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Substantial increase of protein stability by multiple disulphide bonds

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DISULPHIDE bonds can significantly stabilize the native structures of proteins^{1–3}. The effect is presumed to be due mainly to a decrease in the configurational chain entropy of the unfolded polypeptide^{4–7}. In phage T4 lysozyme, a disulphide-free enzyme, engineered disulphide mutants that crosslink residues 3–97, 9–164 and 21–142 are significantly more stable than the wild-type protein^{8–11}. To investigate the effect of multiple-disulphide bonds on protein stability, mutants were constructed in which two or three stabilizing disulphide bridges were combined in the same protein. Reversible thermal denaturation shows that the increase in melting temperature resulting from the individual disulphide bonds is approximately additive. The triple-disulphide variant unfolds at a temperature 23.4 °C higher than wild-type lysozyme. The results demonstrate that a combination of disulphide bonds, each of which contributes to stability, can achieve substantial overall improvement in the stability of a protein.

Unpaired cysteine residue(s) in a protein containing a disulphide bond(s) can lead to oligomerization through thiol/disulphide interchange¹². Previously described single-disulphide mutants (designated as 3C-54T, 9C-164C-wt* and 21C-142C-wt*, see Fig. 1) were, therefore, constructed in an otherwise cysteine-free pseudo wild-type lysozyme (wt*)^{11,13}. For the same reason the two double-disulphide mutants D3-97/9-164 and D9-164/21-142 and the triple disulphide mutant T3-97/9-164/21-142 were also designed and constructed to have no unpaired cysteines. Figure 1 shows the locations of the disulphide bonds and ancillary mutations introduced into T4 lysozyme. Each of the mutant genes was expressed in *Escherichia coli* and the proteins purified to homogeneity. Immediately after purification, the mutant enzymes were found to be a mixture of oxidized (crosslinked) and reduced (noncrosslinked) forms, as judged by ion-exchange high-performance liquid chromatography (HPLC)¹¹, reversed-phase HPLC^{8,13} and titration of protein thiols with Ellman's reagent (data not shown). After exposure of proteins to air under mild alkaline conditions (pH 8.0) for several days, however, the mutant proteins were all converted to the oxidized forms (Table 1). Nonreducing SDS-PAGE showed that the double- and triple-disulphide mutants migrate faster than the disulphide-free wild-type lysozyme, whereas all the multiple-disulphide mutants had mobility identical with wild type in the presence of reducing agent (data not shown). This

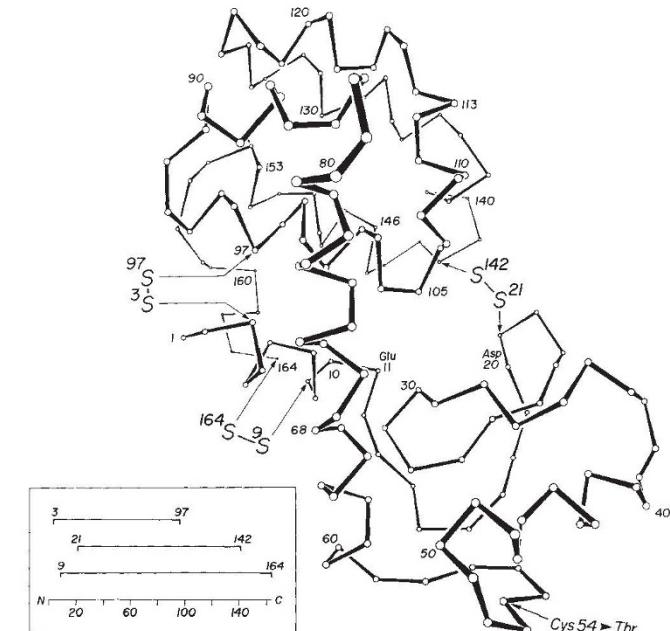


FIG. 1 Backbone of T4 lysozyme showing the locations of the three engineered disulphide bridges. The insert illustrates the loops formed by these bridges. Identification and generation of the mutant lysozymes is as follows:

Variant	Amino-acid replacements								No. of disulphide bonds
	3	9	21	54	97	142	164	No. of cysteines	
wt	Ile	Ile	Thr	Cys	Cys	Thr	Leu	2	0
wt*				Thr	Ala			0	0
3C-54T	Cys			Thr				2	1
9C-164C-wt*		Cys		Thr	Ala		Cys	2	1
21C-142C-wt*			Cys	Thr	Ala	Cys		2	1
D3-97/9-164	Cys	Cys		Thr			Cys	4	2
D9-164/21-142	Cys	Cys	Thr	Ala	Cys	Cys	4	2	
T3-97/9-164/21-142	Cys	Cys	Thr		Cys	Cys	6	3	

Recombinant DNA techniques used in the construction of the disulphide mutants were essentially as described^{4,7}. Mutagenic oligonucleotides (21–23 mer) were synthesized using a model 380B DNA synthesizer (Applied Biosystems) and purified by a C18 Sep-Pak cartridge (Millipore). The single-stranded DNA template for site-directed mutagenesis was an M13mp18 derivative containing the T4 lysozyme gene on a 630 base-pair *Bam*H-I-HindIII fragment. The mutagenesis was performed according to Kunkel *et al.*¹⁸ using *E. coli* strain CJ236 (*dut1*, *ung1*, *thi1*, *relA1*/pCJ105 (Cm^r)). After the repair synthesis of DNA *E. coli* JM101 (ref. 19) was transformed and the mutants identified either by DNA sequencing²⁰ or plaque hybridization with the [³²P]-labelled mutagenic prime²¹. The mutations were verified by sequencing the entire T4 lysozyme gene. The mutated T4 lysozyme gene on M13 was digested with *Bam*H and *Hind*III, and cloned into the expression plasmid pHSe5 that contains *tac* and *lacUV5* promoters, *lacI*^q gene as well as *trp* terminator²². *E. coli* RR1 (ref. 17) was then transformed by the recombinant pHSe5 and the mutant protein was overproduced by addition of isopropyl-β-thiogalactoside. The mutant proteins were purified to homogeneity by CM-Sepharose and SP-Sephadex (Pharmacia) chromatography as described²².

observation indicates that the crosslinked proteins have a more compact structure in the denatured state.

The activity of the D3-97/9-164 mutant (Table 1) is indistinguishable from that of wild-type enzyme in both the oxidized and reduced forms. The result is consistent with the observation that the corresponding single-disulphide mutants have essentially the same activities as wt* in both oxidized and reduced forms¹¹. In addition it indicates that virtually all the D3-97/9-164 molecules have the correct pairing of the disulphide bridges as any mispairing would presumably lead to a loss of activity. The wt* lysozyme loses enzymatic activity at ~55 °C at pH 7.4, whereas the D3-97/9-164 mutant, which is more thermostable than wt* by 15.7 °C at pH 2.0 (see below), retains activity up to

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TABLE 1 Properties of mutant lysozymes

Variant	Ox/Red	Thiols (mol SH/mol prot.)	Relative activity (%)	T_m (°C)	$\Delta T_m(\text{mut-wt}^*)$ (°C)	$\Delta T_m(\text{ox-red})$ (°C)
wt	Ox	1.9	(100)		—	—
	Red	2.0	100	41.9		
wt*	Ox	0.0	(103)			
	Red	0.0	103	41.9	0.0	0.0
3C/54T	Ox	ND	96	46.7	4.8	6.7
	Red	ND	90	40.0	-1.9	
9C/164C/wt*	Ox	ND	106	48.3	6.4	12.9
	Red	ND	99	35.4	-6.5	
21C/142C/wt*	Ox	ND	0	52.9	11.0	13.7
	Red	ND	68	39.2	-2.7	
D3-97/9-164	Ox	0.0	95	57.6	15.7	26.0
	Red	4.1	100	31.6	-10.3	
D9-164/21-142	Ox	0.0	0	58.9	17.0	22.9
	Red	4.1	58	36.0	-5.9	
T3-97/9-164/21-142	Ox	0.2	0	65.5	23.4	31.9
	Red	6.0	43	33.4	-8.5	

Oxidized (crosslinked) mutant lysozymes were prepared on exposure to air *in vitro* by incubating in 0.1 M Tris-HCl, 0.15 M NaCl (pH 8.0) for several days. The reduced (noncrosslinked) forms of the mutant lysozymes were prepared by treating the purified proteins ($\sim 1 \text{ mg ml}^{-1}$) with 6 M guanidine hydrochloride, 20 mM DTT and 1 mM EDTA in 50 mM Tris-HCl (pH 8.3). After incubation for 4 h at 23 °C, the mixture was extensively dialysed under a nitrogen gas purge at 4 °C against 0.2 M NaCl and 1 mM EDTA (pH 2.0). Under the nitrogen atmosphere and at acid pH, the reduced proteins were stable for at least a week at 4 °C. Thiol content was determined by Ellman titration²³. A 0.5 ml fraction of the oxidized or reduced protein (0.02 μmole) was mixed with 2.5 ml of a solution containing 2% SDS, 0.08 M sodium phosphate (pH 8.0), and 0.5 mg ml^{-1} EDTA. To 3 ml of the solution was immediately added 0.1 ml 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) (40 mg DTNB in 10 ml of 0.1 M sodium phosphate, pH 8.0). After 15 min, the absorbance at 410 nm was measured. Lysozyme activities were measured at 23 °C using the turbidity assay²⁴. Protein concentrations were determined spectrophotometrically (molar absorption coefficient for wild-type T4 lysozyme $\epsilon_{280\text{nm}}^{0.1\%} = 1.28$) (ref. 24). T_m is the temperature at the midpoint of the thermal denaturation transition at pH 2.0. $\Delta T_m(\text{mut-wt}^*)$ and $\Delta T_m(\text{ox-red})$ are the differences between mutant and wt* lysozyme and between the oxidized and reduced mutant protein, respectively. Data for wild type and single S-S bridge lysozymes are from refs 10, 11 and 25. Thermal stability was assessed at pH 2.0 by measuring the circular dichroism at 223 nm as a function of temperature^{26,27}. Solutions contained $\sim 0.02 \text{ mg ml}^{-1}$ protein, 0.15 M KCl and 1 mM EDTA, adjusted to pH 2.0 with HCl. To avoid air oxidation, the experiments were performed in a nitrogen atmosphere and the KCl-EDTA solutions were extensively bubbled with nitrogen gas before use²⁸.

~65 °C (data not shown). Mutants D9-164/21-142 and T3-97/9-164/21-142, which include the 21-142 disulphide bond, have virtually no catalytic activity in the oxidized form. This is expected as the 21-142 disulphide bond spans the 'mouth' of the active-site cleft (Fig. 1) leading to the complete abolition of lysozyme activity¹³.

The melting temperatures of the single and multiple disulphide bridge mutants at pH 2.0 are summarized in Table 1 and Fig. 2. Both the double mutants are more heat-stable than their constituent single mutants. In the case of the triple mutant the increase in melting temperature (ΔT_m) is substantial (23.4 °C) and agrees surprisingly well with the sum of the ΔT_m s of the corresponding single disulphide variants (22.2 °C). Measurement of the melting temperature at higher pH values is difficult because the solutions approach boiling point before the unfolding transition is complete. As a guide, however, the estimated reversible melting temperature of the triple disulphide mutant at pH 5.0 is 86 °C, which is 20 °C higher than wild type at the same pH.

It is presumed that a consequence of a disulphide bridge is to reduce the configurational backbone chain entropy of the protein in the denatured state, thus increasing the stability of the folded protein³⁻⁷. Theoretical considerations suggest that the entropic contribution to the free energy of stabilization increases in proportion to the logarithm of the number of residues in the loop formed by the disulphide bridge³⁻⁶. When multiple, overlapping disulphide bonds are introduced into a protein, their effects on the configuration of the unfolded protein are interdependent, and the determination of the entropic term becomes more complicated^{6,7,14}. Nevertheless, reasonable agreement between experiment and theory has been observed for ribonuclease A^{6,14}, ribonuclease T1 (ref. 3) and hen egg-white lysozyme¹⁵. Based on theory¹⁴, the reductions in entropy in the unfolded protein caused by the single 3-97, 9-164 and 21-142 bridges are, respectively, 20.5, 21.9 and 21.2 entropy units. The sum of these three terms, considered independently, is 63.6 entropy units, which corresponds to 20.0 kcal mol⁻¹ at 42 °C, the melting temperature of wild-type lysozyme at pH 2.0. By

contrast, the theoretically estimated¹⁴ entropy reduction when all three bridges are included in the same protein is 56.7 entropy units, corresponding to 17.9 kcal mol⁻¹. Thus, even though the loops formed by the three disulphide bridges have substantial overlap (Fig. 1), this has only a relatively small effect on the

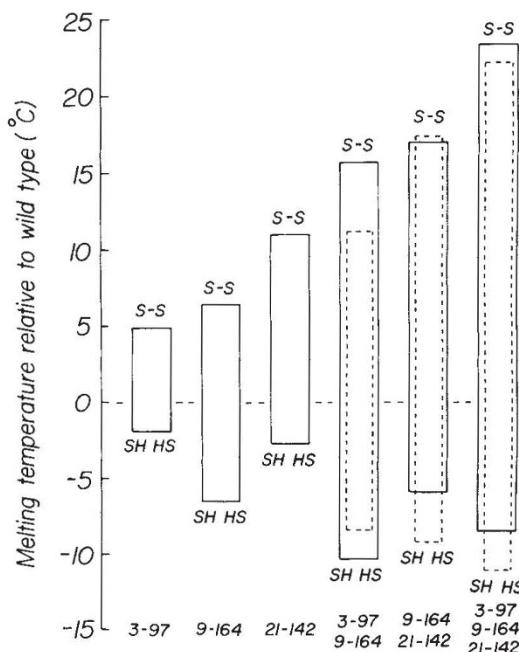


FIG. 2 Melting temperatures of single, double and triple-disulphide bonded lysozymes relative to wild-type lysozyme (ΔT_m) at pH 2.0. The solid bars show the observed melting temperatures of the oxidized and reduced forms of the mutant lysozymes (Table 1). The broken bars for the multiply bridged proteins correspond to the sums of the ΔT_m s for the constituent singly bridged lysozymes.

overall entropy term. Although each disulphide bridge reduces the conformational freedom of the unfolded chain, this restriction represents only a small fraction of the total number of conformational states that are available. Thus, the contributions to stability from each of the three disulphide bridges are still largely additive. The crosslinked lysozymes display a correlation between the number of disulphide bonds and the Stokes radii of the denatured proteins, as judged by gel filtration HPLC in the presence of urea¹⁶. This also indicates that the crosslinks successively decrease the configurational freedom of the unfolded protein.

The above arguments are based on entropy considerations alone. As discussed in ref. 11, the net stabilization of a given disulphide bond depends on other factors, such as the loss of stabilizing interactions associated with introduction of the cysteine(s) and possible introduction of strain associated with the closure of the disulphide bridge. In the case of T4 lysozyme, factors that seem to be helpful in the design of the stabilizing

disulphide bridges include the use of large loops to maximize the entropic effect on the unfolded state and the choice of flexible sites to avoid the introduction of strain into the folded protein¹¹. Exploration of the energetic and theoretical basis of the observed stabilization of the multiply bridged lysozymes will require detailed thermodynamic analysis of these proteins.

In terms of the goal of developing general methods to increase the stability of proteins, the present results are especially encouraging. They demonstrate that individual S-S bridges can be combined together to dramatically enhance stability, even in cases where the disulphide bridges form overlapping loops. It is also encouraging to find that the double-disulphide mutant D3-97/9-164 is fully active and also retains activity at temperatures substantially higher than wild-type lysozyme. This shows that a protein does not have to be marginally stable in order to be fully active. Instead, the present results indicate that the stability of a protein can be substantially increased with no loss in enzymatic activity. □

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Focal points for chromosome condensation and decondensation revealed by three-dimensional *in vivo* time-lapse microscopy

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ALTHOUGH the dynamic behaviour of chromosomes has been extensively studied in their condensed state during mitosis, chromosome behaviour during the transition to and from interphase has not been well documented. Previous electron microscopic studies suggest that chromosomes condense in a non-uniform fashion at the nuclear periphery^{1,2}. But chromosome condensation is a complicated and dynamic process and requires continuous observation in living tissues to be fully understood. Using a recently developed three-dimensional time-lapse fluorescence microscopy technique³, we have observed chromosomes as they relax from telophase, through interphase, until their condensation at the next prophase. This technique has been improved to produce higher-resolution images by implementing new stereographic projection and computational processing protocols⁴. These studies have revealed that chromosomal regions on the nuclear envelope, distinct from the centromeres and telomeres, serve as foci for the decondensation and condensation of diploid chromosomes. The relative positions of the late decondensation sites at the beginning of interphase

appear to correspond to the early condensation sites at the subsequent prophase.

In order to study the chromosomal events that occur between telophase and the ensuing prophase, we have followed embryonic nuclei of *Drosophila melanogaster* during the late syncytial blastoderm period. Chromosomes in living *Drosophila* embryos were visualized by imaging chromatin which had assembled fluorescent-labelled histones into nucleosomes. Calf thymus histones H2A and H2B were conjugated with rhodamine and microinjected into *Drosophila* embryos 30 minutes after oviposition to ensure a uniform distribution of the labelled histones by the time of observation. The injected rhodamine-labelled histones are stably incorporated into chromatin during periods of DNA replication. Under low levels of illumination, these embryos can be observed for more than three hours and develop normally to hatching.

Observing the behaviour of an entire nucleus requires a three-dimensional analysis over time. Imaging at low light levels was accomplished using a cooled, scientific grade charge-coupled device (CCD) imager^{5,6} operated by a self-contained computer workstation. Three-dimensional data sets were obtained by digitally recording a series of five optical sections taken at 1 μm focal intervals every five seconds, as described in the legend to Fig. 1. By repeating this optical sectioning protocol every 25 seconds, a time-lapse series of 80 three-dimensional data sets was recorded. The period of analysis spanned nuclear cycles 12 and 13 of syncytial blastoderm stage embryos. During this stage of embryonic development, the nuclei form an almost synchronously replicating monolayer on the embryo surface^{7,8}. The nuclear cycle length for the embryo shown in Fig. 1 was 22 minutes at 23 °C. Embryos were monitored after data collection to verify that gastrulation occurred normally.

To facilitate analysis of the large amount of three-dimensional data over time, a time-lapse stereo movie was constructed using