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SHORT COMMUNICATION

Steady State Fluorescence Studies of Wild Type Recombinant Cinnamoyl CoA Reductase (Ll-CCRH1) and its Active Site Mutants

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Abstract Fluorescence quenching and time resolved fluorescence studies of wild type recombinant cinnamoyl CoA reductase (Ll-CCRH1), a multitryptophan protein from *Leucaena leucocephala* and 10 different active site mutants were carried out to investigate tryptophan environment. The enzyme showed highest affinity for feruloyl CoA (K_a=3.72×10^5 M^{-1}) over other CoA esters and cinnamaldehydes, as determined by fluorescence spectroscopy. Quenching of the fluorescence by acrylamide for wild type and active site mutants was collisional with almost 100 % of the tryptophan fluorescence accessible under native condition and remained same after denaturation of protein with 6 M GdnHCl. In wild type Ll-CCRH1, the extent of quenching achieved with iodide (f_a=1.0) was significantly higher than cesium ions (f_a=0.33) suggesting more density of positive charge around surface of trp conformers under native conditions. Denaturation of wild type protein with 6 M GdnHCl led to significant increase in the quenching with cesium (f_a=0.54), whereas quenching with iodide ion was decreased (f_a=0.78), indicating reorientation of charge density around trp from positive to negative and heterogeneity in trp environment. The Stern-Volmer plots for wild type and mutants Ll-CCRH1 under native and denatured conditions, with cesium ion yielded biphasic quenching profiles. The extent of quenching for cesium and iodide ions under native and denatured conditions observed in active site mutants was significantly different from wild type Ll-CCRH1 under the same conditions. Thus, single substitution type mutations of active site residues showed heterogeneity in tryptophan microenvironment and differential degree of conformation of protein under native or denatured conditions.

Keywords Active site mutants · Cinnamoyl CoA reductase · Fluorescence · Ligand binding · Solute quenching

Introduction

Lignin biosynthesis is a significant part of phenylpropanoid pathway which leads to formation of monolignols and subsequently lignin [1]. Among several enzymes involved in lignin biosynthesis, cinnamoyl CoA reductase (CCR, EC 1.2.1.44) catalyzes the first committed step and plays a key regulatory role in lignin formation [2]. Hydroxycinnamoyl CoA esters of general phenylpropanoid pathway when acted upon by CCR become destined to form respective monolignols. During the last two decades, significant headway has been made in characterizing CCR genes from variety of plants like *Arabidopsis*, poplar, *Medicago* and wheat [3–8]. Being entry point enzyme in lignin biosynthesis, CCR has proven to be good target for down regulation to reduce the lignin content, to improve saccharification efficiency in bioenergy crops without compromising yields, to enhance the forage quality and produce optimal feedstock plants for biofuel production [9, 10]. CCR exhibits substrate specificity for different hydroxycinnamoyl CoA esters and hydroxycinnamaldehydes [11–13].

Studies on the intrinsic fluorescence properties have been widely used to obtain the information about the protein structure and the conformational changes induced by alteration of the environment and/or ligand binding [14–17]. Among

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several intrinsic fluorescent probes, tryptophan is the most popular probe. The fluorescence of the indole chromophore is highly sensitive to environment, making it an ideal choice for reporting protein conformation changes and interaction with other molecules. Also, indole fluorescence quenching by added solutes have provided valuable information regarding structure and dynamics of protein in solution [15, 18–20].

Structure-functional studies on CCR reported in literature are very few; in fact no studies regarding intrinsic fluorescence and characterization of trp microenvironment have been reported so far. As a first step towards understanding structure-function relationship, we isolated, cloned, overexpressed, purified and characterized cinnamoyl CoA reductase 1 (Ll-CCRH1) from *Leucaena leucocephala* in detail [13, 21, 22]. Active site characterization of Ll-CCRH1 was carried out using modeling/docking, site directed mutagenesis and chemical modification studies [23]. Conformational transitions of Ll-CCRH1 were studied using fluorescence and circualar dichroism (CD) spectroscopy [24]. The present paper describes the exposure and differential environment of tryptophan residues in wild type recombinant Ll-CCRH1 and active site mutants on the basis of steady state fluorescence and solute quenching studies. To the present author's knowledge, this is the first report on fluorescence studies of CCR from plant species.

Materials and Methods

Materials

Acrylamide, potassium iodide, cesium chloride and sodium thiosulphate, were purchased from Sigma-Aldrich, USA. All other reagents used were of high purity and analytical grade. Solutions prepared for spectroscopic measurements were in sterile MilliQ water.

Heterologous Expression and Purification of Recombinant Ll-CCRH1 from *L. Leucocephala*

Heterologous expression, purification and assay of the Ll-CCRH1 enzyme with cinnamoyl coA esters were carried out as described by Sonawane et al. [13].

Steady State Fluorescence

Intrinsic fluorescence of the protein 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.

Ligand Binding Analysis

Fluorescence measurements were carried out as described in Section 2.2. The binding of CoA esters to Ll-CCRH1 was studied by intrinsic fluorescence titrations. The CoA ester/ligand solution was added in 10–12 aliquots (1–10 μl). The ligands were used in the concentration of 1 mM forward substrates (feruloyl CoA and sinapoyl CoA), reverse substrates (coniferaldehyde and sinapaldehyde), and cofactor (NADPH and $NADP^+$). The fluorescence intensity at 352 nm (λ_{max} of the protein) was considered for further analysis. Corrections were also made to compensate the dilution effect upon addition of CoA ester/ligand to the Ll-CCRH1.

The association constants were calculated according to the method described by Chipman et al. [25]. The abscissa intercept of the plot of log $[C]_f$ against log $\{(\Delta F)/(Fc–F_\infty)\}$, where $[C]_f$ is the free ligand concentration, yielded pK_a value for protein-ligand interaction according to the relationship

$$\log[F_o - F_c / F_c - F_\infty] = \log K_a + \log\{[C]_t - [P]_t(\Delta F/\Delta F_\infty)\} \quad (1)$$

where F_c is the fluorescence intensity of the protein at any point during the titration, $[P]_t$ is the total protein concentration, ΔF_∞ is the change in fluorescence intensity at saturation binding, $[C]_t$ is the total ligand concentration, and $[C]_f$ is the free ligand concentration, given by,

$$[C]_f = \{[C]_t - [P]_t(\Delta F/\Delta F_\infty)\} \quad (2)$$

Free energy changes of association (ΔG) were determined by the equation,

$$\Delta G = -RT \ln K_a \quad (3)$$

Solute Quenching Studies

Fluorescence measurements were performed for native and denatured protein with different quenchers like acrylamide (5 M) (neutral quencher), iodide (5 M) and cesium ions (5 M) (charged quenchers), as described above. Small aliquots of quencher stock solutions were added to protein samples and fluorescence spectra were recorded after each addition. Sodium thiosulphate (0.2 M) was added to the iodide stock solution to prevent the formation of tri-iodide ions (I^{-3}). For quenching studies with denatured Ll-CCRH1, the protein was incubated in 6 M GdnHCl overnight at room temperature.

Volume correction was done for fluorescence intensities before analyzing the quenching data.

The steady state fluorescence quenching data obtained with different quenchers were analyzed by Stern-Volmer (Eq. 4) and modified Stern-Volmer (Eq. 5) equations in order to obtain quantitative quenching parameters [19, 26].

$$F_o/F_c = 1 + K_{sv}[Q] \quad (4)$$

$$F_o/\Delta F = f_a^{-1} + 1/[K_a f_a(Q)] \quad (5)$$

Where F_o and F_c are the relative fluorescence intensities in the absence and presence of quencher, respectively, [Q] is the quencher concentration, K_{sv} is Stern-Volmer quenching constant, $\Delta F = F_o - F_c$ is the change in fluorescence intensity at any point in the quenching titration, K_a is the corresponding Stern-Volmer constant for accessible fraction of the total fluorophores and f_a is the fraction of the total fluorophore accessible to quencher. Equation 4 shows that the slope of the plot of $F_o/\Delta F$ versus $[Q]^{-1}$ (modified Stern-Volmer plot) gives the value of $(K_a f_a)^{-1}$ and its Y-intercept gives the value of f_a^{-1}.

Fluorescence Measurements and Quenching Studies of Active Site Mutants

Active site characterization of Ll-CCRH1 was previously carried out by means of molecular, computational and biochemical methods. Putative active site residues identified in homology modeling and docking studies were further confirmed by site directed mutagenesis and chemical modification. Active site of Ll-CCRH1 is made up of 10 residues, that is, Phe30, Ile31, Arg51, Asp77, Ser136, Tyr170, Lys174, Val200, Ser212 and His215 [23]. Respective 10 site directed mutant proteins were generated for each of active site residue. These active site mutants were constructed, cloned, over-expressed and purified similarly as that of wild type recombinant Ll-CCRH1 [23]. Fluorescence measurements and quenching studies of active site mutants were performed as described in Section 2.2 and 2.4.

Lifetime Measurement of Fluorescence Decay

Lifetime fluorescence measurements were carried out on an FLS920 single photon counting spectrometer supplied by Edinburgh instruments. A xenon flash lamp of pulse width 1 ns was used for excitation and a synchronization photomultiplier was used to detect fluorescence. The diluted Ludox solution was used for measuring Instrument Response Function (IRF). Protein sample (0.2 mg/ml) was excited at 295 nm and emission was recorded at 352 nm. Slit widths of 10 nm each were used on the excitation and emission monochromators. The resultant decay curves were analyzed by a multiexponential iterative fitting program provided by instrument.

Results and Discussion

Steady State Fluorescence

The native Ll-CCRH1, a multi tryptophan protein (GenBank: DQ986907) showed fluorescence maximum, λ_{max} at 352 nm indicating the trp residues to be exposed to solvent. However, decomposition analysis of the intrinsic fluorescence profile revealed two populations/conformers of the trp, 1) class A or S (33 %), hydrophobic environment and 2) class III (67 %), polar environment. The denatured protein showed red shift in λ_{max} to 358 nm, indicating more enhanced exposure of trp to completely polar environment due to unfolding of protein (Supplementary Fig. S1)

Tryptophan residues appear to be uniquely sensitive to quenching by variety of solutes as a result of a propensity of the excited indole nucleus to donate electrons while in the excited state. Fluorescence spectra of the native and denatured Ll-CCRH1 recorded in the absence and in presence of increasing concentrations of acrylamide (Supplementary Fig. S2 A and B) displayed higher extent of quenching in the presence of 6 M GdnHCl, clearly revealing that unfolding results in significant increase in the accessibility of the tryptophan residues to the quencher. Also, denaturation led to significant increase in the extent of quenching with other quencher, namely cesium (Table 1). The percentage quenching was calculated from raw data.

Of the three quenchers used, acrylamide was the most effective, quenching 93 % of the total intrinsic fluorescence of the protein (at 0.49 M). Amongst ionic quenchers, iodide and cesium ions, which cannot penetrate into the protein matrix and can access only surface exposed tryptophans, were found to quench only 77 % and 39.5 % respectively, of the total intrinsic fluorescence of Ll-CCRH1. With charged quenchers, higher quenching was observed by iodide ions compared to cesium, with native Ll-CCRH1 indicated surface tryptophan in the protein to have more positively charged amino acids around them. Also, the inherently low quenching efficiency of cesium ions may be responsible for lower quenching observed. On the other hand, the iodide ions could get concentrated in a positively charged environment in the vicinity of surface tryptophans

Table 1 Extent of fluorescence quenching of Ll-CCRH1 with different quenchers

Quenchers	Quenching (%)	
	Native	In 6 M GdnHCl
Acrylamide (0.49 M)	93	100
CsCl (0.5 M)	39.5	53
KI (0.56 M)	77	72

and increased probability of iodide ions colliding with them and quenched the fluorescence.

Denaturation of Ll-CCRH1 resulted in a significant increase in quenching by acrylamide and cesium ions, with extent of quenching observed being 100 % and 53 % respectively. In case of cesium ions, the extent of quenching increased almost by 40 % after denaturation (Table 1). Denaturation of Ll-CCRH1 led to increase in the accessibility of tryptophan fluorescence by cesium ions indicating increase in the density of negative charge around the tryptophan residues. Thus, in native Ll-CCRH1 condition, the environment of tryptophan residues is electropositive; while in denatured state, charge reorientation resulted in electronegative environment around tryptophan residues. This may be the reason for decrease in extent of quenching by iodide ions (72 %) in denatured condition. The presence of negatively charged residues in proximity of trp residues repel the negatively charged iodide ions, resulting in decreased quenching efficiency (Table 1).

Lifetime Measurements

The fluorescence decay of the native Ll-CCRH1 on a nanosecond time scale was obtained from time resolved measurements (data not shown). When fitted into a biexponential curve ($\chi 2=1.076$), two decay times $\tau 1$ (2.27 ns) and $\tau 2$ (7.92 ns) with their relative contributions to the overall fluorescence being 20 % and 80 % respectively, were obtained indicating the presence of two conformers of the tryptophans in Ll-CCRH1. The shorter life time component is supposed to be on the surface of the protein and its fluorescence decays faster, while the longer conformer lies in the interior and decays slowly. The λ_{max} at 352 nm is hence the cumulative intrinsic fluorescence of Ll-CCRH1. The two life times of present protein can be correlated with decomposition analysis of intrinsic fluorescence profile showing polar as well as non polar environment of trp. The average life time was calculated using the following equation [26]:

$$\tau = \sum \alpha_i \tau_i / \sum \alpha_i \quad i = 1, 2, ...$$

Where τ is the average life time and α is the weighting factor. The average τ was found to be 5.28 ns for native Ll-CCRH1.

For the denatured enzyme, again two decay times $\tau 1$ (1.3822 ns) and $\tau 2$ (3.5810 ns) with 41 % and 59 % contribution to the total fluorescence, respectively were observed.

Fluorimetric Analysis of CoA Esters Binding

Cinnamoyl CoA esters binding studies were carried out using fluorescence spectroscopy. Specific binding of CoA ester may change the microenvironment of tryptophan either by enhancing [27] or by quenching of the fluorescence [28]. Titration of Ll-CCRH1 with substrates (feruloyl CoA, sinapoyl CoA, coniferaldehyde and sinapaldehyde) and cofactors (NADPH and NADP) resulted in quenching of fluorescence. Biochemical studies of Ll-CCRH1 showed better affinity of enzyme for feruloyl CoA over other substrates and preference of reduction reaction over oxidation [13]. Here, the enzyme showed association constant, K_a for feruloyl CoA, sinapoyl CoA, coniferaldehyde and sinapaldehyde as 3.72, 3.3, 2.2., 1.99 ($\times 10^5$ M^{-1}) respectively, confirming the differential binding affinity of substrates towards protein (Fig. 1, Table 2). The binding constants for cofactors, NADPH and $NADP^+$ to protein are 11.49×10^6 M^{-1} and 7.46×10^5 M^{-1} respectively showing preference for NADPH over NADP in Ll-CCRH1 mediated reaction (Fig. 2). ΔG values for different ligands are in the range of −31 to −41 $kJmol^{-1}$, indicating spontaneous nature of binding.

Analysis of the Fluorescence Quenching Data of Ll-CCRH1

Quenching with Acrylamide

Acrylamide, being neutral by nature, was found to be the most efficient quencher for intrinsic fluorescence of Ll-CCRH1 as it could penetrate into the interior of protein. Quenching of the Ll-CCRH1 with acrylamide gave a linear Stern-Volmer plot with K_{sv} value as 23.15 M^{-1} for native protein, indicating exceptionally high rate of quenching (Fig. 3a). 100 % of the tryptophan fluorescence is accessible to protein under native condition (Fig. 3b, Table 3).

Accessibility of acrylamide to fluorescence remained same, 100 % upon denaturation of the protein with 6 M GdnHCl as determined from modified Stern-Volmer plot (Fig. 3d, Table 4). An upward curvature was obtained in the Stern-Volmer's plot, indicating the involvement of both collisional and static components (Fig. 3c). The static mechanism is a consequence of complex formation, while the dynamic mechanism involves collisions with acrylamide during the lifetime of tryptophan in excited state [18]. Almost similar K_{sv} value, 21.51 M^{-1} for denatured protein was also observed.

Quenching with Cesium and Iodide Ions

The Stern-Volmer plots for the quenching of Ll-CCRH1 with ionic quenchers (CsCl and KI) are shown in Fig. 3. The quenching profile obtained for iodide ion under native and denatured conditions showed linear dependence. The Stern-Volmer plot obtained for cesium ion under same conditions displayed negative curvature (Fig. 3a, c). Downward/negative curvature of the plots indicated that certain tryptophans are selectively quenched before others. At low concentration of quencher, the slop of the Stern-Volmer plots reflects largely the quenching of the more accessible residues. At higher concentrations, the easily quenched fluorescence has been

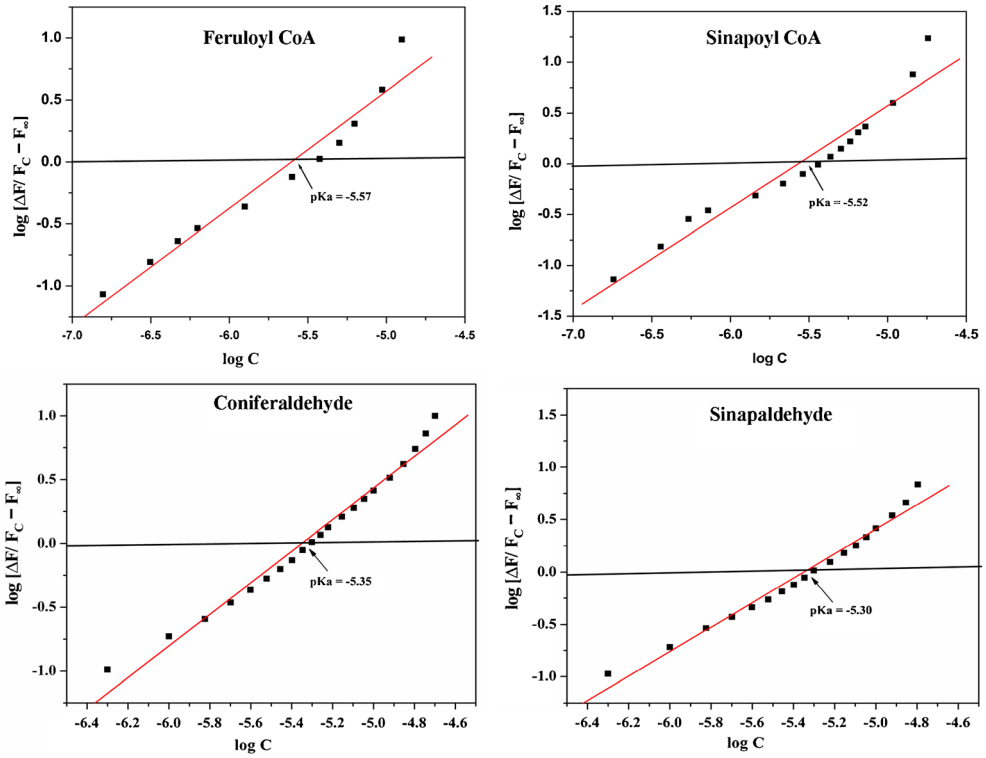

Fig. 1 Determination of the association constants (K_a) for the interaction of Ll-CCRH1 with CoA esters and cinnamaldehydes by fluorescence titration using double log plot of log $\{\Delta F/(F_c - F_\infty)\}$ versus log $[C]_f$. The X-intercept of the plot gives pKa value for the interaction between Ll-CCRH1 and substrates

depleted, and those tryptophans having lower quenching constants become dominant. Similar quenching patterns have been observed for several single or multi-tryptophan proteins [29–35]. Downward curvatures of quenching profiles with cesium ions shown for native and denatured Ll-CCRH1 suggest the heterogeneity in the microenvironment of surface exposed tryptophans. These results also indicate the presence of tryptophans in Ll-CCRH1 in different environments where some tryptophans are partially or fully exposed to solvent while others are present inside hydrophobic environment. These observations are in good agreement with the decomposition analysis profile of native enzyme.

The biphasic curvature obtained for CsCl quenching in native state was split into two linear components and the values of K_{sv1} and K_{sv2} obtained were 1.31 M^{-1} and 0.82 M^{-1} respectively; while K_{sv} of 5.35 M^{-1} was observed for KI (Fig. 3a, Table 3). The higher K_{sv} value of iodide as compared to cesium indicates higher efficiency of quenching by iodide due to presence of an electropositive environment around trp residues in the enzyme.

Modified Stern-Volmer plots obtained with ionic quenchers are shown in Fig. 3b, d, from which f_a or fractional accessibility of the total fluorescence and K_a, quenching constant were obtained and listed in Table 4. From Table 4, it is seen that 33 % and 100 % of the total fluorescence is accessible to cesium and iodide ions, respectively under native condition. These results also confirm higher extent of quenching by iodide ions and suggest more density of positive charge around the surface of trp conformers.

The ionic solute quenching profile was different for denatured protein. The denatured protein gave a linear Stern-Volmer plot (Fig. 3c) and the K_{sv} value observed was 6.86 M^{-1} for iodide ion. From the slopes of the two linear components of the Stern-Volmer constants, K_{sv1} of 3.56 M^{-1} and K_{sv2} of 1.69 M^{-1} were obtained for cesium in denatured conditions. K_{sv1} is considerably greater than K_{sv2}, giving substantial evidence for selective tryptophan quenching. Upon denaturation of protein with 6 M GdnHCl, fraction accessible has been increased to 54 % for cesium ions (from 33 %); while same has been decreased to 78 % (from 100 %) for iodide ions. This may be due to change in conformation of the protein upon unfolding. The extent of quenching achieved with cesium is significantly higher than iodide ions, as evident from Tables 3 and 4. Thus, denaturation of protein with 6 M GdnHCl resulted in the reorientation of charge density around trp from positive to negative, that is, it is quite likely that a

Table 2 Summary of ligand binding to Ll-CCRH1 with fluorescence analysis

Substrates	K_a (M^{-1})	ΔG ($kJmol^{-1}$)
Feruloyl CoA	3.72×10^5	−31.77
Sinapoyl CoA	3.3×10^5	−31.40
Coniferaldehyde	2.23×10^5	−30.51
Sinapaldehyde	1.99×10^5	−30.15
NADPH	11.49×10^6	−40.28
$NADP^+$	7.46×10^5	−33.50

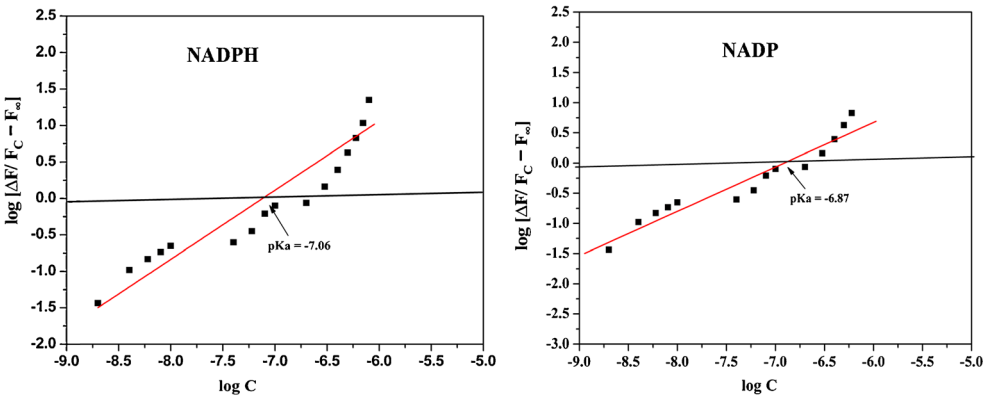

Fig. 2 Determination of the association constants (K_a) for the interaction of Ll-CCRH1 with cofactors ($NADP^+$ and NADPH) by fluorescence titration using double log plot of log $\{\Delta F/(F_c - F_\infty)\}$ versus log $[C]_f$. The X-intercept of the plot gives pKa value for the interaction between Ll-CCRH1 and cofactor

larger fraction of tryptophan residues has positively charged residues in their close proximity.

Solute Quenching Studies for Active Site Mutants

The exposure and environment of the tryptophan residues in active site mutants were investigated by solute quenching technique using steady state fluorescence studies. Ten different mutant proteins, namely F30V, I31N, R51G, D77G, S136A, Y170H, K174M, V200E, S212G and H215L were used in this study. Substitution type mutations were designed in order to have minimal effect on secondary structure and 3D conformation of protein [23]. Tryptophan fluorescence spectra of wild type Ll-CCRH1 and mutants were similar, suggesting that mutant proteins are properly folded. λ_{max} for active site mutants were observed in the range of 350–352.5 nm, suggesting apparently overall unchanged conformation (Fig. S3).

Quenching of Mutants Fluorescence with Acrylamide

Quenching of the intrinsic fluorescence of all active site mutants gave linear Stern-Volmer plots with K_{sv} values as 6–20 M^{-1} and 8–14 M^{-1} for native and denatured proteins, respectively, indicating differential degree of quenching (Tables 3 and 4). Among the three quenchers used, acrylamide was the most efficient quencher for intrinsic fluorescence of Ll-CCRH1

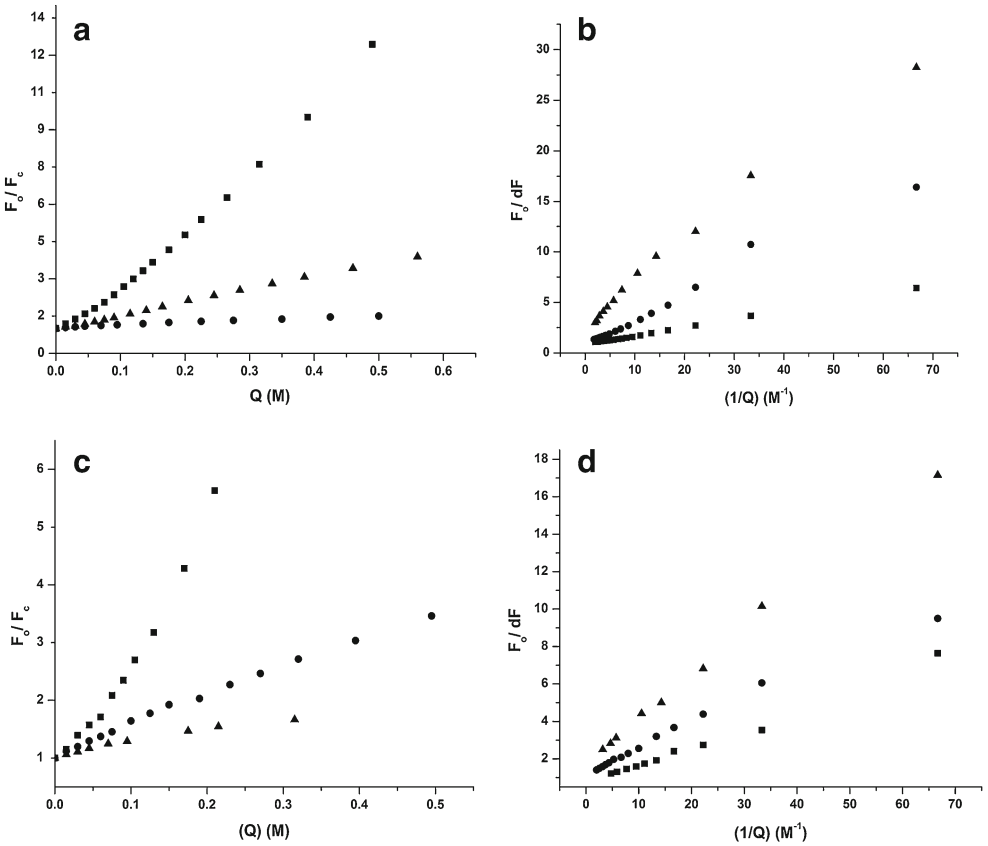

Fig. 3 Stern-Volmer plots (**a**, **c**) and modified Stern-Volmer plots (**b**, **d**) for the quenching of native (**a**, **b**) and 6 M GdnHCl denatured (**c**, **d**) Ll-CCRH1 (0.018 mg/ml). Acrylamide (*filled squares*), cesium (*filled triangles*) and iodide (*filled circles*) was used as quenchers. After fitting the data, the R^2 value in each case was 0.99. The downward curves seen in the quenching profiles of iodide and cesium under native and denatured conditions respectively (**a**, **c**) were split into two linear components

Table 3 Summary of parameters obtained from the intrinsic fluorescence quenching of wild Ll-CCRH1 and different active site mutants under native conditions with different quenchers

	Acrylamide			CsCl			KI		
	K_{sv}	f_a	K_a	K_{sv1}/K_{sv2}	f_a	K_a	K_{sv}	f_a	K_a
Native	23.15	1.17	12.78	1.31/0.82	0.33	5.69	5.35	1.23	5.47
F30V	13.05	1.02	10.08	1.31/0.48	0.27	3.20	3.78	0.69	7.68
I31N	15.09	0.93	15.15	1.26/0.72	0.23	26.02	3.45	0.64	10.68
R51G	20.41	1.05	12.78	1.08/0.85	0.39	11.21	4.17	0.89	5.47
D77G	9.86	0.91	10.62	2.34/0.38	0.20	27.11	2.47	0.66	7.26
S136A	11.37	0.89	13.37	1.31/0.57	0.20	15.37	2.84	0.58	10.29
Y170H	10.37	0.90	11.58	0.40/0.27	0.13	18.9	2.31	0.58	10.60
K174M	14.64	1.01	12.22	0.73/0.47	0.29	4.50	3.42	2.08	1.30
V200E	6.07	0.82	12.1	0.79/0.23	0.13	5.98	2.54	0.59	11.44
S212G	19.17	1.0	13.90	0.94/0.64	0.32	4.77	4.26	1.33	1.71
H215L	8.80	0.91	9.12	1.18/0.42	0.18	17.02	2.40	0.52	13.96

mutants. Almost 100 % of the trp fluorescence of mutants was accessible to acrylamide except V200E mutant, where around 80 % of the total fluorescence was accessible under native condition. Accessibility of acrylamide to fluorescence increased to 100 % upon denaturation of mutant proteins with 6 M GdnHCl (Tables 3 and 4). An upward curvature was obtained for all mutants in the Stern-Volmer plots, indicating the involvement of both collisional and static components.

Quenching with Cesium Ions

The quenching profile obtained for native and denatured Ll-CCRH1 mutants with cesium ion showed downward curvature. The biphasic curvature obtained for CsCl quenching indicated heterogeneous ionic environment around the trp with presence of two conformers- one getting quenched earlier than other. Downwards curves were divided into two linear components and the values of K_{sv1} and K_{sv2} obtained were 0.7–2.4 M^{-1} and 0.2–0.9 M^{-1}, respectively for native state and, 0.9–1.4 M^{-1} and 0.4–1.0 M^{-1}, respectively for denatured state. K_{sv1} is considerably greater than the K_{sv2} in each case, suggesting selective tryptophan quenching. Based on the f_a values, 33 % and 54 % of the total fluorescence was found to be accessible to cesium ions in native and denatured wild type protein respectively. 20–40 % accessibility was obtained for CsCl in F30V, I31N, R51G, D77G, S136A, K174M, S212G and H215L mutants, showing apparently similar quenching profile as that of native wild type protein (Table 3). Mutants Y170H and V200E showed only 13 % accessibility of fluorescence suggesting lower extent of quenching with cesium ions. For cesium ions, the fraction accessible increased to 26 %, 32 %, 52 % and 33 % for mutants (upto 2 fold) Y170H, V200E, S212G and H215L respectively after denaturation. However, analysis of the quenching data shows that there is slight increase in the accessibility of fluorescence (25–40 %) compared to wild type Ll-CCRH1, indicating there may be partial unfolding or minor change in the conformation after denaturation (Table 4).

Table 4 Summary of parameters from Stern-Volmer and modified Stern-Volmer analysis of the intrinsic fluorescence quenching of wild type Ll-CCRH1 and different active site mutants under denatured conditions with different quenchers

	Acrylamide			CsCl			KI		
	K_{sv}	f_a	K_a	K_{sv1}/K_{sv2}	f_a	K_a	K_{sv}	f_a	K_a
Denatured Ll-CCRH1	21.51	1.55	4.47	3.56/1.69	0.54	11.71	6.86	0.78	10.74
F30V	12.59	0.97	9.27	1.33/0.77	0.32	7.72	4.02	0.68	8.33
I31N	12.84	1.02	8.44	1.25/0.83	0.37	7.11	3.75	0.70	9.03
R51G	11.58	1.48	4.47	1.39/0.90	0.31	11.71	3.27	0.59	10.74
D77G	12.65	1.02	8.01	0.97/0.66	0.28	9.83	3.60	0.61	11.21
S136A	11.34	1.37	4.23	1.08/0.41	0.27	10.11	0.96	0.68	9.34
Y170H	8.86	1.21	4.75	0.92/0.73	0.26	11.45	3.24	0.60	9.35
K174M	11.64	0.91	10.70	1.11/0.68	0.36	5.24	3.24	0.60	8.75
V200E	10.04	1.14	6.25	1.20/0.76	0.32	8.30	3.16	0.66	8.91
S212G	12.15	0.94	10.79	1.08/0.85	0.52	2.91	3.83	0.66	10.42
H215L	11.33	1.18	5.43	1.25/0.92	0.33	7.43	3.87	0.64	10.84

Quenching with Iodide

The Stern-Volmer plots for the quenching of Ll-CCRH1 with iodide under native and denatured conditions showed linear dependence. Quenching of the intrinsic fluorescence of active site mutants with K_{sv} values, 2.3–4.3 M^{-1} and 0.8–4.1 M^{-1} for native and denatured mutant proteins respectively, indicating lower rate of quenching as compared to wild type Ll-CCRH1 (Tables 3 and 4). 100 % and 78 % accessibility was observed for KI in native and denatured wild Ll-CCRH1 respectively, showing reorientation of charge density around trp from positive (native state) to negative (denatured state). From Table 3, it is seen that 100 %, 100 % and 89 % of the total fluorescence of the mutants S212G, K174M and R51G is accessible to iodide ions respectively, similar to wild type Ll-CCRH1; and it is decreased to 50–70 % approximately in mutants F30V, I31N, D77G, S136A, Y170H, V200E and H215L. Denaturation of protein with 6 M GdnHCl led to decrease in accessibility (60–70 %) with respect to all mutants, similar to denatured wild Ll-CCRH1 (78 %) (Table 4).

Conclusion

The first reductive committed step in monolignol biosynthesis is catalyzed by cinnamoyl CoA reductase (CCR, EC 1.2.1.44) and considered as a first regulatory point in lignin formation. CCR carries out the NADPH dependent reduction of various hydroxycinnamoyl CoA esters to corresponding hydroxycinnamaldehydes and vice-versa. Previous studies on CCR from various plants were basically focused on isolation, cloning, molecular characterization and downregulation aspects. Structural-functional studies on CCR are very few, and no studies regarding exposure and differential environment of tryptophan residues were reported so far. Here, fluorescence quenching and time resolved fluorescence studies of wild type recombinant cinnamoyl CoA reductase (Ll-CCRH1), a multitryptophan protein from *Leucaena leucocephala* and 10 different active site mutants were carried out to investigate tryptophan environment. These 10 different single substitution type active site mutants were generated by site directed mutagenesis in order to determine the role of amino acid residues either in catalysis or substrate binding. Although tryptophan fluorescence spectra of native and mutant Ll-CCRH1 showed similar overall conformation, it was interesting to see whether these active site mutants showed similar tryptophan microenvironment and heterogeneity as that of wild type Ll-CCRH1.

The tryptophans were found to be differentially exposed to the solvent and were not fully accessible to the ionic quenchers indicating heterogeneity in the environment in both wild type and Ll-CCRH1 and active site mutants. Quenching of the fluorescence by acrylamide for wild type and active site mutant was collisional. Also surface tryptophans were found to have predominantly positively charged amino acids around them and differentially accessible to ionic quenchers. The extent of quenching for cesium and iodide ions under native and denatured conditions observed in active site mutants is significantly different from wild type Ll-CCRH1 under same conditions. Thus, single substitution type mutations of active site residues showed heterogeneity in tryptophan microenvironment and differential degree of conformation compared to native protein under native or denatured conditions. The biophysical studies presented herein may provide insight into comparative structural dynamics of native protein and its mutants in solution using fluorescence spectroscopy. This is an attempt towards the conformational studies of Ll-CCRH1.

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