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Kinetic stability plays a dominant role in the denaturant-induced unfolding of *Erythrina indica* lectin

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Abstract

The urea-induced denaturation of dimeric *Erythrina indica* lectin (EIL) has been studied at pH 7.2 under equilibrium and kinetic conditions in the temperature range of 40–55 °C. The structure of EIL is largely unaffected in this temperature range in absence of denaturant, and also in 8 M urea after incubation for 24 h at ambient temperature. The equilibrium denaturation of EIL exhibits a monophasic unfolding transition from the native dimer to the unfolded monomer as monitored by fluorescence, far-UV CD, and size-exclusion FPLC. The thermodynamic parameters determined for the two-state unfolding equilibrium show that the free energy of unfolding ($\Delta G_{u,\ aq}$) remains practically same between 40 and 55 °C, with a value of 11.8 ± 0.6 kcal mol^{-1} (monomer units). The unfolding kinetics of EIL describes a single exponential decay pattern, and the apparent rate constants determined at different temperatures indicate that the rate of the unfolding reaction increases several fold with increase in temperature. The presence of probe like external metal ions (Mn^{2+}, Ca^{2+}) does not influence the unfolding reaction thermodynamically or kinetically; however, the presence of EDTA affects only kinetics. The present results suggest that the ability of EIL to preserve the structural integrity against the highly denaturing conditions is linked primarily to its kinetic stability, and the synergic action of heat and denaturant is involved in the unfolding of the protein.

Keywords: Erythrina indica lectin; Legume lectin; Equilibrium denaturation; Unfolding kinetics; Thermodynamic and kinetic stability

1. Introduction

Erythrina indica lectin (EIL) is a dimeric protein (M_r = 68,000) of nearly identical subunits [1], and belongs to the legume family of genus *Erythrina*. The *Erythrina* lectins, which are specific for galactose/N-acetylgalactosamine, show remarkable similarity in their physicochemical properties [2]. These are metalloproteins containing Mn^{2+} and Ca^{2+} and composed of two identical or nearly identical glycosylated subunits. Their amino acid composition is very similar and a high degree of homology exists in their N-terminal sequences. The three-dimensional structure of EIL is not available; however, the crystal structures of *Erythrina corallodendron* lectin (ECorL) and *Erythrina cristagalli* lectin (ECL) in complex with lactose have been determined [3,4]. The tertiary structure of ECorL or ECL monomer is similar to that of other legume lectins, but their quaternary structures involve a 'handshake' mode of association which is different from that of the canonical dimer found in concanavalin A [5].

Though extensive studies on lectin structure and lectin–carbohydrate interactions have been carried out, relatively little is known about the folding and association reactions of oligomeric lectins. In the past few years, reports on reversible unfolding of several legume lectins (including ECorL) have appeared in the literature [6–9]. Recently, we have demonstrated the biphasic equilibrium unfolding of soybean agglutinin and concanavalin A involving a structured monomeric intermediate [10,11]. The kinetic pathways of reassociation of these legume lectins to their native quaternary structures have also been reported [12,13]. In this paper, we present the characteristics of urea-induced

Abbreviations: EIL, *Erythrina indica* lectin; HEPES, N-(2-hydroxyethyl) piperazine-N′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid (disodium salt); CD, circular dichroism; FPLC, fast-protein liquid chromatography

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denaturation of EIL at various temperatures under equilibrium and kinetic conditions, and explore the role of thermodynamic and kinetic stability of EIL in the unfolding reaction.

2. Materials and methods

2.1. Materials

Seeds of *Erythrina indica* were purchased from a local company. N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES) and guar gum were obtained from Sigma. Cross-linked guar gum matrix was prepared by cross-linking guar gum with epichlorohydrin [10]. Urea (AR, E. Merck, India) was further crystallized from hot ethanol to remove possible contamination by cyanate ions [14], and its stock solution was prepared on a dry weight basis [15]. All other reagents used were of analytical grade. Double distilled water was used in unfolding reactions.

2.2. Protein purification

EIL was purified from *Erythrina indica* seeds according to the published procedure [1] except that the cross-linked guar gum was used as an affinity matrix [16]. The purity of the preparation was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [17], and the assay of activity was done by hemagglutination assay [18] using 3% suspension of trypsin-treated rabbit erythrocytes. The concentration of EIL was determined spectrophotometrically at 280 nm from its specific extinction coefficient, $A^{1\%,1\ cm} = 13.4$ [1], and expressed in terms of the monomeric protein concentration using monomer mass of 34 000 Da.

2.3. Spectroscopic measurements

Absorption spectra were recorded on a Hitachi U 3210 UV-VIS spectrophotometer using Sigma cuvette (volume: 2 mL; pathlength: 1 cm).

Fluorescence spectroscopy was performed on a Hitachi 4010 spectrofluorometer connected to a Techne (UK) circulation water bath to maintain the sample temperature. The spectra were measured in 10 mM HEPES buffer containing 0.15 M NaCl, pH 7.2 using Sigma fluorometer cuvette (volume: 2 mL; pathlength: 1 cm). The excitation wavelength was fixed at 280 nm and the emission scanned from 300 to 400 nm. The excitation and emission band pass was 5 nm each, and the scan rate was 60 nm/min. The change of emission wavelength maximum (λ_{max}) is expressed as a relative change based on the change of λ_{max} between the native and the unfolded state taken as 100%. The relative change (%) of fluorescence intensity at 325 nm (which was the wavelength maximum of the difference spectrum between native and unfolded protein) is given by $(\Delta I/I_{325}) \times 100$, where ΔI is the change in intensity from that in the native state and I_{325} is the intensity of the native protein at 325 nm.

Far-UV CD measurements were made on a JASCO J-600 spectropolarimeter attached to a Peltier type temperature controller. Spectra were collected using a 1 mm pathlength cell at a scan speed of 20 nm/min, a response time of 2 s and a bandwidth of 2 nm. The spectra were averaged over five scans to eliminate signal noise. The buffer was 10 mM HEPES containing 0.15 M NaCl, pH 7.2. All readings were corrected by subtracting the baseline recorded for the buffer having the same concentration of denaturant under similar conditions.

2.4. Protein denaturation

The equilibrium denaturation experiments in urea were performed in 10 mM HEPES buffer containing 0.15 M NaCl, pH 7.2 at different temperatures between 25 and 55 °C. For each denaturation experiment, a known amount of HEPES buffer was mixed with a fixed amount of the protein stock solution and varying amounts of the concentrated denaturant (both in HEPES buffer) in a final volume of 2 mL, and the mixture was incubated up to 24 h at a specified temperature. The monomeric protein concentration was 1.5 μM. The experiments were also carried out in presence of metal ions (10 mM each of Mn^{2+} and Ca^{2+}) or EDTA (10 mM), and at a high protein concentration of 15 μM at 45 °C. The denaturation reactions were studied by measuring the steady-state fluorescence and the far-UV CD. To test the reversibility of denaturation, the protein solution (30 μM), after complete denaturation in 8 M urea, was diluted with 10 mM HEPES buffer containing 0.15 M NaCl, 1 mM Mn^{2+} and 1 mM Ca^{2+}, pH 7.2, to a residual denaturant concentration of 0.4 M urea. The mixture was incubated at 25 °C for 4 h. The final protein concentration during renaturation was 1.5 μM. Reversibility was checked by fluorescence measurement as described above.

The kinetics of denaturation was measured at various temperatures in the range of 40–55 °C by using the time-based acquisition program. A solution of the native protein preincubated at a specified temperature was rapidly added manually to an 8-M urea solution in HEPES buffer in a cuvette maintained at the same temperature, and the solutions were mixed well. The dead time for this procedure was about 5 s. The monomeric protein concentrations were in the range of 1.5–6 μM. The denaturation kinetics was studied by recording the relative emission intensity at 325 nm or the relative CD signal at 226 nm as a function of time. The signals for the native and unfolded proteins were also checked in order to avoid discrepancies. Data were analyzed using the Origin version 6.0 software provided by Microcal Inc.

2.5. Size-exclusion chromatography

The experiments on size-exclusion chromatography were carried out using a Superose-12 10/300 GL column attached to a Pharmacia FPLC system. The column was preequilibrated with 10 mM HEPES buffer, pH 7.2, containing 0.15 M NaCl and the required concentration of urea at room temperature. 500 μL of a protein solution (15 μM) was incubated with required concentration of urea under equilibrium condition at 45 °C, and an aliquot (200 μL) of the mixture was injected into the column. The flow rate was 0.5 mL/min, and eluent was detected on-line by

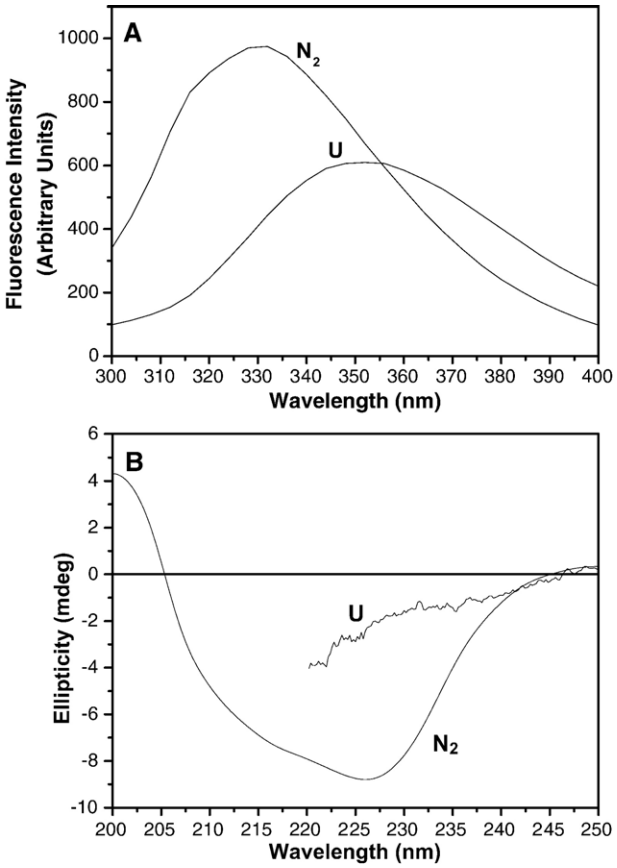

Fig. 1. (A) Fluorescence emission spectra and (B) far-UV CD spectra of EIL in native (N_2) and denatured (U) states at 45 °C. EIL was denatured in 8 M urea in 10 mM HEPES buffer containing 0.15 M NaCl, pH 7.2. The spectra were corrected for the buffers with or without urea. For fluorescence measurements, the monomeric protein concentration was 1.5 μM. Excitation wavelength, 280 nm; excitation and emission band pass, 5 nm each; scan rate, 60 nm/min. For CD measurements, the monomeric protein concentration was 6 μM. The spectra were measured in 1 mm pathlength cell using a scan speed of 20 nm/min, and averaged over five scans.

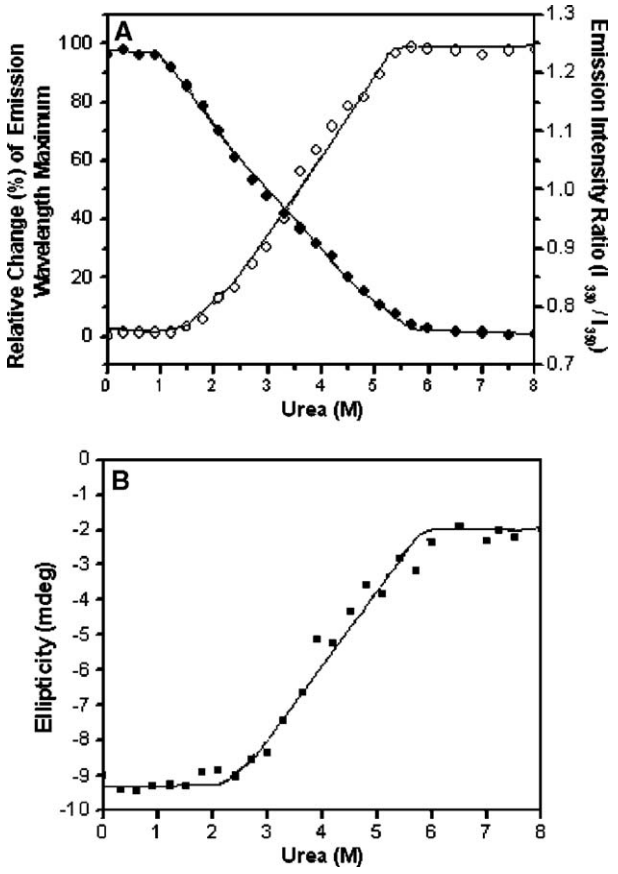

Fig. 2. (A) Urea denaturation curves of EIL at 45 °C measured in terms of relative change of emission wavelength maximum (open circles) and emission intensity ratio at 330 and 350 nm (filled circles) as a function of urea concentration at pH 7.2. Each data point represents average of three determinations. The monomeric protein concentration was 1.5 μM. (B) Urea denaturation profile of EIL at 45 °C as monitored by CD ellipticity at 226 nm. Each data point represents average of two determinations, and the monomeric protein concentration was 6 μM. See Materials and methods for other details.

absorbance at 280 nm. The Superose-12 column was precalibrated with the following marker proteins: bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa) and soybean trypsin inhibitor (20.1 kDa).

3. Results

3.1. Equilibrium denaturation of EIL in urea monitored by fluorescence and circular dichroism

The fluorescence spectra of tryptophan residues are conventionally used as very sensitive probe to the tertiary structure of proteins, as the spectral parameters such as position, shape and intensity are dependent on the electronic and dynamic properties of the chromophore (tryptophan) environment. EIL has 13 tryptophan residues in the dimer [1]. When excited at 280 nm, it exhibited an emission maximum at 330 ± 1 nm. The fluorescence spectra showed progressive red shift along with a decrease in fluorescence intensity upon exposure to gradually increasing concentration of denaturant at a specified temperature. However, the fluorescence spectra of EIL in presence of 8 M urea, after incubation for 24 h at room temperature, did not show any significant change from that in 0 M urea. The protein began to denature partially or completely in 8 M urea at higher temperatures. To study denaturation equilibrium of EIL in a suitable temperature range, we chose experimental conditions where heat, in absence of denaturant, had negligible effect on the structure of the protein as monitored by fluorescence. Thus, under conditions of complete denaturation in urea, the equilibrium unfolding of EIL was investigated in the temperature range of 40–55 °C.

Fig. 1A shows the fluorescence emission spectra of native EIL, and EIL denatured with 8 M urea at 45 °C. As shown, the denatured protein displays a red shift from 330 ± 1 to 350 ± 1 nm, which can be attributed to increased exposure of EIL tryptophans to water upon denaturation in 8 M urea. To confirm the changes in secondary structure of EIL upon denaturation with urea, we examined the far-UV CD spectra of native and denatured protein, which are shown in Fig. 1B. The

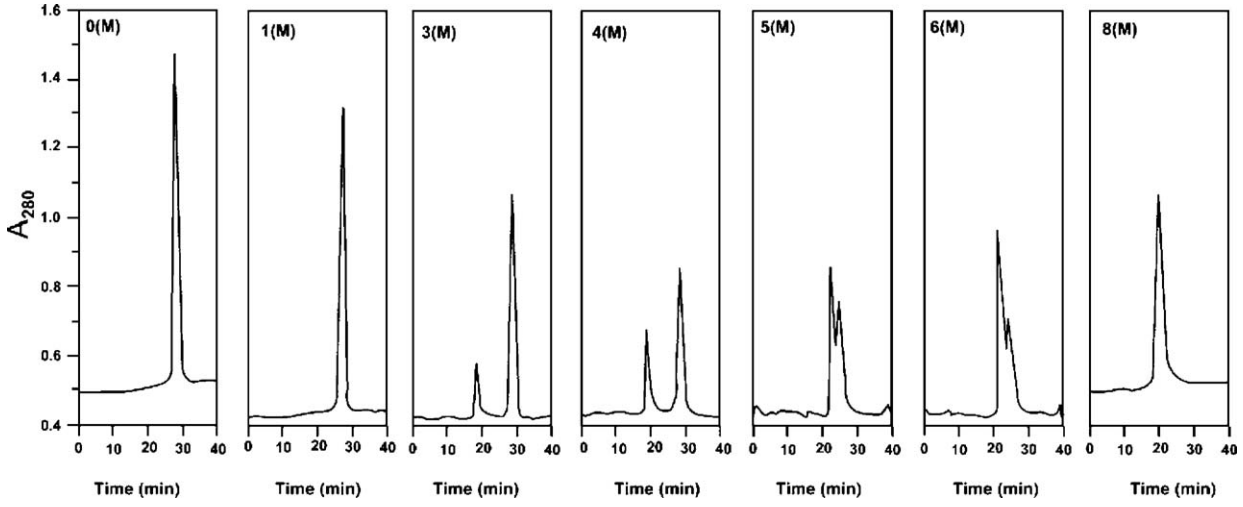

Fig. 3. Elution profiles of EIL from size-exclusion FPLC on Superose-12 10/300 GL column at different concentrations of urea. The protein samples (15 μM) were incubated in presence of requisite concentrations of urea under equilibrium conditions, and an aliquot (200 μL) was loaded onto the column. The flow rate was 0.5 mL/min. The urea concentrations are indicated in the figure. See Materials and methods for other details.

native protein exhibits a far-UV CD band around 226 nm, which is similar to that seen for the galactose-specific legume lectin soybean agglutinin [12]. When denatured in 8 M urea, the CD spectrum of EIL shows the loss of secondary structures for the completely unfolded state of the protein.

The urea denaturation curves for EIL at 45 °C, as monitored by fluorescence, are shown in Fig. 2A. The red shift of emission maximum and the changes in the fluorescence intensity with increasing concentration of urea, serve to delineate the denaturation equilibrium of the protein. The denaturation profile, in terms of relative change of emission maximum as a function of urea concentration, suggests that the EIL unfolding is a two-state process. The denaturation curve monitored by fluorescence intensity ratio at 330 and 350 nm (I_{330}/I_{350}), shown in Fig. 2A, supports the conclusion. Fig. 2B shows the unfolding profile of EIL monitored by far-UV CD measurements. The overall shape of this denaturation curve is similar to that obtained using fluorescence, and there is good agreement between the two spectroscopic techniques. Thus, the urea-induced denaturation of EIL exhibits a monophasic equilibrium.

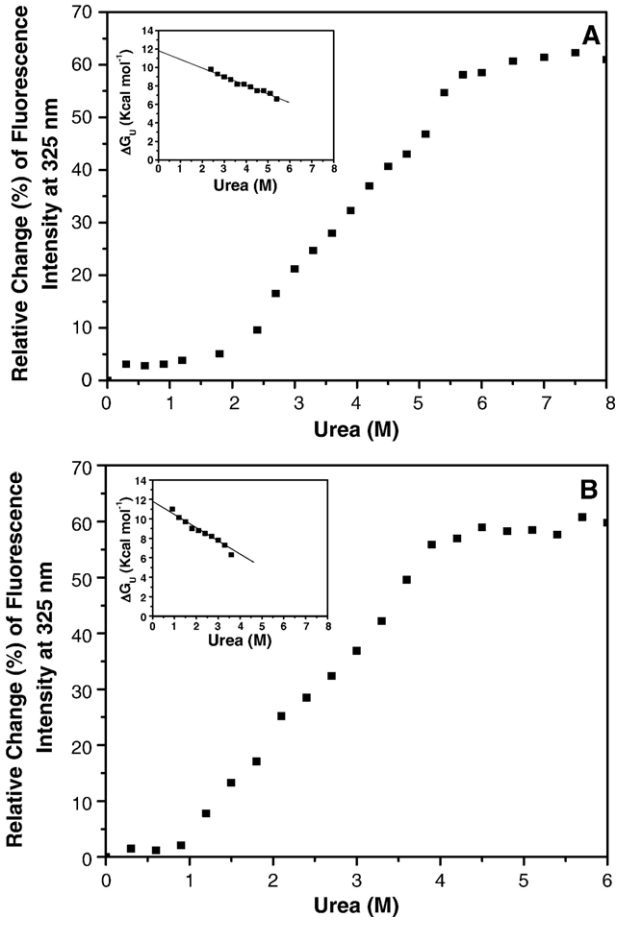

Fig. 4. Equilibrium unfolding transition of EIL measured in terms of relative change of fluorescence intensity at 325 nm as a function of urea concentration at (A) 42 °C and (B) 52 °C. Each data point represents average of three determinations. The monomeric protein concentration was 1.5 μM. (Inset) Plot of ΔG_u versus urea molarity. ΔG_u values were calculated using Eqs. (3) and (4) from the fluorescence data. The line has been drawn according to Eq. (5), using the least squares analysis of the data. See the text for more details.

Table 1
Thermodynamic parameters for equilibrium unfolding of EIL at various temperatures

Temp (°C)	$\Delta G_{u,\,aq}$ (kcal mol^{-1})[a]	m (kcal mol^{-1} M^{-1})	C_m(M, urea)
40	11.5	0.9	3.9
42	11.8	1.0	3.9
45	11.3	0.9	3.4
48	12.4	1.5	2.5
50	11.7	1.4	2.5
52	11.9	1.3	2.5
55	12.3	1.8	2.5

$\Delta G_{u,\,aq}$, and m denote, respectively, the free energy of unfolding in water and the dependence of ΔG on denaturant concentration. C_m is the midpoint denaturant concentration of the unfolding transition. $\Delta G_{u,\,aq}$, m and C_m are calculated from analysis of the fluorescence data as shown in Fig. 4. The protein concentration was 1.5 μM. See the text for details.

[a] Expressed in terms of mole of monomer.

3.2. Equilibrium denaturation of EIL monitored by size-exclusion FPLC

To determine the number of species formed in the equilibrium denaturation pathway of EIL, size-exclusion chromatography on Superose-12 was performed in presence of different concentrations of urea (Fig. 3). As can be seen in the figure, EIL gives a single peak in 0 M and 1 M urea for the native dimer, and a single peak in 8 M urea for the unfolded monomer. It is interesting to note that the unfolded monomer eluted at a lower elution volume than that of the dimeric native protein. This may be due to the extensive randomly coiled conformations of the unfolded monomer compared with that of a compact globular conformation of the native protein [11,19]. It may be mentioned that for a folded, structured monomer, elution volume would be greater than that of the native dimer as was obtained for the marker proteins used in calibration (data not shown). In the pretransition and posttransition regions, a single species corresponding to the native dimer and unfolded monomer, respectively, has been observed. However, in the transition zone, two peaks are observed displaying the unfolding equilibrium (native dimer ⇔ unfolded monomer), and the proportions of dimeric and unfolded proteins vary from 3 to 6 M urea, with gradual decrease in the amount of native protein and consequent increase in the amount of unfolded polypeptide in denaturation equilibrium (Fig. 3). These results thus provide a conclusive evidence for the equilibrium unfolding of EIL as a two-state process.

3.3. Thermodynamic analysis of the unfolding reaction at various temperatures

The urea-induced unfolding of EIL depicts the following two-state mechanism:

$$N_2 \Leftrightarrow 2U \quad (1)$$

where N_2 is the native dimer, and U is the unfolded monomer. These transitions were found to be completely reversible as monitored by fluorescence, and the unfolding process can therefore be treated thermodynamically.

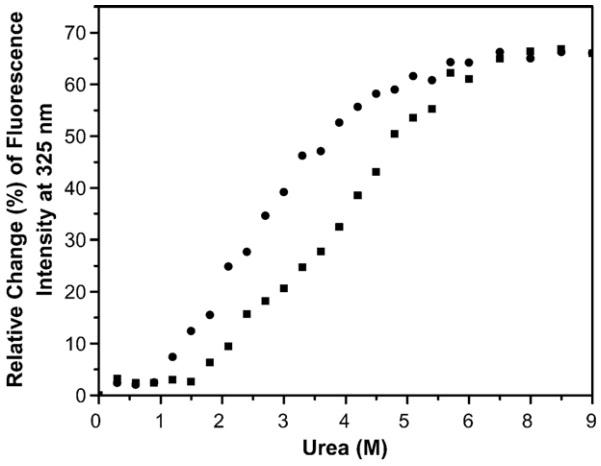

Fig. 5. Effect of protein concentration on the unfolding reaction of EIL monitored by relative changes of fluorescence intensity at 325 nm. The experiments were performed at 45 °C in 10 mM HEPES buffer containing 0.15 M NaCl, pH 7.2. The monomeric protein concentrations used were 0.75 μM (filled circles) and 15 μM (filled squares).

The equilibrium constant for the unfolding reaction (1) is given by:

$$K_u = [U]^2/[N_2] = 2c(f_U)^2/(1-f_U) \quad (2)$$

where $[N_2]$ and [U] are the concentrations of native dimer and unfolded monomer, respectively, c is the total concentration of EIL in monomer units, and f_U is the fraction of protein unfolded.

The free energy of unfolding (ΔG_u) at a given concentration of denaturant is then obtained from the equation:

$$\Delta G_u = -RT1nK_u = -RT1n[2c(f_U)^2/(1-f_U)] \quad (3)$$

f_U is given by:

$$f_U = (Y_{obs} - Y_{N2})/(Y_U - Y_{N2}) \quad (4)$$

where Y_{obs} is the observed value of signal monitored at a particular concentration of denaturant in the transition region, and Y_{N2} and Y_U are the values characteristic of the native dimer and unfolded monomer, respectively.

ΔG_u depends on the denaturant concentration according to the linear free energy model [15,20],

$$\Delta G_u = \Delta G_{u,aq} - m[D] \quad (5)$$

where m is the slope of the plot of free energy of unfolding versus denaturant concentration [D], and $\Delta G_{u,\ aq}$ is the free energy of unfolding reaction in water.

The thermodynamic parameters for the urea-induced EIL unfolding at different temperatures have been determined from the denaturation melts monitored by fluorescence. As the fluorescence intensity rather than emission maximum is the preferred signal for quantitative analysis of the thermodynamics of the unfolding transition [21], the fluorescence intensity signal has been used for the purpose. Native EIL shows an emission maximum at 330 nm, which shifts to 350 nm on denaturation with 8 M urea. The difference spectrum shows a maximum at 325 nm, and this was chosen as the emission wavelength at which the fluorescence intensities were monitored. Figs. 4A and B show the denaturation melts at 42 °C and 52 °C, respectively, measured in terms of the relative change of the fluorescence intensity at 325 nm as a function of urea concentration. From the fluorescence data as shown in the figures, the midpoint of the transition (C_m) where 50% of the protein was unfolded, and the parameters $\Delta G_{u,\ aq}$ and m have been estimated using Eqs. (2), (3), (4) and (5), and the results are shown in Table 1. It is interesting to note that the values of $\Delta G_{u,\ aq}$, a measure of protein stability, are similar in the temperature range of 40–55 °C, and lie within 11.8 ± 0.6 kcal mol^{-1} (monomer units). The temperature dependent EIL unfolding in urea, however, leads to significant changes in the values of m and C_m (Table 1). The C_m values decrease with increase in temperature, being 3.9 M at 40 °C and 2.5 M at 48–55 °C. The m values show a similar pattern. This resemblance in the changes of the C_m and m values with temperature may be attributed to the concomitant increase in the extent of denaturation and solvent-accessible area per unit concentration in the transition region.

Table 2
Effect of protein concentration and metal ions on the thermodynamic properties of EIL unfolding in urea at 45 °C

Condition	$\Delta G_{u,\ aq}$ (kcal mol^{-1})[a]	m (kcal mol^{-1} M^{-1})	C_m (M, urea)
0.75 μM EIL	10.5	1.2	2.7
15.0 μM EIL	11.3	1.1	4.1
1.5 μM EIL + 10 mM Mn^{2+}, Ca^{2+}	10.9	1.2	3.5
1.5 μM EIL + 10 mM EDTA	10.7	1.1	3.4

$\Delta G_{u,\ aq}$, m and C_m are calculated from the fluorescence data. See the text for details.

[a] Expressed in terms of mole of monomer.

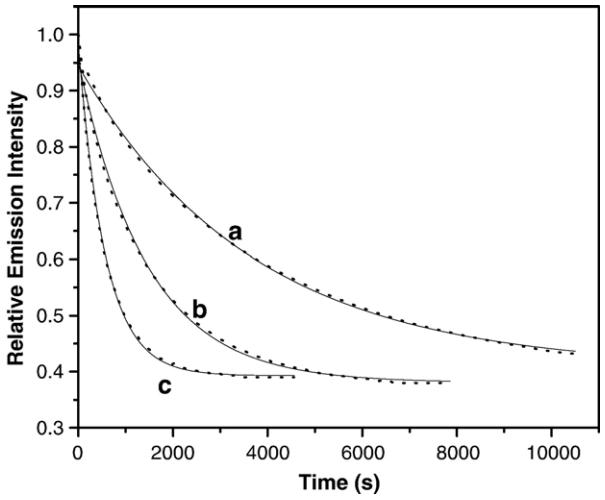

Fig. 6. Kinetics of EIL unfolding at 45 °C (a), 50 °C (b) and 55 °C (c) as monitored by fluorescence. The emission intensities are the values relative to that in the native state at 325 nm. The monomeric protein concentration was 6 μM. The thick line represents the theoretical curve for a single exponential function according to Eq. (6). The best-fit values of the parameters are (a) $A_1 = 0.54$, $A_\infty = 0.41$, $k_1 = 2.8 \times 10^{-4}$ s^{-1}; (b) $A_1 = 0.56$, $A_\infty = 0.38$, $k_1 = 7.1 \times 10^{-4}$ s^{-1}; (c) $A_1 = 0.58$, $A_\infty = 0.39$, $k_1 = 1.8 \times 10^{-3}$ s^{-1}.

Table 3
Rate constants for urea-induced unfolding of EIL at various temperatures

Temperature (°C)	Apparent rate constant, k_1(s^{-1})	
	Fluorescence[a]	Circular dichroism[a]
40	1.2×10^{-4}	n.d.
45	2.8×10^{-4}	4.6×10^{-4}
50	7.1×10^{-4}	1.4×10^{-3}
55	1.8×10^{-3}	5.5×10^{-3}

n.d. = not determined.
[a] The protein concentration was 6 μM.

3.4. Effect of protein concentration and metal ions on equilibrium denaturation of EIL

In order to characterize the unfolding equilibrium further, we examined the dependence of protein concentration and the role of metal ions (Mn^{2+}, Ca^{2+}) on the unfolding reaction of EIL. Fig. 5 shows the denaturation profiles of EIL at 45 °C at a protein concentration of 0.75 and 15 μM, respectively. It is clearly seen that the unfolding transition is dependent on the concentration of the protein. The thermodynamic parameters of these reactions are given in Table 2. While the $\Delta G_{u,\,aq}$ and m values are similar, the most noticeable difference occurs in the C_m values, being 2.7 and 4.1 M at a protein concentration of 0.75 and 15 μM, respectively. These observations are consistent with the two-state mechanism of the unfolding reaction Eq. (1). As the native dimer and unfolded polypeptide populate the transition zone as equilibrium species, the fraction of molecules in the native state increases with protein concentration Eq. (2), and consequently the C_m shifts to higher denaturant concentration.

As EIL is a metalloprotein containing Mn^{2+} and Ca^{2+}, the equilibrium denaturation curves of EIL in presence of external Mn^{2+} and Ca^{2+}(10 mM each), and in EDTA (10 mM) which complexes with the metal ions during unfolding, have been measured, which also display monophasic behavior (data not shown). The thermodynamic properties estimated for these unfolding reactions are shown in Table 2. The values of $\Delta G_{u,\,aq}$, m and C_m are similar in both cases. These results indicate that the metal ions are not involved in modulating the unfolding equilibrium of the protein.

3.5. Kinetic studies of urea-induced EIL denaturation monitored by fluorescence and CD

The unfolding kinetics of EIL was monitored by following changes of fluorescence at 325 nm, and by changes in the CD signal at 226 nm, respectively, in the temperature range of 40–55 °C. Fig. 6 shows typical kinetic traces at three temperatures (45, 50 and 55 °C) measured by fluorescence. The kinetic parameters have been determined by nonlinear least-squares fitting to the equation:

$$A(t) = \sum A_i \exp(-k_i t) + A_\infty \quad (6)$$

where $A(t)$ is the value of fluorescence intensity or CD signal at a given time t, A_i is the amplitude corresponding to each individual phase (i), k_i is the associated rate constant, and A_∞ is the amplitude at infinite time.

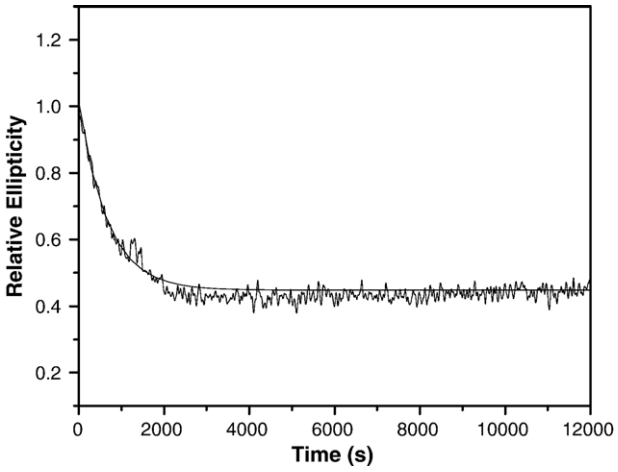

Fig. 7. Kinetics of EIL unfolding at 50 °C as monitored by CD. The ellipticity values are relative to that in the native state at 226 nm. The monomeric protein concentration was 6 μM. The thick line represents the theoretical curve for a single exponential function according to Eq. (6). The best-fit values of the parameters are $A_1 = 0.56$, $A_\infty = 0.44$, $k_1 = 1.4 \times 10^{-3}$ s^{-1}.

The unfolding kinetics, as measured by fluorescence (Fig. 6), follows a single exponential decay pattern, and the apparent rate constants determined are given in Table 3. It is seen that the unfolding reaction becomes 15 times faster when the temperature changes from 40 to 55 °C, and the rate constant (k_1) increases about 2.5 times for an increase of temperature by 5 °C. A typical kinetic trace monitored by CD at 50 °C is shown in Fig. 7. The data fit well to a single exponential equation, and the apparent rate constants at different temperatures are shown in Table 3. The rate constant increases 3–4 fold for a change of temperature by 5 °C. The results monitored by fluorescence and CD thus show reasonable agreement for the temperature dependent EIL unfolding that obeys first-order kinetics.

3.6. Effect of various probes on the unfolding kinetics of EIL

The kinetics of EIL unfolding was compared at 45 °C in presence of several probes (metal ions—Mn^{2+} and Ca^{2+}, EDTA, and the binding ligand—lactose), and the apparent rate constants obtained from the fluorescence data are shown in Table 4. The results show that the external metal ions do not affect the kinetics of EIL unfolding ($k_1 = 2.9 \times 10^{-4}$ s^{-1}); however, the presence of EDTA which complexes with the metal ions of the protein during

Table 4
Comparison of kinetics for EIL unfolding using several probes as monitored by fluorescence at 45 °C

Protein concentration (μM)	Probe	Apparent rate constant, k_1 (s^{-1})
1.5	none	2.9×10^{-4}
1.5	Mn^{2+}, Ca^{2+} (1 mM each)	2.8×10^{-4}
1.5	EDTA (1 mM)	8.8×10^{-4}
1.5	lactose (10 mM)	1.8×10^{-4}

unfolding, makes the reaction 3 times faster ($k_1 = 8.8 \times 10^{-4}$ s^{-1}) whereas the unfolding reaction in presence of lactose becomes slightly slower ($k_1 = 1.8 \times 10^{-4}$ s^{-1}).

4. Discussion

EIL is a dimeric legume lectin belonging to *Erythrina* family. Unlike the legume lectins—concanavalin A (ConA), soybean agglutinin (SBA) and *Erythrina corallodendron* lectin (ECorL) (a member of *Erythrina* family), EIL displays an intriguing structural stability at ambient temperature. Upon incubation in 8 M urea for 24 h, EIL showed no melting of structure at 25 °C. It started to denature partially, and then completely in presence of 8 M urea at physiological pH as the temperature was progressively increased. This leads to the questions: To what does EIL owe its capacity to survive under highly denaturing conditions? Is this problem principally a thermodynamic or kinetic one? In order to address the issue, we have examined in the present study the combined effect of heat and denaturant in the unfolding of EIL. In general, the unfolding studies involve either thermal denaturation [22] or chemical denaturation with urea or guanidine hydrochloride [15]. The present experimental approaches are so designed that heat may influence the denaturant-induced unfolding of EIL but exert negligible effect on its structure in absence of denaturant. Under these conditions, the experiments have been carried out in the temperature range of 40–55 °C for investigating the courses of urea-induced equilibrium and kinetic denaturation of EIL.

The nature of the equilibrium denaturation profiles of EIL as monitored by intrinsic fluorescence and far-UV CD (Fig. 2) suggests a two-state unfolding from the folded dimer to the unfolded monomer. As the two spectroscopic techniques probe different structural characteristics of the protein (tertiary structure by fluorescence and secondary structure by far-UV CD), similar loss in protein structure by the two methods is generally taken as an evidence for a two-state mechanism. However, with oligomeric proteins, care should be taken to apply this criterion. If the dissociation of an oligomeric protein leads to an intermediate monomer with similar secondary and tertiary structures as of native protein, no appreciable difference in fluorescence intensity and CD signal may occur between the native protein and the intermediate, as is seen in case of SBA [12], and consequently a two-state process cannot be ascertained from the similarity of fluorescence intensity and CD profiles. Thus, for oligomeric proteins, other probes are necessary to vindicate a two-state unfolding. The results of size-exclusion FPLC (Fig. 3) rule out the presence of any equilibrium intermediate during unfolding of EIL. The two-state pathway is also supported by the protein concentration dependence of the unfolding reaction (Fig. 5) when the midpoint of transition (C_m) shifts to higher denaturant concentration with increase in protein concentration (Table 2). The absence of intermediate monomer during EIL unfolding suggests that intersubunit interactions play a dominant role in the stability of these proteins. This seems to be generally true for the legume lectins [23]. Even when an intermediate appears in their unfolding pathway, as in the denaturation of ConA [11], the structural stability may still be maintained mostly by association of subunits.

The thermodynamic evaluation of two-state behavior reveals an important feature of the unfolding of EIL. It is seen that the free energy of stabilization ($-\Delta G_{u,\ aq}$) remains similar at different temperatures of the experiment (Table 1). This result implies that the action of heat has no or negligible effect on thermodynamics of the denaturation reaction in the temperature range of 40–55 °C. The present results of equilibrium unfolding of EIL are in sharp contrast to that reported for the guanidine hydrochloride-induced unfolding of the *Erythrina* lectin ECorL [8]. The denaturation of ECorL has been described at temperatures from 7 to 45 °C showing a dependence of free energy with temperature. In comparison, at temperatures below 40 °C the EIL unfolding did not either occur or go to completion. This may be attributed to a greater stability of EIL over ECorL though the unfolding transitions are similar, being two-state, in both cases. The free energy of stabilization of the two *Erythrina* lectins may be compared at a particular temperature. At 45 °C, the free energy of unfolding of ECorL is reported to be 13.8 kcal mol^{-1} while that of EIL in the present study is obtained as 22.6 kcal mol^{-1} (dimer units). This clearly indicates the greater structural stability of EIL compared to ECorL at 45 °C. Further, metal ions (Mn^{2+}, Ca^{2+}) are involved in modulating the folding events of ECorL [8]; in contrast, EIL unfolding is not influenced by the presence of external metal ions or of EDTA which complexes with the metal ions during unfolding, and the thermodynamic parameters of unfolding in two cases are similar (Table 2). It thus appears that the two proteins from the same family of plants of genus *Erythrina*, with similar physicochemical properties, differ remarkably in their stability and unfolding behavior, and in their responses to denaturing conditions. However, in absence of the three-dimensional structure of EIL, these differences cannot be further delineated at present.

As the problem of EIL unfolding with heat and denaturant does not appear to be a thermodynamic one, we turn to the kinetic issue. While studying the unfolding kinetics by fluorescence and far-UV CD, it has been found that the unfolding reaction follows a single exponential decay pattern, and the intermediacy of a monomer prior to denaturation remains undetected. The strong intersubunit interactions may be responsible for the native dimer behaving as a single molecular species that dissociates and unfolds at once. Further, the increase in protein concentration from 1.5 to 6 μM did not change the rate of the reaction as monitored by fluorescence (Tables 3 and 4), which gives further evidence for the decay of the native dimer directly to the unfolded monomer. The increase in temperature of the EIL unfolding reaction, however, unfolds a profound effect on the reaction kinetics. The rate increases 15-fold as the temperature is raised from 40 to 55 °C, and the values of rate constants increase appreciably with increase in temperature (Table 3). This observation reveals that the action of heat in the urea-induced denaturation of EIL is linked to the kinetics of the reaction. It may also be noted that the external metal ions (Mn^{2+}, Ca^{2+}) do not influence the kinetics, but in presence of EDTA, the unfolding rate increases by a factor of 3

(Table 4). During unfolding, metal ions are released from the protein, and the reaction may be favored by complexation of the metal ions with EDTA. Further, the kinetics of the reaction becomes a little slower in presence of the binding ligand, lactose (Table 4). Thus, the unfolding process is influenced by these probes kinetically.

In conclusion, the thermodynamics of urea-induced unfolding of EIL indicates a high conformational stability of the protein. It is evident from the equilibrium and kinetic studies that the EIL dimer behaves as a single molecular species in the unfolding reaction probably due to the strong intersubunit interactions between the monomers. However, the most striking results are with respect to the ability of EIL to resist highly denaturing conditions. The results suggest that the unusual structural stability of EIL under such conditions is primarily linked to its kinetic stability, and the synergy of heat and denaturant operates in the unfolding of the protein. These findings may provide insight into the folding and structural stability of legume lectins, and establish model systems for the investigation of the thermodynamic and kinetic stability of oligomeric proteins in general.

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