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Development of an in vivo method to identify mutants of phage T4 lysozyme of enhanced thermostability

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Abstract

An M13 bacteriophage-based in vivo screening system has been developed to identify T4 lysozyme mutants of enhanced thermal stability. This system takes advantage of easy mutagenesis in an M13 host, the production of functional T4 lysozyme during M13 growth, and the ability to detect lysozyme activity on agar plates. Of several mutagenesis procedures that were tested, the most efficient was based on misincorporation by avian myeloma virus reverse transcriptase. This one-step mutagenesis and screening system has been used to find 18 random single-site mutant lysozymes, of which 11 were heat resistant. Each of these had a melting temperature within 0.8–1.4 °C of wild type, suggesting that the screening system is quite sensitive.

Keywords: mutagenesis; protein stability; screening

Following the pioneering studies of Streisinger et al. (1961), Alber and Wozniak (1985) attempted to develop a convenient method for generating and locating thermostable mutants of T4 lysozyme using the T4 phage system itself. The screening method was based on the ability to detect lysozyme activity as a digestion halo surrounding phage plated on a bacterial lawn, following exposure to chloroform (Streisinger et al., 1961, 1966). One of the initial mutants that was characterized was subsequently identified as Cys 54 → Tyr (Alber & Matthews, 1987). In this case the apparent heat resistance may have been due to elimination of one of the chemically reactive cysteines rather than a thermodynamic effect per se.

Because of the obvious benefits of a system that would allow rapid, in vivo screening of large numbers of mutants for stability, an attempt has been made to develop a similar, phage-based system for mutagenesis and screening of T4 lysozyme, but using instead the single-strand phage M13mp18. Because M13 does not produce lysozyme activity of its own, but will express a T4 lysozyme gene cloned into it, this activity can be visualized using the same halo assay used for T4 phage. In addition, because M13 is a small, single-strand phage, mutagenesis, screening, and sequencing of the gene can be carried out quickly, efficiently, and easily, without the need for intermediate cloning steps.

Methods to identify thermostable variants have previously been developed for kanamycin nucleotidyltransferase (Matsumura & Aiba, 1985; Liao et al., 1986), subtilisin (Bryan et al., 1986), and glucose dehydrogenase (Makino et al., 1989).

Results

Plating assays

The halo assay is based on the fact that, like T4 phage, the M13 vector containing the T4e gene expresses T4 lysozyme activity when plated on bacterial lawns and this activity can be visualized as clear circles, or "haloes," surrounding the phage plaques when the plates are treated with chloroform vapor (Streisinger et al., 1961). When the assay plates are incubated at low temperatures, all mutants and the wild-type protein exhibit haloes of approximately the same size. As the incubation temperature is increased, the haloes increase in size uniformly and the proteins remain undistinguishable, until the temperature reaches a certain critical value, which varies from mutant to mutant, above which the halo size of that mutant rap-

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idly decreases. The critical temperature depends on the reversible melting temperature of the protein. By carefully adjusting the incubation temperature it is possible to distinguish mutants from a reference protein for which the melting temperature differs by less than 2 °C (Fig. 1).

When using WT* as the reference protein (see Materials and methods), plates were incubated for 6 h at 37 °C, followed by 6 h at 42 °C, before exposure to chloroform overnight for ~10–12 h. WT*-containing plaques produced sharp haloes with an average diameter 7 mm, whereas phage containing the mutant N144E/WT*, which has an increase in melting temperature of 1.5 °C at pH 6.5 (Daopin et al., 1991a), produced haloes of 8–9 mm. When using H31N/D70N as reference, a 12-h incubation at 37 °C was used. This produced haloes of 2–2.5 mm average diameter, compared to a 5-mm average diameter halo for WT*.

Hydroxylamine mutagenesis

Treatment of the phage with hydroxylamine for periods ranging from 1 to 8 h gave stocks with surviving titers of 15 to 0.005% of the starting value. In total, approximately 20,000 plaques were examined, of which 49 were selected and phage stocks made for rescreening. Of these, 10 were sequenced and were found to code for WT* lysozyme. On the assumption that mutations elsewhere in the phage may have caused increased levels of expression of the enzyme, one of the putative increased-expression mutations of M13 was used as the cloning vehicle. Also, the background was changed from WT* to H31N/D70N. Thirty-eight thousand plaques were screened, of which 20 were picked for rescreening. Of these, four were sequenced. All four coded for the mutant Asp 41 → Val, which in the wild-type background has an increase in melting temperature, ΔT_m, of 0.6 °C (Table 1).

Thionucleotide misincorporation mutagenesis

Mutagenesis was carried out in the H31N/D70N background. Approximately 3,000–4,000 plaques were screened, of which 29 candidates were picked for rescreening. Of these, eight were judged to be promising after a second round of screening and were sequenced. Four of these were identified as the pseudo-revertant with lysine at position 70 (H31N/D70K), which is 1.7 °C more stable at pH 5.4 than its parent (H31N/D70N) (Table 1). The other four coded for the parent enzyme.

Avian myeloma virus (AMV) reverse transcriptase misincorporation mutagenesis

In a third series of experiments, a variation of the thionucleotide misincorporation method (Fig. 2) was used to try to further increase the mutagenic efficiency. This uses AMV reverse transcriptase instead of the Klenow enzyme for the misincorporation step. This enzyme lacks the proofreading activity of Klenow, and so it is unable to excise a misincorporated nucleotide once it has been inserted into the extended primer sequence. It gave very high levels of mutagenesis. Using the plaque morphologies of the mutated stocks as an indicator, it was estimated that 5–50% of the phage carried mutated T4 lysozyme genes. This made it possible to identify more mutants

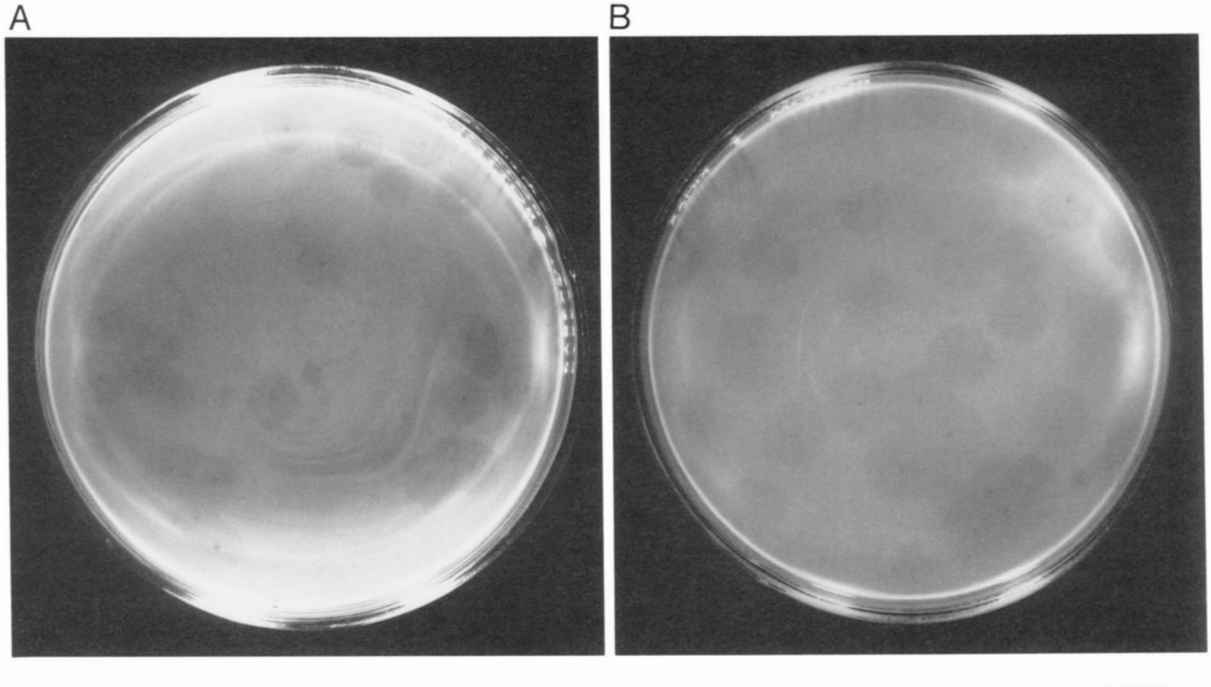

Fig. 1. Assay plates showing the discrimination between WT* and a thermostable mutant. **A:** WT*. **B:** Mutant N144E/WT*.

Table 1. *Activity and stability of mutant lysozymes*[a]

Mutant	Activity (%)	Charge on mutant relative to reference protein	ΔT_m, pH 5.4 (°C)	ΔT_m, pH 6.78 (°C)	ΔH, pH 5.4 (kcal/mol)	ΔS^0, pH 5.4 (e.u.)	$\Delta\Delta G$, pH 5.4 (kcal/mol)
WT*	100	0	0.0	0.0	134	397	0.0
R14K	106	0	−0.08	−0.53	135	401	−0.03
K16E (WT)	142[b]	−2	1.1[c]				
E22K	40	+2	1.37	1.58	141	400	0.57
T26S	75	0	1.35	1.35	143	406	0.57
N40D	124	−1	1.14	1.28	132	378	0.44
A41D	105	−1	0.71	1.10	138	398	0.29
A41V (WT)	ND	0	0.58	ND	NC	NC	0.26
D70K (H31N)	33	+1	1.7[d]	ND	NC	NC	0.36
R80K	44	0	−0.43	−0.34	136	408	−0.17
R80K/R119H	104	0	−1.20	−1.25	133	406	−0.47
A93T	105	0	0.13	0.16	138	408	0.06
G113E	165	−1	0.79	1.03	126	364	0.30
N116D (WT)	ND	−1	1.6[e]				
R119H	105	0	−0.74	−0.83	132	399	−0.29
K147E (WT)	120[b]	−2	−1.6[c]				
T151S	86	0	0.93	0.97	141	407	0.39
F153L	ND	0	0.88	0.51	137	393	0.35
N163D	193	−1	−0.50	−0.49	141	424	−0.21

[a] All mutants are in the WT* background except where "(WT)" is explicitly indicated in the first column. Activity was measured as the rate of hydrolysis of a suspension of *E. coli* cell walls (Tsugita et al., 1968). The charge on the mutant relative to the reference protein is the formal charge change obtained by counting changes in ionizable groups. The mutants identified in this study were most well characterized at pH 5.4 in 0.10 M NaCl, 0.010 M $H_{0.14}Na_{0.86}OAc$ (sodium chloride, sodium acetate buffer). This buffer is at or close to the pH value for which T4 lysozymes that have the His 31–Asp 70 salt bridge intact are most stable (Anderson et al., 1990). At this pH, all measurements (except for footnoted mutants) were the average of 4–8 independent determinations, with WT* having been measured 15 times. The melting temperature, T_m, of WT* in pH 5.4 buffer was 65.15 ± 0.2 °C and in pH 6.78 buffer (0.15 M KCl, 0.01 M KPO_4) was 62.19 ± 0.2 °C. The ΔT_m values were determined by subtraction of the T_m of the reference protein from the T_m of the mutant, and have estimated uncertainty of ±0.3 °C. ΔH, the change in the enthalpy of unfolding at the melting temperature, and the melting temperature itself were both determined from van't Hoff analysis of the melting curves (Becktel & Baase, 1987; Dao-pin et al., 1990). ΔS^0 is the isothermal change in the entropy of unfolding calculated at the melting temperature of WT*, assuming a constant value for ΔC_p of 3.5 kcal/mol-deg. $\Delta\Delta G$ is the isothermal difference in the change in the free energy of unfolding, ΔG (mutant) minus ΔG (reference), determined at the T_m of the reference protein. Since ΔT_m values are small, the $\Delta\Delta G = \Delta T_m * \Delta S$ formula of Becktel and Schellman (1987) was used. Errors for ΔH, ΔS^0, and $\Delta\Delta G$ are estimated to be ±5 kcal/mol, ±15 e.u., and ±0.08 kcal/mol, respectively, from the averaging of the standard deviations of the means for sets of individual mutants. ND, not determined; NC, not comparable due to the change in background from WT* to WT or to H31N/D70N.

[b] Dao-pin et al. (1991b).

[c] 25 mM KCl, 20 mM KPO_4, pH 5.3 (Dao-pin et al., 1991b).

[d] Relative to the T_m of the reference protein H31N/D70N (57.2 °C).

[e] Nicholson et al. (1991).

while screening fewer phage. It also made it possible to return to the WT* background, and so avoid the obvious complication arising from revertants associated with the H31N/D70N background.

Three different sets of stocks were made with this method, each using a different primer located either upstream from, or within, the T4 coding sequence. By adjusting the conditions of the initial primer extension reaction, it was possible to localize the sites of misincorporation to areas within about 200 bases of the coding sequence downstream from each primer. Approximately 11,000 plaques from stocks made from the three different primers were screened, of which 150 were selected for rescreening. From these, 66 were picked for sequencing. About a third of these were mutants and consisted of one or more isolates of 16 different mutants. These are listed in Table 1 and shown in Figure 3 and Kinemage 1. The rest were pseudo-wild type.

Stability and activity

It was found that five of the mutants isolated (K16E, A41V, N116D, K147E, F153L) had previously been constructed by site-directed methods, although usually in the background of WT rather than WT*. Four of these have been shown to be more stable than wild type (Table 1). These were not studied further.

The other 13 variants were transferred into the expression vector pHSE5 (Muchmore et al., 1989) and trans-

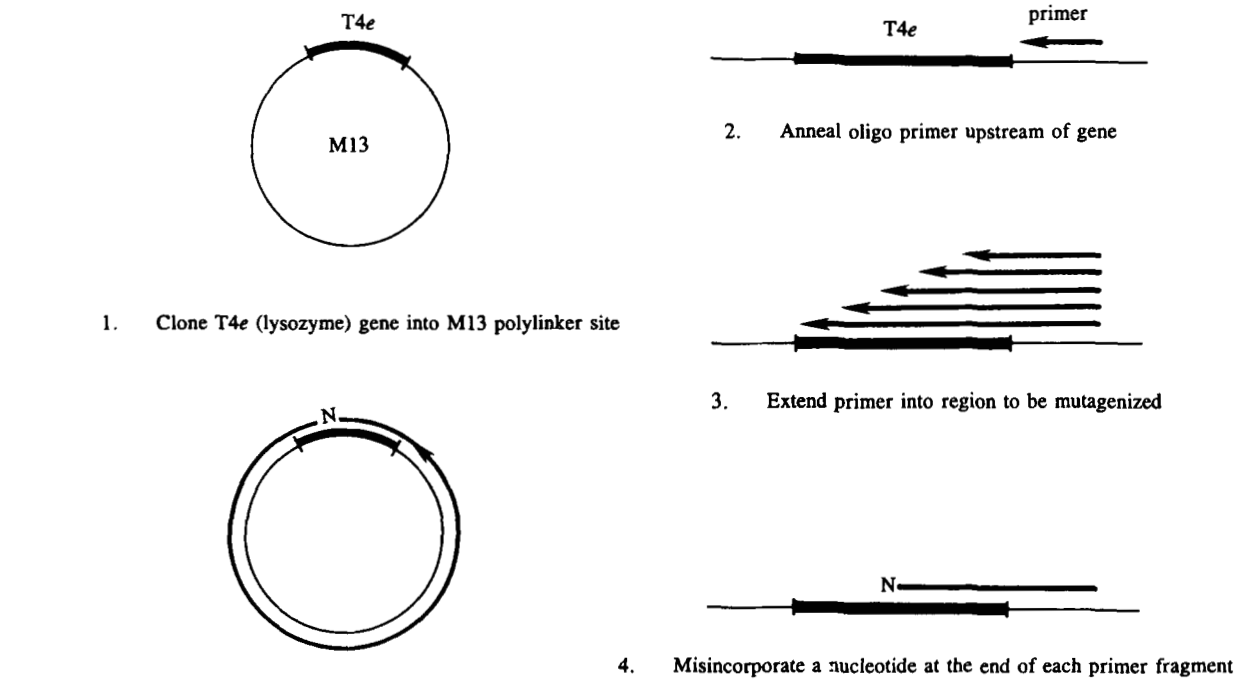

Fig. 2. Misincorporation mutagenesis scheme. The basic scheme is the same for both the thionucleotide and AMV reverse transcriptase protocols, with the major difference being the polymerase used for the misincorporation reaction. In both cases, uracil-containing M13 template is used to eliminate replication of the wild type-containing copy of the parent phage genome.

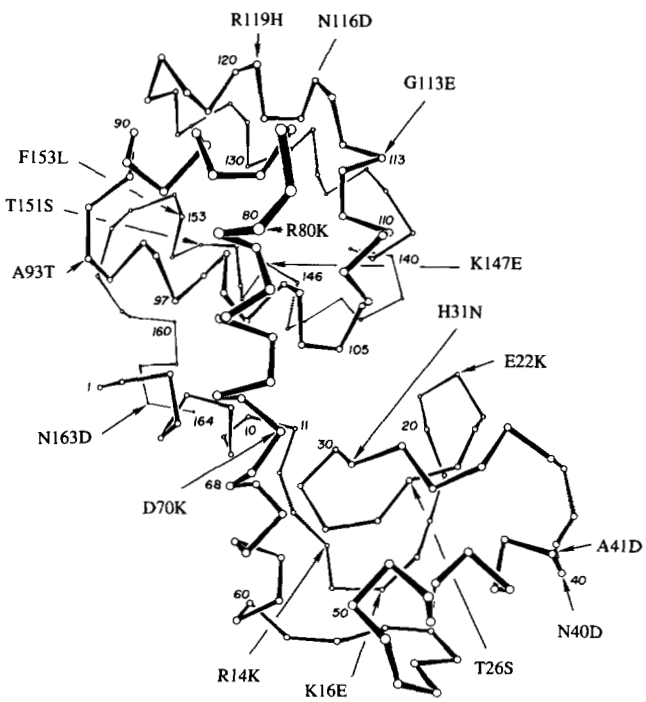

Fig. 3. Backbone of phage T4 lysozyme, showing the locations of all mutants discussed in the text.

formed into *Escherichia coli* strain RR1, and the mutant proteins were purified (Poteete et al., 1991). Thermal stabilities were determined by measuring the thermal denaturation of the proteins under reversible conditions by monitoring the change in circular dichroism at 223 nm as a function of temperature (Dao-pin et al., 1990; Eriksson et al., 1993). Activities were determined by the method of Tsugita et al. (1968). The results are summarized in Table 1.

Ignoring H31N/D70K, of the 17 mutants selected, 12 are bona fide thermostable variants, with melting temperatures 0.8–1.4 °C higher than wild type (the estimated error in ΔT_m is ±0.3 °C; Table 1). The other seven typically have either approximately the same stability as WT* or are somewhat less stable, with K147E (ΔT_m = −1.6 °C) being least stable. The single mutants R80K and R119H, as well as the double mutant R80K/R119H, were each identified as independent isolates. All are slightly less stable than WT*, with ΔT_m for the double mutant (−1.2 °C) essentially equaling the sum of the two singles (−0.4 °C and −0.7 °C) (Table 1). Activities are all within a factor of two of WT*.

The structures of three representative mutants are described in the companion paper (Pjura & Matthews, 1993).

Discussion

M13 mutagenesis/screening system

The overall objective was to develop a convenient system for the identification of thermostable mutants of T4 lysozyme. The most difficult requirement for this system is sensitivity. Except for variants that introduce disulfide bridges (Matsumura et al., 1989), the most thermostable mutant of T4 lysozyme that has been characterized to date has a melting temperature 2.8 °C above WT* at pH 5.4 (Anderson et al., 1993). Most thermostable variants are within 1–2 °C of wild type (e.g., Bell et al., 1990; Matthews, 1993). The assay therefore was required to detect stability differences of as little as 1 °C in order to identify typical thermostable mutants. This mitigated against the use of a selection protocol that relied upon large differences in activity or stability and required the use of a screening method that allowed the detection of more subtle differences.

Although substantial experience has suggested that the halo assay for lysozyme activity is a useful guide, it is not easy to discriminate between small differences in stability. In the present case the screen using the plate assay was the most difficult part of the protocol to develop because it has a number of variables that are difficult to control. The halo size can be quite variable, even for the plating of a single mutant stock. Conditions such as the particular batch of plates, the temperature of the incubator, the length of time after the plates are chloroformed before they are examined, and other factors can affect average halo size. The most troublesome difficulty, however, is the limited range of temperatures within which the phage and bacteria will grow. M13 phage will not grow below 32 °C, and the bacteria will not grow above 42 °C. This limits the usefulness of the assay to this range of temperature. This is a particular problem when WT* is used as background, because the temperature at which its halo size starts to decrease, which is the optimum temperature for discriminating between differences in stability, appears to be at or perhaps slightly above 42 °C. It was in part for this reason that an attempt was made to use the alternative H31N/D70N background. This mutant has a melting temperature of about 5 °C below that of WT* and allows good discrimination at an incubation temperature of 37 °C. However, selection in a background of H31N/D70N has obvious disadvantages, including frequent selection of revertants such as H31N/D70K. On balance, it was preferable to use WT* and to accept the slightly lower level of discrimination that it provided.

There is also an important difference between the M13-based and T4-based assays (Alber & Wozniak, 1985) in regard to the point in time at which the lysozyme begins to diffuse into the agar plate. Bacteriophage T4 is lytic, so each round of virus production is accompanied by the release of lysozyme. This lysozyme can diffuse outward, but the surrounding bacterial lawn has not yet been exposed to chloroform and so is resistant to attack by the enzyme. In the M13-based technique described here, however, the addition of chloroform synchronizes the release of lysozyme to a relatively well-defined time point. This means that different phenotypes tend to be distinguished by changes in halo diameter rather than in the degree of clarity of the halo.

Optimization of mutagenesis

An objective was to find a method of mutagenesis that was quick and easy to use, was efficient, would be generally applicable over the entire gene, and would give predominantly single-site mutants.

Of the methods tested, the most useful was a variation on the Klenow thionucleotide misincorporation method using AMV reverse transcriptase for the primer extension and misincorporation reactions. It provided most of the mutants isolated. One disadvantage is that it is prone to making more than one mutation in the gene. In one case this resulted in the isolation of the double mutant R80K/R119H. Another problem is that mutations occasionally can also occur outside of the intended area of the M13 genome targeted by the initial primer-extension reaction. In a number of cases where putative mutants were found to code for WT* lysozyme, the phage genome upstream from the T4 coding region was also sequenced. In all such cases, mutations were found in the *lac* operator gene and were presumably the cause of the observed overexpression of the protein. In several other cases tested, where mutants were identified in the coding sequence, no mutations were found in the upstream region.

Each of the methods tested would be expected to generate single-base-change mutants, which limits the range of amino acid substitutions possible at a given codon.

Mutants isolated

In total, 18 candidates for thermostable mutants were isolated (Fig. 3; Kinemage 1). Of these, five had previously been constructed by site-directed mutagenesis. Because four out of five of these site-directed mutants were constructed in different backgrounds, it obviates any possible concern that they might have been picked up by contamination. This also gave confidence that the overall approach was effective. Of the 18 candidates, 11 (K16E, E22K, T26S, N40D, A41D, A41V, H31N/D70K, G113E, N116D, T151S, F153L) are in fact thermostable mutants, 4 (R14K, R80K, A93T, N163D) are essentially equivalent to the background mutant, and 3 (R80K/R119H, R119H, K147E) are slightly (0.8–1.2 °C) less stable. As shown in Figure 3, the mutants come from a number of contexts in the protein: 11 (R14, K16, N40, R80, A93, G113, N116, R119, R80/R119, K147, N163) are surface-exposed residues (≥60% of side chain exposed), 4 (E22, A41, D70,

T151) are partially buried (20–60% exposed), and 2 (T26, F153) are core-packing residues (≤20% exposed); 5 come from the N-terminal domain (residues 1–59), 7 from the C-terminal domain (residues 81–164), and 1 from the long helix joining the two (residues 60–80); 2 are acidic residues (E22, D70), 6 are basic residues (R14, K16, R80, R80/K119, R119, K147), 5 are neutral polar residues (T26, N40, N116, T151, N163), and 4 are hydrophobic (A41, A93, G113, F153).

A number of false positives were found using the assay, and these fall into one of two categories: either candidates that were subsequently found to code for the wild-type (or WT*) gene, or actual mutants that had melting temperatures the same as or slightly less than wild type. The first group may in part be due to inadvertent mutations created outside of the T4e region leading to increased expression of the T4 gene.

The second group of false positives probably result from a number of different factors. The first is the creation of surface mutations that alter activity and/or the mobility of the protein through the agar substrate. Dao-pin et al. (1991b) observed that mutants of T4 lysozyme that included Lys → Glu or Arg → Glu substitutions had apparent activities ranging from 90 to 170% of wild type when measured with the cell wall turbidity assay of Tsugita et al. (1968). When measured on agar–peptidoglycan plates using an in vitro halo assay (Becktel & Baase, 1985) related to the one used here, however, the apparent activities increased dramatically, ranging from 270 to 13,600% that of wild type. It appeared that the mutants reduced the net positive charge on T4 lysozyme and thereby enhanced its mobility. Thus, a halo of larger than usual size on a lysozyme assay plate might occur for several reasons. The lysozyme might have enhanced stability, it might have enhanced activity, or it might simply have enhanced mobility through the gel. Table 1 shows evidence for a correlation between charge and activity. Six of the mutants cause a reduction in positive charge, and all of these are slightly more active than wild type (105–193%). This is very comparable with the observations of Dao-pin et al. (1991b). Only two of the mutants cause an increase in positive charge, and both lead to a reduction in activity (40% and 33% of WT).

Of the seven false positives (R14K, R80K, R80K/R119H, A93T, R119H, K147E, N163D; Table 1), all but one cause changes in the distribution of surface charge. K147E has been shown by Dao-pin et al. (1991b) to be substantially more mobile in agarose than WT, which might also explain why it appeared as a false positive. N163D also appears to be more active than WT by about a factor of two. The other false positives do not decrease positive charge; neither are they more active than wild type. Possibly the redistribution of surface charge affects mobility on the assay plate, but this is speculation.

Another factor in the generation of false positives is the limitation of the assay. As noted, the combination of phage and host strain limits the maximum temperature of incubation to 42 °C. This is a problem for WT* because the temperature at which its halo size begins to decrease, which is the most sensitive for detecting changes in melting temperature, is above this value. As a result, the discrimination between WT* and the more stable mutants is relatively poor. For mutant N144E, which is 1.5 °C more stable than WT*, the typical increase in halo diameter is from 7 mm to 8 or 9 mm. This is very close to the average variation in halo size. If a mutant lysozyme with lower melting temperatures is used as the "parent" or "reference," the discrimination is much greater. Mutant H31N/D70N is 1.7 °C less stable at pH 5.4 than H31N/D70K, but the halos differ in diameter by a factor of two (2.5 mm vs. 5 mm). This is in fact part of the reason why this mutant was explored as the "reference" protein (ultimately WT* was preferred because it is the standard reference, even though it allows poorer discrimination). Yet another possible factor in the generation of false positives is that the conditions used for the plate assay are not the same as those used for the CD measurements (see below).

All this aside, however, a relatively simple procedure has been developed to generate a library of mutants and to assay them directly. Using this system, approximately 120 candidates were selected. All that had mutations within the lysozyme gene were found to have melting temperatures within 1.5 °C of WT*.

The entropies of unfolding of two of the mutants, namely N163D and G113E, stand out relative to WT* (Table 1). For N163D, ΔS^0 is about two standard deviations above WT*, whereas for G113E it is about two standard deviations below. In the crystal structure of WT*, residues 162–164 at the C-terminus of the molecule are very mobile (Weaver & Matthews, 1987). In mutant N163D, however, the introduced aspartate apparently interacts favorably with the dipole of the C-terminal helix, and, as a result, in the crystal structure of this mutant residues 162–164 are better ordered than in WT* (data not shown). This decrease in entropy of the folded mutant structure could explain the observed increase in the entropy of unfolding. For G113E, the likely effect of the mutant is on the unfolded structure (Matthews et al., 1987). Substitution of Glu for Gly would be expected to decrease the entropy of the unfolded state, which in turn could explain the observed decrease in the entropy of unfolding for this variant.

Finally, what is the physical basis of the assay? The observed initial increase in halo size with increasing temperature, followed by a sudden decrease in halo size above a certain temperature, is consistent with the behavior that one would expect of an enzyme at or near a critical temperature. The melting temperature, T_m, is measured for the purified enzyme, in buffer, at a defined pH. In contrast, in the plate assay the enzyme is in the presence of substrate and a host of other factors, and is at a temper-

ature 20 °C or so below T_m. The plate assay also extends over 12–24 h, very much longer than the refolding rate of the protein. It is therefore likely that the plate assay is sensitive not only to changes in T_m but also to kinetic and other factors as well.

Materials and methods

Reference proteins and construction of M13 vector system

The principal reference protein used in this work was the cysteine-free double mutant C54T/C97A, referred to as WT* (Matsumura & Matthews, 1989). This eliminates the chemically reactive cysteines present in wild-type lysozyme but has little effect on structure or stability. Another reference or background strain was obtained by introducing two additional mutants, H31N and D70N, and is referred to as H31N/D70N. In wild-type lysozyme the salt bridge between His 31 and Asp 70 contributes 3–5 kcal/mol to the stability of the protein (see Kinemage 1; Anderson et al., 1990). The fragments were cloned into the polylinker region of the single-strand phage M13mp18 between the BamHI and HindIII restriction sites, using standard methods (Maniatis et al., 1982).

M13 lysis plate assay

Phage stocks were assayed as follows. Typically, 100 μL of a stock, diluted to a titer of 250–1,000 pfu/mL with either Low TE buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA) or UV buffer (21.1 mM Na_2HPO_4, 11.1 mM KH_2PO_4, 68.4 mM NaCl, 28.7 mM K_2SO_4, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.01% gelatin, pH 7.0), was combined with 200 μL of a JM101 overnight culture, grown in YT broth (5 g NaCl, 8 g tryptone, 5 g yeast extract per liter, pH 6.8–7.0) or 2× YT broth (5 g NaCl, 16 g tryptone, 10 g yeast extract per liter, pH 6.8–7.0), and 5 mL of top YT agar (5 g NaCl, 8 g tryptone, 5 g yeast extract, 7 g agar per liter, pH 6.8–7.0), at approximately 55 °C, and plated onto 3.5-inch glass YT agar plates (5 g NaCl, 8 g tryptone, 5 g yeast extract, 15 g agar per liter, pH 6.8–7.0). The plates were allowed to set for 30 min at room temperature, then were inverted and incubated in a dry plate incubator at time(s) and temperature(s) as described. After incubation, the plates were allowed to cool for 20–30 min at room temperature. Finally, 1 mL of $CHCl_3$ was added to the lid of each, and the plates were incubated overnight at room temperature. Representative examples are shown in Figure 1.

Hydroxylamine mutagenesis

Mutagenesis reactions were prepared by combining 10 μL of an M13 phage stock, 65 μL of 0.2 M Na_3PO_4, pH 6.0, and 25 μL of 1 M hydroxylamine-HCl, pH 6.0. The reaction mixture was incubated at 37 °C in a water bath. At zero time and at successive intervals, 10-μL aliquots were taken and diluted 10,000-fold in 50% UV buffer/50% glycerol and stored at −20 °C. Stocks were assayed for titer by serial dilution and plating onto lawns of JM101 (Messing, 1983), as for the lysis plate assay.

Thionucleotide misincorporation mutagenesis

The procedure is adapted from that of Abarzua and Marians (1984) and is summarized in Figure 2. Because it proved not to be very effective in the present case, details will not be given.

Reverse transcriptase misincorporation mutagenesis

The procedure followed that of Lehtovaara et al. (1988), with modifications. First, an annealing reaction was prepared containing 3.3 μL of 1.5 pmol/μL kinased mutagenic primer, 2.8 μL of 0.15 pmol/μL U* M13 template, 2.5 μL of 10× Klenow buffer (0.5 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 10 mM dithiothreitol, 1 mg/mL bovine serum albumin), and 16.4 μL sterile H_2O in a 1.5-μL Eppendorf tube. The reaction was heated to 65–75 °C in a water bath and allowed to cool to room temperature over approximately 0.5–2.5 h.

Next, the annealed primer/template mix was split and used in two primer-extension reactions, each containing 12.5 μL of the primer/template mix, 3.75 μL of 10× Klenow buffer, either 5 or 12 μL of 2 μM each dATP, dCTP, dGTP, and dTTP, and water to make 49 μL total volume. Primer extension was carried out by adding 1 μL of 1 U/μL Klenow enzyme to each and incubating for 5 min at room temperature. The reactions were stopped by adding 2 μL of 0.5 M NaEDTA, pH 7.0, to each. The reactions were each extracted with 1:1 phenol/chloroform, the two supernatants combined, and the pooled reactions extracted with chloroform. The recovered supernatant was then ethanol precipitated overnight at −20 °C. Finally, the primer/template reaction was recovered by spinning at 4 °C in a microfuge, washed in 80% ice-cold ethanol, and dried in a Speedvac. The pellet was resuspended in 80 μL of Low TE buffer.

The mixture was then divided and used in four misincorporation reactions, each containing 20 μL of the extended primer/template mix, 2.5 μL of 10× AMV buffer (0.5 M Tris-HCl, pH 8.3, 60 mM $MgCl_2$, 0.6 M KCl, 10 mM dithiothreitol, 0.9 mg/mL bovine serum albumin), and 2.5 μL of 2 mM dNTP (one type only per reaction). Misincorporation was performed by adding 1 μL of 7 U/μL AMV reverse transcriptase (Promega) to each. The reactions were incubated for 90 min in a 37 °C water bath, 0.5 μL of reverse transcriptase was added to each, and the reactions were continued for another 90 min. Finally, the templates were extended beyond the misincorporation sites ("chased") by adding, to each, 2.5 μL of

5 mM each of all four dNTPs and incubating for another 20 min at 37 °C.

Next, double-strand synthesis and ligation reactions were performed on each of the four misincorporation mixes by combining each with 2.5 μL of 10× AMV buffer, 5 μL of 10 mM ATP, and 13 μL of sterile H_2O. The reactions were carried out by adding, to each, 0.5 μL of 1 U/μL Klenow enzyme (Boehringer Mannheim) and 0.5 μL of 2 U/μL T4 DNA ligase (Boehringer Mannheim). The reactions were carried out overnight at 15 °C in a circulating water bath. Finally, the reactions were stopped by adding 2 μL of 0.5 M NaEDTA, pH 7.0, to each, and the mixes were stored at −20 °C.

Finally, the four misincorporation reaction mixes were used to transform competent cells and to raise phage libraries for subsequent screening. Competent JM101 cells were made using the $CaCl_2$ method, as previously described. The Ca-treated cells were kept in ice water for approximately 90 min before use. Each of the separate dNTP misincorporation reactions was added to a 2-mL portion of the competent cells and incubated for 30 min in ice water. The cells were heat shocked by transferring to a 37 °C water bath for 7 min, then returned to room temperature. A fresh 4-mL volume of 2× YT broth was added to each and incubated for 6 h at 37 °C on a rocker table. Finally, phage stocks were made from the incubations by spinning down 1 mL of each for 5 min at room temperature in a microfuge and combining 0.5 mL of each supernatant with 0.5 mL of glycerol and storing at −20 °C.

In a variation on the above, the initial primer extension reaction was performed using AMV reverse transcriptase instead of Klenow as follows: following annealing as described above, the mixture was used in the two primer extension reactions as before, except that 10× AMV buffer was used in place of Klenow buffer, 1 μL of 7 U/μL reverse transcriptase was used in place of Klenow, and the reaction was incubated for 10 min instead of 5 min. After the incubation, the misincorporation reaction was performed directly, without the extraction and precipitation steps, by combining for each misincorporation reaction 12.5 μL of each molarity extension reaction with 2.5 μL of 2 mM of one dNTP. They were incubated for 90 min in a 37 °C water bath, an additional 0.5 μL of reverse transcriptase was added to each, and the incubations continued for another 90 min. The reactions were chased for 20 min at 37 °C by adding, to each, 2.5 μL of a solution 5 mM in each of the four dNTPs. Finally, 10× AMV buffer and 10 mM ATP were added as above with water to make 49 μL total volume, and the mixtures were incubated overnight with Klenow enzyme and T4 ligase as before.

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