Spectroscopy and Microscopy Studies of the Recognition of Amino Acids and Aggregation of Proteins by Zn(II) Complex of Lower Rim Naphthylidene Conjugate of Calix[4]arene

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A lower rim naphthylidene conjugate of calix[4]arene [L] has been synthesized and characterized, and the structure has been established on the basis of single crystal XRD. The L has been found to be selective toward Zn^{2+}, which induces appropriate changes in the arms of L so that the N_2O_2 coordination results in the formation of a tetrahedral complex; namely, [ZnL]. [ZnL] recognizes Asp, Cys, His, and Glu from among the naturally occurring amino acids owing to the protonation and chelating ability of the amino acid and the $\pi-\pi$ interaction ability of the side chain of the amino acid with [ZnL]. All of these features have been demonstrated on the basis of fluorescence, absorption, and lifetime measurements. The rechelation of Zn^{2+} by the amino acid used in the titration has been demonstrated on the basis of ESI-MS studies in the case of Cys to result in a Zn^{2+} complex having either 5- or 6-coordination. [ZnL] has also been shown to be selective toward glutathione and glutathione oxidized. The amino acids present in the proteins also interact with [ZnL], resulting in dechelation of [ZnL] as well as aggregation of the protein, as demonstrated on the basis of absorption and fluorescence spectroscopy and atomic force microscopy. The α-helical proteins (namely, albumins) exhibit greater conformational changes, as compared to the β-sheet proteins (namely, lectins), as studied on the basis of CD spectroscopy. The aggregation of the proteins when treated with [ZnL] follows a trend: peanut agglutinin < bovine serum albumin < jacalin < human serum albumin. The present studies clearly demonstrated the recognition features of [ZnL] toward Asp, Cys, His, and Glu, and the peptides and proteins containing these by spectroscopy and microscopy studies.

Introduction

Recognition of amino acids is an important phenomenon in biology. Amino acids, the constituents of proteins, can take part in various physiological processes, and the deficiency of some of these amino acids causes diseases.[1−3] As a result, molecules or molecular systems that can selectively recognize amino acids are of paramount importance to provide analytical quantification of these in proteins. Therefore, molecular systems that can report the interaction of amino acids through changes in either their emission or absorption properties could act as good candidates for the same.[4−10] Derivatives of calix[4]arenes are known in the literature for the recognition of amino acids. These include upper-rim-modified peptide,[11−13] sulfanato calixarenes,[14−16] and lower-rim-substituted calix[4]arene[17,18] with a carboxylic group[19] and a phosphoryloxy derivative[20] that functions in the presence of the Zn^{2+} ion. Some of the calix[4]arene derivatives have also shown recognition toward peptides[21] and proteins.[22] In addition to the phosphoryloxy derivative, to our knowledge, there has been no report on the recognition of amino acids by metal ion complexes of calix[4]arene derivatives, although a few non-calixarene metal ion complexes were reported for the same.[23] In view of this, we have studied the amino acid recognition aspects of a Zn(II) complex of 1,3-dinaphthylidene calix[4]arene conjugate [ZnL] with naturally occurring amino acids, tripeptides (viz., GSH and GSSG), albumin proteins (viz., BSA and HSA), and lectins [viz., jacalin and peanut agglutinin (PNA)] using steady state as well as time-resolved fluorescence spectroscopy. The species formed has also been studied by absorption spectroscopy. The results obtained in the case of proteins have been further augmented by atomic force microscopy studies. The conformational changes brought about in the protein have been studied by circular dichroism spectrosocpy. Results of all these studies are reported in this paper. To our knowledge, this is the first report on the recognition of amino acids by a metal ion complex of a conjugate of calix[4]arene.

Results and Discussion

A 1,3-dinaphthylidene conjugate of calix[4]arene, L, was prepared and characterized and was shown to be selective toward Zn^{2+} through the formation of a 1:1 complex in solution wherein the isolated complex [ZnL] has been further characterized as reported by us earlier (Scheme 1, the Experimental Section, Supportin Information (SI) 01).[24] The structure of L has been established by single-crystal XRD, and that of the [ZnL] has been derived on the basis of computational studies as reported in this paper.

Structure of L. The single-crystal XRD structure of L exhibits a cone conformation for the calixarene, as can be seen from the stereo view of L shown in Figure 1, as the lower rim intramolecular circular hydrogen bonding is being retained. The metric data of L does not show any abnormalities (SI 02). Most of the dihedral angles of the two arms observed for the structure of L were found to be the same, except for one (C59−N4−C60−C61 = −173.9° and C46−N2−C47−C48 = 175.0°). This results in the arrangement of the imine and naphthylic−OH of the two arms in the opposite directions, as can be seen from SI

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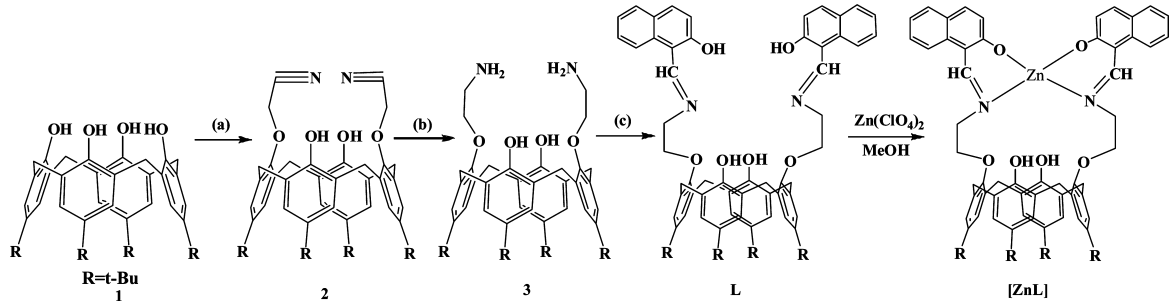
SCHEME 1: Synthesis of L and [ZnL][a]



[a] (a) K_2CO_3, NaI, $ClCH_2CN$, dry acetone reflux 12 h; (b) $LiAlH_4$, dry THF, reflux for 7 h; and (c) 2-hydroxy-1-naphthaldehyde, C_2H_5OH, stirred at RT for 3 h.

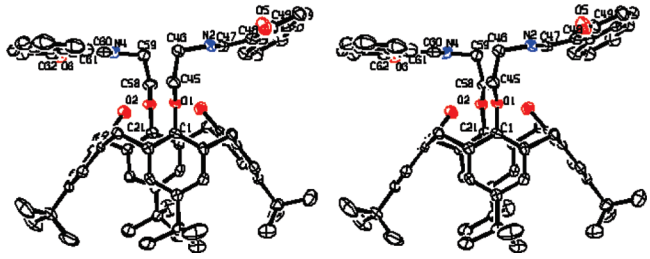


Figure 1. Stereo view of the crystal structure of L.

02. The naphthylidene moieties lie almost perpendicular to the principal axis of the calixarene platform. The interstrand N-to-O distances are in the range of 6.8−11.5 Å, indicating that the structure of L does not have a preformed binding core congenial for metal ions. This necessitates that the incoming metal ion must bring changes in the dihedral angles so as to bring the N_2O_2 core into the binding mode. An analogue of L possessing salicilidene having similar structure exhibited a 1:1 complex with Cu^{2+} that binds through the N_2O_2 core, whose crystal structure has been established by us as a pyridine-bound 5-coordinated complex.[25]

Structure of [ZnL]. The analytical data and the mass spectrum suggested the formation of a 1:1 complex for [ZnL]. The molecular ion peak observed at a *m*/*z* of 1107 is indicative of the formation of a 1:1 complex, and the isotopic peak pattern reveals the presence of zinc. Since there is no crystal structure for [ZnL], the structural features have been addressed by computational calculations. The crystal structure of the ligand L was optimized after replacing the *tert*-butyl groups in L with hydrogen to result in L′ in a cascade fashion starting from AM1 → HF/6-31G levels of theory. Replacement of a *tert*-butyl group with a hydrogen has been done to reduce the computational times without compromising the chemical and conformational features of the molecule. The optimization of [ZnL′] was carried out by simply placing the zinc far above the binding core so that there are no interactions present between L′ and Zn^{2+} in the beginning. The optimized geometry of this complex has been found to be a highly distorted tetrahedron wherein the bond angles at Zn^{2+} vary from 92 to 140° (Figure 2). Even in [ZnL], the naphthylidene moieties are placed almost perpendicular to the principal axis of the calixarene platform, like that found for L. Interstrand N-to-O distances now fall in the range of 2.7−3.7 Å, indicating that the distances were shortened by about 6−8 Å on going from the crystal structure of free L to [ZnL′], suggesting the movement of the strands toward each other upon complexation. The complexation also affected the dihedral angles of the arms to bring the binding groups in proximity [SI 03], which would result in the effective binding of Zn^{2+} with the N_2O_2 core. The orientation of the naphthylidene moieties from each arm in the complex differs from that in L to bring these into the binding mode, as can be understood from the space-filling diagrams given in Figure 2b in comparison with that of the uncomplexed L (Figure 2c).

Solution Studies of the Recognition of Amino Acids by [ZnL]. To carry out the amino acid recognition studies in solution, a 1:1 complex of L with Zn^{2+} was generated *in situ* freshly by mixing equimolar quantities of $Zn(ClO_4)_2$ and L in methanol. That solution was then used in all the studies reported in this paper. Formation of [ZnL] has been monitored in solution by observing several-fold (viz., 15−20-fold) enhancement in the fluorescence intensity of L upon addition of Zn(II). Fluorescence studies have also been repeated with the isolated [ZnL] to confirm these results.

(i) Fluorescence Titration of [ZnL] by Simple Amino Acids. The titration of [ZnL] with amino acids has been carried out by exciting the solutions at 320 nm and measuring the emission in the range 330−550 nm. The fluorescence intensity of the 440 nm emission band was diminished gradually by the addition of the amino acid. However, the extent and rate of quenching seems to be dependent upon the nature of the amino acid added. Plots of relative fluorescence intensity as a function of the amino acid concentration can be seen from Figure 3a for Asp, Cys, His, and Glu, which showed maximum fluorescence quenching as compared to all other amino acids. The ability of each amino acid to quench the fluorescence of [ZnL] can be clearly judged from the histogram given in Figure 3b that exhibits a trend, namely, Asp > Cys ≫ Glu ∼ His, which cannot be strictly fitted either to the pK_a of the C^{α}-carboxylic group or to the chelating ability; rather it should be attributed to a combination of both. This suggests that the protonation followed by chelation by the amino acid are important factors in quenching the fluorescence of [ZnL]. Photo electron transfer (PET) from the lone pair of electrons present on the imine moiety of L is *off* in the [ZnL] complex owing to the binding of Zn^{2+}. The trend in the fluorescence reverses to PET-*on* in the presence of added amino acid, suggesting the dechelation of Zn^{2+} from [ZnL] via the possible formation of a ternary complex. Zinc(II)-mediated ternary complexes formed between diphosphoryloxy-calix[4]arene and amino acid (viz., His, Cys and Ser) have been reported by Coleman et al. on the basis of ESI-MS.[20] This seems to be largely favored in the case of Trp, His, Tyr, and Phe, which can extend $\pi-\pi$ type interaction with the naphthalene moiety of [ZnL], which has been delineated on the basis of absorption spectral studies, as discussed in this paper. To see whether the nature of interaction of the amino acid with the *in-situ*-generated [ZnL] and the isolated [ZnL] are the same, fluorescence titrations were performed between the isolated [ZnL] and Asp, Cys, His, and Glu, and the results were the same (inset of Figure 3a).

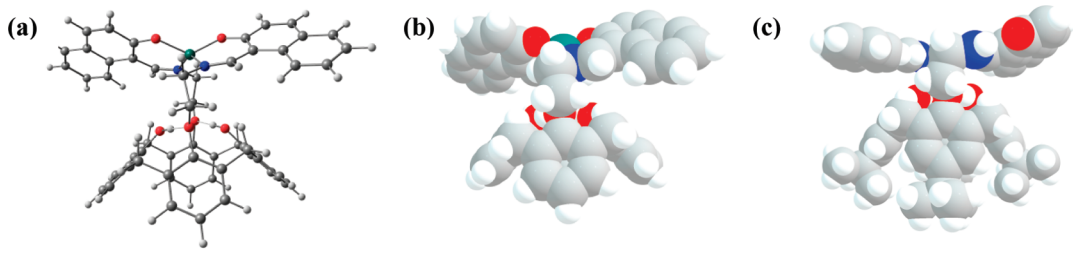


Figure 2. (a) HF/6-31G optimized structure of [ZnL]. (b) Space filling model for [ZnL]. (c) Space filling model of crystal structure L. Bond lengths (Å) and bond angles (°) in the coordination sphere are Zn−O6 = 1.880, Zn−O5 = 1.880, Zn−N2 = 1.967, Zn−N4 = 1.967; N2−Zn−O5 = 92.1, N4−Zn−O6 = 92.1, N2−Zn−O6 = 104.8, N4−Zn−O5 = 104.7, O5−Zn−O6 = 129.9, N1−Zn−N2 = 139.8. The atom numbering can be seen from Figure 1.

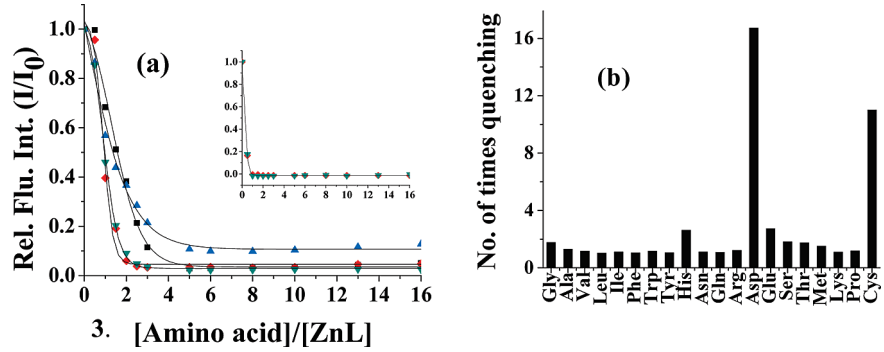


Figure 3. Fluorescence titration of [ZnL] (10 μM) with amino acids in methanol: (a) Plot of relative fluorescence intensity of [ZnL] as a function of the mole equivalents of His (black ■), Asp (red ◆), Glu (blue▲), Cys (green▼). Inset: Results obtained from the titration of the isolated [ZnL] with Asp (red ◆) and Cys (green ▼). (b) Histogram representing the number of times of quenching of fluorescence of [ZnL] by amino acids as taken at two equivalents. The bulk concentration of amino acids used is 6×10^{-4} M.

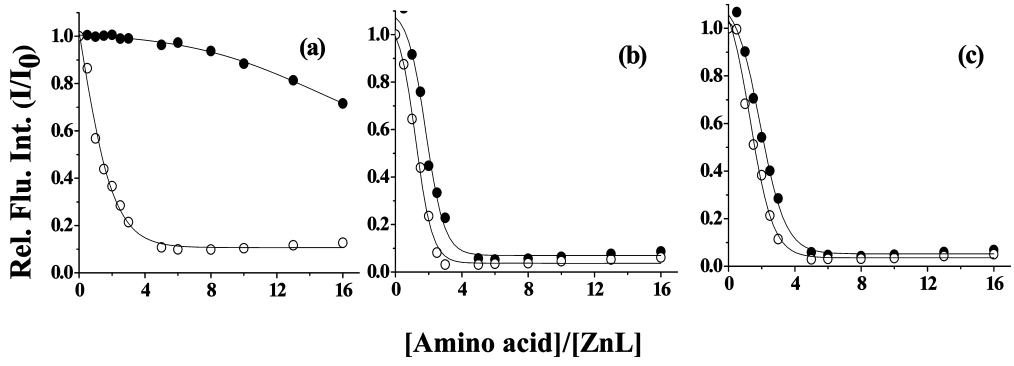


Figure 4. Relative fluorescence intensity plots for the titration of [ZnL] (10 μM) in methanol by amino acids (open symbols) and their esters (closed symbols): (a) Glu; (b) Cys; (c) His. The bulk concentration of the amino acids used was 6×10^{-4} M.

(ii) Comparison of the Fluorescence Titration Data of [ZnL] among Amino Acids and Their Esters. To understand the effect of the side chain of the amino acid on the fluorescence behavior of [ZnL], titrations were also performed with some esters of amino acids (Figure 4, SI 06). The differences observed in the quenching behavior of amino acids and their esters is large in the case of Glu, Ser, Gly, Ala, Phe; significant in the case of Trp and Tyr; and least in case of Cys and His. This is attributable to the protonating capacity of the free amino acids and chelating ability of their esters. Protonation of [ZnL] by amino acids has been further confirmed by carrying out the titrations with simple acids not possessing amino function; namely, acetic acid, benzoic acid, and phenylacetic acid (SI 07). Thus, the protonation and dechelation of [ZnL] followed by rechelation of the released Zn^{2+} by the amino acid has been addressed by different studies. The rechelation of Zn^{2+} by the amino acid has been demonstrated on the basis of ESI-MS (SI 01) carried out between [ZnL] and Cys to result in the formation of a [Cys + Zn + Cys + CH_3OH − H]$^+$ by exhibiting a peak at *m*/*z* of 338. The presence of a zinc center in this peak has been confirmed on the basis of the isotopic peak pattern. Various structural forms of Zn^{2+} bound to amino acids have been established on the basis of ESI-MS and DFT studies in the literature, whereas the primary focus of these studies has been with glycine, although a few other amino acids (viz., Asp and Asn) have also been studied.[26−29] The various structural forms reported involve the binding of α-carboxylic either as mono- or as bidentate or in conjunction with the binding of α-amino moiety or the side chain moiety, or both, like that in aspartic acid.[30] On the basis of these, the species observed in the present case may be viewed as 5- or 6-coordinated ones (SI 08). In some, tripeptides possessing terminal cystine and/or histidine have been shown to act as bindentate through the side chains of terminal moieties.[31]

(iii) Titration of [ZnL] by Simple Amino Acids and Their Esters Using Absorption Spectroscopy. Absorption studies were performed by titrating [ZnL] with the amino acids, and the spectra exhibited a number of isosbestic points (Figure 5, SI 09), indicating a transition of the species formed between [ZnL] and amino acid. Although the absorption spectral changes

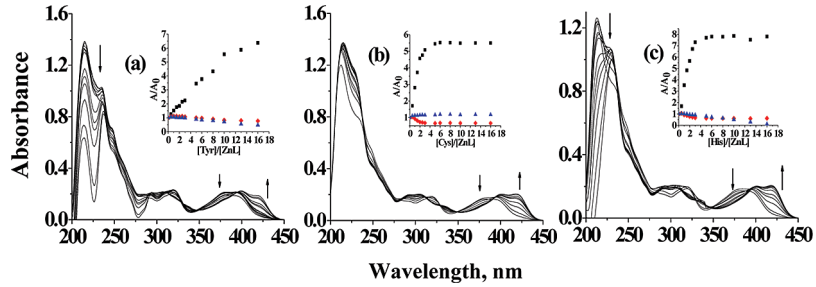


Figure 5. Absorption spectral traces for the titration of [ZnL] (10 μM) with amino acids in methanol: (a) Tyr, (b) Cys, (c) His after subtracting the corresponding control data. Inset: Relative absorbance plots for 420 (■), 375 (red ◆) and 215 (blue ▲) nm bands. The bulk concentration of amino acids used was 6×10^{-4} M.

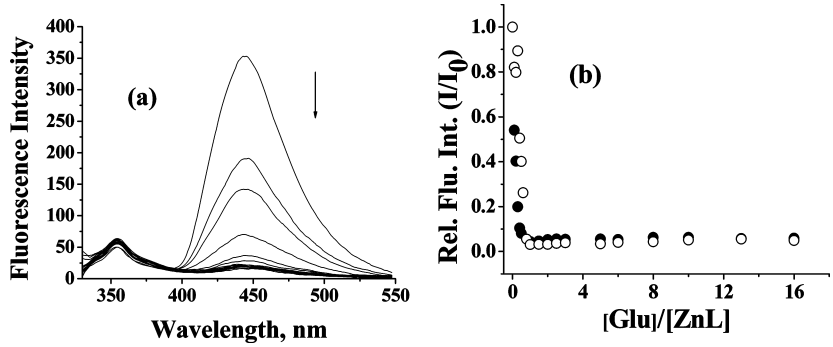


Figure 6. (a) Spectra obtained during the fluorescence titration of [ZnL] (10 μM) with glutathione reduced (GSH) in methanol. (b) Relative fluorescence intensity plots for the corresponding titrations of GSH (●) and GSSG (○). The bulk concentration of GSH and GSSG used was 6×10^{-4} M.

observed in the region 340−450 nm are characteristic of free L vs Zn^{2+}-bound L with a preference of demetalation of His > Cys > Tyr > Asp, the spectral changes observed in the region 200−250 nm are characteristic of the involvement of $\pi-\pi$ interactions present between the aromatic side chains of these amino acid and the naphthalene moiety present in [ZnL] in the order Trp ≫ Tyr > His. The dechelation or the presence of $\pi-\pi$ interactions or both have been reconfirmed by carrying out the absorption studies with the esters of amino acids as well as aromatic nonamino acids; namely, benzoic acid and phenyl acetic acid (SI 09).

(iv) Titration of [ZnL] by Amino Acids Using Time-Resolved Fluorescence Spectroscopy. The time-resolved fluorescence spectroscopy studies have been carried out by a time-correlated, single-photon count (TCSPC) method for simple L; [ZnL]; and [ZnL] titrated with Asp, Glu, His or Cys and the corresponding decay curves. The data are given in SI (SI 10). Although the lifetimes of the species of [ZnL] differ substantially from simple L, the same when treated with these amino acids results in a species whose lifetime characteristics are similar to that of L, indicating the release of L from [ZnL] when the latter is titrated against the amino acid. In fact, the studies based on ESI-MS, absorption, and fluorescence spectroscopy clearly indicated the release of L in the titration, and the first two techniques (viz., ESI-MS and absorption spectra) further supported the formation of Zn^{2+} complex of the amino acid. The release of L and the formation of the Zn^{2+} complex of amino acid may go via a ternary complex formed among L, Zn^{2+}, and the amino acid, as has also been reported in the literature in the case of lower rim calix[4]arene derivatives in the presence of Zn^{2+}.[20] Thus, the lifetime measurements of the titration of [ZnL] with amino acids strongly support the results obtained from ESI-MS, absorption, and steady state fluorescence measurements.

Titration of [ZnL] by Reduced and Oxidized Glutathione (GSH and GSSG). Since [ZnL] recognizes amino acids such as Glu and Cys, peptides possessing such amino acids (viz., GSH and GSSG) have been studied for their interaction with [ZnL]. Titration with glutathione (Figure 6) shows behavior similar to that of the individual amino acids (viz., Cys and Glu): the 445 nm band quenches upon adding higher amounts of GSH. Similar behavior was observed in the GSSG case, indicating that the chelation by amino acid plays an important role in leaching out the Zn^{2+} from [ZnL].

Steady State and Lifetime Fluorescence Titrations of [ZnL] with Proteins. Since [ZnL] recognizes amino acids as well as GSH and GSSG, the present studies have been extended to proteins, as well. The proteins that we have chosen were highly α-helical (viz., BSA and HSA) and β-sheet type (viz., jacalin and Peanut agglutinin, PNA). Fluorescence emission spectra were measured at two different excitations, 280 and 320 nm. Whereas the excitation at 320 nm gives emission only from [ZnL], the excitation at 280 nm gives emission from both the Trp of the protein and [ZnL]. Titration of [ZnL] with BSA exhibited a linear decrease in the intrinsic Trp emission observed at ∼344 nm (Figure 7). The emission from [ZnL] observed at ∼450 nm has been found to be quenched at both excitations. Similar behavior has also been observed even with lectins (viz., jacalin and PNA (Figure 8)). The extent of decrease in the intrinsic fluorescence emission of Trp of these lectins has been found to be less when compared to that of BSA. These results were suggestive of dechelation of Zn^{2+} from [ZnL], in addition to some changes in the conformation of the protein that makes the Trp residues more exposed. The results of the titration of [ZnL] with HSA are similar to that observed in the case of BSA (Figure 7). A control experiment carried out by titrating BSA or HSA with Zn^{2+} showed quenching of the Trp emission. The slope of the fluorescence quenching in the case of {[ZnL] + protein} is double in the case of BSA, as compared to HSA, and a similar trend was observed between PNA and jacalin (Figure 8); however, the magnitude of quenching is only about one-half in the case of lectins as compared to albumin proteins,

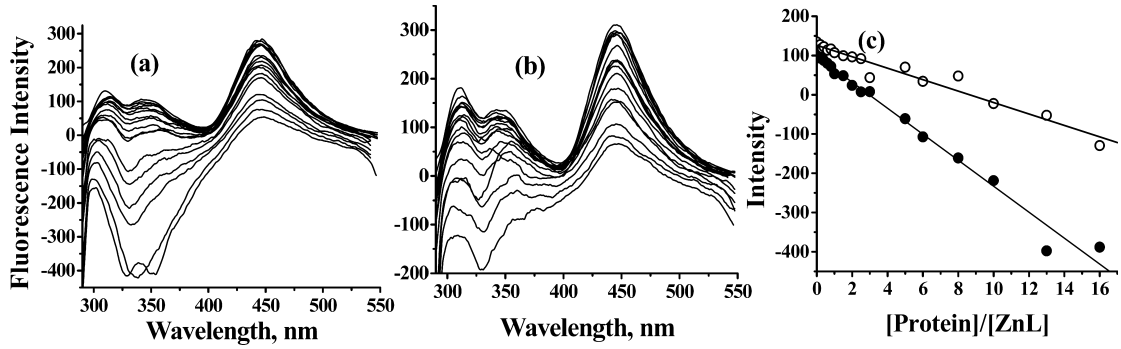


Figure 7. Intrinsic fluorescence emission of the protein during the titration of [ZnL] (10 μM) with BSA and HSA in methanol: Spectra given in part a for BSA and part b for HSA are those obtained after subtracting the control spectra. (c) Plots of fluorescence intensity (at ~345 nm) as a function of concentration of the added protein: BSA (●) and HSA (○). The bulk concentration of the proteins used was 1.5×10^{-6} M.

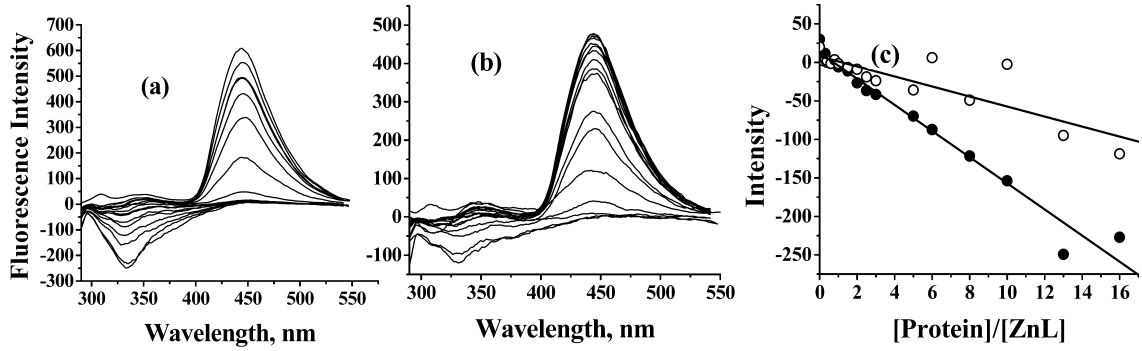


Figure 8. Intrinsic fluorescence emission of the protein during the titration of [ZnL] (10 μM) with PNA and jacalin in methanol. Spectra given in part a for PNA and part b for jacalin are those obtained after subtracting the control spectra. (c) Plots of fluorescence intensity (at ~345 nm) as a function of concentration of the added protein: PNA (●) and jacalin (○). The bulk concentration of the proteins used was 1.5×10^{-6} M.

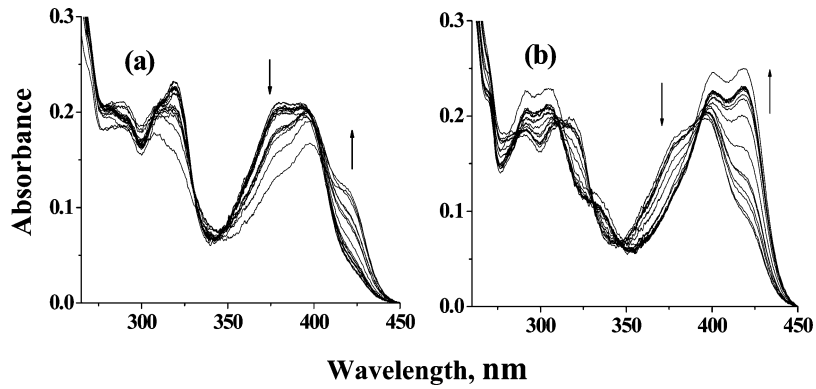


Figure 9. Absorption spectral traces for the titration of [ZnL] (10 μM) with proteins in methanol: (a) BSA, (b) PNA. The bulk concentration of the proteins used was 1.5×10^{-6} M.

indicating that the α-helices are more affected by [ZnL] as compared to a β-sheet. This has been further checked by CD studies.

On the basis of the TCSPC results obtained from the titration of [ZnL] with proteins, it is possible to conclude that L is liberated from its complex, at least partly, during its interaction with the protein (SI 11). On the other hand, the excited state characteristics of the protein exhibit only marginal changes in its lifetimes upon interaction with [ZnL], since no major changes are expected in the protein.

Absorption Titration Studies of {[ZnL] + Protein}. The inferences drawn on the basis of the fluorescence studies have been further checked by absorption spectral studies. Absorption studies carried out between the [ZnL] and protein exhibited an isosbestic point around 400 nm. The increase in absorbance of the 420 nm band is because of the dechelation of zinc from [ZnL], which is evident from the control experiments, including those of simple carboxylic acids. The demetalation of Zn^{2+} from [ZnL] follows a trend: PNA > BSA ≥ HSA > jacalin. These studies suggest that both the conformational changes in the protein and dechelation of Zn^{2+} from [ZnL] are responsible for the changes observed. (Figure 9, SI 12) The conformational changes have been further addressed by CD studies.

Circular Dichroism (CD) Titrations. The conformational changes that occurred during the interaction of [ZnL] with BSA, HSA, jacalin, and PNA have been studied by CD at different concentrations of [ZnL] (Figure 10, SI 13). These studies exhibited considerable changes in the ellipticity during the interaction. The changes observed in the ellipticity in the case of BSA was greater by about 2-fold as compared to that observed in HSA, indicating greater secondary structural changes in BSA. However, only marginal changes were observed with lectins when compared to the albumin proteins. This shows that secondary structural changes occurring during

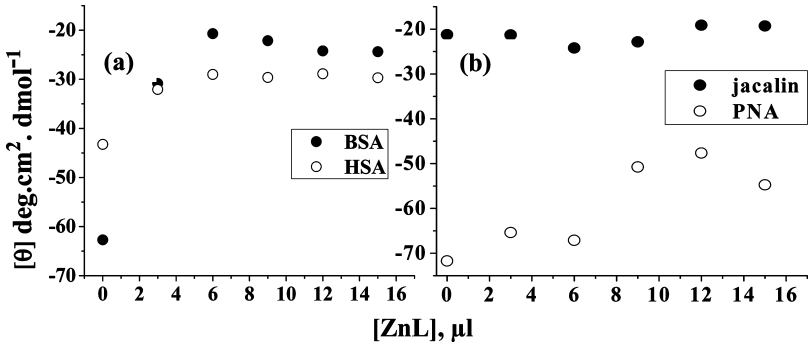


Figure 10. Titration of proteins with [ZnL] (10 μM) by CD spectroscopy: (a) Plots of ellipticity as a function of [ZnL] concentration in the case of albumin proteins. (b) Plots of ellipticity as a function of [ZnL] concentration in the case of lectins. The bulk concentration of the proteins used was 1.5×10^{-6} M.

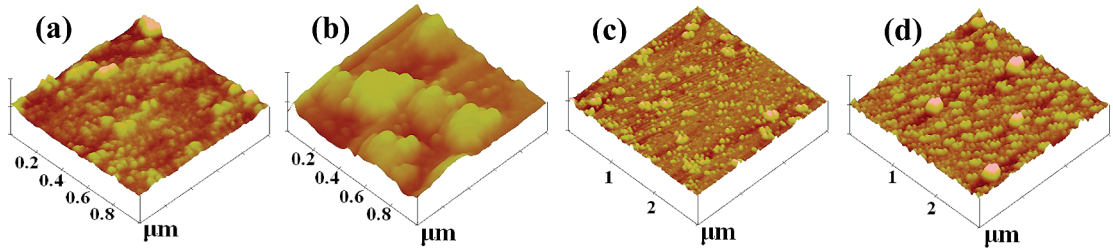


Figure 11. AFM pictures of (a) HSA, (b) [ZnL] + HSA, (c) BSA, and (d) {[ZnL] + BSA}.

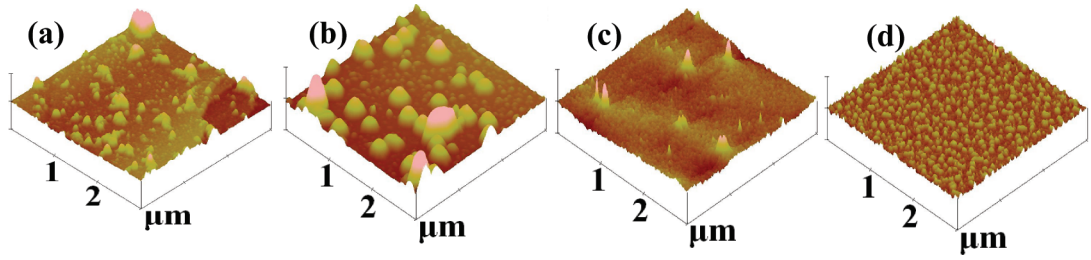


Figure 12. AFM pictures of (a) jacalin, (b) {[ZnL] + jacalin}, (c) PNA, and (d) {[ZnL] + PNA}.

the interaction of [ZnL] with lectins is smaller than that occurring in BSA and HSA, indicating that the α-helices are more affected than the β-sheets. The observed trends in the CD studies were found to support the inferences drawn on the basis of the fluorescence as well as absorption studies.

Atomic Force Microscopy Studies. All the studies clearly indicated that the interaction of [ZnL] with amino acids, peptides, and proteins resulted in the dechelation of Zn^{2+} and its chelation by the later. Therefore, it is of great interest to see how such changes reflect on the nanostructural features of proteins when treated with [ZnL]. Hence, AFM studies were carried out with {[ZnL] + BSA}, {[ZnL] + HSA}, {[ZnL] + jacalin}, and {[ZnL] + PNA}, and the data were compared with the data obtained for the appropriate control systems for L, [ZnL], BSA, HSA, jacalin, and PNA.

In AFM, the [ZnL] exhibited polydispersity of particles with sizes ranging from 70 to 190 nm and heights ranging from 4 to 9 nm, as compared to simple L (Figure 11a, b). Clusters of such particles have also been found. On the other hand, the AFM micrographs of BSA exhibited rather uniformly distributed particles of 90−120 nm size in addition to a few clusters of such particles. These particles also exhibited a height of 4−8 nm. When BSA was treated with [ZnL], the resulting {[ZnL] + BSA} also exhibited uniformly distributed particles having sizes of 160−280 nm, which is roughly double/triple the size of the precursor BSA (Figure 11c, d). Even the height of these particles also got doubled (from 10 to 20 nm), thereby suggesting the aggregation of the protein in the presence of [ZnL].

To check whether the observed aggregation is due to [ZnL] or otherwise, control experiments were carried out, and it was found that there was no aggregation when BSA was treated with either L or with Zn^{2+} (SI 14). Similar studies carried out with HSA resulted in a much larger aggregation of the protein upon addition of [ZnL], as compared to the simple HSA, as can be judged from the size, height and shape of the particles formed. Even the control experiments carried out supported the aggregation of HSA as resulting from the interaction of [ZnL]. The individual particle sizes of jacalin and PNA were found to be in the range of 85−115 nm. Upon treating these lectins with [ZnL], the particle size increases dramatically for jacalin (~350 nm), whereas a nominal increase was observed (~165 nm) in the case of PNA (Figure 12). Particle size analysis of all these is shown in Figure 13 as a histogram. In the literature, a sulfonato-calix[4]arene derivative has been found to be responsible for the aggregation behavior of albumin proteins; namely, BSA.[32,33]

The fact that the proteins exhibit considerable changes only in the presence of [ZnL], as observed in the particle size, and not in the presence of either Zn^{2+} or L is suggestive of the interaction of the ubiquitous nature of [ZnL] with these proteins, including possible binding through Zn^{2+}. Thus, the ubiquitous nature of [ZnL] seems to be affecting the protein aggregational

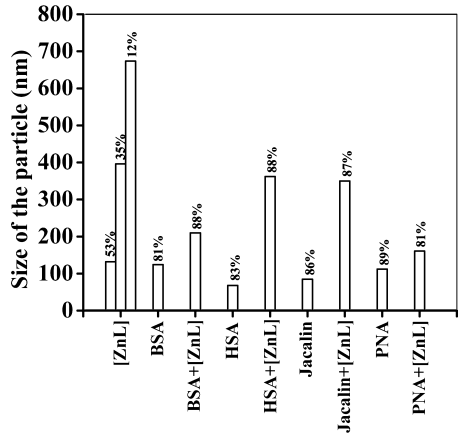


Figure 13. Histogram indicating the percent of particles having a particular size as shown for [ZnL], proteins, and their complexes with [ZnL]. Number of particles analyzed ranges from 100 to 200 in each case.

behavior. The aggregation of the protein when treated with [ZnL] follows a trend: PNA < BSA < jacalin < HSA, which is almost the reverse of that observed for demetalation.

Correlations

A lower-rim naphthylidene conjugate of calix[4]arene, L has been synthesized and characterized, and the structure has been established by single-crystal XRD. The L has been found to be selective toward Zn^{2+} and forms a [ZnL] complex that exhibits high fluorescence enhancement owing to PET-*off* of the imine lone pair and also shows an increase in the absorbance of the 375 nm band characteristic of the formation of a Zn^{2+}-bound complex. The Zn^{2+} induces some changes in the arms of L so that the N_2O_2 coordination results in the formation of a highly distorted tetrahedral complex of Zn^{2+} ([ZnL]).

[ZnL] recognizes Asp, Cys, His, and Glu from among the naturally occurring amino acids by exhibiting large fluorescence quenching. The recognition of these amino acids by [ZnL] results in dechelation of Zn^{2+} and rechelation of this ion by the corresponding amino acid on the basis of the changes observed in the 340−450 nm region in absorption spectra. The recognition features of [ZnL] toward amino acids seem to have bearing on protonation and chelating ability (His > Phe > Asp > Cys ≫ Glu), $\pi-\pi$ interaction ability (Trp ≫ Tyr > His) of the side chain with the [ZnL], and the dechelation of [ZnL] (His > Cys > Tyr > Asp ∼ Trp), as demonstrated on the basis of fluorescence, absorption, and ESI-MS studies, indicating that the incoming amino acid may approach the zinc center from the top, perhaps through the formation of a ternary complex. [ZnL] has also been found to be selective toward the amino acids present in GSH and GSSG.

Extension of this to proteins also resulted in dechelation of [ZnL] as well as aggregation of the protein, although the extent to which these occur differs from one protein to the other. These features have been demonstrated on the basis of absorption and fluorescence spectroscopy and atomic force microscopy. The conformational changes occurring in the protein by the interaction of [ZnL] have been established on the basis of CD spectroscopy. The fact that the proteins exhibit considerable changes only in the presence of [ZnL], as observed in the particle size, is suggestive of the interaction of the ubiquitous nature of [ZnL] with these proteins, including possible binding through Zn^{2+} that results from the dechelation of [ZnL]. The aggregation of the protein when treated with [ZnL] follows a trend, PNA < BSA < jacalin < HSA, which is almost a trend in the reverse of that observed for demetalation. The aggregation observed in the case of albumin proteins in the presence of [ZnL] in the present study is in conformity with that reported for BSA in the presence of a sulfonato-calix[4]arene reported in the literature.[33] Thus, [ZnL] is not only capable of recognizing the amino acids, it is also capable of interacting and recognizing the corresponding amino acids present in α-helical proteins (BSA and HSA) as well as *β*-sheet lectins (jacalin and PNA).

Experimental Section

All chemicals were purchased from Sigma-Aldrich, and the solvents were obtained from Merck (India). All the solvents were distilled before use. ^1H and ^{13}C NMR spectra were measured on a Varian Mercury NMR spectrometer working at 400 MHz. The mass spectra were recorded on a Q-TOF micromass (YA-105) using the electrospray ionization method. The elemental analyses were performed on a Thermoquest microanalysis instrument. FT IR spectra were measured on a Perkin-Elmer spectrometer using KBr pellets. Absorption measurements were recorded using a Jasco UV−vis spectrophotometer. Emission measurements were made by using a Perkin-Elmer LS55 fluorescence spectrophotometer. Lifetime measurements were recorded on a time-resolved fluorescence spectrometer from IBH, U.K. CD studies were performed on a JASCO circular dichroism spectrometer. AFM studies were performed in multimode Veeco Dimensions 3100 SPM with a Nanoscope IV controller instrument. All the computational calculations were performed using the Gaussian03 package.[34]

Synthesis and Characterization of L. A mixture of calix[4]arene-1,3-diamine **3** (100 mg, 0.136 mmol) and 2-hydroxy-1-naphthaldehyde (46.8 mg, 0.272 mmol) were stirred in ethanol at room temperature for 12 h. A yellow precipitate thus formed was isolated through filtration, washed with methanol, and dried under vacuum. Yield, 67%. $C_{70}H_{78}N_2O_6$ (1042): C, 80.58; H, 7.54; N, 2.68%. Found: C, 80.20; H, 7.27; N, 2.31%. FTIR (KBr, cm^{-1}): 3438 (υ_{OH}), 1631 ($\upsilon_{C=N}$). ^1H NMR ($CDCl_3$, δ ppm): 0.87 (s, 18H, CMe_3), 1.27 (s, 18H, CMe_3), 3.3 (d, 4H, J = 13.2 Hz, Ar−CH_2−Ar), 3.9 (t, 4H, Ar−CH_2−Ar), 4.17 (d, 4H, J = 13.2 Hz, Ar−CH_2−Ar), 4.13 (t, 4H, OCH_2, Ar−CH_2−Ar), 6.68 (s, 4H, Ar−H), 6.70 (d, 2H, Ar−H), 6.88 (s, 4H, Ar−H), 6.98 (d, 4H, Ar−H), 7.20 (d, 2H, Ar−H), 7.40 (t, 2H, Ar−H), 7.58 (t, 4H, Ar−H), 8.02 (d, 2H, Ar−H), 8.97 (s, 2H, HC═N), 14.6 (s, 2H, Ar−OH); *m/z* (ESI-MS) 1043 M^+, 100%). ^1H NMR and ESI-MS spectra are given in Supporting Information (SI 01).

Synthesis and Characterization of [ZnL]. To a solution of L (0.105 g, 0.1 mmol) in CH_2Cl_2 (5 mL), zinc(II) acetate (0.022 g, 0.1 mmol) in methanol was added dropwise. After stirring for 1 day, the volume was reduced to half by rota-evaporation under vacuum, and the solid formed was filtered, washed with cold methanol (3 mL), and dried under vacuum to give the product [ZnL]. Yield, 70%. NMR ($CDCl_3$, 300 MHz): 0.82 (s, 18H, $C(CH_3)_3$), 1.27 (s, 18H, $C(CH_3)_3$), 3.25 (d, 4H, J = 13 Hz, Ar−CH_2−Ar), 3.88 (br, 4H, NCH_2), 4.12 (d, J = 13 Hz, 4H, Ar−CH_2−Ar), 4.15 (br, 4H, OCH_2), 6.68 (s, 4H, calyx-Ar−H), 6.88 (d, J = 9.2 Hz, 2H, naphthyl-Ar−H), 6.97 (s, 4H, calyx-Ar−H), 7.22 (t, J = 7.2 Hz, 2H, naphthyl-Ar−H), 7.40 (t, J = 7.6 Hz, 2H, naphthyl-Ar−H), 7.55 (d, J = 8.0 Hz, 2H, naphthyl-Ar−H), 7.59 (d, J = 9.2 Hz, 2H, naphthyl-Ar−H), 8.00 (d, J = 8.8 Hz, 2H, naphthyl-Ar−H), 8.95 (s, 2H, HC−N), 14.6 (s, 2H, Ar−OH). Anal. (% found): C, 76.20; H, 6.50; N, 2.11. $C_{70}H_{76}N_2O_6Zn$ (% required): C, 75.98; H, 6.92; N, 2.53. *m/z* (ESI-MS) 1106 (M^+). ^1H NMR and ESI-MS spectra are given in Supporting Information (SI 01).

Isolation and Purification of Lectins from the Seeds of Jack Fruit and Peanut. Seeds (jack fruit or peanut) were ground to make a fine powder, and the powder was treated with acetone to defat the material. This material was extracted with 25 mM Tris buffer saline (TBS), pH 7.4; 5 mM $CaCl_2$; and 5 mM $MnCl_2$ and was stirred overnight at 4 °C. The supernatant was collected by centrifugation at 12 000 rpm for 20 min at 4 °C. To the supernatant, ammonium sulfate was added to get 0−80% saturation. Precipitated protein was collected by centrifugation at 12 000 rpm for 20 min at 4 °C. The pellet was dissolved in the minimum amount of TBS buffer and dialyzed overnight. The dialyzed mixture was loaded onto the corresponding column,[35] a galactose-affinity column in the case of jacalin and a lactose-affinity column in the case of PNA. The column was washed, and the protein was eluted with the elution buffer (Tris, 25 mM; NaCl, 0.15 M; galactose/lactose, 0.3 M). The protein was concentrated and dialyzed. The dialyzed protein obtained from the affinity chromatography was further purified by gel filtration chromatography using Sephacryl-100 (S-100) in the case of jacalin and S-200 in the case of PNA. The purity of the protein was checked by SDS−PAGE analysis. The activity of the lectins was confirmed by hemagglutination assay.[36]

Fluorescence Titrations. Fluorescence emission spectra were measured on a Perkin-Elmer LS55 by exciting the samples at 320 nm, and the emission spectra were recorded in the 330−550 nm range. The bulk solutions of L, zinc perchlorate, and amino acid were prepared in MeOH in which a small amount of $CHCl_3$ was used for dissolving the ligand L. All the measurements were made in a 1 cm quartz cell, and the effective concentration of L was maintained as 10 μM. During the experiment, the zinc complex of ligand L was made *in situ* by adding a 1:1 mixture of L and zinc perchlorate. During the titration, the concentration of amino acid was varied accordingly to result in the requisite mole ratios of amino acid to L, and the total volume of the solution was maintained constant at 3 mL in each case by adding an appropriate solvent. Amino acid ester titrations were performed after neutralizing the hydrochloride salt by using triethylamine. In the case of proteins, emission was followed by excitation at two different wavelengths. One was at 280 nm, where the protein tryptophan excites, and the other was at 320 nm for zinc complex excitation. Similar titrations were carried out even between [ZnL] and the protein by the following method. Bulk solutions of proteins were made at a concentration of 1.5×10^{-6} M. [ZnL] was prepared *in situ* by adding equimolar quantities of L and $Zn(ClO_4)_2$. Fluorescence titration was carried out by adding increasing concentrations of the protein. Fluorescence spectra were measured by exciting at two wavelengths, one at 280 nm where the Trp of protein as well as [ZnL] are excited and the other at 320 nm where only the [ZnL] is excited. The emission spectra were recorded in the range of 290−550 nm. In the case of the aromatic amino acids (Trp, Phe, Tyr and His), the background fluorescence was subtracted before making the (I/I_o) plots.

Absorption Titrations. Bulk solutions were prepared by adapting a procedure similar to that given for fluorescence studies. Titrations were performed by varying the equivalents of amino acid in the range 0−16 by fixing the [ZnL] concentration at 10 μM (constant throughout), and all the solutions were diluted to 3 mL by using MeOH. Similar titrations were carried out between [ZnL] and the protein by using the procedure given under fluorescence studies. Since simple Trp has absorbance in the UV region, to find out the effect of this amino acid on [ZnL], the absorbance of the former was subtracted from the latter. The negative absorbance observed at ~215 nm indicates that in the presence of Trp, the absorbance of [ZnL] decreases dramatically.

Circular Dichroism Studies. Protein solutions were prepared for CD studies by dissolving 1 mg/mL in methanol. Titrations were performed by adding increasing amounts of freshly prepared [ZnL] to the protein solution, and the measurements were made in the region 180−300 nm.

Atomic Force Microscopy Studies. Tapping mode with a phosphorus-doped Si probe having a sharp, fine tip at the end was used in all cases. The sample of [ZnL] for AFM was prepared from a 3×10^{-4} M solution in methanol. The solution for BSA, HSA, jacalin, and PNA were prepared to have 1 mg/10 mL in each case. Sample solutions were prepared by mixing 200 μL of [ZnL] and 200 μL of the corresponding proteins in methanol. All the samples for AFM studies were made from solutions sonicated for ~30 min. About 50 μL of the sonicated solutions was spread on mica sheets as the substrate and were allowed to air-dry. The dried substrates were used for AFM measurements.

Conclusions

A lower-rim naphthylidene conjugate of calix[4]arene [L] was synthesized and characterized, and the structure was established by single-crystal XRD. [ZnL] recognizes amino acids (Asp, Cys, His, and Glu) and peptides and proteins containing these that occur through protonation of [ZnL], chelation of Zn^{2+}, and $\pi-\pi$ interactions between amino acid and [ZnL], as studied on the basis of absorption, steady state, and time-resolved fluorescence spectroscopy. Present studies demonstrated the dechelation of [ZnL] in the presence of amino acids and proteins and the aggregation of the latter by atomic force microscopy study. The α-helical proteins (albumins) exhibited greater conformational changes as compared to the β-sheet proteins (lectins).

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Supporting Information Available: Characterization and spectral data (SI 01), crystal data (SI 02), computational data (SI 03, SI 04), fluorescence data (SI 05, SI 06, SI 07), absorption data (SI 09, SI 12), TCSPC data (SI 10, SI 11), CD data (SI 13), and AFM data (SI 14). This material is available free of charge via the Internet at http://pubs.acs.org.

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