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Thermodynamics of replacing an α-helical Pro residue in the P40S mutant of *Escherichia coli* thioredoxin

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Abstract

Escherichia coli thioredoxin is a 108 amino acid oxidoreductase and contains a single Met residue at position 37. The protein contains a long α-helical stretch between residues 32 and 49. The central residue of this helix, Pro40, has been replaced by Ser. The stabilities of the oxidized states of two proteins, the single mutant M37L and the double mutant M37L,P40S, have been characterized by differential scanning calorimetry (DSC) and also by a series of isothermal guanidine hydrochloride (GuHCl) melts in the temperature range of 277 to 333 K. The P40S mutation was found to stabilize the protein at all temperatures upto 340 K though both proteins had similar T_m values of about 356 K. At 298 K, the M37L,P40S mutant was found to be more stable than M37L by 1.5 kcal/mol. A combined analysis of GuHCl and calorimetric data was carried out to determine the enthalpy, entropy, and heat capacity change upon unfolding. At 298 K there was a large, stabilizing enthalpic effect in P40S though significant enthalpy-entropy compensation was observed and the two proteins had similar values of ΔC_p. Thus, replacement of a Pro in the interior of an α helix can have substantial effects on protein stability.

Keywords: proline mutant; stability; thioredoxin

The free energy difference (ΔG°) between the folded and unfolded states of a globular protein is a quantitative measure of protein stability. Typical values of ΔG° at room temperature and neutral pH range from 5–20 kcal mol^{-1}. A quantitative understanding of the various interactions that stabilize a folded protein relative to the unfolded state is essential in protein design and protein engineering efforts. An obvious approach to increase protein stability is to identify possible destabilizing interactions in a given protein structure and then make appropriate residue substitutions that replace such interactions by favorable ones.

Pro residues in proteins are generally not found in the hydrogen bonded, central regions of α-helices or β-strands (MacArthur & Thornton, 1991). In an α-helix the amide proton of residue i forms a hydrogen bond with the carbonyl group of residue $i - 4$. Pro residues lack an amide proton and hence cannot form such a hydrogen bond. However, it has recently been suggested (Chakrabarti & Chakrabarti, 1998) that Pro residues in helices can form C—H$\cdots$O hydrogen bonds. Most Pro residues in α-helices are found in the first turn of the helix. Pro residues have been introduced into helical regions of T4 lysozyme (Sauer et al., 1992; Gray et al., 1996) and have been shown to destabilize the protein by about 3–8 kcal/mol.

A recent study (Gunasekaran et al., 1998) of the occurrence of Pro residues in helices showed that 14% of the helices examined contained a Pro residue in the central region. In such helices, the bulky pyrrolidine ring also constrains the conformation of the residue preceding Pro and causes a kink in the helix of around 20–30° (Ramachandran et al., 1963). In the majority of these cases, Pro is found on the solvent exposed surface of the helix and it is thought that kinking of the helix may help in packing of the helix against the rest of the protein (Woolfson & Williams, 1990).

Several studies have clearly shown that substitution of a residue in the interior of a helix with a Pro is destabilizing (O'Neil & DeGrado, 1990; Strehlow et al., 1991; Sauer et al., 1992; Blaber et al., 1993). In contrast, there have been few studies that have examined the potential stabilizing effects of replacing Pro in a helix with other amino acids. Replacement of the Pro61 residue in the middle of a long helix in the *Escherichia coli* Fis protein with Ala and Ser resulted in significant increases in the T_m of the protein

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Abbreviations: CD, circular dichroism; DTT, dithiothreitol; DSC, differential scanning calorimetry; GuHCl, guanidine hydrochloride; PCR, polymerase chain reaction; trx, thioredoxin; wt*, single mutant (M37L) trx; P40S, double mutant (M37L, P40S) trx; UV, ultraviolet.

(Yuan et al., 1994). However, the crystal structure of the P61A mutant revealed that the helix was still kinked in the mutant protein, showing that factors other than the presence of Pro were responsible for kinking of the helix. In contrast, in T4 lysozyme (Alber et al., 1988) replacement of a helical residue, Pro86, with a variety of other residues did not affect the T_m of the protein. All thermodynamic studies of Pro replacements have so far focused only on changes in T_m and $\Delta G^\circ(T_m)$. To obtain further insight into the stability changes associated with such mutations, it is essential to also measure additional thermodynamic parameters such as $\Delta H(T_m)$ and ΔC_p, and to study the effects of the mutation on $\Delta G^\circ(T)$ over an extended temperature range instead of at a single temperature.

E. coli thioredoxin is a stable monomeric protein of 108 amino acid residues. It catalyzes reduction of disulfide bonds in the cytosol and performs a wide variety of biological functions. It folds reversibly, is small enough for NMR studies, and can be overexpressed in *E. coli* (Kelley & Stellwagen, 1984; Hellinga et al., 1992; Santoro & Bolen, 1992). The structure of the oxidized form of the wild-type protein has been solved to high resolution by X-ray crystallography (Katti et al., 1990). Thioredoxin contains a single Met residue at position 37 and five Pro residues at positions 34, 40, 64, 68, and 76. Of these only Pro76 is in the cis conformation. Pro40 occurs in the middle of a long α-helix that extends from residue 32 to 49. Pro40 is conserved in thioredoxins obtained from 17 different species, while Met37 is conserved in 11 species (Eklund et al., 1991). We have constructed the mutant M37L as a first step to introducing single Met residues at a variety of sites throughout the protein with the ultimate goal of cleaving such single Met containing mutants to generate a series of fragment complementation systems for *E. coli* thioredoxin (Ghoshal et al., 1999). In the present work, we have characterized the stability of the M37L single mutant (hereafter referred to as wt*) and the P40S double mutant (hereafter referred to as P40S) by carrying out DSC studies as well as GuHCl denaturation studies of the two proteins as a function of temperature. The P40S mutation was constructed to study the thermodynamics associated with replacement of Pro in the interior of a helix. Pro was replaced by Ser as these two residues have similar hydrophobicities (Rose et al., 1985).

Results and discussion

Both mutants have similar structure and activity to wild-type thioredoxin

Both the wt* single mutant and the P40S double mutant could be purified to homogeneity with yields of 70 and 100 mg/L of culture, respectively. The molecular weights of the two proteins determined by mass spectrometry were 11,653 and 11,643, respectively. These compare favorably with the calculated molecular weights of 11,657 and 11,647 for the two proteins. The insulin assay showed the wt* mutant to be 40% less active than wild-type. In contrast, both proteins showed similar activity in the thioredoxin reductase assay. The tertiary CD spectra for wild-type and both mutants were quite similar (data not shown) showing there were no substantial changes in tertiary structure introduced as a result of the mutations. We have also crystallized the P40S mutant and the structure is quite similar to that of the wild-type protein (R. Jain, S. Ramakumar & R. Varadarajan, unpubl. results).

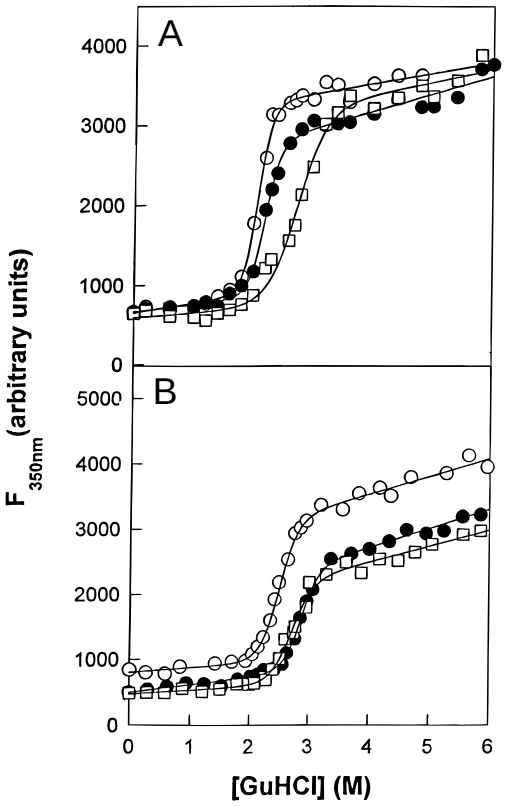

Fig. 1. Representative GuHCl-induced denaturation curves of oxidized states of (**A**) wt* at 278 K (○), 298 K (●), 313 K (□) and (**B**) P40S at 278 K (○), 298 K (●) and 313 K (□). The unfolding transition was monitored by the change in fluorescence emission at 350 nm with excitation at 295 nm.

GuHCl denaturation studies

Both mutants were reversibly denatured by GuHCl. The data fit well to a two state model as previously shown for the wild-type protein (Kelley & Stellwagen, 1984; Wilson et al., 1986). A series of isothermal GuHCl-induced denaturation curves in the temperature range 278 to 338 K were obtained for oxidized wt* and for oxidized P40S. A few representative curves are shown in Figures 1A and 1B, respectively, and the thermodynamic parameters derived from the data are in listed in Tables 1 and 2. Denaturation was also monitored at 298 K using far-UV CD instead of fluorescence. The transitions observed using CD and fluorescence were superimposable (data not shown). Data were analyzed according to the linear free energy model (Schellman, 1987; Pace & Laurents, 1989) essentially as described previously (Sheshadri et al., 1999). The values of ΔG°, m, and C_m at each temperature for both proteins are presented in Table 1. At each temperature, the data in the transition zone were analyzed to obtain the value of ΔG_D as a function of denaturant concentration and temperature in the transition zone. Each stability curve was used to obtain values of T_m, $\Delta H_D(T_m)$, and ΔC_{pD}. These values of $\Delta H_D(T_m)$ and ΔC_{pD} were used to calculate ΔH_D at all temperatures used for the isothermal GuHCl denaturation experiments (Table 1). The calculated values of ΔH_D at each temperature were fit to Equation 1 obtain ΔH° and ΔH_i as a function of temperature. Values of ΔH_i thus obtained were used to obtain ΔC_{pi}. Values of ΔC_{pD} as a function of denaturant concentration were also fit to Equation 1 to obtain alternate estimates of ΔC_p and ΔC_{pi} (Table 2).

Table 1. *Parameters obtained from isothermal GuHCl denaturation studies as a function of temperature*

P40S(Ox)				wt*			
T (K)	ΔG° (kcal mol^{-1})	$-m$ (kcal mol^{-1}M^{-1})	C_m (M)	T (K)	ΔG° (kcal mol^{-1})	$-m$ (kcal mol^{-1} M^{-1})	C_m (M)
278	8.6 ±.0.8	3.5 ± 0.3	2.47	277	8 ± 0.9	3.8 ± 0.4	2.09
283	9.2 ± 0.6	3.4 ± 0.2	2.71	279	8 ± 1.3	3.9 ± 0.6	2.11
288	7.3 ± 1.1	2.6 ± 0.4	2.76	281	6.2 ± 1.1	2.9 ± 0.5	2.15
293	10.4 ± 0.8	3.6 ± 0.3	2.85	283	5.9 ± 0.5	2.4 ± 0.2	2.47
298	11.5 ± 1.1	4.0 ±.4.0	2.87	288	8.0 ± 0.9	3.4 ± 0.4	2.34
303	10.1 ± 0.7	3.6 ± 0.2	2.84	298	9.1 ± 1.3	3.7 ± 0.7	2.58
310	8.1 ± 0.8	3.0 ± 0.3	2.73	313	6.1 ± 1.2	2.2 ± 0.4	2.74
313	9.4 ± 1.3	3.4 ± 0.5	2.71	318	7.6 ± 1.1	3.1 ± 0.5	2.45
318	8.6 ± 1.7	3.4 ± 0.7	2.53	328	5.3 ± 0.8	2.1 ± 0.4	2.29
323	5.0 ± 1.5	2.1 ± 0.6	2.41	333	2.9 ± 0.8	2.0 ± 0.5	1.40
328	6.6 ± 2.3	3.1 ± 1.0	2.10				
333	3.4 ± 0.8	1.6 ± 0.3	2.10				
338	2.2 ± 1.0	1.5 ± 0.4	1.44				

Calorimetric studies of oxidized states of wt and P40S*

DSC scans were carried out as a function of protein concentration. The temperature at which the excess heat capacity was maximal decreased only slightly at higher concentrations. The data were therefore fit to a simple two state model. The values of T_m, $\Delta H(T_m)$, and ΔC_p obtained for the various scans are presented in Table 3. The average values of ΔC_p are 2.5 and 2.6 kcal mol^{-1} K^{-1} for wt* and P40S, respectively. However, values of ΔC_p obtained from the DSC scans are very sensitive to the choice of baseline parameters. A more reliable estimate of ΔC_p was obtained by carrying out a joint fit of calorimetric and GuHCl denaturation data (see below). The calorimetric data show that both proteins have similar values of T_m and $\Delta H(T_m)$.

Joint fit of calorimetric and GuHCl data

The data from the calorimetric scans were integrated as described (Kirchoff, 1993) to obtain the value of ΔG° as a function of temperature in the transition zone (temperature range 350–363 K for wt* and 351–361 K for P40S). Values of ΔG° at different temperatures in the transition zone were obtained for each calorimetric scan listed in Table 3. At each temperature the average value and standard deviation of ΔG° were calculated for both wt* and P40S. These values were combined with values of ΔG° derived from isothermal GuHCl denaturation studies and listed in Table 1. The combined set of ΔG° values was fit to Equation 2 (Fig. 2) with T_o = 298 K to obtain values of $\Delta G^\circ(298)$, $\Delta H^\circ(298)$, and ΔC_p listed in Table 2. The values of ΔC_p are similar to that obtained previously for the wild-type protein (Santoro & Bolen, 1992).

Comparison of thermodynamic parameters for oxidized wt and P40S*

The thermodynamic data summarized in Table 1 and Figure 2 clearly demonstrate that the P40S mutation results in an increase in ΔG_D at temperatures below 340 K. The m value for both proteins is similar and relatively independent of temperature over a large range of temperature (Table 1). Hence, the increased C_m value of P40S relative to that of wt* is an indication of the increased stability of the former protein.

The stabilization due to the P40S substitution at 298 K results primarily from a large increase (16.3 kcal mol^{-1}) in the value of

Table 2. *Thermodynamic parameters derived from joint analysis of GuHCl denaturation and DSC studies*

	P40S	wt*
$\Delta G^{\circ a}$ (kcal mol^{-1})	9.3 ± 0.3	7.8 ± 0.2
$\Delta H^{\circ a}$ (kcal mol^{-1})	20.0 ± 4.2	3.7 ± 0.4
$\Delta C_p{}^a$ (kcal mol^{-1} K^{-1})	1.4 ± 0.25	1.6 ± 0.25
$\Delta C_p{}^b$ (kcal mol^{-1} K^{-1})	1.8 ± 0.6	2.1 ± 0.3
$\Delta C_{pi}{}^b$ (kcal mol^{-1} K^{-1})	−0.005 ± 0.2	0.008 ± 0.14
$\Delta S^{\circ c}$ (cal mol^{-1} K^{-1})	36 ± 13	−14 ± 1

[a] From ΔG° vs. T plot (Fig. 2).
[b] From Equation 1.
[c] Using equation $\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T$.

Table 3. *Thermodynamic parameters for the thermal unfolding of oxidized wt* and P40S obtained from DSC*

[Trx] (mM)	T_m (°C)	ΔH° (kcal mol^{-1})	ΔC_p (kcal mol^{-1} deg^{-1})
wt*			
0.090	84.1 ± 0.7	88.6 ± 2.5	2.47 ± 1.3
0.206	83.6 ± 0.2	101.0 ± 1.0	2.87 ± 0.6
0.360	83.7 ± 0.2	105.0 ± 0.8	2.08 ± 0.5
P40S			
0.080	83.7 ± 0.5	96.4 ± 2.1	2.45 ± 1.0
0.112	83.6 ± 0.7	109.0 ± 3.1	2.23 ± 2.1
0.183	82.4 ± 0.5	103.0 ± 2.1	2.38 ± 1.2
0.238	81.9 ± 0.5	109.0 ± 3.7	4.04 ± 1.7

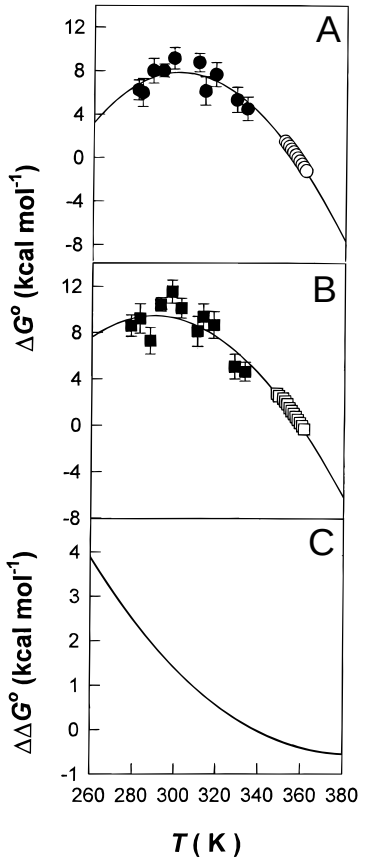

Fig. 2. Temperature dependence of ΔG° for oxidized states of (**A**) wt* and (**B**) P40S. The values of ΔG° were obtained from individual isothermal guanidine hydrochloride denaturation curves at different temperatures for wt* (●) and P40S (■), and DSC scans for wt* (○) and P40S (□). The error bars for DSC data lie within the size of the data points. The solid line is a fit of the data to Equation 2. The temperature dependence of $\Delta\Delta G^\circ$ is shown in **C**.

ΔH°. The large increase in ΔH° is offset by a concomitant increase in the value of ΔS. The increased enthalpic stabilization is probably due to the additional hydrogen bonds resulting from the substitution. There are no major rearrangements upon substitution, and the main-chain amide nitrogen of Ser is appropriately placed to form a hydrogen bond with the carbonyl groups of residues 36 and 37 (R. Jain, S. Ramakumar, R. Varadarajan, unpubl. results). The carbonyl of residue 37 does not form a hydrogen bond in the wild-type protein. The P40S mutation is also expected to result in an increase in the conformational entropy of the unfolded state (Reidhaar-Olson et al., 1990). Hence, the signs of changes in both ΔH° and ΔS are as expected. Large entropy–enthalpy compensations (as observed here) are often taken to imply that solvent contributions are important. In the present instance, there is no evidence to suggest this is the case though the magnitude of the enthalpy–entropy compensation observed is surprisingly large. The favorable enthalpic effect of additional hydrogen bonding in the folded state of P40S relative to wt* is sufficient to compensate for the unfavorable increase in the entropy of the unfolded state at room temperature. The value of ΔC_p for wt* appears to be marginally higher than that of P40S. At high temperatures (greater than 340 K), the effects of the $T\Delta S$ term dominate and consequently both wt* and P40S have similar values of T_m. The experiments also demonstrate that T_m alone is not an appropriate quantity for characterizing mutational effects on protein stability. The introduction of Pro residues into an α-helix has been shown in a number of studies to lead to substantial destabilization of the protein by as much as about 10 kcal/mol (Gray et al., 1996). The converse experiment of replacing an α helical Pro residue in *E. coli* trx results in a smaller stabilization of 1.5 kcal/mol at 298 K. The difference between the numbers may be rationalized as follows. Introduction of Pro into the interior of an α helix leads to both loss of at least one hydrogen bond and to unfavorable steric interactions that in turn lead to kinking of the helix. In contrast, substitution of an existing α-helical Pro with another residue can lead to formation of an additional hydrogen bond. However, since the helix is already kinked and the remaining protein structure has adapted to the kink, there is unlikely to be any energetic benefit obtained by removal of the kink.

The present study shows that replacement of a Pro residue in the middle of an α-helix by Ser can lead to an increase in protein stability. Such an increase in stability was also observed in similar Pro replacements in the *E. coli* fis protein suggesting that replacements of such helical Pro residues might serve as general method of stabilizing proteins. An attractive feature of such an approach is that the increase in stability is expected to result primarily from effects of the substitution on secondary rather than on tertiary structure. As a caveat to this, no increase in thermal stability was observed when Pro was replaced with a variety of other amino acids in an α-helix of T4 lysozyme (Alber et al., 1988). However, as seen above, thermal stability is not necessarily a good indicator of changes in ΔG° at physiological temperatures in proteins. It should be noted that the vast majority of single amino acid substitutions has either no or destabilizing effects (Richards & Lim, 1993). Hence, it is encouraging that at least in two out of three proteins studied so far, α-helical Pro residue replacements result in increased stability.

Materials and methods

Mutagenesis, expression, and protein purification

Site-directed mutagenesis of the *trx* gene in pBS-SK was carried out using a PCR-based strategy (Ho et al., 1989). The identities of the single mutant wt* and the double mutant P40S were confirmed by sequencing the entire *trx* coding region. The two mutants were amplified by PCR, subcloned into the vector pET20b and expressed under control of the *T7* promoter in strain BL21(DE3) (Studier & Moffatt, 1986). Protein purification and activity assays were carried out as described previously (Ghoshal et al., 1999).

GuHCl-induced denaturation

Denaturation studies and data analysis were carried out in 0–6 M GuHCl (ultra pure grade from U.S. Biochemical, Cleveland, Ohio) in 50 mM phosphate buffer (pH 7) as described previously (Sheshadri et al., 1999). In accordance with the linear free energy model (Schellman, 1987), it was assumed that values of the unfolding free energy (ΔG_D), enthalpy (ΔH_D), entropy (ΔS_D), and heat capacity (ΔC_{pD}) depend linearly on the denaturant concentration. It was also assumed that ΔC_p and ΔC_{pD} are independent of temperature. Hence,

$$\Delta X_D = \Delta X^\circ + \Delta X_i[D] \quad (1)$$

$$\Delta G^\circ(T) = \Delta H^\circ(T_0) - T\Delta S^\circ(T_0) + \Delta C_p(T - T_0 - T\ln(T/T_0)) \quad (2)$$

where ΔX° ($X = G, H, S, C_p$) is the value of the thermodynamic parameter X at zero molar denaturant, ΔX_i are the changes in X associated with preferential interaction of the denaturant with the denatured state relative to the native state of the protein, and T_0 is some reference temperature. All data were analyzed using Sigmaplot™ for Windows™ scientific graphing software.

Differential scanning calorimetry

DSC scans of the change in excess heat capacity of thioredoxin as a function of temperature were measured using the MC-2 ultrasensitive scanning calorimeter. DSC measurements were carried out as a function of protein concentration at pH 7.0 in 50 mM phosphate buffer using a scan rate of 60 °C/h as described previously (Ladbury et al., 1993). DSC data were fit to a two state model using the Origin™ DSC software provided by Microcal Inc.

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