the alternative core-packing mutants (Fig. 7a) are comparable to those due to alternative crystal-packing forces (Figure 7b). It suggests that backbone shifts of this magnitude cannot be of high energy. Rather, it would appear that the “native” backbone structure of a protein can be perturbed by ~0.5 Å or so with a relatively small energy cost (~1–2 kcal/mol).

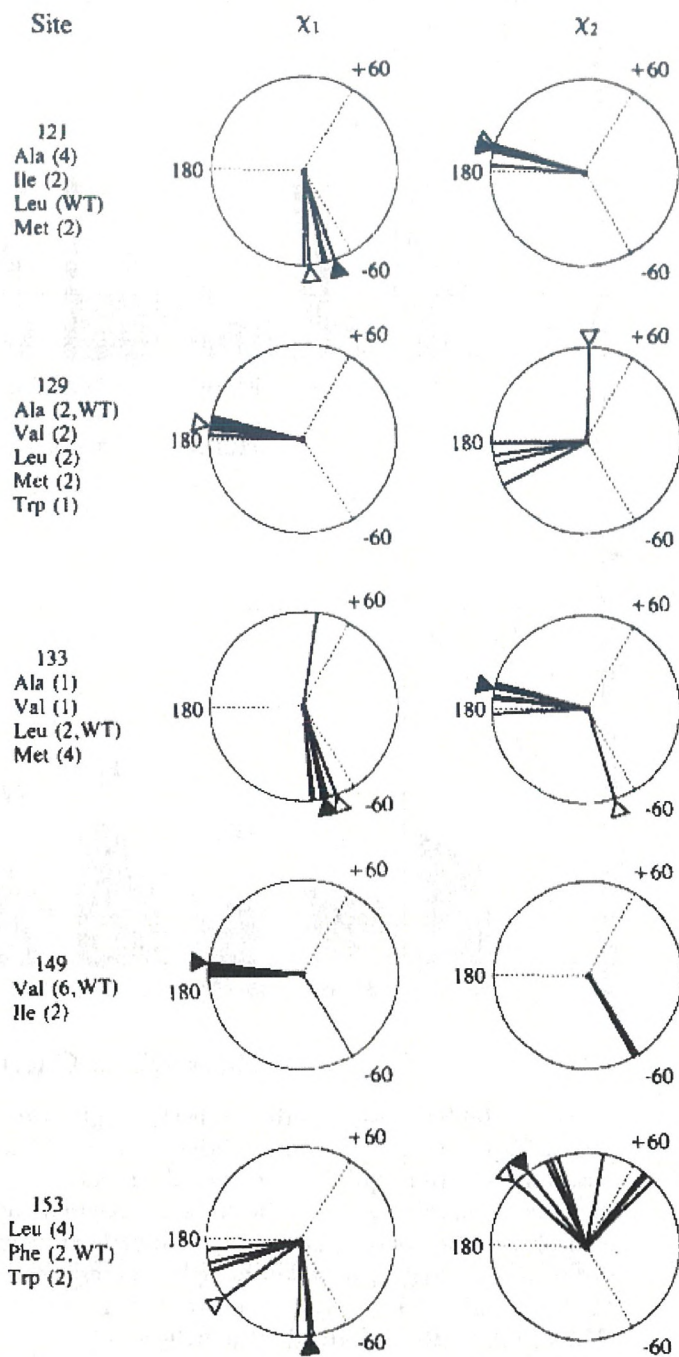


FIG. 6. Newman projections of the side-chain torsion angles of five sites that are substituted in eight different core-packing mutants. The sites of substitution and the different amino acids that occur at each site are given on the left, with the number of occurrences in parentheses. The torsion angles in the wild-type structure are indicated by the solid arrowheads; the torsion angles in variant V, which has the largest structural changes of all mutants, are indicated by the open arrowheads. After, with permission, Baldwin, E. P., Hajiseyedi, O., Baase, W. A., and B. W. Matthews. The role of backbone flexibility in the accommodation of variants that repack the core of T4 lysozyme. *Science* 262, 1715–1718 (1993), © AAAS.

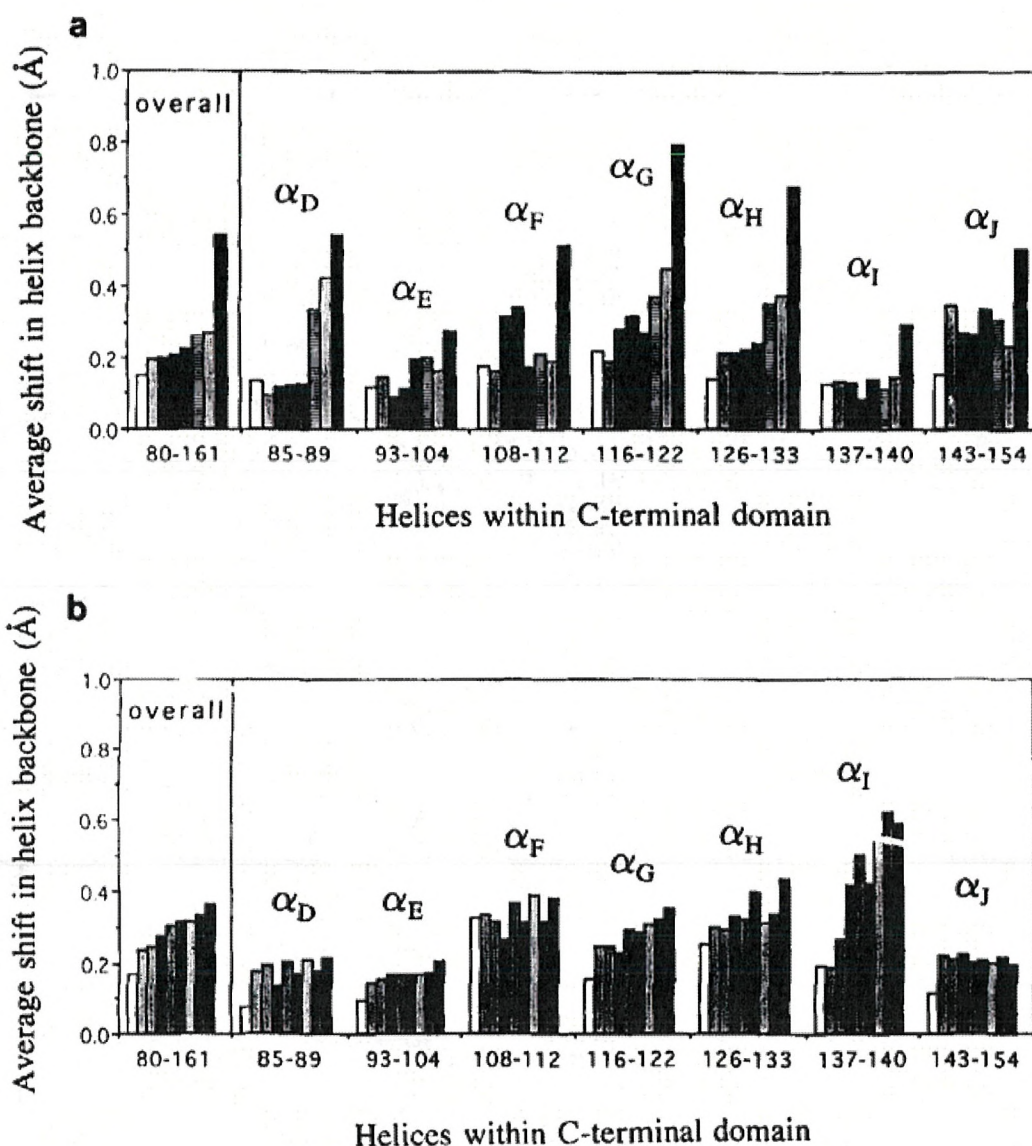


FIG. 7. (a) Shifts in individual helices in eight core-repacking variants. For each variant the backbone atoms within the carboxy-terminal domain (residues 81–161) were superimposed on the wild type. The average differences for the backbone atoms within each of the seven α helices (α_D – α_J) in the carboxy-terminal domain are shown. The average differences for all backbone atoms within the carboxy domain are also shown at the left. The order of the variants, from left to right, corresponds to their relative stabilities. Based on Baldwin *et al.* (1993), except that average rather than root-mean-square differences are shown. (b) Shifts in individual α helices, relative to wild type [as in (a)], seen in the nonisomorphous crystal structures of the mutant lysozymes Ser-44 $\rightarrow$ Glu (space group $P2_1$, one independent molecule), Ser-44 $\rightarrow$ Trp (space group $P1$, four molecules per asymmetric unit), and Ser-44 $\rightarrow$ Phe (space group $P2_1$, two independent molecules) (Blaber *et al.*, 1993a). In each case the site of the amino acid substitution is in the amino-terminal domain, remote from the helices shown, which are in the carboxy-terminal domain. The shifts shown are presumably due to the effect of the different crystal contacts that occur for each of these seven crystallographically distinct lysozyme molecules. Based, with permission, on Baldwin, E. P., Hajiseyedi, O., Baase, W. A., and B. W. Matthews. The role of backbone flexibility in the accommodation of variants that repack the core of T4 lysozyme. *Science* **262**, 1715–1718 (1993), © AAAS.

VI. HELIX PROPENSITY

Blaber *et al.*, (1993a, 1994) have attempted to understand the structural basis of α -helix propensity by making a series of substitutions at two different sites (positions 44 and 131) within α -helices in T4 lysozyme.

With the exception of proline, the amino acid substitutions caused little if any perturbation of the α -helix backbone. Also, the β -branched residues Thr, Val, and Ile show no indication of either side-chain or backbone distortion. Therefore, other than proline, there is no evidence that differences in helix propensities are associated with different amounts of strain introduced into the helix.

The changes in protein stability for the replacements at site 131 in general agreed well with those at site 44, suggesting that these may be representative of substitutions at fully solvent-exposed sites in the middle of α helices. The free energy values also agreed quite well with those observed for equivalent replacements in a number of soluble α -helical model peptides and with data from "host-guest" studies and statistical surveys (correlation coefficients $r = 0.69$ – 0.93). The results suggested that helix propensity scales derived from model peptides can be applicable to proteins, but energy differences due to helix propensity per se are small, and can easily be masked by a myriad of context-dependent factors.

The observed changes in free energy associated with different replacements within an α helix are, however, small (0–1.0 kcal/mol). At the same time, relatively small changes in the conformation of the folded structure, or different assumptions as to the nature of the unfolded state, can easily alter estimates of surface area or side-chain entropy by amounts equivalent to several tenths of a kilocalorie. This highlights perhaps the major problem in understanding the underlying basis of α helix propensity. Lacking truly reliable values for the hydrophobic effect and for side-chain entropy, among other factors, it is difficult to be sure as to the relative importance of these terms.

Subject to these caveats, the data suggested (Blaber *et al.*, 1993) that the buried hydrophobic surface estimated from the crystal structures of the site 44 and site 131 substitutions correlated with observed $\Delta\Delta G$ values except for a couple of amino acids (Phe and Trp) that, in the crystal, displayed (weak) side-chain–side-chain contacts.

The possible importance of the hydrophobic effect in promoting α -helix formation has been appreciated for some time (Chou *et al.*, 1972; Ptitsyn, 1972; Richards and Richmond, 1978). As a helix forms, the access of solvent to a given side chain is partly restricted by atoms within the next turn of the helix [typically backbone atoms within the $(i - 3)$

and ($i - 4$) residues]. Because the side-chain surface area involved is relatively small, the equivalent free energy of hydrophobic stabilization is expected to be modest ($\Delta\Delta G \lesssim 1$ kcal/mol), but this is on the order of magnitude of $\Delta\Delta G$ values due to differences in α -helix propensity. Richards and Richmond (1978) attempted to estimate the contribution of the hydrophobic effect by surface area calculations, although at that time the experimental data against which they could test their model were extremely limited. [Rather than publishing solvent-accessible surface areas, for which transfer free energies of 20–33 cal/mol/Å² have been suggested, Richards and Richmond (1978) reported contact areas, for which corresponding transfer free energies are 70–115 cal/mol/Å².] The correlations between the buried hydrophobic contact area estimated by Richards and Richmond (1978) and the various scales now available average 0.49 (or 0.64 if Gly is included). This suggests that hydrophobic stabilization plays a significant role in determining α -helix propensity. The contact area can be converted into an equivalent energy of hydrophobic stabilization, $\Delta\Delta G_H$, and added to the estimated side-chain entropy, $T\Delta S$ (Blaber *et al.*, 1994), to give the free energy of unfolding (relative to Gly) due to the combination of the hydrophobic effect plus side-chain entropy. This combined energy term, $\Delta\Delta g_H + T\Delta S$ (Blaber *et al.*, 1994), agrees somewhat better with the various helix propensity scales (average correlation 0.57) than does the hydrophobic effect (average correlation 0.49) or entropic effect (average correlation 0.39) alone. Thus, even though the differences in helix propensity for the naturally occurring amino acids are generally small, it is becoming possible to understand these differences in structural terms.

VII. SUMMARY

A series of mutants of phage T4 lysozyme have been constructed and have permitted a systematic analysis of different aspects of protein stability and folding. One striking result has been the ability of the protein to accommodate changes at many sites, yet still fold and retain activity. This shows that many amino acids in the sequence of the protein are nonessential for protein folding and stability. Such amino acids appear, predominantly, to be those that are mobile and/or largely exposed to solvent. Even though buried residues seem to be more important, it is still possible to change these as well. In some cases individual amino acids have been replaced. In other cases it has been found that a combination of substitutions permits repacking of the core. Such repacking is associated with adjustments of both the main chain and side chains. Only rarely do side chains rotate into radically new orientations.

Taken together, substitutions of core residues confirm the overall importance of the hydrophobic effect as the dominant factor in stabilizing the folded structures of proteins. "Cavity-creating" substitutions of the form Leu $\rightarrow$ Ala show, as expected, that the burial of the bulky leucine side chain within the core of the protein confers greater hydrophobic stabilization than is the case for the smaller alanine side chain. Leu $\rightarrow$ Ala substitutions that create large cavities are especially destabilizing because they result in a loss of both hydrophobic and van der Waals interactions. In cases where the protein relaxes to reduce the size of the putative cavity, alternative van der Waals interactions are generated and the overall destabilization of the protein may be less severe than in cases where a large cavity is formed.

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