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Biophysical Chemistry 115 (2005) 105–107

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

The effect of charge-introduction mutations on *E. coli* thioredoxin stability

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Received 21 June 2004; received in revised form 29 October 2004; accepted 10 December 2004
Available online 23 December 2004

Abstract

Technological applications of proteins are often hampered by their low-stability and, consequently, the development of procedures for protein stabilization is of considerable biotechnological interest. Here, we use simple electrostatics to determine positions in *E. coli* thioredoxin at which mutations that introduce new charged residues are expected to lead to stability enhancement. We also obtain the corresponding mutants and characterize their stability using differential scanning calorimetry. The results are interpreted in terms of the accessibility in the native structure of the mutated residues and the potential effect of the mutations on the residual structure of the denatured state.

Keywords: Protein stability; Mutation effect; Electrostatics

1. Introduction

Practical and technological applications of proteins are very often limited by their low-stability. Thus, proteins may denature when exposed to the comparative harsh conditions (relatively high temperature, for instance) under which some processes must be carried out. In addition, low stability may cause the gradual loss of function during storage, a fact that may limit the shelf-life of, for instance, protein pharmaceuticals. Clearly, the development of approaches to enhance protein stability is a major biotechnological goal. It has become apparent in recent years (see Ref. [1] for a review) that proteins may be stabilized trough the optimization of interactions between charged groups on the protein surface and, furthermore, that such optimized interactions may provide structural determinants for the enhanced stability of proteins from thermophilic organisms [1]. However, most of the experimental studies along this line have relied on the detection (using simple electrostatic methods) of charged residues involved in destabilizing charge–charge interactions, followed by the introduction of charge-deletion or charge-reversal mutations in those positions. Although this approach has been shown to be reasonable successful [1–5], it appears that most charged groups in proteins are arranged in such a way that charge–charge interactions are favorable and, therefore, stabilization via removal of unfavorable interactions is probably of limited applicability. It is likely that the achievement of large stabilizations trough optimization of charge interactions will have to rely upon the creation of additional favorable charge–charge interactions via the introduction of new charged groups at adequate positions. Here we explore the effect of new positively charged groups on protein stability using *E. coli.* thioredoxin as a model system.

2. Materials and methods

2.1. Site-directed mutagenesis

Oligonucleotides used for mutagenesis were obtained from Genotek. Mutations in the codons corresponding to positions 14, 59 and 63 in the amino acid sequence of thioredoxin were introduced by the QuikChange™ Site-Directed Mutagenesis method developed by Stratagene®.

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doi:10.1016/j.bpc.2004.12.012

Briefly, the QuikChange method is based on a PCR amplification using two complementary oligonucleotide primers containing the desired mutation. The parental nonmutated DNA is finally digested by an endonuclease. Mutation was verified by DNA sequence analysis.

2.2. Protein expression and purification

Plasmid pTK100 encoding thioredoxin (a gift from Dr. Maria Luisa Tasayco) was transformed into *E. coli* JF521 strain for protein overexpression. Cells were grown, starting from single colonies, at 37 °C in Luria broth with 40 μg/mL of kanamycin to select for the plasmid-bearing cells. The final 750-fold dilution of the cell broth was allowed to grow during 12 h after stationary phase was reached. After centrifugation, cell pellets were frozen at −20 °C until purification. Protein purification protocol was as follows. Briefly, cells were thawed, resuspended in 1 mM EDTA, 30 mM TRIZMA buffer, pH 8.3 and lysed using a French press. The cell debris was centrifuged and the supernatant was collected and stirred with streptomycin sulfate (10% w/v) at 4 °C overnight, in order to precipitate nucleic acids. The filtered supernatant was then loaded onto a 2-L Sephacryl™ S-100 High Resolution (Amersham Pharmacia Biotech AB) gel filtration column equilibrated in 1 mM EDTA, 30 mM TRIZMA buffer, pH 8.3. Thioredoxin fractions were identified by SDS-PAGE, pooled and applied to a 250-mL Fractogel® EMD DEAE(M) (Merck) ion exchange column equilibrated in 1 mM EDTA, 30 mM TRIZMA buffer, pH 8.3. The protein was eluted by a linear gradient between 0 and 0.5 M NaCl. The proteins were pure as measured by SDS-PAGE gel densitometry. The molecular weight of pure proteins was confirmed by mass spectrometry. Thioredoxin concentration was determined spectrophotometrically at 280 nm using a published value of the extinction coefficient [6].

2.3. Differential scanning calorimetry

DSC experiments were carried out with a VP-DSC calorimeter from MicroCal (Northhamton, MA) at a scan-rate of 1.5 K/min. Protein solutions for the calorimetric experiments were prepared by exhaustive dialysis against the buffer (5 mM HEPES, pH 7.0). The samples were degassed at room temperature before the calorimetric experiments. Calorimetric cells (operating volume ~0.5 ml) were kept under an excess pressure of 30 psi to prevent degassing during the scan. In all measurements, the buffer from the last dialysis step was used in the reference cell of the calorimeter. Several buffer–buffer baselines were obtained before each run with a protein solution in order to ascertain proper equilibration of the instrument. In most experiments, a reheating run was carried out to determine the reversibility of the denaturation process. High reversibility was found for all the protein variants studied in this work. Finally, an additional buffer–buffer baseline was obtained immediately after the protein runs to check that no significant change in instrumental baseline had occurred. A protein concentration dependence for thioredoxin denaturation temperature has been reported in the literature and attributed to protein dimerization [7]. Therefore, we carried out all the DSC experiments at comparatively low protein concentrations: about 0.5 mg/mL or below in some cases. We found no protein concentration effects on denaturation energetics within the 0.1–0.5 mg/mL range. Fittings of theoretical models to the heat capacity profiles were performed using programs written by us in the MLAB environment (Civilized Software). The general approach used in the two-state fittings was as described previously [8].

3. Results and discussion

In order to design potentially stabilizing charge-introduction mutations, we considered all positions with serine, threonine or asparagine residues in WT thioredoxin, regardless of the accessibility to solvent of those residues, and we used the Swiss Viewer v3.7b2 program to model thioredoxin variants in which lysine residues had been introduced in those positions. We then selected those positions at which at least one rotamer of the newly introduced side-chain could be accommodated without steric clashes. Finally, for the variants including mutations

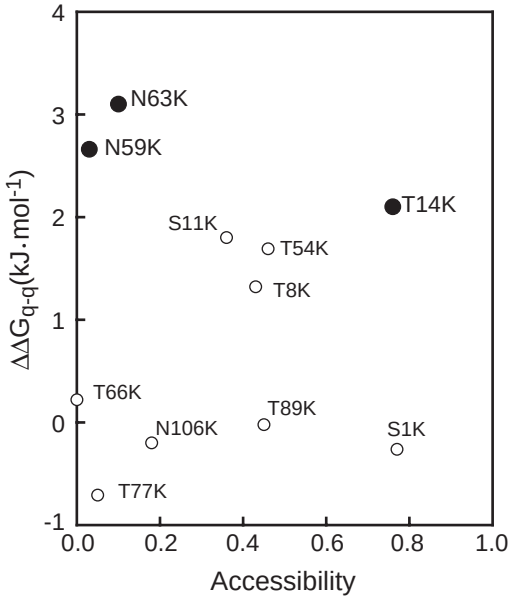

Fig. 1. Effect of polar neutral to lysine mutations on *E. coli* thioredoxin stability, as calculated from a simple electrostatic model that takes into account charge–charge interactions [2]. $\Delta\Delta G_{qq}$ is the calculated effect of the mutation on denaturation Gibbs energy. Accessibilities are calculated as the ratio between the side-chain accessible surface area (ASA) in the native structure and that in Gly-X-Gly tripeptide; ASA values were obtained using a modification of the Shrake–Rupley algorithm, as we have previously described [2].

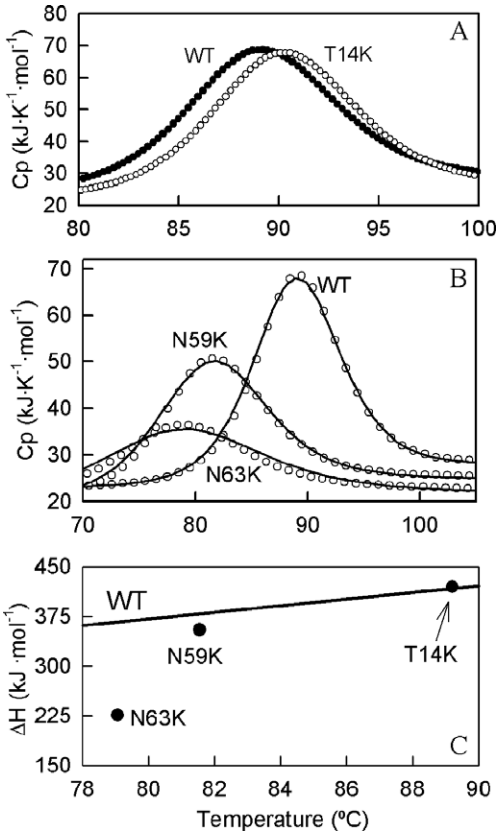

Fig. 2. (A) Heat capacity versus temperature profiles for WT thioredoxin and the T14K variant at pH 7. (B) Heat capacity versus temperature profiles for WT thioredoxin and the N63K and N59K variants. The continuous lines represent the best fits of the two-state equilibrium model. (C) Plot of denaturation enthalpy versus temperature. The line is the ΔH versus T dependence for WT thioredoxin and the points are the ΔH values for the variants T14K, N63K and N59K at the corresponding transition temperatures.

at the selected positions, we performed electrostatic calculations using our implementation of the Tanford–Kirkwood model with the solvent accessibility correction of Gurd, as we have previously described in detail [2].

The results of the above calculations are given (Fig. 1) as a plot of $\Delta\Delta G_{qq}$ (the calculated effect of the mutation on denaturation free energy, as arising from charge–charge interactions exclusively) versus the accessibility to solvent of the mutated residue in the WT protein.

In view of the results shown in Fig. 1, we deemed of interest to study the mutations T14K, N59K and N63K. T14K appears to be an excellent candidate for stabilizing mutation, since the calculated $\Delta\Delta G_{qq}$ is significant and positive and T14 is well-exposed to the solvent in the WT structure. N59K and N63K were chosen because these mutations show the highest calculated values of $\Delta\Delta G_{qq}$, although the fact that N59 and N63 have low solvent accessibility suggests that other factors, besides charge–charge interactions, may play a role in this case.

Variants of thioredoxin with the mutations T14K, N59K and N63K were purified and studied by differential scanning calorimetry. Variant T14K was found to be slightly more stable than WT (Fig. 2A), in qualitative agreement with the theoretical prediction of Fig. 1. On the other hand, variants N59K and N63K were less stable than the WT form (Fig. 2B); however, the two following points must be noted: (1) the denaturation enthalpy (ΔH) values of these two variants (in particular N63K) are smaller than the ΔH value for the WT form, even when the temperature dependence of the latter is taken into account (see Fig. 2C); (2) the denaturation heat capacity change values for the variants are also smaller than the WT value and, furthermore, the heat capacity versus temperature profiles suggest that this difference may be put down, at least in part, to the heat capacity of the denatured state (see Fig. 2B). These results suggest that the N63K and N59K mutations may induce residual structure in the denatured state. We are currently carrying out structural studies (circular dichroism and X-ray crystallography) to determine the effect of these mutations on native and denatured state structure, as well as additional mutagenic studies aimed at delineating the region of the thioredoxin molecule in which mutations have the largest effect on denatured state properties.

Acknowledgements

This work was supported by Grant BIO2003-02229 from the Spanish Ministry of Science and Technology and by FEDER funds. Raul Perez-Jimenez is a recipient of a predoctoral fellowship from the Spanish Ministry of Science and Technology.

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