Thermodynamic and kinetic analysis of the *Escherichia coli* thioredoxin-C′ fragment complementation system

Alokesh K. GHOSHAL*, Chittoor P. SWAMINATHAN*, Celestine J. THOMAS*, Avadhesha SUROLIA*
and Raghavan VARADARAJAN*†[1]

*Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India, and †Chemical Biology Unit, Jawaharlal Nehru Center for Advanced Scientific
Research, Jakkur P. O., Bangalore 560 004, India

Escherichia coli thioredoxin was cleaved with CNBr at its single Met residue at position 37, which lies in the middle of a long α-helix. The two fragments, 1–37 and 38–108, were purified and characterized by using CD and fluorescence spectroscopy. Both fragments lack structure at neutral pH and room temperature. The secondary and tertiary structural contents of the non-covalent complex formed on the mixing of the two peptide fragments are 47% and 35% of the intact protein respectively. The thermodynamics and kinetics of fragment association were characterized by titration calorimetry and stopped-flow fluorescence spectroscopy. Single phases were observed for both association and dissociation, with rate constants at 298 K of k_{on} = 4971 ± 160 $M^{-1} \cdot s^{-1}$ and k_{off} = 0.063 ± 0.009 s^{-1} respectively. The ratio k_{on}/k_{off} was very similar to the binding constant

determined by titration calorimetry, suggesting that binding is a two-state process. The values for ΔC_p, ΔH^0 and ΔG^0 at 298 K for dissociation of the complex were 5.7 $kJ \cdot mol^{-1} \cdot K^{-1}$, 45.3 $kJ \cdot mol^{-1}$ and 29.8 $kJ \cdot mol^{-1}$ respectively. The value for ΔH^0 was linearly dependent on temperature from 8 − 40 °C, suggesting that ΔC_p is independent of temperature. The values for ΔC_p and ΔG^0 are very similar to the corresponding values for the unfolding of intact thioredoxin at 25 °C. However, both ΔH^0 and ΔS are significantly more positive for dissociation of the complex, suggesting a decreased hydrophobic stabilization of the complex relative to the situation for intact thioredoxin.

Key words: heat capacity, isothermal titration calorimetry, peptide fragments, stopped-flow kinetics.

INTRODUCTION

Fragment complementation systems consist of two or more protein fragments that associate through non-covalent inter-actions to give a complex with a similar structure and activity to the uncleaved protein. Such systems are a useful tool for the study of protein folding and protein stability in the absence of denaturants. Several fragment complementation systems have been constructed *in vitro* (reviewed in [1]).

Thermodynamic and kinetic parameters for the unfolding of globular proteins are typically measured in the presence of either a high concentration of denaturants such as urea, guanidinium chloride or protons or at elevated temperatures. It is therefore necessary to extrapolate these parameters to zero denaturant concentration or room temperature [2]. In contrast, for fragment complementation systems, it is possible to study the thermo-dynamics and kinetics of fragment association and dissociation in the absence of any denaturant as a function of temperature. It is also of interest to characterize the structures of the individual protein fragments because they might be good models of the denatured state in aqueous solution [3].

A single methionine residue is present at position 37 in *Escherichia coli* thioredoxin. This residue occurs in the N-terminal portion of a long α-helix that extends from residue 32 to residue 49 [4]. On cleavage with CNBr, two peptide fragments of 71 and 37 residues are generated [5]. These two peptides assemble to a native-like complex, thioredoxin-C′ (Trx-C′), in solution. The complex has 10–15% of the activity of the intact protein [6].

However, detailed information on the thermodynamics and kinetics of binding is not available. RNase S is the only fragment complementation system for which extensive thermodynamic [7], kinetic [8] and structural information [9] are all available.

Here we report the thermodynamic and kinetic charac-terization of complex formation between the 37-residue and 71-residue fragments of thioredoxin (peptides 37 and 71 respectively) with the use of titration calorimetry and stopped-flow fluor-escence respectively. In RNase S, the larger S-protein fragment has some residual structure at neutral pH and above [10]; however, in Trx-C′ both fragments are completely unfolded initially, so it was of interest to perform a titration calorimetry study of a fragment complementation system in which both peptide fragments are initially unfolded and become folded on complex formation.

MATERIALS AND METHODS

Protein expression and purification

The plasmid paltrxa-781 carrying the wild-type thioredoxin gene, which is under the control of P_L promoter [11], was transformed into an *E. coli* lambda lysogen N4830-1 [12] carrying the temperature-sensitive cI repressor for the P_L promoter. Cells were grown to an A_{600} of 1.0 in Luria–Bertani medium containing 100 μg/ml of ampicillin at 30 °C, followed by temperature induction at 42 °C for 3–4 hours. Purification of thioredoxin was performed as described [6]. Protein purity was more than 95% as judged by SDS/PAGE. The activity was checked by the thio-

redoxin-reductase-coupled 5,5′-dithiobis-(2-nitrobenzoic acid) assay, as described [6].

Peptide purification and characterization

Peptides 37 and 71 were obtained by CNBr (Spectrochem, Bombay, India) cleavage of wild-type *E. coli* thioredoxin and purified as described [6]. Fragment purity was checked by reverse-phase HPLC with a C_{18} column (0.46 cm × 15 cm; Vydac). Peptides 37 and 71 eluted at 55 % and 50 % (v/v) acetonitrile respectively. The peptides also exhibited mobilities consistent with their molecular masses in tricine/SDS/PAGE [13]. Concentrations of peptides 71 and 37 were determined on the basis of their reported ϵ_{280} values at 280 nm, namely 3700 and 10 000 $M^{-1} \cdot cm^{-1}$ respectively [6]. Mass spectrometry of peptides 37 and 71 was performed in a Kratos matrix-assisted laser desorption ionization system. We observed two mass populations for peptide 37, at 4104 and 4133 Da respectively. The former mass is in excellent agreement with the calculated mass for peptide 37 in homoserine lactone form (4108.7 Da). The latter mass could be due to formylation of the peptide during HPLC purification. Peptide 71 also exhibited two masses: 7547 and 7577 Da. The calculated mass for peptide 71 is 7536.7 Da and is in good agreement with the first mass value. The higher-mass peak for peptide 71 probably resulted from formylation at the N-terminus. For both peptide 37 and peptide 71, formylation did not seem to affect binding, because the binding stoichiometry was close to 1 for all titrations.

Spectroscopy and gel filtration

All CD spectra were recorded on a Jasco J-500A spectropolarimeter. Far-UV (250–190 nm) and near-UV (300–250 nm) CD spectra were recorded in cuvettes with path lengths of 2 mm and 10 mm respectively. All fluorescence measurements were performed in a Jasco FP-777 spectrofluorometer with slit widths of 1.5 nm for excitation and 3 nm for emission. For monitoring FITC fluorescence, excitation and emission wavelengths were fixed at 495 and 525 nm. Gel filtration experiments were performed with a Superose-6 column (1 cm × 30 cm; Pharmacia) at a flow rate of 0.5 ml/min on a Beckman 112 HPLC system, with detection at 226 nm. To detect Trx-C′, a mixture of 800 μM peptide 37 and 800 μM peptide 71 was injected. For binding experiments with 8-anilino-1-naphthalenesulphonic acid (ANS) (Sigma Chemical Co., St. Louis, MO, U.S.A.) the excitation wavelength was 370 nm and emission was monitored between 400 and 550 nm. Typical concentrations used were 2 μM peptide and 35 μM ANS. All spectra were corrected for fluorescence of free ANS.

Isothermal titration calorimetry (ITC)

All ITC measurements were performed with a MicroCal Omega titration calorimeter [14]. Both peptide 37 and peptide 71 were dialysed extensively against 60 mM Hepes, pH 8.1, at 4 °C with dialysis bags of molecular mass cut-off 1000 Da before calorimetric titrations. For all ITC measurements, peptide 71 was placed in the cell and was titrated with peptide 37 in the syringe. A 5 min time difference was given between each injection with a typical injection volume of 20 μl. Dilution heats of peptide 37 into buffer and buffer into peptide 71 were measured and subtracted from the observed heats. Typical concentrations of peptides 37 and 71 used were 1.2 mM and 60 μM respectively. The shape of the titration curve is determined by the unitless parameter $c = K[M_T]$, where K is the binding constant and $[M_T]$ is the total concentration of the molecule in the cell [14]. Accurate estimation of binding parameters is possible for titrations in which c lies between 1 and 1000. All c values for the present titrations were between 3 and 15. All ITC data were analysed by using the ORIGIN™ software package.

Labelling and fluorescence titration

The labelling of peptide 71 with FITC was performed as described [15]. Stoichiometric amounts of peptide 71 and FITC (Sigma) were mixed in 50 mM carbonate buffer pH 9.1, and kept for 6 h with stirring. The reaction mixture was then loaded on a Sephadex G-25 column to separate free FITC from the labelled peptide. The labelled peptide fraction exhibits a single fluorescent band on SDS/PAGE and also on electrophoresis under non-denaturing conditions. MS of the labelled peptide was performed in a Hewlett-Packard electrospray system (model SD 1091) to confirm that most of the peptide was labelled at a single site. The ϵ_{488} of the labelled peptide was taken to be the same as that reported for FITC-labelled S-peptide, namely 77000 $M^{-1} \cdot cm^{-1}$ [16]. All fluorescence measurements were performed at 22 °C in 50 mM Hepes buffer, pH 7.5. The excitation and emission wavelengths were 495 and 525 nm respectively. For fluorescence titrations, 1.2 μM FITC-labelled peptide 71 in a cuvette was titrated with peptide 37. The Scatchard plot was constructed on the assumption of 1:1 binding between peptides 71 and 37. All fluorescence intensities were corrected for dilution.
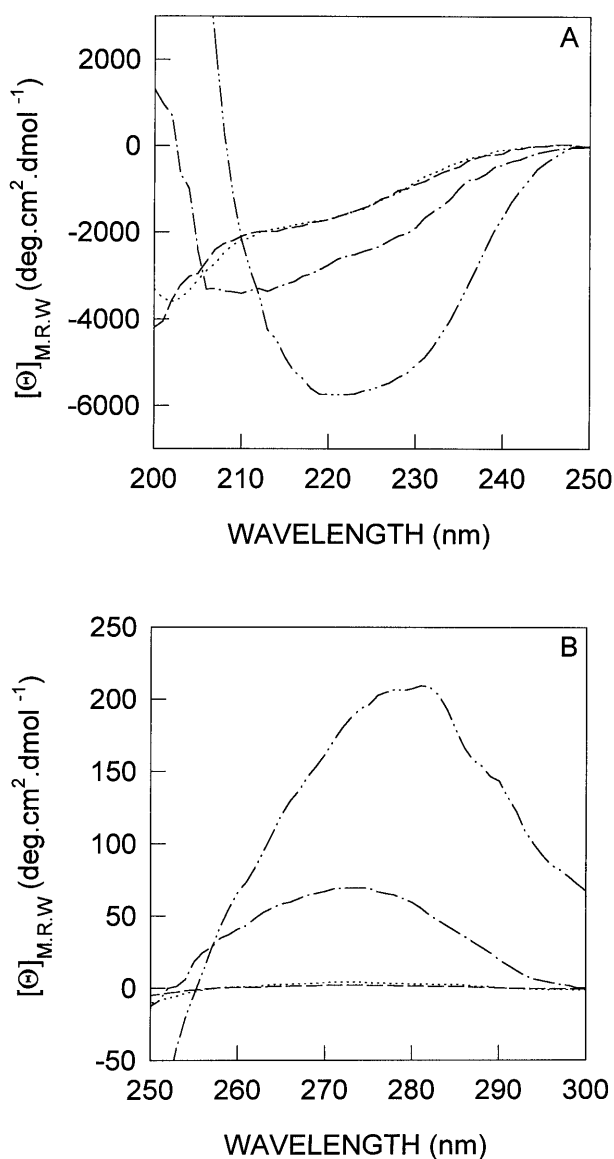
Stopped-flow kinetics

All rapid mixing measurements were performed at 25 °C in an Applied Photophysics (model 05-139) stopped-flow spectrometer. A tungsten lamp (450 W) was used for excitation at 495 nm; emission at less than 500 nm was detected with the use of a cut-off filter. Each kinetic trace is an average of at least eight experimental mixings. A control experiment was performed in which labelled peptide 71 was diluted with the buffer (50 mM Hepes, pH 7.5). No photobleaching of the sample was observed.

RESULTS

Spectroscopic studies for complex formation between peptides 37 and 71

Secondary structures for individual peptide fragments, the complex (Trx-C′) and intact thioredoxin were monitored by recording individual far-UV CD spectra at 25 °C in 20 mM phosphate buffer, pH 7.5. Peptides 71 and 37 both lack secondary structure, as shown in Figure 1(A). The spectrum of the complex was recorded by mixing 10 μM peptide 71 and 70 μM peptide 37, whereas for the control experiment a 10 μM solution of thioredoxin was used. The spectrum of the complex was corrected for the presence of free peptide 37. The change in sign of molar ellipticity for the complex Trx-C′ from negative to positive in the range 210–200 nm clearly demonstrates the formation of secondary structure, as both α-helices and β-strands have maxima at approx. 190–195 nm [17]. The complex exhibited a flat minimum in the region of 208–220 nm, which is similar to intact thioredoxin. The concentration of the complex formed was calculated from the binding constant between peptides 37 and 71, determined from titration calorimetry (see below). At 220 nm, Trx-C′ exhibited 47 % of the molar ellipticity of intact thioredoxin. Thioredoxin contains two Trp residues at positions 28 and 31: Trp-28 is at the C-terminus of a β-strand; Trp-31 forms part of the active site loop. Both of these residues are located close to the site of cleavage and it is possible that they are highly mobile in the complex. A reduced far-UV CD contribution from
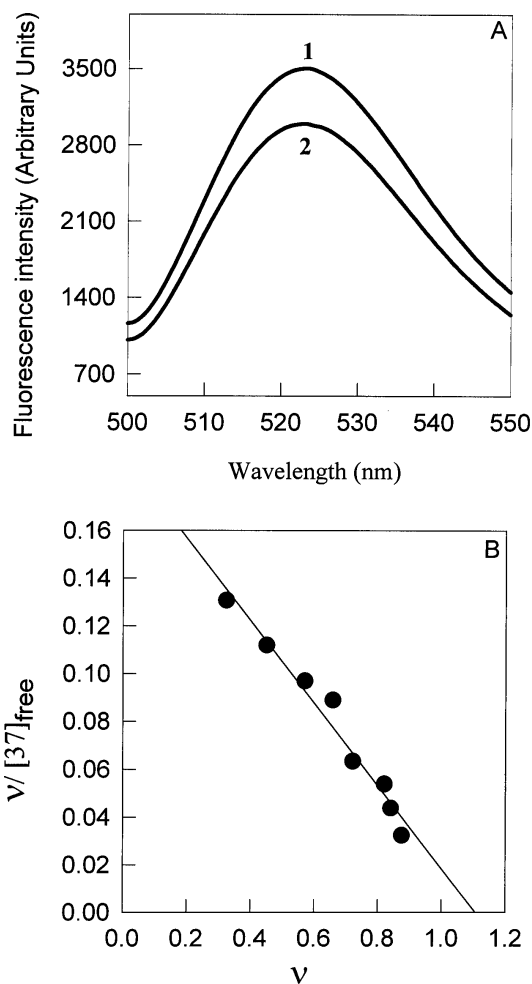
Figure 1   CD spectra

(**A**) Far-UV CD spectra in 20 mM phosphate buffer, pH 7.2. (**B**) Near-UV CD spectra in 20 mM phosphate buffer pH 7.2. Key to both panels: dashed line, peptide 37; dotted line, peptide 71; dot–dashed line, Trx — C′; double-dot–dashed line, intact thioredoxin.

Figure 2   Fragment complementation detected by fluorescence spectroscopy

(**A**) Fluorescence emission spectra with excitation wavelength at 495 nm. Curve 1, 1 μM peptide 71 labelled with FITC; curve 2, as in curve 1, plus 40 μM peptide 37. (**B**) Scatchard plot for the binding of peptide 37 to FITC-labelled peptide 71: ν is the fraction of peptide 71 bound; $[37]_{free}$ is the concentration of free peptide 37.

these two residues might partly explain the decreased far-UV CD signal in the complex.

The isolated peptides 37 and 71 did not exhibit any near-UV CD signal (Figure 1B). However, on mixing of the two fragments, a near-UV signal with a broad maximum between 270 and 275 nm was observed. This shows distinct tertiary packing of aromatic residues in Trx-C′. At 275 nm the mean residue relative-molecular-mass ellipticity ($[\theta]_{MRW}$) for the complex was 35 % of that of intact thioredoxin. The near-UV CD spectrum of *E. coli* thioredoxin, however, showed a flat maximum between 275 and 280 nm, indicating that the tryptophan residues are in a rigid tertiary environment. As with the far-UV CD, a decreased near-UV CD contribution from the two Trp residues would explain the decrease in the maximal value of the $[\theta]_{MRW}$ as well as the shift in position of the maximum to lower wavelength.

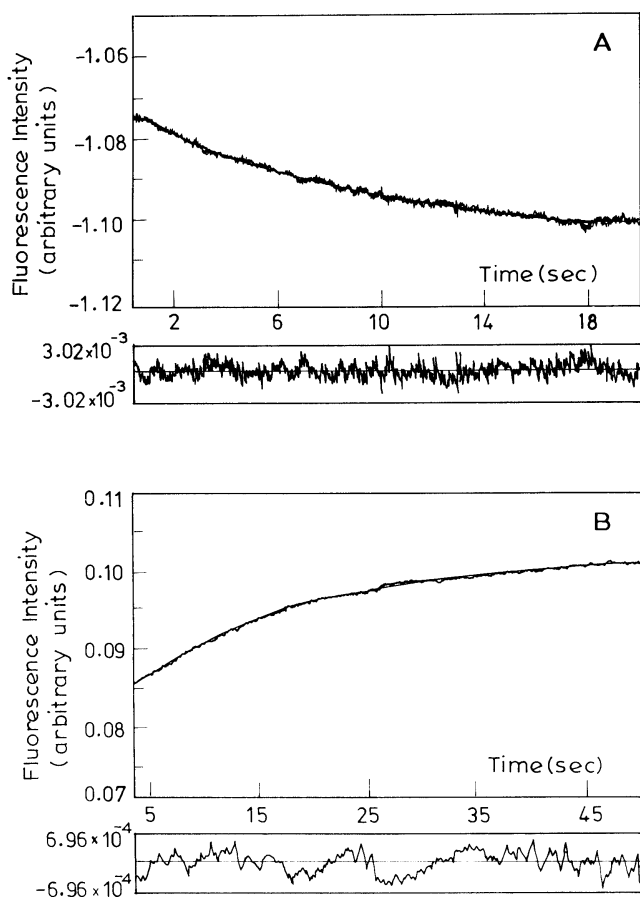Peptide 37 contains two tryptophan residues; on excitation at 295 nm its fluorescence emission spectrum shows a maximum at 355 nm, as reported previously [5]. This shows that both tryptophan residues are completely solvent-accessible. However, unclipped *E. coli* thioredoxin has an emission maximum at 345 nm, suggesting the partial burial of tryptophan residues on folding. Peptide 71 has two tyrosine residues but no tryptophan residue. As expected, peptide 71 showed tyrosine fluorescence (emission maximum 308 nm) but no tryptophan fluorescence. There was no change in tryptophan fluorescence (either in intensity or wavelength of emission maximum with excitation at 295 nm) on mixing peptide 37 (containing both tryptophan residues) with peptide 71 under conditions favouring complex formation. This also suggests that the two Trp residues are solvent-exposed in the complex. However, we did observe a small quenching of tyrosine fluorescence (4–5%) on complex formation.

We examined ANS binding properties for individual peptide fragments and the complex, because this dye is often used as a probe for exposed hydrophobic patches on the surface of proteins
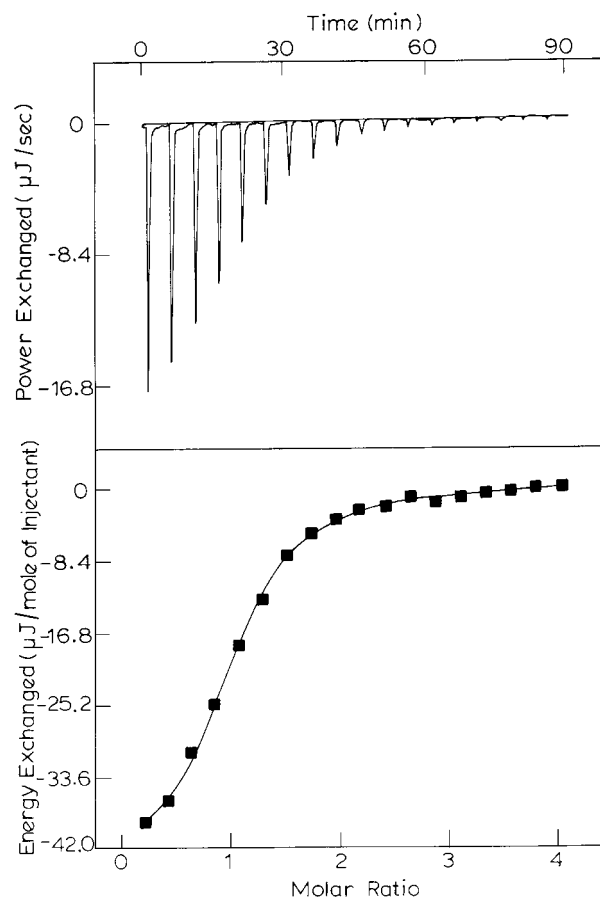
Figure 3 Stopped-flow fluorescence kinetics

(**A**) Stopped-flow kinetic trace for the mixing of 0.5 μM FITC-labelled peptide 71 with 10 μM peptide 37. The solid line fitted through the data points yields a rate constant of 0.104 s^{-1}. The residuals for the fitted line are given below the trace. (**B**) Stopped-flow kinetic trace for the mixing of 50 μM peptide 71 with 1 μM preformed Trx-C′. The solid line fitted through the data points yields a k_{off} value of 0.06 s^{-1}, with residuals as shown below the trace.

Figure 4 Calorimetric titration of peptide 71 against peptide 37

Top panel: data obtained for 20 injections (15 μl each) of peptide 37 (1.2 mM) into peptide 71 (60 μM). Bottom panel: plot of total energy exchanged as a function of molar ratio of ligand (peptide 37). The solid line is a non-linear regression fit to the data [14], which yielded a binding constant of 1.6 × 10^5 M^{-1}, a binding enthalpy of −45.2 kJ · mol^{-1} and a binding stoichiometry of 0.96.

[18]. In 50 mM Tris/HCl buffer, pH 7.5, on being mixed with excess ANS both peptides 37 and 71 exhibited enhanced fluorescence and a blue shift of emission maximum from 520 nm to 475 nm. Peptide 71 exhibited a 5-fold greater fluorescence enhancement than peptide 37. The Trx-C′ complex also bound ANS, suggesting that the complex has some exposed hydrophobic surface.

Gel-filtration chromatography

The compactness of individual fragments as well as the complex was studied by gel-filtration chromatography with a Superose-6 gel-filtration column (bed volume 25 ml) equilibrated with 50 mM Tris/HCl, pH 8.0. An equimolar mixture of peptides 71 and 37 (800 μM) was eluted at 16.85 ml. This is very close to the elution volume of wild-type *E. coli* thioredoxin (17 ml), suggesting that both species are similarly compact. Peptides 71 and 37 individually were eluted as single peaks at 17.5 and 18.0 ml respectively. We did not observe any peak close to the void volume (8 ml) in any elution profile. This shows that neither the free peptides nor the Trx-C′ complex were aggregated.

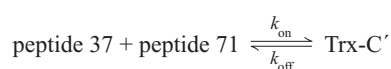## Kinetics of complex formation between peptides 37 and 71

We observed no detectable changes in intrinsic tryptophan fluorescence on complex formation. To monitor the kinetics of the binding reaction we therefore covalently modified peptide 71 with FITC and monitored conjugate fluorescence on complex formation between peptide 37 and FITC-labelled peptide 71. As shown in Figure 2(A), significant quenching of fluorescence (21 %) was observed on complex formation. Scatchard analysis of a fluorescence-quenching titration (Figure 2B) gave a value of 5.5 μM for the dissociation constant of the complex at 25 °C, which is very close to that obtained from ITC (6.2 μM). In a manual mixing protocol it was observed that a significant fraction of the fluorescence change was complete within the dead time of mixing (10 s). No slow phase was detected during the manual mixing experiment. Rapid mixing experiments were performed in the presence of excess peptide 37. The FITC-labelled peptide 71 concentration was 0.5 μM and the concentration of peptide 37 was varied between 10 and 35 μM. Under these conditions the reaction is pseudo-first-order and both the association and the dissociation rate constants can be obtained from the observed relaxation time. A typical kinetic trace is shown in Figure 3(A). No slow phase was observed in the stopped-flow experiments.

Table 1 Thermodynamic parameters for binding between peptide 37 and 71 obtained from titration calorimetry

Fitting errors (S.E.M.) are shown for each parameter.

Temperature (K)	n	$10^{-4} \times K_b$ (M^{-1})	ΔG^0 (kJ/mol)	ΔH^0 (kJ/mol)	$T\Delta S^0$ (kJ/mol per K)
281.3	0.99 ± 0.02	4.3 ± 0.2	-24.8 ± 1.7	49.6 ± 1.3	74.4 ± 2.9
286.0	0.95 ± 0.05	9.9 ± 1.9	-27.3 ± 2.4	17.2 ± 1.3	44.5 ± 10.5
293.2	1.07 ± 0.02	11.3 ± 1.0	-28.6 ± 2.1	-21.4 ± 1.2	7.2 ± 2.9
298.1	0.96 ± 0.01	16.0 ± 1.0	-29.8 ± 2.1	-45.4 ± 1.0	-15.6 ± 2.9
303.5	0.99 ± 0.02	16.9 ± 2.1	-30.2 ± 2.5	-70.1 ± 1.7	-39.9 ± 4.2
308.1	1.01 ± 0.03	8.6 ± 0.7	-29.0 ± 2.1	-99.9 ± 3.3	-70.9 ± 5.9
313.3	1.05 ± 0.03	3.4 ± 0.1	-27.3 ± 1.7	-147.0 ± 4.3	-119.7 ± 6.7

The error associated with fitting for each fluorescence decay was less than 5%. For the following bimolecular reaction

$$\text{peptide 37} + \text{peptide 71} \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} \text{Trx-C}'$$

the observed pseudo-first-order rate constant can be written as [19]

$$k_{\text{obs}} = k_{\text{off}} + k_{\text{on}} [\text{peptide 37}] \tag{1}$$

where k_{obs}, k_{off} and k_{on} are the observed, off and on rate constants respectively. The slope and intercept of a plot of k_{obs} against peptide 37 concentration therefore provided values for k_{on} and k_{off} respectively: at 25 °C these were 4971 ± 160 M$^{-1} \cdot$s^{-1} and 0.063 ± 0.009 s^{-1}. To measure the dissociation rate constant directly we followed the enhancement of conjugate fluorescence on the addition of a 50-fold excess of unlabelled peptide 71 to the preformed complex between peptide 37 and FITC-labelled peptide 71. The fluorescence enhancement could be fitted to a single exponential rate constant of 0.070 ± 0.008 s^{-1} (Figure 3B). This value is in excellent agreement with the same rate constant obtained from pseudo-first order kinetics as that mentioned above.

Calorimetric titrations

ITC was performed to study the thermodynamics of binding between peptides 71 and 37 in 60 mM Hepes buffer, pH 8.1. It was possible to perform ITC over a broad range of temperatures (8–40 °C). A representative calorimetric titration is shown in Figure 4. The binding stoichiometry for all titrations was very close to 1 (Table 1). The binding constant (K_b) and thermodynamic parameters for binding (ΔG^0, ΔH^0, $T\Delta S^0$) for each temperature obtained from ITC are given in Table 1. Binding enthalpies were found to vary linearly with temperature. The temperature dependencies of ΔG^0 and ΔH^0 are given by:

$$\Delta H^0(T) = \Delta H^0(T^0) + \Delta C_p(T - T^0) \tag{2}$$

$$\Delta G^0(T) = \Delta H^0(T^0) + \Delta C_p(T - T^0) - T\{[\Delta H^0(T^0)$$
$$- \Delta G^0(T^0)]/T^0 + \Delta C_p \ln(T/T^0)\} \tag{3}$$

A value of ΔC_p of 5.7 ± 0.4 kJ$\cdot$mol$^{-1} \cdot$K^{-1} was obtained from a fit of $\Delta H^0(T)$ to Eqn. (2). The value of K_b as a function of temperature varied between 4×10^4 and 1.7×10^5 M^{-1} and had a maximum at 30 °C. On the basis of the calorimetric titration data we calculated the dissociation constant for the complex Trx-C' at 25 °C as 6.5 μM. This value agrees well with the earlier value of

2 μM reported by Holmgren and Slaby [6], who independently derived a value for the dissociation constant from activity measurements of Trx-C'.

DISCUSSION

The present study shows that peptides 37 and 71 are unstructured in solution and combine to form a complex with significant tertiary structure. These results are in agreement with a recent two-dimensional NMR study [20], which showed that the Trx-C' complex is structurally similar to the intact protein except near the cleavage site. Met-37, the site of CNBr cleavage, lies in the middle of a long α-helix that extends from residues 32 to 49. The main-chain and side-chain accessibilities calculated by the procedure of Lee and Richards [21] are 26% and 80% respectively. Although the main chain is appreciably buried and the site of cleavage lies in a region of secondary structure, it is still possible to obtain fragment complementation. The dissociation constants obtained from titration calorimetry agree well with those calculated either from fluorescence titrations or from the measured on and off rates of the binding of peptide 37 to peptide 71, suggesting that the binding is a two-state process.

The value of k_{on} (4980 ± 900 M$^{-1} \cdot$s^{-1}) reported here is slightly greater than that reported recently (1330 ± 54 M$^{-1} \cdot$s^{-1}) for another set of disordered fragments (residues 1–73 and 74–108) derived from the tryptic cleavage of *E. coli* thioredoxin at Arg-73 in the Trx-T' complementation system [22]. The fact that two completely different sets of disordered Trx fragments have similar k_{on} values suggests that complex formation does not occur between small subpopulations of ordered fragments but rather that the two disordered fragments form a compact native-like complex in a single step. With the small protein CI2, efficient complementation was observed only when the two fragments corresponded to subdomains of the protein [23]. For thioredoxin this is clearly not true, so co-operative interactions between subdomains are not a necessary prerequisite for complementation. Our value of k_{off} for the CNBr derived fragments (0.07 s^{-1}) is considerably higher than the reported value for the trypsin-derived fragments of *E. coli* thioredoxin (10^{-9} s^{-1}) [22]. Arg-73 is located on a short solvent-exposed loop and has main-chain and side-chain accessibilities of 95% and 102% respectively. This increase in the value of k_{off} in Trx-C' relative to Trx-T' is probably because the site of cleavage is considerably more buried for the CNBr fragments than for the tryptic fragments. In addition, in Trx-C' the cleavage site is in the middle of a long helix. Because the generation of charged chain termini at a buried location in a region of regular secondary structure is expected to have a greater destabilizing effect than at a surface loop, Trx-C' is expected to be less structured around the cleavage site than the Trx-T' complex. This decreased structure and

stability is the probable cause of the increased value of k_{off} in the Trx-C′ complex.

Only a single phase was observed for the binding reaction. The slow phase of the refolding reaction of intact *E. coli* thioredoxin (300–500 s) has been shown to be due to *trans* to *cis* isomerization at Pro-76 [24]. It is possible that the fluorescent probe FITC is insensitive to the slow isomerization step. Alternatively it is possible that Pro-76 is already in the *cis* conformation in fragment 71. With RNase S also, no slow binding phase was reported, though the final structure contains two *cis*-proline residues [8]. The ITC results summarized in Table 1 show that below 17 °C the binding is endothermic, whereas above this temperature it is exothermic. At low temperatures the driving force for the binding reaction is driven by entropy and indicates the dominant contribution of the hydrophobic driving force for complex formation. The positive entropy change is likely to be caused by the release of water molecules from the fragments on complex formation. On the basis of peptide sequence at neutral pH the estimated charges on peptides 71 and 37 are 0 and −3.5 respectively. To examine the role of electrostatics in binding between peptides 71 and 37, ITC experiments were performed in the presence of 500 mM NaCl at 25 °C. The values of K_b and ΔH decreased by 50 % and 30 % respectively. This suggests that electrostatic interactions in the complex are stabilizing. The heat capacity change on binding is negative and large (-5.7 kJ·mol^{-1}·K^{-1}), suggesting the formation of a compact folded complex from unfolded peptide fragments. This ΔC_p is 81 % of the ΔC_p of folding observed for intact *E. coli* thioredoxin with the use of differential scanning calorimetry [25]. In fact this heat capacity value for folding (52.5 J·mol^{-1}·K^{-1} per residue) is comparable to those for several monomeric globular proteins. The total surface area buried on the formation of the Trx-C′ complex was estimated by using the equation proposed by Myers et al. [26]:

$$\Delta A = 1.19(\Delta C_p + 500) \tag{4}$$

where ΔC_p is in units of J·mol·K^{-1} and ΔA is the total surface area buried, in Å². On the basis of the calorimetrically determined ΔC_p for the formation of the complex Trx-C′ (5.7 kJ·mol^{-1}·K^{-1}), the calculated ΔA value is 7378 Å². The ΔA value for intact oxidized *E. coli* thioredoxin, corrected for the single cross-link between residues 32 and 35, was calculated to be 8223 Å² from the crystal structure [21]. Therefore on the basis of heat-capacity data alone, Trx-C′ is 89 % as compact as unclipped thioredoxin.

ΔC_p is an important thermodynamic parameter that determines the temperature dependence of ΔG, ΔH and ΔS and is closely linked to the hydrophobic effect [27,28]. ΔC_p values for proteins are typically measured either by using DSC or by performing chemical denaturation studies as a function of temperature. The ΔC_p values measured from individual calorimetric scans are subject to considerable experimental uncertainty. Hence ΔC_p values are estimated from measurements of ΔH as a function of temperature by performing calorimetric scans at different pH values. Such ΔC_p values are typically measured at temperatures of 50–60 °C, considerably above room temperature. Because the accessible range of temperature is often limited, it is difficult to measure ΔC_p accurately. It is generally assumed that ΔC_p is independent of temperature but it is difficult to verify this experimentally in view of the large experimental errors. The present study shows that it is possible to measure the association thermodynamics of disordered protein fragments by using ITC to obtain an accurate value of ΔC_p that is close to the corresponding value for folding of the intact protein. The only previous ITC studies of the association of protein fragments were for RNase S [9,28,29]. However, in the RNase S system, one of the fragments (S-protein) has considerable structure at room

Table 2 Comparison of the structural and thermodynamic properties of Trx-C′ and thioredoxin

$[\theta]_{MRW}$ is mean residue relative-molecular-mass ellipticity.

Parameter	Trx-C′	Thioredoxin
Far-UV CD $[\theta]_{MRW}$ at 220 nm (degrees·cm²·dmol^{-1})	−2753	−5857
Near-UV CD $[\theta]_{MRW}$ at 275 nm (degrees·cm²·dmol^{-1})	69.4	198.2
ΔC_p (for dissociation/unfolding) (kJ·mol^{-1}·K^{-1})	5.7	6.9*
ΔG^0 (for dissociation/unfolding)† (kJ·mol^{-1})	29.8	34.0*
ΔH^0 (for dissociation/unfolding)† (kJ·mol^{-1})	44.9	−20.2
ΔS^0 (for dissociation/unfolding)† (J·mol^{-1}·K^{-1})	12.3	−180.6
ΔA (Å²)	7378‡	8223§

* From [25].
† At 298 K.
‡ Calculated from observed ΔC_p.
§ Calculated from crystal structure.

temperature and hence the value of ΔC_p for binding is approx. 70 % of that for RNase unfolding. In addition, the ΔC_p of binding becomes strongly temperature-dependent in the range 25–40 °C because of the unfolding of S-protein [28]. In contrast, for the Trx-C′ system, the enthalpy of binding increases linearly with temperature (correlation coefficient 0.98) from 8 to 40 °C, which clearly shows that the heat capacity of binding is temperature-independent over this entire range.

Table 2 compares various properties of intact thioredoxin and Trx-C′. The thermodynamic estimates for thioredoxin at 25 °C were based on the data of Santoro and Bolen [25]. Although Trx-C′ has less secondary and tertiary structure than intact thioredoxin, the ΔG^0 and ΔC_p values are remarkably similar. The temperature of maximal stability of Trx-C′ is 30 °C, which is very similar to the corresponding value of 31 °C for the intact protein [30]. The ΔG^0 value for the unfolding of Trx-C′ is slightly less than that for thioredoxin. This decreased stability is primarily due to an increase in ΔS for dissociation relative to that for unfolding. The increase in translational entropy due to dissociation of the two fragments (cratic entropy) has previously been estimated to be 33.6 J·mol^{-1}·K^{-1} [31]. This is much smaller than the observed difference (approx. 231 J·mol^{-1}·K^{-1}) between values of ΔS(25 °C) for Trx-C′ and thioredoxin. Another surprising observation is that ΔH^0(25 °C) for the dissociation of Trx-C′ is considerably more positive than the corresponding value for unfolding of thioredoxin. Because enthalpic interactions within the complex are unlikely to be more stabilizing than in the intact protein, the reason for these large differences is not clear. Such effects might arise from a decreased hydrophobic stabilization in Trx-C′ relative to thioredoxin. At 25 °C, the signs of ΔH^0 and ΔS for the transfer of non-polar aliphatic solutes from organic solvents to water are both negative [32]. The lower ΔC_p, and hence the lower ΔA, for Trx-C′ dissociation relative to thioredoxin unfolding will result in more positive values of ΔH^0 and ΔS for the former process.

REFERENCES

1 Prat-Gay, G. (1996) Protein Eng. **9**, 843–847
2 Pace, C. N. (1986) Methods Enzymol. **131**, 266–280
3 Shortle, D. and Meeker, A. K. (1989) Biochemistry **28**, 936–944
4 Katti, S. K., LeMaster, D. M. and Eklund, H. (1990) J. Mol. Biol. **212**, 167–184

5   Holmgren, A. and Reichard, P. (1967) Eur. J. Biochem. **2**, 187–189
6   Holmgren, A. and Slaby, I. (1979) Biochemistry **18**, 5591–5599
7   Connelly, P. R., Varadarajan, R., Sturtevant, J. M. and Richards, F. M. (1990) Biochemistry **29**, 6108–6114
8   Goldberg, J. M. and Baldwin, R. L. (1998) Biochemistry **37**, 2546–2555
9   Kim, E. E., Varadarajan, R., Wyckoff, H. W. and Richards, F. M. (1992) Biochemistry **31**, 12304–12314
10 Filippi, B., Borin, G., Moroder, L. and Marchiori, F. (1976) Biochim. Biophys. Acta **454**, 524–538
11 LaVallie, E. R., DiBlasio, E. A., Kovacic, S., Grant, K. L., Schendel, P. F. and McCoy, M. (1992) Bio/Technology **11**, 187–193
12 Gottesman, M. E., Adhya, S. and Das, A. (1980) J. Mol. Biol. **140**, 57–75
13 Schagger, H. and von Jagow, G. (1987) Anal. Biochem. **166**, 368–379
14 Wiseman, T., Williston, S., Brandts, J. F. and Lin, L. N. (1989) Anal. Biochem. **179**, 131–137
15 Heuck, A. P. and Wolosiuk, R. A. (1997) Anal. Biochem. **248**, 94–101
16 Labhardt, A., Ridge, J. A., Lindquist, R. A. and Baldwin, R. L. (1983) Biochemistry **22**, 321–327
17 Woody, R. W. (1996) in The Theory of CD and the Conformational Analysis of Biomolecules (Fasman, G. D., ed.), pp. 25–157, Plenum Press, New York
18 Ptitsyn, O. B. (1994) in Protein Folding (Creighton, T. E., ed.), pp. 243–300, W. H. Freeman, New York
19 Fersht, A. R. (1985) Enzyme Structure and Mechanism, W. H. Freeman, New York
20 Yang, X. M., Yu, W. F., Li, J. H., Fuchs, J., Rizo, J. and Tasayco, M. L. (1998) J. Am. Chem. Soc. **120**, 7985–7986
21 Lee, B. and Richards, F. M. (1971) J. Mol. Biol. **55**, 379–400
22 Chaffotte, A. F., Li, J. H., Georgescu, R. E., Goldberg, M. E. and Tasayco, M. L. (1997) Biochemistry **36**, 16040–16048
23 Ladurner, A. G., Itzhaki, L. S., de Prat Gay, G. and Fersht, A. R. (1997) J. Mol. Biol. **273**, 317–329
24 Kelley, R. F. and Richards, F. M. (1987) Biochemistry **26**, 6765–6774
25 Santoro, M. M. and Bolen, D. W. (1992) Biochemistry **31**, 4901–4907
26 Myers, J. K., Pace, C. N. and Scholtz, J. M. (1995) Protein Sci. **10**, 2138–2148
27 Spolar, R. S., Livingstone, J. R. and Record, Jr., M. T. (1992) Biochemistry **31**, 3947–3955
28 Varadarajan, R., Connelly, P. R., Sturtevant, J. M. and Richards, F. M. (1992) Biochemistry **31**, 1421–1426
29 Thomson, J., Ratnaparkhi, G. S., Varadarajan, R., Sturtevant, J. M. and Richards, F. M. (1994) Biochemistry **33**, 8587–8593
30 McCrary, B. S., Edmondson, S. P. and Shriver, J. W. (1996) J. Mol. Biol. **264**, 784–805
31 Kauzmann, W. (1959) Adv. Protein Chem. **15**, 1–28
32 Tanford, C. (1980) The Hydrophobic Effect: Formation of Micelles and Biological Membranes, John Wiley, New York