A single point mutation (Glu85Arg) increases the stability of the thioredoxin from *Escherichia coli*

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Introduction

Although structural determinants of protein stability have been the object of numerous studies on model proteins, no universal stabilization mechanism has yet been found (Jaenicke, 1998). To identify better the structural determinants of protein stability, thioredoxin (Trx) was chosen as a model system. Trx, a small (12 kDa) protein found in all living cells from Archaea to humans (Eklund et al., 1991), is a highly structured molecule with 90% of its residues involved in secondary structural elements. Trx has a central core of five strands of β-sheet enclosed by four α-helices with the active-site disulphide (Cys32–Cys35) localized in a short loop at the N-terminal of the α2-helix. The physicochemical features of Trx from *Bacillus acidocaldarius* (BacTrx) and its primary structure have already been reported (Bartolucci et al., 1997). An analysis of the structural stability of BacTrx using circular dichroism (CD), differential scanning calorimetry and nanogravimetry showed that it is endowed with a higher conformational heat capacity than Trx from *Escherichia coli*, which shows 49% sequence identity. Molecular dynamic (MD) simulations on *E. coli* Trx and BacTrx, carried out in vacuo and in water solution at different temperatures (Pedone et al., 1998), showed that the residue replacements observed in BacTrx gave rise to networks of H-bonds, thus making it more stable than *E. coli* Trx (Frankenberg et al., 1999). It also revealed high preservation levels of the hydrophobic core as well as the structural importance of the C-terminal tail. To gain more insight into the structural features responsible for the thermodynamic stability of BacTrx, four mutants were designed and produced using site-directed mutagenesis (Pedone et al., 1999). Lys18 and Arg82, which seem to be involved in stabilizing hydrogen bonds and ion pairs, were substituted with the corresponding residues (glycine and glutamate, respectively) present in the less stable *E. coli* Trx. Following the deletion of the last four amino acids from Asp102, investigations were then carried out on the structural role of the C-terminal helix, which is anchored by many salt bridges to the rest of the BacTrx core. The effects of the substitutions on the stability were monitored by a wide array of techniques showing that the mutants were significantly less stable ($T_m <12–15^\circ$C).

Recently, the NMR solution structure of BacTrx was reported (Nicastro et al., 2000), confirming that the increased thermostability of BacTrx is achieved by various improvements at many locations within the proteins. The structure analysis revealed the importance of electrostatic interactions for obtaining a more rigid structure, in agreement with simulation data. In addition, the NMR study confirmed the MD predictions on the structural features for the decreased stability of the four designed mutants. In particular, for the Arg82Glu mutant, the destabilizing effect stemmed from the lack of the Arg82 side-chain H-bonds, also supported by the slow exchange data of the side-chain NHs.

With a view to confirming the ability of MD to direct protein engineering to ‘increase’ the stability of proteins also, in this study we mutagenized the less stable *E. coli* Trx by substituting the residue Glu85 with the arginine present in the corresponding thermophilic BacTrx (Arg82), thus producing the mutant E85R *E. coli* Trx that was expressed at a high level; the physicochemical behaviour was compared by means of MD simulations, CD, spectrofluorimetry and limited proteolysis with the *E. coli* Trx. In line with our expectations, the single point mutation E85R led to an increase in thermostability compared with the wild-type Trx, in addition to a more rigid structure and some local conformational constraints.

Materials and methods

**Molecular dynamic simulations**

All calculations and graphical analyses were run on a Silicon Graphics Indigo2 workstation. The InsightII/Discover program (Biosym Technologies, 1998) was used to build the preliminary structure and to perform energy minimizations and MD simulations in vacuo, at pH 7.0, using the AMBER force field (Weiner et al., 1986). In all simulations, the Arg, Glu, Gln, His, Lys, Asp and Asn side chains carry a charge related to the pH value. The E85R *E. coli* Trx mutant was built using the coordinates of the *E. coli* Trx X-ray structure at 1.6 Å resolution (Eklund et al., 1998), obtained from the Protein Data Bank (Bernstein et al., 1977) (1TRX) (Brookhaven National Laboratory, Upton, NY) and replacing the glutamate residue in position 85 with an arginine residue. Energy minimizations were carried out using the conjugate gradient algorithm on the E85R mutant. These procedures were stopped when the maximum derivative was $<0.001$ kcal/mol.

The energy-minimized structure was used as the initial structure for the MD simulations in vacuo at 500 K. The computational conditions were chosen to avoid boundary
effects (Saviano et al., 1991) according to the procedures applied to E.coli Trx (Pedone et al., 1998).

**Construction of E.coli Trx and E85R mutant**

Based on the E.coli Trx amino acid sequence from residues 1–8 and that from residues 103–108, the oligonucleotides were designed (Pedone et al., 1998) and used as primers in the PCR gene amplification procedure, using the chromosomal DNA (100 ng) as template. The amplification was performed according to Saiki (Saiki, 1990), on a Perkin-Elmer Cetus Cycler Temp using Pfu polymerase (Stratagene). The amplified DNA fragment (E.coli Trx), opportunistically digested, was inserted into the pTrc99A plasmid. The recombinant clone, designated pEcTrx, represented the expression vector.

The mutation Glu85Arg (E85R) was introduced in the E.coli Trx DNA following the methodology of Kunkel (Kunkel, 1987). The insertion of the correct mutation was confirmed by DNA sequencing with Sanger’s dideoxy method, with a Sequenase Sequencing Kit from Amersham (Sanger et al., 1977).

**Expression and purification of recombinant thioredoxins**

E.coli Rb791 (AMS Biotechnology) competent cells were transformed with pEcTrx and competent E.coli BL21DE3 cells (AMS Biotechnology) were transformed with pEcTrxE85R and grown at 37°C to different densities in 500 ml of Luria–Bertani medium. E.coli Rb791 and BL21DE3 cells transformed with pTrc99A (Pharmacia) represented a negative control. The optimized overexpression of both the proteins was obtained by exposing the cells to 1 mM isopropyl β-d-thiogalactoside (IPTG) at a cell density of OD_{600} nm = 1 for 20 h. Cell pellets from 500 ml cultures were re-suspended in 5 ml of 10 mM Tris–HCl pH 8.4 and crude extracts were prepared by disrupting the cells with 20 min pulses at 20 Hz (Sonicator Ultrasonic liquid processor; Heat System Ultrasonics) and ultracentrifugation at 160 000 g for 30 min. The purification of the recombinant wild-type protein and its mutant was carried out in a similar way. The crude extracts were subjected to different heat treatments depending on the protein and then centrifuged at 5000 g at 4°C for 15 min, at 60°C for 20 min for wild-type Trx and at 70°C for 20 min for E85R, removing almost 30% of the mesophilic host proteins. The supernatants were extensively dialysed against 10 mM Tris–HCl pH 8.4 and then applied to a 2.6×60 cm column (HiLoad Superdex 75; Pharmacia) connected to an FPLC system (Pharmacia) and eluted with 10 mM Tris–HCl pH 8.4, 0.2 M NaCl at a flow-rate of 2 ml/min. The active fractions were pooled and concentrated; only one peak was observed on reversed-phase high-performance liquid chromatography (RP-HPLC) and only one protein band was revealed by SDS–PAGE.

**Analytical methods for proteins**

Protein concentration was determined (Smith et al., 1995) using BSA as the standard. Thioredoxin activity was assessed according to Holmgren’s method (Holmgren, 1979). Protein homogeneity was estimated using SDS–PAGE 15% (w/v) gels with the silver staining procedure of Rabilloud et al. (Rabilloud et al., 1994). In addition, the proteins were analysed using a non-denaturing electrophoresis 15% (w/v) polyacrylamide slab gel.

The molecular mass of the proteins was estimated using electrospray mass spectrometry (ESMS) with a Bio-Q triple-quadrupole instrument (Micromass) (Pedone et al., 1999).

**CD studies**

CD spectra were recorded on a JASCO J-710 spectropolarimeter equipped with a Peltier thermostatic cell holder (model PTC-343; JASCO) in 10 mM sodium phosphate pH 7.0, under the conditions described previously (Pedone et al., 1999).

**Denaturation/renaturation studies**

Thermal denaturation of each protein was monitored by following the change in dichroism at 223 nm and recording spectra at 185–260 nm under the conditions described previously (Pedone et al., 1999). The reversibility of the transition was then checked by lowering the temperature.

The temperature of the midpoints of the transitions, T_m, for each protein was determined from curve fitting of the f (θ = θ_{obs} − θ_{nat}/θ_{nat} − θ_{den}) against temperature plots. The results were processed using the Curve Fit program for IBM computers.

**Fluorescence measurements**

Fluorescence spectra were recorded with a thermostatically controlled Perkin-Elmer LS-50B spectrophotofluorimeter; samples were excited at 295 nm and the emission spectra were recorded from 310 to 410 nm every 10°C from 25 to 95°C. The proteins (40 μg/ml) were dissolved in 10 mM sodium phosphate pH 7.0.

**Limited proteolysis experiments**

Limited proteolysis experiments were conducted by incubating E.coli Trx and E85R with subtilisin (Sigma). Enzymatic digestion was performed in 50 mM sodium phosphate pH 7.5 at 37°C. The final substrate concentration was 0.25 mg/ml and the reaction mixture was analysed at different reaction times ranging from 1 to 24 h. Proteolytically digested samples were fractionated by RP-HPLC on a Vydac C18 column; peptides were eluted by a linear gradient of 5–75% acetonitrile in TFA (0.1%) over 35 min; elution was monitored at 220 nm. Individual fractions were collected and proteolytic fragments were analysed by ESMS using a VG BIO-Q triple quadropole instrument equipped with an electrospary ion source (Pedone et al., 1999) or by matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS) using a Voyager DE instrument (Perkin-Elmer). A 1 μl volume of analyte solution was mixed with 1 μl of α-cyano-4-hydroxycinnamic acid, 10 mg/ml in acetonitrile–TFA (0.2%), 70:30 (v/v), containing 250 fmol of bovine insulin. The mixture was applied to the metallic sample plate and air dried. Mass calibration was performed with the mass signals of insulin at m/z 5734.5 and a matrix peak at m/z 379.1. Raw data were analysed using a computer program provided by the manufacturer; all mass values are reported as average masses.

An SDS–PAGE analysis at 15% was performed to follow the degradation of the intact protein over 24 h of incubation. A densitometric analysis was conducted on the gel using a Bio-Rad GS710 calibrated imaging densitometer.

**Results**

**Choice of mutation**

In a previous paper (Pedone et al., 1999), the Arg82 of BacTrx was substituted with the corresponding residue, a glutamate (Glu85) present in the less stable E.coli Trx, using the data on the thermal mobility of the two proteins at high temperature. The comparative analysis of the trajectories during the simulations at 300 K revealed that in the thermophilic protein the arginine was able to have a high occurrence of H-bonds and ion pairs, given that the two NHs of Arg82 were H-bonded to
the CO Gln15, to the CO Gly16 and to Oδ Asp17 groups. In contrast, at high temperatures, the mutant R82E presented the Glu82 hydrogen bonded with high occurrence levels only to Lys85. Using spectroscopic and thermodynamic approaches, we demonstrated that the Glu82 mutant was 12°C less stable than the wild-type BacTrx. To demonstrate that MD simulation at different temperatures can also predict how to stabilize the mesophilic E.coli Trx, we designed a mutant, E85R, by substituting the glutamate present in E.coli Trx with the corresponding arginine residue present in BacTrx.

**MD analysis**

To understand better the dynamic behaviour of E85R compared with the wild-type protein, we used MD simulation in vacuo at 500 K. In order to gain some insight into the structural determinant of the different stability, it is necessary to compare the behaviour of the two proteins at high temperature given that the solid-state (Katti et al., 1990) and NMR (Jeng et al., 1994) structures of E.coli Trx were not solved at high temperature, only molecular dynamics techniques can be used to explain the stability difference in E.coli Trx and its mutant. Figure 1 reports the mean square displacement of and the distance travelled by the set of atoms for the wild-type and for the E85R mutant at 500 K expressed as a function of time. The analysis of the mean square displacement reveals that in E85R, a decrease in mobility can be observed in the β4 and β5 secondary structures and in the loop between these secondary structures. In addition, the mutant presents a decrease in flexibility in the C-terminal tail. This feature underlines the importance of the C-terminal α4 helix in the stability of this class of proteins and in the protection of their hydrophobic core. The two proteins show similar thermal behaviour around the active site (17–21 region), indicating that the substitution in position 85 should not reduce the activity of the Trx mutant.

The analysis of the average structures at 500 K in vacuo clarifies the structural basis of the stability. Indeed, the NHs of the Arg85 side chain act as H-bond donors with the oxygens of the terminal carboxylic group (Figure 2). The simulations show the strong influence of hydrogen bond and electrostatic interactions in stabilizing the β-strand core. Indeed, the additional H-bonds between the β5 and the α4 regions found in the E85R mutant give rise to a more compact and less flexible structure. The comparison of the solid-state structure of E.coli Trx and the average structures from MD simulations at 500 K underlines that, at high temperature, there is a distortion in the structures, as expected, due to the denaturation effect of temperature. This distortion gives rise to the occurrence of salt bridges and H-bond interactions not present in the solid-state structure involving the Arg85 residue linking the β5 and the α4 regions.

**Production and characterization of wild-type E.coli Trx and the mutant E85R**

The protein yields from 500 ml of cultures were 35 mg for E.coli Trx and ~20 mg for E85R. An analysis of native PAGE revealed only one band with different electrophoretic mobility for the mutant compared with the E.coli Trx according to the ionic nature of the substituton introduced (Figure 3).

The molecular mass of the proteins was analysed by ESMS. The measured mass of the recombinant E.coli Trx was 11 674.11 ± 0.74 Da, (calculated mass 11 675.4 Da); the measured mass of E85R was 11 700.3 ± 0.7 (calculated mass 11 688.0), thus confirming that the mutant protein contained only the expected modification. In addition, these data demonstrated that the purified E85R preparation was no longer contaminated by the wild-type protein.

The E.coli Trx and the mutant E85R both proved to be fully active and the substitution was not disruptive. Moreover, the increased temperature (70°C instead of 60°C) for the thermal precipitation used for E85R purification was already a preliminary demonstration of a different thermostability.

**Denaturation of wild-type and mutant thioredoxins**

In order to compare the stabilities of the mutant and wild-type proteins in terms of thermal denaturation, CD spectra in the 185–260 nm region were recorded and the magnitude of the CD band at 223 nm was followed at increasing temperature from 25 to 98°C. Positive increases in the rotation values at 223 nm are assumed to be a measure of the decrease in the α-helical content of a polypeptide. The reversibility of the transition was then checked by lowering the temperature.

The denaturation curves of E.coli Trx and E85R, obtained by plotting the percentage of fraction unfolded against temperature, are shown in Figure 4. The E.coli mutant E85R was less stable than the wild-type, with a Tm of 90°C compared with 85°C; this value was in line with that obtained by differential scanning calorimetry (Bartolucci et al., 1997). Figure 4 reports the fusion curves of the thermophilic BacTrx and its mutant R82E (Bartolucci et al., 1997). Clearly, the thermophilic BacTrx is still the most resistant, with a Tm of 103°C, whereas a progressive decrease in stability for the other Trxs is observed. As predicted, the two mutants are stabilized (E85R) or destabilized (R82E) by the substitution.

All proteins exhibited reversible denaturation and activity was completely restored when tested using the Holmgren assay.

The fluorescence emission spectra of E.coli Trx and its mutant at neutral pH and 25°C revealed a single maximum at 347 nm. When the temperature was increased, a change in tryptophanyl fluorescence intensity and a red shift from 347 to 356 nm of the emission maximum were observed, indicating the unfolding of the proteins. The effect of increasing temperature on the tryptophanyl fluorescence intensity at 350 nm is shown in Figure 5. A conformational change is observed for E85R at about 90 and 85°C for the wild-type E.coli Trx.

**Topological studies of E.coli Trx and E85R**

Limited proteolysis experiments were carried out to determine protease-accessible sites on the protein surface and to probe the tertiary structure of the two proteins (Caporale et al., 1999). A 1:10 protease:substrate ratio was used in all cases, as preliminary results had shown that both proteins were very resistant to proteolytic attack. Figure 6 shows the results after
Fig. 2. Stereodrawing of the β5-α4 region of *E.coli* Trx (A) and E85R (B) as obtained from MD simulation in vacuo at 500 K. The H-bond interactions are indicated as dashed lines.

Fig. 3. Native PAGE analysis of *E.coli* Trx and E85R after the purification procedure. Lanes: A, *E.coli* Trx; B, E85R.

6 and 24 h of incubation with subtilisin. As expected, most of the Trxs (peak T) remained undigested and only a few fragments were detected in the chromatograms. Peptides were analysed by ESMS and located within the protein sequences on the basis of their molecular mass. Fraction 1 yielded molecular masses of 769.9 and 824.9 Da, which were identified as peptides 72–78 and 71–78, respectively, generated by a proteolytic cleavage at Gly71 and Tyr70. Fraction 2 yielded a molecular mass of 1301.7 Da and was identified as peptide 62–73 generated by a cleavage at Asp61. The mass value of fraction 3 was measured as 915.4 Da, corresponding to the peptide 8–15 generated by a cleavage occurring at Leu7 and observable only after 24 h of incubation, which can only be explained by a previous cleavage at Gly71 and Tyr70. Fraction 4 yielded a molecular mass of 1318.1 Da, generated by a proteolytic cleavage at Lys96. Fraction 5 represented an amount of undigested protein with one oxidized methionine. The data from the limited proteolysis experiments were summarized by identifying primary sites (Gly71, Tyr70, Asp61, Lys96) and secondary sites (Leu7). Compared with the wild-type, E85R shows similar proteolytic sites but slower hydrolysis kinetics, as expected. Indeed, after 6 h of incubation, the amount of E85R decreases by 20%, compared with 30% for *E.coli* Trx and after 24 h the amount of intact protein decreased by 60% for the mutant, compared with 70% for the wild-type, as measured by densitometric analysis.

After 24 h of incubation, it is possible to observe the disappearance of some peaks in E85R as a consequence of a sub-digestion of the pre-existing peptides owing to their slow
Stability of thioredoxin from E. coli hydrolysis kinetics. These observations can be explained by the less flexible structure of E85R compared with that of E.coli Trx.

**Discussion**

One of the major goals of current protein engineering studies is to design protein mutants with improved enzyme properties, such as resistance to heat, organic solvents, proteolytic enzymes, etc. Knowledge of structure–function–stability relationships in thermophilic enzymes is thus of great help in devising strategies for improving mesophilic enzymes currently used in biotechnology. In our previous work the mutant R82E of BacTrx proved to be particularly interesting as it became highly destabilized upon the substitution of Arg82, involved in numerous H-bonds and electrostatic interactions, with a glutamate residue present in E.coli Trx. In the present work, on the basis of molecular dynamic simulations, we substituted the Glu85 residue of E.coli Trx with the corresponding residue (Arg) present in the more stable BacTrx. The predictions were in total agreement with the experimental data, obtaining a more stable mutant. The results obtained by comparing the wild-type and the mutant E85R clearly confirm the important role both of the substitution in position 85 and of the C-terminal α-helix region in stabilizing the whole structure of E.coli Trx as also demonstrated in BacTrx.

In a previous paper (Pedone et al., 1999), structural investigation using an extrinsic fluorophore, Nile Red, detected differing exposures of hydrophobic areas on protein surfaces among BacTrx and its mutants. In this study, spectrofluorimetric analysis using the intrinsic fluorophore tryptophan revealed conformational changes, detected by an increase in emission intensity and a red shift from 347 to 356 nm, at different temperatures for E.coli Trx and E85R.

Limited proteolysis also was used to reveal structural differences between E.coli Trx and E85R. Indeed, the native conformation of proteins provides some stereochemical barriers to the accessibility of specific regions, which can be correlated with stability.
to enzymatic attack, leaving the exposed and flexible regions accessible to proteases and preventing the occurrence of proteolytic cleavages within the highly structured core of the protein molecule or at least slowing their kinetics. Preferential cleavage sites were classified as primary and secondary sites based on qualitative kinetic evaluation according to their rate of appearance during the time-course experiments. All the primary sites detected are located within the helix 3_10, indicating that in E.coli Trx this region is endowed with considerable conformational flexibility, whereas in E85R it adopts a more rigid and compact conformation, showing an increasing resis-
tance to proteolytic cleavages. Leu7 may be classified as a secondary site showing much slower hydrolysis kinetics since it is detected only at a later incubation stage (24 h), indicating that the N-terminal portion is poorly exposed and accessible only after the primary cleavage sites.

These results indicate that there is an inverse correlation between the flexibility and stability of protein molecules and that H-bonds and ion pairs play an important role in stabilizing proteins. It is also interesting to note how only one mutation, Glu85 in E.coli Trx replaced by an arginine, can increase the $T_m$ by 5°C, whereas the opposite modification, Arg82 in BacTrx replaced by a glutamate, gives rise to high destabilization and lowers the $T_m$ by 12°C, even though CD spectra indicate no changes in the overall conformation among the molecules.

It is worth noting that the simulations at high temperature revealed a distortion in the structures and that the Arg85 residue was involved in preventing ‘fraying’ of the C-terminus. These details cannot be obtained from solid-state and NMR structures not resolved at high temperature. This underlines the importance of the use of computational approaches to clarify the behaviour of proteins near denaturation.

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