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Conformational transitions of cinnamoyl CoA reductase 1 from *Leucaena leucocephala*

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ABSTRACT

Conformational transitions of cinnamoyl CoA reductase, a key regulatory enzyme in lignin biosynthesis, from *Leucaena leucocephala* (Ll-CCRH1) were studied using fluorescence and circular dichroism spectroscopy. The native protein possesses four trp residues exposed on the surface and 66% of helical structure, undergoes rapid structural transitions at and above 45 °C and starts forming aggregates at 55 °C. Ll-CCRH1 was transformed into acid induced (pH 2.0) molten globule like structure, exhibiting altered secondary structure, diminished tertiary structure and exposed hydrophobic residues. The molten globule like structure was examined for the thermal and chemical stability. The altered secondary structure of L1-CCRH1 at pH 2.0 was stable up to 90 °C. Also, in presence of 0.25 M guanidine hydrochloride (GdnHCl), it got transformed into different structure which was stable in the vicinity of 2 M GdnHCl (as compared to drastic loss of native structure in 2 M GdnHCl) as seen in far UV-CD spectra. The structural transition of Ll-CCRH1 at pH 2.0 followed another transition after readjusting the pH to 8.0, forming a structure with hardly any similarity to that of native protein.

1. Introduction

Leucaena leucocephala, a multipurpose nitrogen fixing tropical legume tree, has a tremendous prospective as a raw material for paper and pulp industry, nutritious forage, timber, organic fertilizer, firewood, industrial fuel and depilatory agent due to its adaptability to thrive under farthest agro-climatic conditions [1–3]. Although lignin plays a major role in growth and development of plants, an agro-economical opinion considers lignin as an obstacle for utilization of plant biomass as it affects many industrial processes [4]. The biosynthesis of lignin precursors proceeds through common phenylpropanoid pathway, starting with conversion of phenylalanine to cinnamate and subsequent formation of hydroxycinnamoyl CoA esters [5]. These esters are the end products of common phenylpropanoid metabolism. In monolignol biosynthesis, cinnamoyl CoA reductase (CCR, EC 1.2.1.44) catalyzes the NADPH dependent reduction of cinnamoyl CoA esters to corresponding hydroxycinnamaldehydes. As the first committed step in monolignol biosynthesis, CCR plays a key regulatory role by controlling the overall carbon flux of metabolites towards lignin [6].

The characterization of unfolded and partly folded conformations is vital to understand the principles governing protein folding and its stability [7,8]. Many studies have shown that protein folding is a discrete pathway with distinct intermediate states between native and denatured one. These intermediate states have been observed under different conditions from native to completely unfolded states and analysis of these intermediates provides an insight into protein folding pathway [9–12].

Although CCR is one of the most investigated enzymes in lignin biosynthesis pathway, its three dimensional crystal structure remains to be determined [13–19]. Also, hardly any reports on CCR conformation and structure-function relationship are available. The recombinant Ll-CCRH1 from *L. leucocephala* has already been purified and characterized by us [20,21]. The enzyme was able to catalyze CoA ester moieties which had OH, OCH_3 substituents at 3 and 5 positions with different rates. Also, the enzyme showed stringent conditions of pH optimum for forward (pH 6.5) and reverse (pH 7.8) catalysis. In the present study, as a step towards structural characterization, the Ll-CCRH1 was subjected to thermal, chemical as well as acid and alkali induced denaturation. Structural transitions in Ll-CCRH1 were monitored using biophysical techniques to understand its conformational stability. Here, we also report the existence and characterization of an acid induced molten globule like intermediate at pH 2.0.

2. Materials and methods

2.1. Materials

Guanidine hydrochloride (GdnHCl) and 1-anilino-8-napthalenesulfonate (ANS) were obtained from Sigma, USA.

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All other reagents were of analytical grade. Solutions prepared for spectroscopic measurements were in sterile Milli Q water.

2.2. Heterologous expression and purification of recombinant CCRH1 from L. leucocephala

Heterologous expression, purification and assay of the Ll-CCRH1 enzyme with cinnamoyl CoA esters were carried out as described earlier [20].

2.3. Fluorescence measurements

Intrinsic fluorescence of the Ll-CCRH1 enzyme was measured at 30 °C using a Perkin–Elmer LS 50B spectrofluorimeter connected to a Julabo F20 circulating water bath. The protein solution (0.47 μM) was excited at 295 nm and emission was recorded in the range of 310–400 nm. Both the excitation and emission spectra were obtained by setting the slit width at 7 nm, and a scan speed of 100 nm/min. To eliminate the background emission, the signal produced by either buffer solution, or buffer containing the desired quantity of denaturants was subtracted.

2.4. Decomposition analysis of fluorescence spectra

The decomposition analysis of trp fluorescence spectra was carried out using PFAST program (http://pfast.phys.uri.edu/pfast/) based on the SIMS and PHREQ algorithm as described elsewhere [22].

2.5. Circular dichroism (CD) measurements

The CD spectra of the enzyme were recorded on a Jasco 815-1505 (Jasco, Tokyo, Japan) spectropolarimeter connected to a Peltier Type CD/FL cell circulating water bath (Jasco, Tokyo, Japan) at 25 °C. Far UV CD measurements of protein (1.44 μM) were recorded in the wavelength range of 190–250 nm with a 1 mm path length cell. Each CD spectrum was accumulated from five scans of 50 nm/min with a 1 nm slit width and a time constant of 1 s for a nominal resolution of 0.5 nm. Near UV CD measurements were recorded at 0.5 mg/ml protein concentration in the wavelength range of 250–300 nm with a 1 cm path length cell.

All spectra were corrected by subtracting buffer contributions and observed values were converted to mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ defined as:

$$\text{MRE} = \frac{M\theta_\lambda}{10dcr}$$

where M is the molecular weight of protein, θ_λ is CD in millidegree, d is the path length in cm, c is the protein concentration in mg/ml and r is the average number of amino acid residues in the protein. Secondary structure elements were calculated by using CD pro software available online.

2.6. Effect of pH

Ll-CCRH1 samples (0.47 μM) were incubated in an appropriate buffer over the pH range (2–12) at 25 °C. The following buffers (20 mM) were used for these studies: Glycine–HCl (pH 2–3), acetate (pH 4–5), phosphate (pH 6–7), Tris–HCl (pH 8–9) and glycine–NaOH (pH 11–12). Fluorescence spectra were recorded as described in Section 2.3. For refolding experiments, the pH of each sample was adjusted back to pH 8.0 and incubated for 25 °C for 1–2 h before recording the spectral measurement. Effect of pH on the secondary structure was studied by incubating the protein samples in 20 mM buffers in pH range 2.0–12.0 and taking the far UV and/or near UV CD scans as described above.

2.7. Thermal denaturation of Ll-CCRH1

Effect of temperature on Ll-CCRH1 was monitored using a thermostatic cuvette holder connected to an external constant temperature circulation water bath in the range of 25–90 °C. The protein sample (0.47 μM) was incubated for 10 min at specified temperature before taking fluorescence scan. For renaturation, the samples were cooled to 25 °C in two ways (90 °C to 25 °C and in other, 50 °C to 25 °C) and left for 1–2 h before recording the spectra. Fluorescence measurements were performed as described in Section 2.3. The effect of temperature on the protein was studied by two methods. In first method, the protein samples were incubated for 5 min at different temperatures ranging from 25 to 90 °C and CD scans were recorded independently. In other method, the temperature of the protein sample was increased at the rate of 1 °C/min for the temperature ranging from 25 to 90 °C and ellipticity was recorded at 208 nm.

2.8. Hydrophobic dye binding studies

The intermediate states of Ll-CCRH1 during unfolding and refolding under different denaturing conditions (pH, temperature and GdnHCl) were analyzed by performing hydrophobic dye (ANS) binding studies. ANS emission spectra were recorded in the range of 410–550 nm with excitation at 375 nm using slit widths of 7 nm each for excitation and emission monochromators. The final ANS concentration used was 50 μM. The spectrum of ANS in the buffer of respective pH or in desired concentration of GdnHCl was subtracted from the combined protein-ANS spectrum.

2.9. Guanidine hydrochloride (GdnHCl) mediated unfolding

Protein samples (0.47 μM) were incubated in 0–6 M GdnHCl solution at pH 8.0 for 4 h to attain the equilibrium. For refolding experiments, the protein samples in 10-fold excess concentration was first denatured in 6 M GdnHCl at 25 °C for 16 h and subsequently diluted ten times in buffer containing 0–5.5 M GdnHCl and, incubated at 25 °C for 2 h. Fluorescence were recorded as described above. Effect of GdnHCl on the secondary structure was studied by incubating the protein samples (1.44 μM) in 20 mM buffers at pH 8.0 in the presence of 0.25–6.0 M GdnHCl and taking the far UV CD scans as described above.

2.10. Light scattering

Protein aggregation upon thermal or pH denaturation was monitored using Rayleigh light scattering with fluorescence spectroscopy. Both excitation and emission wavelengths and slit widths were set at 400 nm and 5 nm and 2.5 nm, respectively. Scattering was recorded for 60 s.

3. Results and discussion

Biochemical properties of recombinant Ll-CCRH1 were studied in detail and have been reported by Sonawane et al. [20]. Conformational transition studies of the enzyme are presented here.

3.1. Monitoring conformation of Ll-CCRH1 at different pH

3.1.1. Fluorescence measurements

The native Ll-CCRH1, a multi tryptophan protein (Trp 34, 169, 156 and 182; GenBank ID: DQ986907) showed maximum intensity of intrinsic fluorescence, λ_{max} at 352 nm (Supplementary Fig. S1A) indicating the trp residues to be exposed to solvent. Fluorescence intensities of the enzyme incubated in the ranges of pH 2–5 and pH 11–12 were significantly reduced as compared to that at pH