The Hydrophobic Core of *Escherichia coli* Thioredoxin Shows a High Tolerance to Nonconservative Single Amino Acid Substitutions†

H. W. Hellinga,*,‡ R. Wynn, and F. M. Richards

Department of Molecular Biophysics and Biochemistry, Yale University, P.O. Box 6666, 260 Whitney Avenue, New Haven, Connecticut 06511

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ABSTRACT: A set of single amino acid substitutions has been constructed at positions Leu42 and Leu78 in the hydrophobic core of *Escherichia coli* thioredoxin. This protein is required for the in vivo assembly of filamentous bacteriophages such as M13. Almost all the mutants retain this activity regardless of the change in size, hydrophobic nature, or charge of the substitution. Determination of the free energies of unfolding of the mutants containing charged residues shows that these are significantly destabilized as would be expected from simple considerations of the hydrophobic effect. Thioredoxin therefore represents a class of proteins where the often observed correlation between a particular biological activity and thermodynamic stability is not evident for single mutants in the all-or-none assay used. Native thioredoxin is very stable. Thus, structurally single mutants may not perturb the folding equilibrium or the dynamic behavior sufficiently for the effects to be sensed in vivo.

The hydrophobic effect has been known to be one of the primary factors in determining the thermodynamic stability of a globular protein (Kauzmann, 1959; Dill, 1990). Equally, the packing in the interior is an important constraint in determining the precise three-dimensional structure of the final folded structure (Richards, 1977), although there is some debate on how powerful a constraint it is for the determination of the set of allowed sequences in a particular protein fold (Behe et al., 1991). The range of allowed amino acid substitutions in proteins is relatively large, indicating that proteins have a plastic structure that can adapt to a variety of perturbations (Matthews, 1987). Nevertheless, systematic analysis of mutants in a variety of different proteins has shown that the hydrophobic and packing constraints are qualitatively correct [reviewed in Shortle (1989), Alber (1989) and Pakula and Sauer (1989)]. Hydrophilic or charged substitutions are deleterious in the interior positions of a protein (Yatani et al., 1987; Matsumara et al., 1988; Lim & Sauer, 1989), while surface positions either are more permissive in the type of acceptable mutation (Reidhaar-Olson & Sauer, 1988, 1990) or are destabilized by hydrophobic residues (Pakula & Sauer, 1990). Similarly, mutations in the interior that create bad steric contacts or cavities, by increasing or decreasing the interior volume too much, are deleterious (Kellis et al., 1989; Lim & Sauer, 1991; Eriksson et al., 1992).

Investigations into the nature of the set of sequences that give rise to folded proteins are an important component of the protein folding problem. Such studies aim to establish the link between the physical constraints imposed by the thermodynamics and structural geometry of the system and the informational content contained within the amino acids. Genetic studies which take advantage of an easily selected or screened for phenotype have provided a very powerful tool in measuring this informational content of amino acid sequences (Smith et al., 1980; Hecht et al., 1983; Shortle & Lin, 1985, Alber et al., 1987; Yu & King, 1988; Reidhaar-Olson & Sauer, 1988, 1990; Lim & Sauer, 1989, 1991; Auld & Pielak, 1991). This approach assumes a strong correlation of mutants that retain activity with their thermodynamic stability and the preservation of their original structure. While this has been true in many experimental systems studied to date, which have involved small globular proteins, it has been pointed out that this assumption may not hold for fibrous or unusually stable globular proteins (King, 1986). In this paper, we present results on single-point mutations in the hydrophobic core of *Escherichia coli* thioredoxin. For these mutations, the thermodynamic stability of the mutant proteins follows the expected trend, but the activity does not. Thioredoxin presents a clear case of a small globular protein where the usual link between stability and biological activity does not apply.

E. coli thioredoxin is a monomeric protein of 108 residues. It is found in many organisms and assumes a wide variety of biological functions (Holmgren, 1985, 1989). The single disulfide bridge in the protein acts as a redox center in a number of reactions. Thioredoxin reductase catalyzes the reduction of this disulfide bond to a dithiol using NADPH (Berglund, 1969). Thioredoxin can serve as a powerful general protein disulfide reductase and isomerase (Holmgren, 1979; Pigiet & Schuster, 1986). It has been found to have a high degree of sequence homology with the eukaryotic chaperonin protein disulfide isomerase (Edman et al., 1985). It also interacts specifically as a reducing reagent for a number of proteins such as ribonucleotide reductase (Laurent et al., 1960) and methionine sulfoxide reductase (Black et al., 1960) and is required for the reduction of sulfate (Wilson et al., 1961). Recently, thioredoxin has been found to be important in the regulation of protein activity by thiol redox control in a variety of systems ranging from light-modulated reduction of various enzymes in the Calvin cycle (Crawford et al., 1988) to the inhibition of cell proliferation in mammalian cells (Deiss & Kimchi, 1991). In prokaryotes, it has been found to be important for the growth of several bacteriophages. It is required for the replication of phage T7, where it acts as a cofactor for T7 DNA polymerase (Holmgren et al., 1981). It is also required for the assembly of filamentous phages such as M13 and fd (Russel & Model, 1985; Lim et al., 1985). The redox activity of the protein is not required in either case (Huber et al., 1986), although it has been shown that only the

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‡ Present address: Department of Biochemistry, Box 3711, Duke University Medical Center, Durham, NC 27710.

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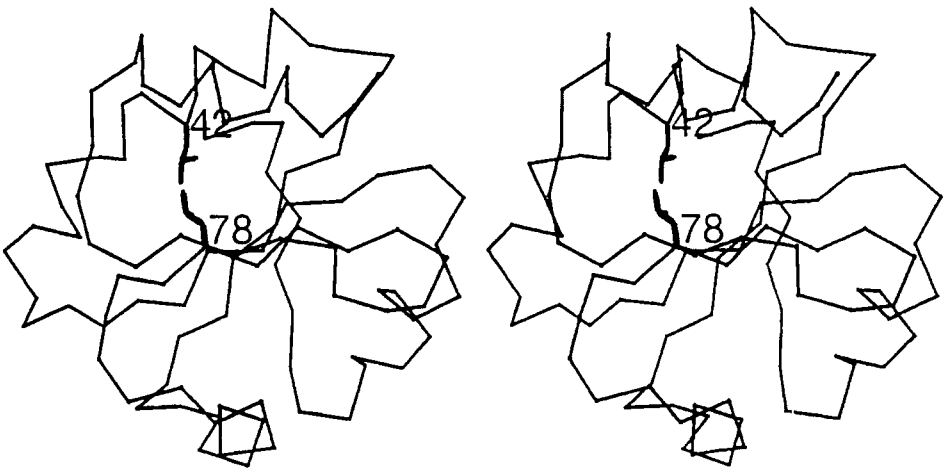

FIGURE 1: Structure of *E. coli* thioredoxin. The molecule is oriented to show that the two positions that are mutated in this study are located in the hydrophobic core that forms part of the interface between the central β sheet and two α helices. The two residues are in van der Waals contact with each other.

reduced conformation of the protein is competent to catalyze phage assembly (Russel & Model, 1986).

Thioredoxin is a good model for studies on protein folding and related biophysical studies. Its requirement for the growth of the cloning vector phage M13 provides a readily manipulated system for mutagenesis and genetic selection of potential folding mutants. The structure of the oxidized form of the protein has been solved to high resolution by X-ray crystallography (Katti et al., 1990) and assigned in NMR spectra (Dyson et al., 1989). High-resolution NMR studies have shown that the changes that occur upon reduction are small and are mostly confined to the region in the immediate vicinity of the disulfide bridge (Dyson et al., 1990). The stability of the protein decreases by 2.4 kcal/mol upon reduction (Kelley et al., 1987), and there are significant changes in its dynamic properties as demonstrated by differences in the hydrogen exchange behavior (Kaminsky & Richards, 1992a) and partial specific volume (Kaminsky & Richards, 1992b). Studies on the kinetics of refolding from denaturants have shown the presence of at least three nonnative transient intermediates (Kelley & Stellwagen, 1984; Kelley et al., 1986; Shalongo et al., 1987; Langsetmo et al., 1989). One of these has been shown to be due to the slow isomerization of Pro76 (Kelley & Richards, 1987). Thioredoxin is a very stable protein (Holmgren, 1985). An even more stable form of the protein was isolated by mutating Asp26 which forms a buried negative charge in the hydrophobic core of the protein. Mutation of this residue to an alanine stabilizes the oxidized form of the protein by 5 kcal/mol at neutral pH (Langsetmo et al., 1991). Since it affects the stability of the oxidized form differently from the reduced form, it is thought that it may play a role in the tuning of the disulfide redox potential (Langsetmo et al., 1990). The thermodynamic linkage of the redox potential to the stability of the various states of the system (Lin & Kim, 1989) has been exploited to evaluate the effect of free energy changes of mutations on both the folded and denatured states of the protein (Lin & Kim, 1991). The ability to partition the free energy changes between these states is an important feature in the interpretation of the effect of mutations (Dill & Shortle, 1991) and is not possible in systems lacking a disulfide bridge.

In this paper, we present studies on mutations of two residues in the hydrophobic core of the protein. Thioredoxin consists of a center β sheet flanked on either side by helices (Figure 1). The hydrophobic core of the molecule is formed by packing interactions of the helices onto the sheet, and is divided into two domains separated by the sheet. Leu42 and Leu78 are both completely buried residues in van der Waals contact with each other, and form part of the core of a $\beta\alpha\beta$ supersecondary fold. Leu42 is located on an α helix and points toward the center of the protein. Leu78 is located on the β sheet and points toward the surface of the protein. These 2 residues were each separately changed to 12 or 15 other amino acids. The ability of these mutants to catalyze the assembly of phage M13 was measured. Selected mutants were recloned into expression vectors and protein-purified, and the stability with respect to denaturants was studied. It was found that although almost all mutations retained biological activity, their thermodynamic stability changed in the expected manner.

MATERIALS AND METHODS

Mutagenesis. A 0.477-kb *Hin*cII fragment bearing the coding region for *E. coli* thioredoxin, *trxA*, was recloned from a pUC19 construct (Kelley & Richards, 1987) into the *Hin*cII site of M13mp19. A clone that had the *trxA* promoter oriented such that it is expressed from the *lac* promoter in the vector (Norrander et al., 1983) was chosen to give the *φHH1* construct, which was used as the starting point in all subsequent mutagenesis experiments. Oligonucleotide-directed mutagenesis was carried out using the procedure described by Kunkel (1985), using pools of mixed oligonucleotides. Four pools of oligonucleotides were synthesized, each with a different base at the first position in the codon to be mutagenized, and having mixed nucleotides at the second and third positions. To avoid reisolating the wild-type residue multiple times, nucleotide mixes were chosen of the pool that could potentially contain the wild type such that their combinations did not include this sequence.

Assay of in Vivo Thioredoxin Activity. The M13mp19 thioredoxin mutants were plated on a bacterial host strain deleted for wild-type thioredoxin (A307: Russel & Model, 1986). This strain does not support growth of wild-type M13mp19, but will support the growth of a M13mp19 recombinant phage into which an active thioredoxin has been cloned.

Expression and Mutant Protein Purification. Mutants constructed in the M13 system were recloned into the *Hpa*I site of $pP_L\lambda$, under the control of the inducible λp_L promoter (Pharmacia LKB Technology; Remaut et al., 1981). Bacterial growth was carried out on a 10-L scale in a small fermenter (New Brunswick Scientific Co.). Ten liters of medium was

inoculated with 400 mL of a fresh culture, which had been started from a single colony. The initial growth was carried out at 30 °C until absorbance at 600 nm reached a value of 0.9. The temperature was then switched to 42 °C, and growth continued another 3 h. Cells were harvested by centrifugation, resuspended in buffer A [100 mM NaCl/20 mM Tris·HCl (pH 7.4)], and lysed by sonication. Cell debris was removed by centrifugation at 12000*g* for 30 min at 4 °C. Nucleic acids were precipitated by the addition of 20% (v/v) poly(ethylenimine) in water, which had been adjusted to pH 7.4, to a final concentration of 0.5% (v/V), followed by a 30-min centrifugation at 12000*g* after 30 min stirring at 4 °C. A 35% (w/v) saturated ammonium sulfate cut of the resulting supernatant was discarded, and the remaining protein was precipitated with 75% saturated ammonium sulfate. This pellet was resuspended in buffer A, applied to a Bio-Gel P-30 acrylamide gel filtration column (Bio-Rad Laboratories), and eluted with buffer A. This step separates most proteins from thioredoxin. The fractions that contained thioredoxin (as judged by SDS/polyacrylamide electrophoresis) were then applied to a DE52 ion-exchange column (Pharmacia) and eluted with a 0–0.3M NaCl salt gradient in buffer A. The fractions containing thioredoxin were stored frozen in buffer at −20 °C. Protein concentrations were determined by the absorption at 280 nm (ϵ_{280} = 13 400 cm^{-1}; Holmgren & Reichard, 1967).

Circular Dichroism Measurements. The CD spectra of mutants were determined with an AVIV Model 60DS circular dichroism spectropolarimeter using a 1-mm path-length quartz cell at 25 °C at a protein concentration of approximately 10 μM, in a 20 mM phosphate buffer (pH 7.0).

Denaturation Curves. Protein unfolding was monitored by following the tryptophan fluorescence (excitation, 295 nm; emission, 350 nm), which increases 3-fold upon unfolding of the protein (Holmgren, 1972), as a function of denaturant concentration. Guanidine hydrochloride (GuHCl) was used as the denaturant. The concentration of GuHCl was determined by the refractive index of the solution (Nozaki, 1972). Measurements were taken at 25 °C in a 20 mM phosphate buffer (pH 7.0).

RESULTS

Twelve substitutions at position Leu42 and 15 at Leu78 were constructed by oligonucleotide-directed mutagenesis of the thioredoxin gene cloned into M13mp19. Both of these positions are deeply buried inside the hydrophobic core of the protein. The ability of these mutants to catalyze the assembly of phage M13 was measured by plating mutant phages on a nonpermissive *E. coli* strain lacking the gene for thioredoxin. The results presented in Table I show that virtually all the mutants are biologically active, regardless of their hydrophobicity, charge, or size. Only proline results in inactive protein at both sites. It is not clear why tyrosine is inactive at position 78, whereas phenylalanine remains active.

The charged substitutions at these two positions (L78D, L78E, L78K, L78R, etc.) were chosen for further physical characterization as these presented the mutations that deviated most radically from the results expected on the basis of a simple consideration of the hydrophobic effect. These eight mutants were recloned into an overexpression system, and protein was purified for five of these. It was not possible to purify protein for three of the four substitutions at position 42 (L42D, L42K, and L42R). Wild-type thioredoxin and also some of the mutants express at very high levels in this system (~150 mg of purified protein for a 1-L culture).

Table I: Biological Activity of Mutant Thioredoxins[a]

amino acid	position 42	position 78	amino acid	position 42	position 78
Ala	+	+	Leu (wt)	+	+
Arg	+	+	Lys	+	+
Asn			Met		+
Asp	+	+	Phe	+	+
Cys		+	Pro	–	–
Gln	+	+	Ser	+	+
Glu	+	+	Thr	+	+
Gly		+	Tyr		–
His			Val	+	+
Ile	+				

[a] Blank, mutant was not isolated; +, supported phage assembly; –, did not support phage growth. Note that Trp was not present in the mutagenic oligonucleotide pools.

However, some of the mutants express 1 or 2 orders of magnitude less well. This probably reflects a difference in the resistance to proteolysis between these mutants, rather than a difference in mRNA stability, since their is no obvious correlation between the DNA sequence and expression. We observe a rough correlation between the stability of the mutant and its level of expression (as determined by the final amount of purified protein), as has also been observed in other systems (Parsell & Sauer, 1989). The mutants at position 42 that we were unable to isolate are therefore probably quite unstable.

Circular dichroism spectra were measured for the mutants that were isolated (Figure 2). Thioredoxin shows a broad, shallow transition with a minimum at 220 nm, typical of a mixed βαβ secondary structure. The L78D mutant shows less ellipticity in this region. It is not possible to tell whether this corresponds to loss of a particular secondary structure element or overall loss of structure. It does indicate that the structure of this mutant is perturbed relative to wild type. None of the other mutants show any significant deviations from the wild-type spectrum, indicating that their secondary structure has not been greatly perturbed.

Unfolding curves were determined by measuring the change in fluorescence as a function of GuHCl concentration (Figure 3). The unfolding curves were analyzed as described in Pace (1986). The fraction of unfolded protein, F_U, is given by

$$F_U = (Y_N - Y)/(Y_N - Y_U) \quad (1)$$

where Y is the fluorescence signal observed at a given GuHCl concentration in the unfolding transition region and Y_N and Y_U are the values of the native and unfolded fluorescence, respectively. Y_N and Y_U are themselves linearly dependent on GuHCl concentration outside the folding transition region, and follow the equations:

$$Y_N = Y_{N,0} + a[\text{GuHCl}] \quad (2a)$$

$$Y_U = Y_{U,0} + b[\text{GuHCl}] \quad (2b)$$

where $Y_{N,0}$ and $Y_{U,0}$ are the fluorescence intensities in the absence and at saturating denaturant concentrations, respectively. Accurate determination of the base lines for these curves is therefore important in the calculation of the fraction of unfolded protein. In each of the curves shown in Figure 3, base lines were calculated on 5–10 points in the pre and posttransitional regions. The unfolding equilibrium constant, K_U, is related to the fraction unfolded in the following manner:

$$K_U = F_U/(1 - F_U) \quad (3)$$

From the equilibrium constant of unfolding, a free energy can be calculated, ΔG_U (i.e., $-RT \ln K_U$). It has been found

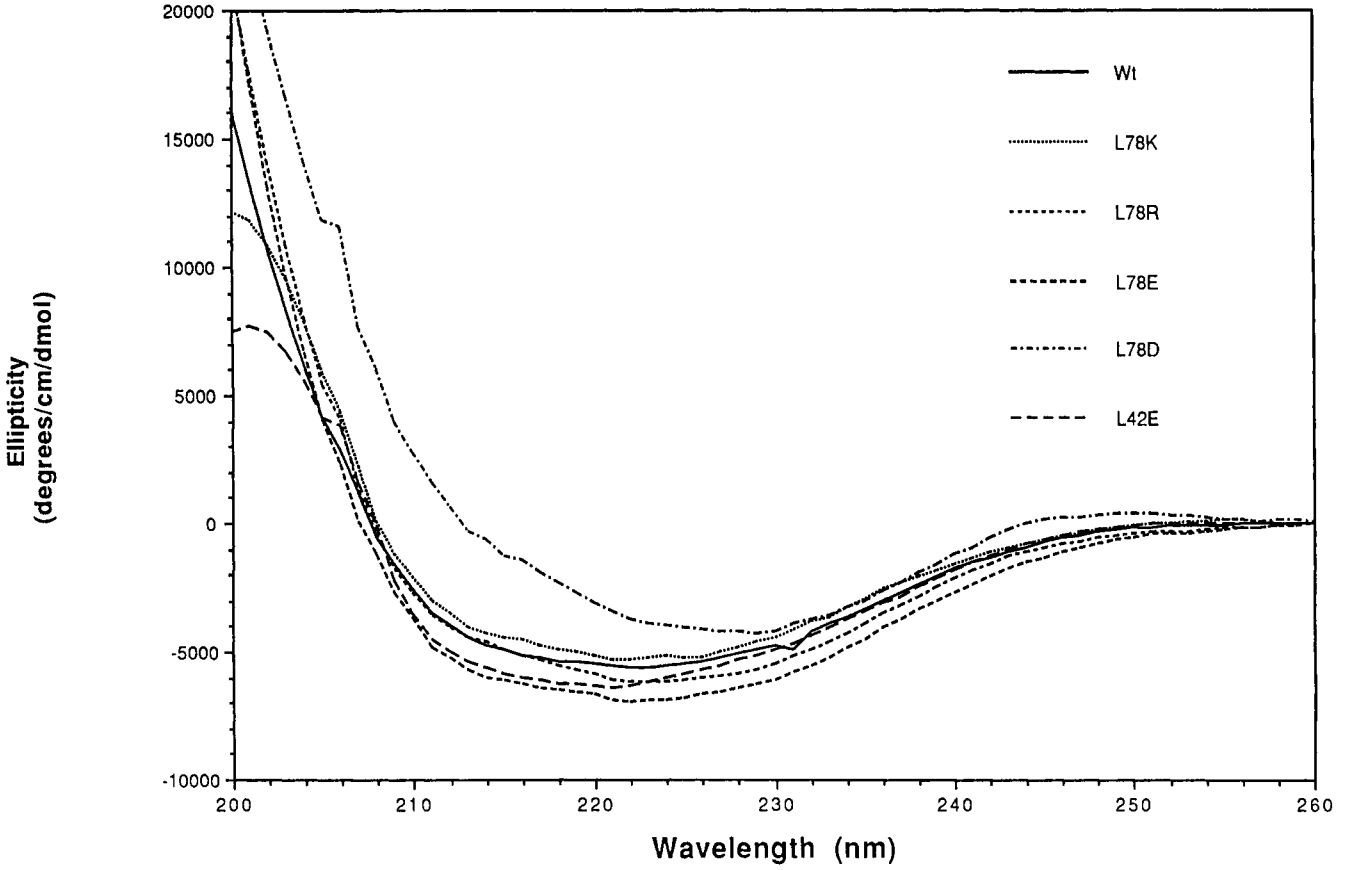

FIGURE 2: CD spectra of wild-type and mutant thioredoxins.

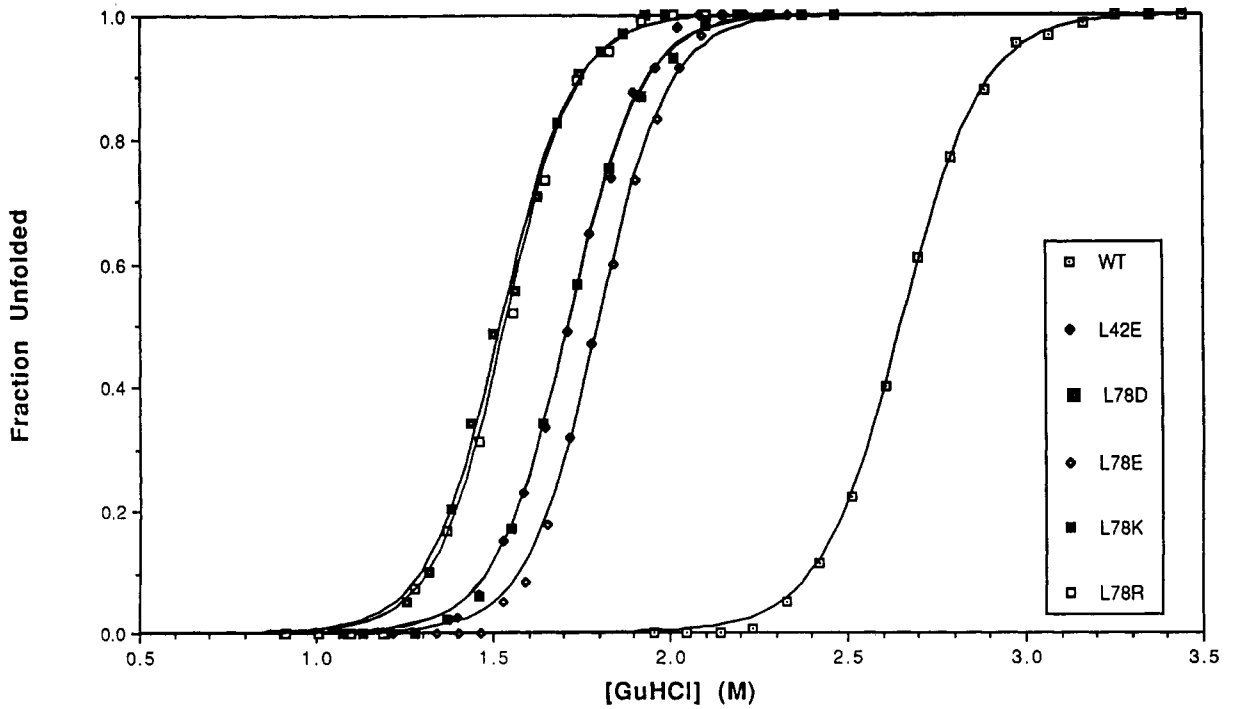

FIGURE 3: Plot of the fraction of unfolded molecules as a function of GuHCl concentration.

empirically that this energy is linearly dependent on the concentration of denaturant (Pace, 1986), so that a free energy in the absence of a denaturant can be calculated:

$$\Delta G_{H_2O} = \Delta G_u - m[\text{GuHCl}] \quad (4)$$

ΔG_{H_2O} and m were calculated by linear regression of the data points in the transitional region of the unfolding curve. In all cases, the correlation coefficient was 0.99 or better. The free energy of unfolding which is a consequence of the mutation is then given by

$$\Delta\Delta G_{mut} = \Delta G_{H_2O}(\text{mutant}) - \Delta G_{H_2O}(\text{wild type}) \quad (5)$$

The free energy of unfolding was determined at neutral pH for those charged mutants that could be isolated. The values for $\Delta\Delta G_{mut}$ presented in Table II show that all these mutations destabilize the protein relative to wild type, as would be

Table II: Stability Parameters for Selected Mutant Thioredoxins

mutant	C_m[a] (M)	m[b]	ΔG_{H_2O}[c] (kcal/mol)	$\Delta\Delta G_{mut}$[d] (kcal/mol)	$\Delta m/m$[e]	yield[f] (mg/L)
wild type	2.65	5.1	13.6	0		150
L78K	1.71	5.6	9.7	3.9	0.10	80
L78R	1.54	5.9	9.1	4.5	0.16	30
L78D	1.52	5.7	8.7	4.9	0.12	4
L78E	1.80	5.9	10.5	3.1	0.16	15
L42E	1.71	5.7	9.8	3.8	0.12	18

[a] Concentration of GuHCl at which the unfolding transition midpoint is observed. [b] Proportionality constant between free energy and GuHCl concentration. [c] Free energy of unfolding extrapolated to zero denaturant. [d] Change in free energy of mutant structures relative to wild-type protein. [e] Relative change in proportionality constant. [f] Amount of pure protein purified per liter of culture medium.

expected. It should be noted that although the midpoint concentration of the unfolding transition for wild-type thioredoxin is similar to previously published values, there is significant variation in the slope parameter m and hence in the derived value for ΔG_{H_2O}. The origin of these differences in the wild-type values from those in the literature is not yet clear. The conditions for all the experiments reported in this study were accurately matched so that the differences between wild-type and mutant values are likely to be reliable.

An important component in the analysis of the effects of mutations on the thermodynamics of folding is determining relative contributions of changes in the folded versus the denatured state (Dill & Shortle, 1991). A change in m, the slope of the dependence of ΔG_U as a function of denaturant concentration, as a consequence of a mutation indicates that there has been a change in the denatured state (Shortle & Meeker, 1986; Shortle et al., 1990, 1992). The data presented in Table II show that $\Delta m/m$ changes by 10–14% for the charged residues. Theoretical analysis (Shortle et al., 1992) and experimental data on a large number of staphylococcal nuclease mutants (Shortle & Meeker, 1986) have shown that the magnitude of these changes should be considered significant. Changes in m reflect a change in the difference in solvent-accessible surface area in the native and denatured states (Dill & Shortle, 1991). All the mutants analyzed here show an increase in m consistent with an expected increase in the exposed surface area of the denatured state due to the solvation of the extra charged residue.

DISCUSSION

A set of single-site mutations was constructed at two positions in the hydrophobic core of *E. coli* thioredoxin. The effect of these mutations was measured in vivo by testing their activity in an assay in which thioredoxin catalyzes the assembly of phage M13. Although this assay is only qualitative and binary in nature (active versus inactive), it is able detect thresholds at which a mutation becomes disruptive. Surprisingly, it was found that most mutations still showed activity. Although it is known that proteins are quite plastic and are able to adapt a variety of sizes of amino acid side chains in their hydrophobic cores, most systems studied so far show some limits as to size and the hydrophobic nature of the changes that are acceptable.

The most extreme demonstration of the tolerance of thioredoxin to substitutions in the hydrophobic core is that both positions accept all four of the charged residues that were constructed. In an extensive study of the core of λ repressor where a pool of random muations at several loci was selected for active sequences, it was found that hydrophobic residues within a certain size distribution, some hydrophilic, but no charged residues were allowed (Lim & Sauer, 1989).

Examination of the changes in free energy does show that the mutant thioredoxins with the charged residue replacements are significantly less stable than the wild type (destabilized by 3–5 kcal/mol). These measurements were made at neutral pH so that the groups either were charged or have a highly perturbed pK_a, either of which is energetically expensive (Honig et al., 1986). The magnitude of these changes is similar to the changes found in other systems where buried charges have been measured [2–9 kcal/mol, depending on the pH for the M102K mutation in the hydrophobic core of T4 lysozyme (Dao-pin et al., 1991); 5–8 kcal/mol for the V66K mutant in staphylococcal nuclease (Stites et al., 1991)]. The thermodynamic response of thioredoxin to these mutations is therefore not unusual.

Why is the link between stability and function not as strong for thioredoxin as has been found in other systems? One reason might be that the structural requirements for the activity may be fairly loose. The mechanism by which thioredoxin functions to catalyze phage assembly remains obscure. It is known that a protein/protein contact is involved with the gene *I* product of the phage (Russel & Model, 1983, 1984). It is also known that the redox activity of the protein is not required but that there is a requirement for the reduced rather than the oxidized conformation of the protein (Russel & Model, 1986). Since the structural changes involved in the change of redox states are small and localized (Dyson et al., 1990), it appears that only a few conformations give activity. The site of the mutations is not in a region where conformational changes have been observed. Thus, if structural perturbations are localized, or if they occur mainly in a region not involved in determining the function, thioredoxin may be able to accomodate relatively large changes. Another factor is that thioredoxin is a very stable protein. The wild type shows a ΔG_{H_2O} of 13.6 kcal/mol. The mutation with the largest measured change (L78D) still has a ΔG_{H_2O} of 8.7 kcal/mol. It is clear that thioredoxin has a large thermodynamic range for the accomodation of deleterious mutations. This may have been an important constraint for the evolution of the control of the redox potential in thioredoxin which involves the burial of charged aspartic acid (Asp26). Mutation of this residue to Ala increases the stability of the protein by 5 kcal/mol at neutral pH (Langsetmo et al., 1991).

The mechanisms by which the structure adapts to the changes in the core are probably not the same at positions 42 and 78, even though the two wild-type residues are in van der Waals contact with each other in the native structure (Figure 4). Position 78 is located on the central β sheet, and it points toward the surface of the protein. It is buried in the hydrophobic core of the protein by two α helices which pack on top of it. If these two helices were to move apart from each other through a combined bending and rolling motion, the resulting gap between them could open up a channel through which long side chains such as lysine, glutamate, and arginine could reach out, leaving their hydrophobic stalks buried in the hydrophobic core of the protein, and allowing their charged, hydrophilic heads to be solvated. In contrast, position 42 is located on one of the α helices that cover Leu78, and points toward the center of the protein. There is no obvious mechanism by which a relatively small structural perturbation could allow the charged moieties of the mutant residues to become exposed to solvent. The charges would therefore have to be buried in the hydrophobic core itself. Such structures have been observed in staphylococcal nuclease (Stites et al., 1991) and T4 lysozyme (Dao-Pin et al., 1991).

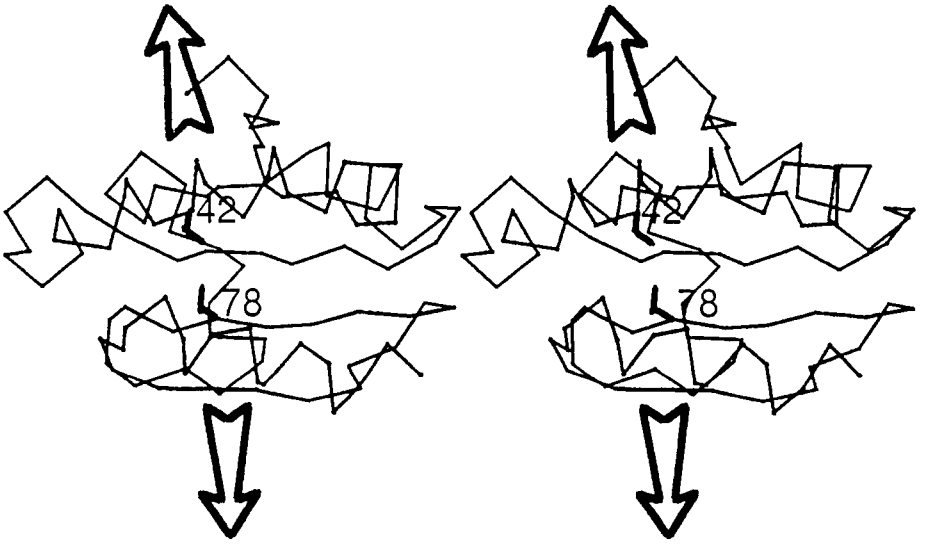

FIGURE 4: Diagram of the thioredoxin structure indicating that the two helices covering Leu78 could move apart to allow a channel to the solvent to open up.

A consequence of the difference in the topology of packing for these two positions is that charged mutants at position 42 would be predicted to be less stable than their equivalent at position 78. This is true when we compare L42E and L78E. Proteins for the other three charged residues at position 42 were not isolated, because they could not be expressed. If we accept that this is because they are proteolytically degraded, and that this degradation is linked to their stability, as has been observed in some other systems (Parsell & Sauer, 1989), then this would suggest that charged mutations at position 42 are indeed less stable than at position 78. This lends further credence to the notion that charges at position 78 could be stabilized by helix movements. Detailed structural studies on the mutants are necessary to test this hypothesis. Unfortunately, so far the mutants have persistently resisted our attempts to crystallize them.

Analysis of the protein folding problem through the use of mutants has led to impressive advances in our understanding. The assumption that biological activity reflects structural stability has allowed the use of rapid genetic selection and screening techniques so that large numbers of mutants can be examined. Successful application of these techniques has allowed the "informational content" of a region to be examined through single and multiple mutations. This approach has been successfully inverted, and the rules derived from studies on proteins of known structure have been applied to predicting the structure of a protein by interpreting the pattern of mutations that gave biologically active mutants (Bowie & Sauer, 1989). It has always been recognized that the mutational spectrum of a protein based on the measurement of biological activity may be biased by problems with expression of particular mutant proteins due to such factors as mRNA stability and resistance to proteolysis, which are not necessarily dependent on thermodynamic stability. The mutations in thioredoxin described in this paper demonstrate that the informational content of a region in a protein is also difficult to measure genetically if the protein is unusually stable or plastic.

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