site for base-unpairing, mimicked MAR properties and function.

The results from the MARs surrounding the IgH enhancer (*15*), the MAR 5′ of the huIFN-β gene, and synthetic oligonucleotides rich in A+T that show contrasting structural properties indicate that the potential for extensive unwinding is required for MAR function. This notion is also supported by the observation that the autonomously replicating sequence (ARS) of the yeast histone H4 gene unwinds under superhelical strain (*18*), and yeast ARS elements are MARs (*19*). The nucleation sites for unwinding vary depending on MARs; the sites did not necessarily contain the AATATATTT motif (*20*).

In prokaryotes, DNA supercoiling is important for various genetic functions (*21*). However, it is unclear whether unconstrained superhelical strain exists at eukaryotic loci. In vitro experiments show that a number of genes are optimally transcribed when supercoiled to various degrees (*22*); for example, formation of the fibroin gene transcriptional initiation complex was greatly facilitated by negative supercoiling (*23*). The transcriptional process generates local negative supercoiling 5′ of a transcribing gene and positive supercoiling 3′ of a transcribing gene (*24*). MARs, together with topoisomerases, may participate in controlling the topological state of chromatin domains. On the basis of the observed tendency of MARs to be base-unpaired, we speculate that negative superhelical strain generated in a chromatin loop domain could be relieved by unwinding of MARs. Thermodynamic energy could then be stored by an association of the unwound MAR with the proteinaceous scaffold and released at other times to generate negative supercoiling at a remote site within the domain. In support of this is the observation that the nuclear scaffold preferentially binds with supercoiled DNA (*25*). MARs could thus support the formation of a transcriptionally active complex, relax the positively supercoiled part of a transcriptional domain, help to accept any histones released from such a region (*26*), aid in the recruitment of topoisomerases (*27*), and prevent superhelical strain from being transmitted to neighboring domains.

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Site-Specific Incorporation of Novel Backbone Structures into Proteins

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A number of unnatural amino acids and amino acid analogs with modified backbone structures were substituted for alanine-82 in T4 lysozyme. Replacements included α,α-disubstituted amino acids, *N*-alkyl amino acids, and lactic acid, an isoelectronic analog of alanine. The effects of these electronic and structural perturbations on the stability of T4 lysozyme were determined. The relatively broad substrate specificity of the *Escherichia coli* protein biosynthetic machinery suggests that a wide range of backbone and side-chain substitutions can be introduced, allowing a more precise definition of the factors affecting protein stability.

SITE-DIRECTED MUTAGENESIS IS A powerful tool for probing the effects of amino acid structure on protein stability and folding. The ability to introduce amino acids with novel backbones and side chains not restricted by the genetic code would likely lead to a more detailed understanding of these phenomena. Substitutions might include amino acid analogs with altered cis-trans rotational barriers, with restricted conformations, or with modified hydrogen-bonding, van der Waals, electrostatic, or covalent interactions. We have recently developed a general biosynthetic method that allows site-specific incorporation of unnatural amino acids into proteins (*1, 2*). We report the application of this method to the preparation and characterization of T4 lysozyme (T4L) mutants containing modifications in the polypeptide backbone. These mutants not only provide an opportunity to assess the effects of backbone structure on protein stability, but they also provide insight into the selectivity of the *Escherichia coli* protein biosynthetic machinery.

T4 lysozyme is a structurally well-characterized protein that has served as a model for a number of mutational studies on protein stability (*3*). To survey the scope of novel backbone structures that can be incorporat-

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ed into a functional protein, Ala^{82} was chosen as the initial mutagenesis target. Mutation of Ala^{82} to Pro, the only natural backbone-constrained amino acid, has been shown to result in increased thermal stability (*4*). Ala^{82} is a surface residue at a break between two helices, with the NH group hydrogen-bonded to water. Therefore, one might expect this site to tolerate backbone and side chain modifications better than other sites within secondary structures that require backbone hydrogen-bonding or introduce steric constraints on amino acid side chains. Moreover, because this residue is distant from the active site, mutations are less likely to directly affect catalytic activity.

Introduction of novel amino acids into position 82 of T4L was accomplished by in vitro suppression of an amber nonsense mutant at position 82 with a chemically aminoacylated suppressor tRNA derived from yeast $tRNA^{Phe}$ (*2, 5*). An amber mutant at the active site (Asp^{20}) can be efficiently suppressed with an aspartic acid derivative to produce a photoactivable T4L (*6*). In addition, wild-type T4L has been synthesized and purified to homogeneity from a cell-free coupled transcription-translation system programmed with the plasmid pHSe54,97.TA (*7*) in sufficient quantity for catalytic assays and circular dichroism (CD) measurements. In this study, an amber codon [$Ala^{82} \rightarrow$ TAG (pT4LA82am)] was introduced into the T4L gene at position 82 by Eckstein mutagenesis (*8*). When an in vitro protein synthesis system programmed with plasmid pT4LA82am was supplemented with chemically acylated alanyl-$tRNA^{Phe}_{CUA}$, a significant amount of protein was produced that corresponds to T4L (lane 7, Fig. 1A). The purified protein suppressed with alanine was found to have the same chromatographic properties and specific activity as wild-type protein expressed in vitro (*9*). Scintillation counting of the bands from the polyacrylamide gel afforded a suppression efficiency of 28%, which is consistent with the suppression efficiency determined by lytic activity toward *E. coli* cell walls (*10*).

The following controls insured that mutant proteins were not heterogeneous because of partial incorporation of any of the 20 natural amino acids. In the absence of suppressor tRNA, pT4LA82am-directed in vitro protein synthesis afforded no observable readthrough of the amber codon as determined by polyacrylamide gel electrophoresis (PAGE) of [^{35}S]Met-labeled protein (lane 2, Fig. 1A). Scintillation counting of gel slices revealed that at 4 mM of added $Mg(OAc)_2$ (Ac = acetyl), the pT4LA82am-directed system yielded less than 1% full-length T4L as compared to in vitro wild-type expression (by pHSe54,97.TA). Addition of 15 μg of full-length unacylated suppressor $tRNA_{CUA}$ to the pT4LA82am-programmed reaction again resulted in less than 1% full-length T4L at 4 mM of added $Mg(OAc)_2$ (lane 3, Fig. 1A). In accord with the lack of full-length protein, neither of these two control reactions yielded detectable lytic activity. In contrast, in vitro synthesis reactions primed with plasmid pHSe54,97.TA produced 40 to 50 μg of active enzyme per milliliter of in vitro reaction mixture (lane 1, Fig. 1A).

A number of amino acid analogs were then tested for their ability to be incorporated at position 82 of T4L (Fig. 2). These included D-alanine (**2**); lactic acid (**3**) and thiolactic acid (**4**), the isoelectronic oxygen and sulfur isosteres of **1**, respectively; the *N*-alkyl amino acids azetidine 2-carboxylic acid (**6**), pipecolic acid (**8**), *N*-methyl alanine (**9**), and *N*-ethyl alanine (**10**); and the two α,α-disubstituted amino acids cyclopropylglycine (**11**) and α-aminoisobutyric acid (Aib) (**12**). The cyanomethyl esters of the nitroveratryl- and nitrobenzyl-protected amino acids **1** to **12** were coupled to pdCpA, and the aminoacyl dinucleotides were then ligated to $tRNA^{Phe}_{CUA}$(-CA) with T4 RNA ligase (*2, 11*). After brief photolysis to

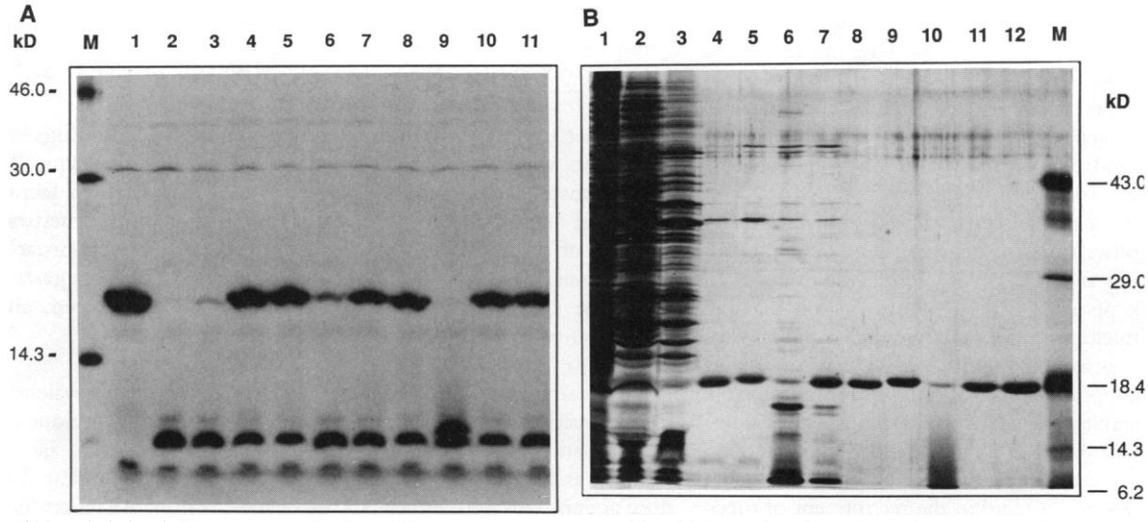

Fig. 1. (**A**) Autoradiogram of in vitro suppression reactions labeled with [^{35}S]Met and containing the following plasmids and tRNAs: lane 1, pHSe54,97.TA (wild type); lane 2, pT4LA82am without $tRNA_{CUA}$; lane 3, pT4LA82am and 7.5 μg of full-length unacylated $tRNA_{CUA}$; lane 4, pT4-LA82am and 7.5 μg of prolyl-$tRNA_{CUA}$; lane 5, pT4LA82am and 7.5 μg of pipecolyl-$tRNA_{CUA}$; lane 6, pT4LA82am and 7.5 μg of azetidine-2-carboxyl-$tRNA_{CUA}$; lane 7, pT4LA82am and 7.5 μg of alanyl-$tRNA_{CUA}$; lane 8, pT4LA82am and 7.5 μg of *N*-methyl-alanyl-$tRNA_{CUA}$; lane 9, pT4LA82am and 7.5 μg of lactyl-$tRNA_{CUA}$; lane 10, pT4LA82am and 7.5 μg of cyclopropylglycyl-$tRNA_{CUA}$; and lane 11, pT4LA82am and 7.5 μg of α-aminoisobutyryl-$tRNA_{CUA}$. Lane M contains ^{14}C-methylated molecular mass standards. Cleared supernatants (20 μl) from terminated 50-μl in vitro reactions were incubated with 4 μl of 2.5 mg ml^{-1} ribonuclease (RNase) A for 15 min at 37°C and then analyzed by 15% SDS-PAGE. Each 30-μl reaction contained 7 μCi of [^{35}S]Met. Suppression efficiencies were determined by comparison of the number of counts obtained by scintillation counting of the bands from lane 1 (wild-type plasmid) with those from lanes 4 to 11 (suppressed proteins). All values were corrected for background counts from a blank gel slice. The suppression efficiency of lactic acid (lane 9) was determined by comparison of the rate of hydrolysis of *E. coli* NAPIV cells from an in vitro suppression reaction programmed with the wild-type plasmid to the in vitro suppression reaction programmed with T4LA82am suppressed with lactyl-tRNA. (**B**) Silver-stained 15% SDS-PAGE showing purification of wildtype and mutant T4Ls containing unnatural amino acids at position 82. Lanes 1 to 3 are the crude in vitro reaction mixture, PEI · HCl supernatants, and DEAE-plus CM-cellulose tandem cartridge wash, respectively, of pT4L82am suppressed with pipecolyl-$tRNA^{Phe}$. Lanes 4 to 7 are the pooled fractions exhibiting T4L lytic activity from a salt-gradient elution of a CM-cellulose cartridge bound by wild-type T4L (lane 4) or by pT4LA82am suppressed with pipecolic acid (lane 5), lactic acid (lane 6), or α-amino isobutyric acid (lane 7). Lanes 8 to 11 are the purified enzymes after a second round of cation exchange chromatography with a mono S column on a Pharmacia Smart system: wild-type (lane 8), $Ala^{82} \rightarrow$ pipecolic acid (**8**) (lane 9), $Ala^{82} \rightarrow$ lactic acid (**3**) (lane 10), and $Ala^{82} \rightarrow$ α-amino isobutyric acid (**12**) (lane 11). Lane 12 contains 1.5 μg of wild-type T4L purified from overexpression of pHSe54,97.TA in *E. coli* strain NM522, and lane M is a molecular mass standard marker. The lactic acid–suppressed protein is contaminated with a small amount (~5%) of T4L containing some natural amino acid, as shown by the remnant of a full-length band; in contrast, most of this protein hydrolyzed under the electrophoresis conditions to give a smear (lane 10).

remove the nitroveratryl and nitrobenzyl protecting groups (*2*), the individual aminoacyl suppressor $tRNA_{CUA}$s were added to the in vitro protein synthesis system directed by pT4LA82am. Suppression efficiencies were determined by scintillation counting of dried SDS polyacrylamide gel slices (Fig. 1A, legend). Several of the amino acids that contained secondary amines (**7**, **8**, and **9**) were efficiently incorporated (46, 43, and 24%, respectively). In contrast, **6** and **10** suppressed poorly (less than 5%). Although suppression by **2** was not detected (*12*), the α,α-disubstituted amino acids, **11** and **12**, were efficiently incorporated into the protein (28 and 23%, respectively). The four-, five-, and six-membered ring analogs of **11** also suppress pT4L82am with roughly the same efficiency as **11**. β-Alanine, **5**, suppressed less than 5%, whereas lactic acid, **3**, was incorporated into position 82 with 30% suppression efficiency, and preliminary results suggest that the thiol analog of alanine, **4**, is also incorporated, although not as efficiently. The mutant T4L containing **3** hydrolyzed completely at the ester bond under standard SDS-PAGE conditions (lane 9, Fig. 1A); consequently, the suppression efficiency was determined by enzyme activity. This result confirms that protein suppressed with **3** is not contaminated with any of the natural amino acids at position 82, because the resulting amide bond linkage would be stable to the standard SDS-PAGE conditions and would give a band corresponding to full-length protein.

Table 1. Thermodynamic stability of in vitro wild-type T4L and mutant T4L that contains unnatural amino acids at position 82 (T_m = melting temperature). CD melts were performed in triplicate according to the procedure of Becktel and Baase (*18*) with a Jasco 600 polarimeter for CD readings and a 5831a thermistor meter with an ON-402 thermistor probe (Omega Engineering) for sample temperature readings. Before use, purified proteins were dialyzed four times against a 20 mM potassium phosphate buffer containing 25 mM KCl at pH 2.51 (*19*). Protein concentrations used in the CD measurements were between 5 and 10 μg ml^{-1}.

Protein	T_m (°C)	ΔT (°C)
Wild-type	43.4 ± 0.25	
Ala^{82} → **3**	39.7 ± 0.25	−3.7 ± 0.25
Ala^{82} → **8**	41.3 ± 0.25	−2.1 ± 0.25
Ala^{82} → **12**	44.6 ± 0.25	1.2 ± 0.25

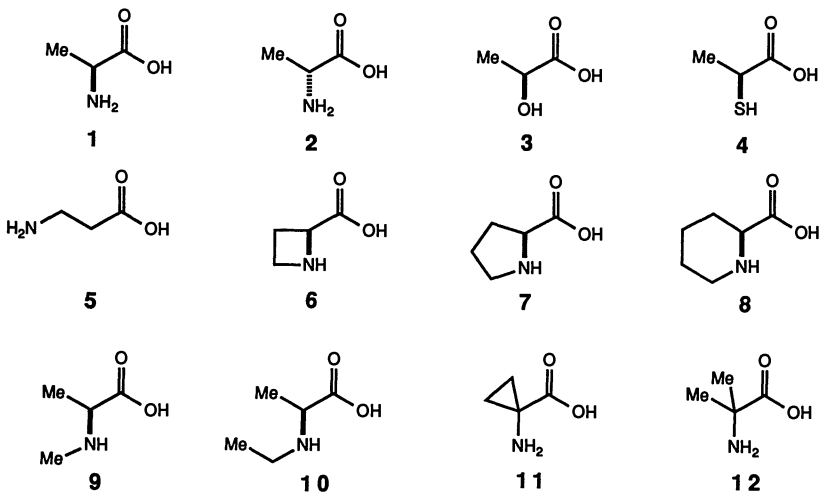

Fig. 2. Amino acids tested for the ability to be incorporated into T4L at position 82 with the *E. coli* protein biosynthesis machinery (Me = methyl).

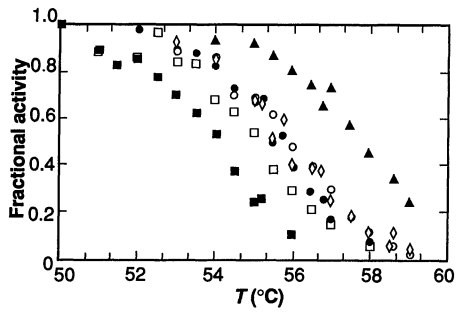

Fig. 3. Residual activity of suppressed proteins as a function of constant time and variation of temperature: (◇), Ala^{82} → alanine; (■), Ala^{82} → lactic acid; (●), Ala^{82} → pipecolic acid; (▲), Ala^{82} → proline; (○), Ala^{82} → cyclopropylglycine; (□), Ala^{82} → *N*-methylalanine. Cleared supernatants (25 μl) from terminated 50 μl in vitro reactions were diluted to 500 μl at a final buffer concentration of 100 mM NaCl, 200 mM Na_2PO_4, and 1 mM EDTA and a pH of 6.5. The solution was divided into 50-μl aliquots. Each aliquot was heated for exactly 10 min at the specified temperature (0.5° ± 0.1°C increments) and then was immediately placed on ice. We evaluated the retained activity by determining the rate of lysis of NAPIV cells (~600 μg ml^{-1} of 50 mM tris · HCl, pH 7.4, and 1 mM EDTA) monitored at 450 nm. Each mutant protein was assayed in triplicate. The data in this figure represent the average values of these experiments.

Efficient incorporation of the α,α-disubstituted amino acids **11** and **12** was unexpected in light of the fact that D-alanine was not incorporated. In addition, previous work had shown that **12** was unable to function as a peptidyltransferase acceptor in a model system (*13*). However, the α,α-disubstituted aminoacyl tRNA derivatives have been shown to bind productively to EF-Tu (*14*), which is consistent with our results. Discrimination against D-alanine may arise in part at the level of aminoacyl tRNA · EF-Tu complex formation, as seen for the D-tyrosyl tRNA, where addition of EF-Tu failed to protect the aminoacyl linkage from hydrolysis (*15*). Efficient incorporation of α-hydroxy acids into proteins is intriguing, both because lactyl and phenyllactyl tRNAs are documented to bind poorly to EF-Tu (*16*) and because the hydroxyl group is a much poorer nucleophile than the amino group of the corresponding amino acids. Our results are in agreement with studies that demonstrate the incorporation of α-hydroxy acids into short peptides and polyesters (*17*).

The thermal stabilities of the mutant proteins containing the representative amino acids **3**, **8**, and **12** were then assayed relative to the wild-type protein. In vitro synthesized wild-type and mutant enzymes were purified to homogeneity (Fig. 1B), and we determined their stabilities by monitoring the CD spectra at 223 nm as a function of temperature (*18, 19*). Mutant enzymes were isolated (*6, 7*) by addition of polyethyleneimine · HCl (PEI · HCl) to the in vitro reaction mixture and then purification of the enzyme from the supernatant on a DEAE-plus CM-cellulose tandem cartridge (see Fig. 1B). Cation-exchange chromatography of the pooled T4L active fractions yielded 10 to 20 μg of purified mutant T4L from a 5.0-ml scale in vitro suppression reaction. For each mutant, the CD melting curves were repeated in triplicate and were highly reproducible as well as reversible (Table 1). The mutant enzyme containing Aib, **12**, is ~1°C more stable than the wild-type enzyme. This α,α-disubstituted amino acid is highly constrained, with a conformational minimum close to the geometry observed for Ala^{82} (ϕ and ψ angles of −67° and −24°, respectively) (*20*). This result is consistent with that of Matthews *et al.*, who showed that substitution of Ala^{82} by Pro (of which the angle ϕ is restricted from −50° to −70°) leads to a 2°C increase in protein stability (*4*). In contrast, the six-membered ring Pro analog **8** destabilizes the protein by ~2°C.

This decrease reflects the fact that the angle ϕ corresponding to the conformational minimum of pipecolic acid, while also negative, has a much greater absolute magnitude than that observed for Ala at this position (*21*). The significantly lower stability of the mutant enzyme containing the isoelectronic hydroxy analog of Ala, **3**, is surprising because the NH of Ala is hydrogen-bonded to water and not to another residue in the protein. In addition, although ester bonds have lower barriers to cis-trans isomerization than amides, they are strongly biased toward the planar trans conformation (*22*). The lower melting temperature may reflect the fact that the ester carbonyl is a much weaker hydrogen-bond acceptor than the analogous amide carbonyl (*23*).

The stabilities of most of the mutant enzymes were also evaluated by correlation of heat and time of inactivation (Fig. 3) (*4, 24*). Although less accurate than the CD determination, this method was more convenient because the assay could be carried out directly with the unpurified in vitro reaction supernatant. Control experiments demonstrated that protein obtained by in vitro suppression of the amber 82 mutation with Pro was approximately 1° to 2°C more stable than wild-type protein, in agreement with previous reports (*4, 25*). Qualitatively, the results agreed with the relative stabilities obtained by the CD experiments, with lactic acid as the least stable mutant. The two mutant enzymes containing **9** and **11** were both close in stability to the wild-type enzyme. It appears from these and the CD melting study results that the electronic properties of substitutions at position 82 of T4L have a greater effect on protein stability than the conformational restrictions caused by substitutions.

We have demonstrated that proteins containing significant modifications to the polypeptide backbone can be synthesized and purified and that their thermal properties can be characterized. These results suggest that it should be possible to systematically survey the effect of a wide range of amino acids containing backbone and side-chain perturbations in order to more precisely evaluate the factors that are important for protein stability.

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Optical Analysis of Synaptic Vesicle Recycling at the Frog Neuromuscular Junction

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The fluorescent dyes FM1-43 and RH414 label motor nerve terminals in an activity-dependent fashion that involves dye uptake by synaptic vesicles that are recycling. This allows optical monitoring of vesicle recycling in living nerve terminals to determine how recycled vesicles reenter the vesicle pool. The results suggest that recycled vesicles mix with the pool morphologically and functionally. One complete cycle of release of transmitter, recycling of a vesicle, and rerelease of transmitter appears to take about 1 minute.

SECRETION OF ACETYLCHOLINE (ACh) from motor nerve terminals occurs through exocytosis of synaptic vesicles at specific sites (active zones), after which vesicle membranes are recaptured, refilled with transmitter, and returned to the pool of vesicles clustered near the active zone. This process, known as vesicle recycling (*1*), is common to all chemically transmitting synapses but is not well understood. We wanted to determine whether recycled vesicles freely mix with the vesicle pool or are preferentially routed to a specific region of the pool (Fig. 1A). To study vesicle recycling, we used styryl dyes to label nerve terminals in an activity-dependent fashion; the dyes mark recycled vesicles (*2, 3*). Transmitter release during or just preceding exposure to the dye must occur for the terminal to be labeled.

The fluorescent labeling pattern in living frog cutaneus pectoris preparations consists of a series of discrete spots 1 to 2 μm in diameter, distributed like beads along the length of motor nerve terminals (*2*). The spots align with postsynaptic ACh receptors and disappear if the nerve is stimulated in dye-free medium. The size and number of spots match the immunocytochemical distribution of synapsin I (*4*) and ultrastructural observations of synaptic vesicle clusters.

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