Substitution of Charged Residues into the Hydrophobic Core of *Escherichia coli* Thioredoxin Results in a Change in Heat Capacity of the Native Protein†

John E. Ladbury,*,‡ Richard Wynn, James A. Thomson,§ and Julian M. Sturtevant

Department of Chemistry and Department of Molecular Biophysics and Biochemistry, Yale University, P.O. Box 208107, 225 Prospect Street, New Haven, Connecticut 06520

Received October 24, 1994; Revised Manuscript Received December 13, 1994⊗

ABSTRACT: Two site-directed mutants of *Escherichia coli* thioredoxin (L78K and L78R) were designed to study the effect of placing a charged residue in the hydrophobic core of the protein. Both mutants retain catalytic activity in the assembly of phage M13. Thermal denaturation of both these mutant proteins at pH 7.0 shows a reduction of stability of approximately 4 kcal·mol^{-1} with respect to the oxidized wild-type form. The thermal denaturation of the protein fits a dimeric state model. A significant reduction in the change in heat capacity (ΔC_p) on unfolding is observed compared to oxidized wild-type thioredoxin. We present data to indicate that this reduction in ΔC_p is attributable to structural perturbations resulting in localized unfolding of the native protein and exposure to solvent of residues that are buried in the wild-type protein.

Structural and mutational studies indicate that the stability and enzymatic activity of globular proteins are dictated by intimate packing of amino acids. Disruption of the packed core of a protein, even in the form of quite subtle mutations, can result in dramatic effects [reviewed by Shortle (1989), Fersht & Serrano (1993), and Sturtevant (1994)]. Although structural studies indicate that rearrangements of the main-chain can allow accommodation of substituted residues, this apparent plasticity is clearly limited (Matthews, 1987; Lim & Sauer, 1989; Kitamura & Sturtevant, 1989; Ladbury et al., 1992).

Major disruptions in the packing interactions of proteins appear to occur when polar or charged residues are introduced into or removed from the protein core. For example, substitution of hydrophobic for charged residues in T4 lysozyme and staphylococcal nuclease resulted in a reduction in protein stability of 2−9 kcal·mol^{-1} and led to a greatly reduced or a complete loss of enzymatic activity (Dao-pin et al., 1991; Stites et al., 1991). Also, insertion of charged residues into the hydrophobic core of the λ repressor was forbidden, and although polar groups were permitted, their presence led to complete loss of biological activity and caused structural changes which led to highly noncooperative thermal denaturation (Lim & Sauer, 1989; Lim et al., 1992).

Escherichia coli thioredoxin has been shown to be able to withstand major mutations in the hydrophobic core and retain stability and activity comparable to that of the wild-type protein (Langsetmo et al., 1991; Hellinga et al., 1992). Substitutions for leucine residues at positions 42 and 78 showed that the hydrophobic core will allow major packing changes without precluding folding of the protein or inhibition of activity required in the assembly of the phage M13. Several unnatural amino acids have been substituted into the hydrophobic core at position 78 via a cysteine mutation for a leucine and subsequent oxidation in the presence of thiol-terminated alkanes (Wynn & Richards, 1993). This mutation strategy showed that the structure of thioredoxin allows even the insertion of bulky hydrophobic groups such as *n*-pentane and cyclopentane (Wynn and Richards, unpublished data). The most extreme demonstration of the tolerance of thioredoxin to substitutions in the hydrophobic core, however, is its ability to accept charged residues without preventing protein folding or complete loss of activity.

E. coli thioredoxin is a small (11.7 kDa) protein which consists largely of a five-stranded twisted β-sheet, four α-helices, and a 3_{10}-helix. Thioredoxin has several biological functions in a variety of organisms generally centered around redox action of the exposed disulfide/dithiol active site [for reviews on the biological roles of thioredoxin, see Holmgren (1985, 1989)]. The structures of both the oxidized and reduced forms of *E. coli* thioredoxin have been determined by X-ray crystallography and NMR (Katti et al., 1990; Dyson et al., 1989, 1990). Furthermore, oxidized thioredoxin has the highest compressibility reported for any protein concordant with it possessing a flexible structure (Kaminsky & Richards, 1992).

We prepared mutant forms of *E. coli* thioredoxin where we substituted the leucine in position 78 for a lysine or an arginine (L78K and L78R, respectively) to investigate the thermodynamic effects of accommodation of these charged residues in the hydrophobic core of the protein. We determined this effect by using differential scanning calorimetric (DSC) thermal denaturation of the mutant forms and compared the data obtained to those for wild-type thioredoxin (Ladbury et al., 1993). In the wild-type protein, position 78 is distal from the active site, but is a part of the hydrophobic core. The leucine 78 is located in the central β-sheet with its side chain pointing toward the surface but

† This work was supported in part by Grant GM-04725 from the National Institutes of Health and Grant DMB-8810329 from the National Science Foundation. J.E.L. is a Career Development Fellow of the Wellcome Trust.

* Author to whom correspondence should be addressed.

‡ Current address: The Oxford Centre for Molecular Sciences, University of Oxford, New Chemistry Laboratory, South Parks Rd., Oxford OX1 3QT, U.K.

§ Current address: Agouron Pharmaceuticals Inc., 3565 General Atomic Court, San Diego, CA 92121.

⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1995.

0006-2960/95/0434-2148$09.00/0

Table 1: Data from DSC Scans of L78K and L78R Mutated Forms of Thioredoxin[a]

concn (mM)	concn (mg·mL^{-1})	$t_{1/2}$ (°C)	% dimer denatured	ΔH_{cal} (kcal· mol^{-1})	β/ MW	ΔC_p (kcal· mol^{-1}·K^{-1})	SD (%)
L78K Mutated Form of Thioredoxin							
(A) pH 6.50 (K_N = <5 M^{-1}; K_D = 2.5 × 10^3 M^{-1})							
387	4.53	71.78	49.4	86.6	1.03	0.69	0.9
239	2.75	72.28	41.2	85.3	1.05	1.00	0.8
229	2.68	72.22	40.5	87.8	1.01	1.07	0.5
86	1.01	72.58	24.5	89.3	1.03	1.96	1.4
76	0.88	73.09	22.7	77.2	1.21	0.55	1.7
(B) pH 7.00 (K_N = <5 M^{-1}; K_D = 4.8 × 10^2 M^{-1})							
817	9.56	70.32	34.2	89.4	1.07	0.79	0.7
481	5.63	71.18	25.7	90.7	1.05	0.64	0.6
302	3.53	72.11	19.1	96.3	1.00	0.75	0.3
235	2.75	72.00	16.0	90.6	1.06	0.65	0.5
152	1.78	72.49	11.5	92.2	1.04	1.38	0.7
109	1.28	72.77	8.8	98.1	0.95	1.13	0.8
78	0.91	72.89	6.6	82.5	1.13	1.11	1.1
(C) pH 7.50 (K_N = <5 M^{-1}; K_D = <5 M^{-1})							
463	5.41	71.79		92.6	1.11	0.65	0.8
325	3.80	71.98		93.1	1.07	0.56	0.6
240	2.81	72.33		98.4	1.03	0.64	0.7
74	0.86	73.70		86.2	1.13	1.48	0.8
L78R Mutated Form of Thioredoxin							
(A) pH 6.50 (K_N = <5 M^{-1}; K_D = 5.0 × 10^2 M^{-1})							
455	5.32	68.72	22.1	60.8	1.52	1.01	1.0
266	2.65	69.48	13.5	60.5	1.53	1.35	1.0
172	2.01	69.85	10.9	62.4	1.47	1.39	1.7
113	1.32	70.15	7.7	60.6	1.52	1.65	1.4
91	1.07	70.13	6.4	63.9	1.47	1.31	1.3
(B) pH 7.00 (K_N = <5 M^{-1}; K_D = 2.5 × 10^3 M^{-1})							
531	6.22	68.60	54.6	74.1	1.11	0.85	0.8
365	4.27	68.94	48.5	70.2	1.16	0.92	1.3
253	2.96	69.31	42.2	76.6	1.07	0.89	0.6
229	2.68	69.58	40.5	80.4	1.02	0.86	0.7
188	2.22	69.86	37.1	75.6	1.06	1.04	1.1
103	1.21	70.43	27.3	78.0	1.07	0.59	0.7
(C) pH 7.50 (K_N = <5 M^{-1}; K_D = 1.0 × 10^2 M^{-1})							
455	5.32	68.71	7.7	62.1	1.69	0.94	1.3
266	3.13	69.37	4.8	63.9	1.61	1.01	1.3
178	2.08	69.54	3.3	60.8	1.65	1.59	1.6
87	1.02	70.33	1.7	70.8	1.36	0.98	1.6

[a] The accuracy of the model fit to the experimental data for the individual DSC scans is reported as a cumulative standard deviation (SD) of all the points recorded.

buried in the core by two α-helices. The separation of these two α-helices, forming a channel that leads to the surface, is believed to confer the ability of the protein to accept major structural changes on substitutions at this position (Hellinga et al., 1992).

We observe a significant change in the heat capacity on thermal unfolding of the L78K and L78R mutants with respect to wild type, which appears to be attributable to changes in the native state of the protein. In the absence of structural data, our results support the hypothesis that the substitued residues cause structural perturbations that lead to partial unfolding of the native state while resulting in limited reduction of biological activity.

MATERIALS AND METHODS

Materials. Mutant forms of *Escherichia coli* thioredoxin were expressed and purified as described by Hellinga et al. (1992). Protein solutions were stored at −20 °C in Tris buffer at pH 7.00 at concentrations of up to 25 mg·mL^{-1}.

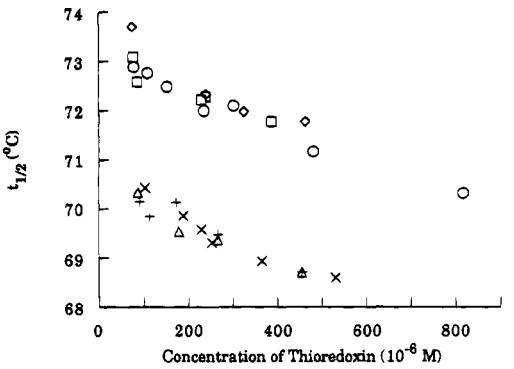

FIGURE 1: Plot of $t_{1/2}$ against concentration of L78K and L78R mutants of thioredoxin indicating the effect of pH. L78K mutant: (□) pH 6.50; (○) pH 7.00; (◇) pH 7.50. L78R mutant: (+) pH 6.50; (×) pH 7.00; (△) pH 7.50.

These solutions were dialyzed exhaustively in 50 mM sodium phosphate buffer at the required pH prior to use. The concentration of thioredoxin was determined by optical density based on an extinction coefficient at 280 nm of 13.7 mM^{-1}·cm^{-1} (Reutimann et al., 1981).

Differential Scanning Calorimetry. All scans were obtained using the MC-2 calorimeter (MicroCal Inc., Northampton, MA). Details of experimental procedures have been described elswhere (Ladbury et al., 1993)

The reversibility of the unfolding process was confirmed by heating the protein to between 5 and 10 °C beyond $t_{1/2}$ and rescanning following rapid cooling. Unfolding of all forms of thioredoxin herein described was found to be between 90% and 100% reversible.

Data Analysis. Calorimetric scans obtained were fit to a two-state model with incomplete dissociation/association to a dimer in both the native and denatured states of the protein as previously described (Sturtevant, 1987; Ladbury et al., 1993).

Anilinonaphthalenesulfonic Acid (ANS) Binding Studies. Fluorescence measurements were carried out on a SPEX DM3000 spectrofluorometer in 0.05 M sodium phosphate at pH 7.0. ANS concentrations were determined by the absorbance at 350 nm. Samples were excited at 400 nm, and emission was followed from 410 to 710 nm in 1 nm steps.

Polyacrylamide Gel Electrophoresis (PAGE) Analysis of Native Protein. PAGE was carried out using a Bio-Rad mini gel apparatus. Electrophoresis in 15% acrylamide gels was carried out at 20 V·cm^{-1} for 2 h, and bands were visualized by staining with Coomassie blue (Sambrook et al., 1989).

RESULTS AND DISCUSSION

In this work, we investigate the changes in thermodynamic parameters resulting from the L78K and L78R substitutions with respect to wild-type thioredoxin in the pH range 6.5−7.5. Reversible thermal denaturation of these mutants was performed using DSC, and the data are shown in Table 1.

The data were best-fit to a model where reversible dimerization of both native and denatured protein is assumed. This model accounts for the inverse dependence of $t_{1/2}$ on the concentration of the protein at all the pH values studied, as shown in Figure 1. This is indicative for increased association of the denatured form. As in previous reports of thioredoxin thermal denaturation, we have attempted to use models involving higher oligomeric forms of the protein;

however, the inclusion of the additional parameters for these models, although slightly improving the data fit, gives a number of best-fit options (Ladbury et al., 1993, 1994).

Our adopted model, based on the potential for thioredoxin to dimerize in both native and denatured forms, has been suggested in several studies. Holmgren (1985) described dimer formation of thioredoxin below its isoelectric point, and Laurent et al. (1964) demonstrated reversible oligomerization at low pH by ultracentrifugation studies. Association of the denatured protein has also been demonstrated by the existence of residual intermolecular hydrogen bonds at temperatures in the posttransition region by Fourier transform infrared spectroscopic methods (J. E. Ladbury, unpublished results). Thermal denaturation of oxidized and reduced forms of wild-type thioredoxin was also best described by a model in which the protein formed reversible dimers in both native and denatured states (Ladbury et al., 1993, 1994).

The magnitudes of the dimerization constants for the native form of both mutated thioredoxins denatured below pH 7.5 are reduced to a level whereby they are experimentally indeterminable ($<5\ M^{-1}$). This is in contrast to data obtained for other forms of thioredoxin in this pH range. This indicated that the mutations in some way significantly reduce the dimerization of the native protein. PAGE analysis at pH 7.0 of native mutated proteins suggests that the substituted side chains are protonated at neutral pH (data not shown). The native wild-type thioredoxin is not protonated at this pH. The presence of this charge in the mutant forms of thioredoxin might interfere with the dimerization reaction by virtue of repulsion between native proteins. Another possibility is that the positioning of a charge in the hydrophobic core of the protein causes some structural perturbation of the dimerization interface.

On the other hand, in the presence of this presumably repulsive charge, the denatured state of the mutant protein is able to form dimers. The removal of hydrophobic surface area from exposure to solvent is expected to be a dominant force in dimerization of the denatured thioredoxin. The determinants of the structure of denatured proteins are, however, poorly understood.

The fitted data show differences between the various forms of thioredoxin. The dimerization constants for the denatured L78K mutated form of thioredoxin shown in Table 1 are inversely dependent on pH, following the same trend as previously observed for wild-type thioredoxin. This, however, is not observed for the L78R mutant form where the denatured form of the protein has a greater propensity to form dimers at pH 7.0. This indicates that there are definite differences between the two mutated forms of thioredoxin. These may well reflect the differences in the charge density of the unfolded forms of the protein.

For a strictly two-state unfolding/folding process, the ratio of β/MW ($=\Delta H_{vH}/\Delta H_{cal}$) is unity, whereas values greater than this are representative of intermolecular cooperativity or slow unfolding (Sturtevant, 1987). For the 16 scans of the L78K mutant thioredoxin, this ratio is 1.06 ($\pm$0.06) over the pH range investigated. At pH 7.0, a simple two-state unfolding process can also be assumed for the arginine mutant, since the mean value of β/MW is 1.08 ($\pm$0.05). However, at the pH values higher and lower than this, the value of the ratio strays significantly from unity (1.50 ± 0.03 at pH 6.5 and 1.58 ± 0.13 at pH 7.5). This effect is similar to that observed for the oxidized wild-type thioredoxin where the value of β/MW increases with pH from pH 6.5.

Plots of ΔH_{cal} against $t_{1/2}$ for both forms of thioredoxin investigated indicated that the unfolding reaction was accompanied by a positive change in heat capacity, ΔC_p. The limited temperature range over which the $t_{1/2}$ changed between pH 6.5 and pH 7.5 resulted in large standard deviations in the attempted fits of these plots. To establish the apparent ΔC_p for this reaction, the mean value from the individually fit scans for each form of the protein was used. This gave values of 0.94 ($\pm$0.39) kcal·mol^{-1}·K^{-1} from 16 individual scans and 1.09 ($\pm$0.29) kcal·mol^{-1}·K^{-1} from 15 individual scans of L78K and L78R, respectively. The contribution to ΔC_p from dimerization is assumed to be negligible. There is no significant change in ΔC_p values as the amount of dimer increases at a given pH (Table 1).

The ΔC_p values obtained for the two mutant forms of thioredoxin are significantly less than the value of 1.93 ($\pm$0.67) kcal·mol^{-1}·K^{-1} (Ladbury et al., 1994) or 1.66 ($\pm$0.05) kcal·mol^{-1}·K^{-1} (Santoro & Bolen, 1992) for the wild-type protein. Since the change in heat capacity can be represented by the equation $\Delta C_p = C_{p(\text{denatured})} - C_{p(\text{native})}$, the reduction in heat capacity for an unfolding reaction is the result of (i) a reduction in the heat capacity of the denatured form and/or (ii) an increase in the heat capacity of the native form.

In the first case, this would require some gross structural rearrangement of the denatured form (for example, the removal of residual structure which was present in denatured wild type). There was no evidence of this from CD or FTIR spectroscopic studies performed at temperatures in the posttransition region of the DSC scan (data not shown). The formation of residual structure has been described as being associated with the presence of rapidly exchanging hydrophobic regions of the denatured protein (Griko et al., 1994), in which case the substitution of two charged residues would not be expected to affect this type of behavior.

In the second case, it has been reported that water forms an ordered structure over hydrophobic surfaces (Du et al., 1994) which results in an increase in the heat capacity of the solution (Kauzmann, 1959; Tanford, 1973). The increase in heat capacity of the native form of the protein could result from the exposure of hydrophobic residues to solvent that normally would be buried in the wild-type protein. This would mean that the L78K and L78R mutations cause a structural perturbation in thioredoxin that allow hydrophobic surfaces to be in contact with the solvent.

These hypotheses are supported by NMR structural data on the mutant protein L78K. Spectral assignments reveal that residues proximal to these substituted side chains that are also close to the protein surface are significantly more mobile than in wild-type thioredoxin, suggesting that exposure to solvent is possible (Spicer, De Lorimier, and Hellinga, personal communication).

To test further the idea that the hydrophobic core of the protein is exposed to the solvent in these mutated forms of thioredoxin, binding studies with ANS were performed. ANS binds and gives a fluorescent signal on interaction with exposed hydrophobic surface areas. It should be noted that ANS is negatively charged and has the potential to form charge−charge interactions. ANS does not bind to the wild-type protein as shown by the lack of fluorescence in the wavelength range studied (Figure 2). There is also no

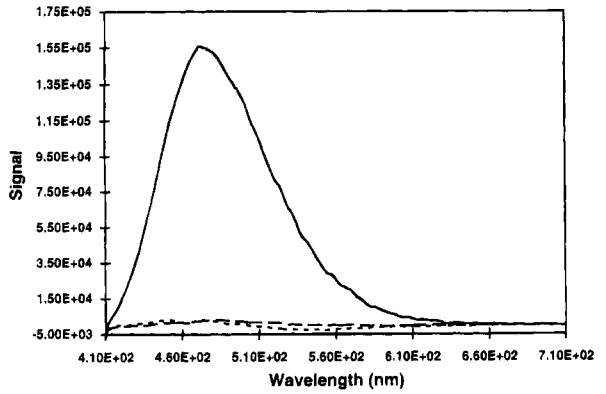

FIGURE 2: ANS binding study of WT, L78K, and L78R forms of thioredoxin. (- - -)Wild-type thioredoxin; (— —) L78K; (—) L78R.

apparent change for the L78K mutant; however, there is a large fluorescent signal for the L78R mutant, indicating that interaction has occurred. The native protein gel shows that the charge on the mutant forms of thioredoxin is the same; thus, the differences in ANS binding propensity are likely to be a result of the differences in hydrophobic surface area exposed. The conclusion drawn from this experiment is that the L78R mutant is capable of binding a hydrophobic probe, suggesting that some additional hydrophobic surface is present which does not appear in the wild-type protein. L78K behaves differently and is able to accommodate the charged side chain in an alternative manner. The sizes and localization of the charges of the respective side chains are significantly different. The side chain of the arginine residue has a bulky guanidine group at its end which may be responsible for the opening of the two helices described above to such an extent that the hydrophobic core becomes accessible to the solvent. ANS itself is a bulky molecule and would require a significant opening in the protein surface to be able to interact.

Calculation of the standard free energies of thermal denaturation for the two mutant forms relative to that of the oxidized wild type by means of the Gibbs–Helmholtz equation is complicated by the fact that, for the equilibria involving partial formation of dimeric species, the free energy at $t_{1/2}$ is no longer zero, but depends on the values for K_N and K_D in the model employed in our curve fitting:

$$^1/_2N_2 \overset{K_N}{\rightleftharpoons} N \overset{K_D}{\rightleftharpoons} D \rightleftharpoons {}^1/_2D_2$$

We have therefore calculated approximate free energies for the mutant forms at their respective temperature of half-completion of denaturation, $T_{1/2,m} = t_{1/2} + 273.15$, relative to the oxidized wild-type at $T_{1/2,o}$ using the equation previously employed by Ladbury et al. (1994):

$$\Delta G°(\text{at } T_{1/2o}) = \Delta H_m(\text{at } T_{1/2,m})[(T_{1/2m} - T_{1/2o})/T_{1/2m}] - \Delta C_p[T_{1/2m} - T_{1/2o} + T_{1/2o} \ln(T_{1/2o}/T_{1/2m})]$$

For both mutant forms of the protein, $T_{1/2}$ at the above conditions was obtained from the linear relationship between $1/T_{1/2}$ and the natural logarithm of concentration as described by Tanaka et al. (1993) [$t_{1/2}$(L78K) = 71.66 °C; $t_{1/2}$(L78R) = 69.21 °C] and ΔH_m from the plot of calorimetric enthalpy, ΔH_{cal}, and concentration [ΔH(L78K) = 91.41 kcal·mol^{-1}; ΔH(L78R) = 75.52 kcal·mol^{-1}]. The ΔC_p value used for the L78K and L78R mutants in determination of $\Delta G°$ were 0.94 and 1.09 kcal·mol^{-1}·K^{-1}, respectively. The thermodynamic parameters obtained for the denaturation of the mutant forms are compared to data for the oxidized wild-type form of thioredoxin under the same conditions, where $t_{1/2}$ is 85.32 °C in Table 2 (Ladbury et al., 1993).

Table 2: Thermodynamic Data for the Thermal Unfolding of L78K and L78R Mutant Forms with Respect to Wild-Type Thioredoxin[a]

protein	$\Delta t_{1/2}$ (°C)	$\Delta\Delta G°$ (kcal·mol^{-1})	$\Delta\Delta C_p$ (kcal·mol^{-1}·K^{-1})
L78K	−13.7	−3.9	−1.0
L78R	−16.1	−4.0	−0.8

[a] pH 7.00; 300 mM protein; 85.32 °C (the $t_{1/2}$ of oxidized thioredoxin). The values are calculated based on the general equation $\Delta\Delta J = \Delta J_{mutant} - \Delta J_{wild\ type}$, where J is the thermodynamic parameter.

Mutations to charged residues at position 78 result in a reduction of $t_{1/2}$ (Table 2). The decreases in the $t_{1/2}$ by 13.66 °C for the L78K mutant and 16.11 °C for the L78R mutant are large, but are comparable to that resulting from the reduction of the disulfide bond in thioredoxin (12.1 °C) which has been shown to involve no major structural perturbations (Ladbury et al., 1994). It should also be noted that the values of $t_{1/2}$ of these mutant proteins are still above average for proteins in general.

Both mutated forms are destabilized with respect to the oxidized wild type (Table 2). This destabilization is comparable to that observed in other studies (approximately 4 kcal·mol^{-1}). The burial of charge in a hydrophobic core of a protein has been shown to be detrimental to the overall stability of the protein. Indeed, at neutral pH, oxidized wild-type thioredoxin can be significantly stabilized (5 kcal·mol^{-1}) by replacing the negatively charged buried aspartic acid in position 26 with alanine (Langsetmo et al., 1991). The valine for lysine substitution in staphylococcal nuclease resulted in a reduced stability of 5−8 kcal·mol^{-1} (Stites et al., 1991). Substitution of the methionine in position 102 for a lysine in T4 lysozyme results in a reduction of $\Delta G°$ of between 2 and 9 kcal·mol^{-1} depending on pH. This last example is particularly relevant to this work, since the crystal structure of the T4 lysozyme mutant, although being very similar to that of wild-type protein, reveals that residues proximal to the charged substitution become substantially more mobile (Dao-pin et al., 1991).

In summary, we observe significant tolerance to substitution of charged residues into the hydrophobic milieu of the core of thioredoxin. This seems to be the result of the capacity of this protein to undergo structural alterations that can accommodate these dramatic changes while still allowing folding to a stable state and retaining enzymatic activity. This structural perturbation has been hypothesized as being due to the possible opening up of a cleft between the β-sheets and the α-helices of the protein (Hellinga et al., 1992). Structural studies underway will help clarify this unique behavior. Our data appear to support this hypothesis to the extent that the L78K and L78R mutations lead to significant changes of ΔC_p in their native states and in the case of the arginine mutant the possible exposure of the hydrophobic core of the protein to solvent.

ACKNOWLEDGMENT

We express our gratitude to Prof. F. M. Richards for his continued support of this work and Prof. H. W. Hellinga for helpful comments. We are also indebted to Dr. S. Petanceska for critical reading of the manuscript.

REFERENCES

Dao-pin, S., Anderson, D. E., Baase, W. A., Dahlquist, F. W., & Matthews, B. W. (1991) *Biochemistry 30*, 11521−11529.

Du, Q., Freysz, E., & Shen, Y. R. (1994) *Science 264*, 826−828.

Dyson, H. J., Gippert, G. P., Case, D. A., Holmgren, A., & Wright, P. E. (1990) *Biochemistry 29*, 4129−4136.

Dyson, H. J., Tennant, L. T., & Holmgren, A. (1991) *Biochemistry 30*, 4262−4268.

Fersht, A. R., & Serrano, L. (1993) *Curr. Opin. Struct. Biol. 3*, 75−83.

Griko, Y. V., Freire, E., & Privalov, P. L. (1994) *Biochemistry 33*, 1889−1899.

Hellinga, H. W., Wynn, R., & Richards, F. M. (1992) *Biochemistry 31*, 11203−11209.

Holmgren, A. (1985) *Annu. Rev. Biochem. 54*, 237−271.

Holmgren, A. (1989) *J. Biol. Chem. 264*, 13963−13966.

Kaminsky, S. M., & Richards, F. M. (1992) *Protein Sci. 1*, 22−30.

Katti, S. K., LeMaster, D. M., & Eklund, H. (1990) *J. Mol. Biol. 212*, 167−184.

Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1−63.

Kitamura, S., & Sturtevant, J. M. (1989) *Biochemistry 28*, 3788−3792.

Ladbury, J. E., Hu, C.-Q., & Sturtevant, J. M. (1992) *Biochemistry 31*, 10699−10702.

Ladbury, J. E., Wynn, R., Hellinga, H. W., & Sturtevant, J. M. (1993) *Biochemistry 32*, 7526−7530.

Ladbury, J. E., Kishore, N., Hellinga, H. W., Wynn, R., & Sturtevant, J. M. (1994) *Biochemistry 33*, 3688−3692.

Langsetmo, K., Fuchs, J., & Woodward, C. (1991) *Biochemistry 30*, 7603−7609.

Laurent, T. C., Moore, E. C., & Reichard, P. (1964) *J. Biol. Chem. 239*, 3436−3444.

Lim, W. A., & Sauer, R. T. (1989) *Nature 339*, 31−36.

Lim, W. A., Farrugio, D. C., & Sauer, R. T. (1992) *Biochemistry 31*, 4324−4333.

Matthews, B. W. (1987) *Biochemistry 26*, 6885−6887.

Reutimann, H., Straub, B., Luisi, P.-L., & Holmgren, A. (1981) *J. Biol. Chem. 256*, 6796−6803.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Santoro, M. M., & Bolen, D. W. (1992) *Biochemistry 31*, 4901−4907.

Shortle, D. (1989) *J. Biol. Chem. 264*, 5315−5318.

Stites, W. E., Gittis, A. G., Lattman, E. E., & Shortle, D. (1991) *J. Mol. Biol. 221*, 7−14.

Sturtevant, J. M. (1987) *Annu. Rev. Phys. Chem. 38*, 463−488.

Sturtevant, J. M. (1994) *Curr. Opin. Struct. Biol. 4*, 69−78.

Tanaka, A., Flanagan, J. J., & Sturtevant, J. M. (1993) *Protein Sci. 2*, 567−576.

Tanford, C. (1973) *The Hydrophobic Effect*, Wiley, New York.

Wynn, R., & Richards, F. M. (1993) *Protein Sci. 2*, 395−403.

BI942480A