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An insight into the biophysical characterization of different states of cefotaxime hydrolyzing β-lactamase 15 (CTX-M-15)

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Cefotaxime hydrolyzing β-lactamase-15 (CTX-M-15) is encoded by bla_{CTX-M-15} gene present on plasmid of various Gram-negative bacteria, such as *E. coli*, *E. cloacae*, *K. pneumoniae*, etc. The widespread dissemination of CTX-M-15 harboring bacteria in hospital as well as community settings is a universal threat as they are resistant to various clinically significant antibiotics. In order to gain an insight into the folding mechanism of CTX-M-15, we carried out pH-induced denaturation study by monitoring Trp fluorescence, far-UV circular dichroism (CD), and ANS fluorescence. We found that the pH-induced denaturation of CTX-M-15 was a three-step process with the accumulation of two stable folding intermediates (X_{II} at pH 2.5 and X_{I} at pH 1.5) in the folding pathway. The intermediates were further characterized by far-UV and near-UV CD analysis, Trp fluorescence, ANS fluorescence, three-dimensional fluorescence, acrylamide quenching, dynamic light scattering, and thermal denaturation studies. We found that X_{I} state lacked tertiary structure but retained most of the secondary structure, its Trp residues were partially exposed to the solvent and its hydrophobic patches were highly accessible to ANS. On the other hand, a complete disruption of tertiary structure along with more than 50% loss in secondary structure was observed in X_{II} state. We conclude that the X_{I} state of CTX-M-15 at pH 2.5 had all the characteristics of a molten globule (MG) state, while its X_{II} state at pH 1.5 was more similar to pre-molten globule (PMG) state. ANS fluorescence also showed that the binding of ANS in X_{II} state was lower than that in the X_{I} state. We propose that the accumulation of MG- and PMG-states was due to separation (at pH 2.5) and then unfolding (at pH 1.5) of the ββαα-fold of CTX-M-15, respectively.

Keywords: protein folding; protein stability; three-dimensional fluorescence; non-native intermediates; extended spectrum β-lactamase

1. Introduction

The discovery of penicillin is one of the most significant achievements in medicine (Fleming, 1929). Since then, large-scale production of penicillin and other antibiotics had revolutionized the way bacterial infections were treated and had promised the beginning of an infection-free environment. However, over the past few decades, the indiscriminate use and over the counter sale of antibiotics has put a positive selection pressure on bacteria to develop resistance against commonly used antibiotics. Several resistance mechanisms have been described in bacteria *vis-a-vis* production of β-lactamases, modification of cellular targets, expression of drug efflux pumps, and change in the metabolic pathways (Chambers, 1999; Ma, Cook, Hearst, & Nikaido, 1994; Nikaido, 1994; Tondi, Morandi, Bonnet, Costi, & Shoichet, 2005). The rapid dissemination of resistance markers by horizontal plasmid transfer in *Enterobacteriaceae* is the major cause for the emerging resistance phenotype in bacteria which are otherwise susceptible to antibiotics. One of the most potent forms of resistance mechanisms is the production of β-lactamases that can cleave β-lactam ring of β-lactam antibiotics (Bush, 2010a). In the recent past, it has been established that plasmid borne *bla*_{CTX-M} (derived its name from being highly active on CefoTaXime and isolated in Munich) is the most prevalent ESBL, which can disseminate in the environment by horizontal gene transfer (Shakil & Khan, 2010). It belongs to Ambler molecular class A or functional class 3β ESBL and is capable of conferring resistance to all antibiotics except carbapenems and cephamycins (Bush, Jacoby, & Medeiros, 1995). *bla*_{CTX-M-15} gene encodes a 31 kDa β-lactamase (CTX-M-15) which can cleave β-lactam group containing antibiotics, such as penicillins and different generations of cephalosporins. CTX-M-15, together with its parent variant CTX-M-3, has become prevalent not only in India but also in other countries, such as Poland, United Kingdom, Bulgaria, Romania, and Turkey (Gniadkowski et al., 1998; Karim, Poirel, Nagarajan, & Nordmann, 2001; Paterson & Bonomo, 2005). The
widespread dissemination of CTX-M-15 in hospitals and community-settings has a significant socioeconomic impact and poses a serious threat to treat bacterial infections (Bush, 2010b; Shakil & Khan, 2010). Although, CTX-M-15 plays a central role in conferring resistance to various antibiotics, the mechanism by which it attains native conformation is poorly understood. Therefore, the characterization of folding intermediates of CTX-M-15 is indispensable for the better understanding of the folding mechanism and determining the factors that affect protein stability and functional activity (Anfinsen, 1973; Brockwell & Radford, 2007).

Previously, we have cloned \( \text{bla}_{\text{CTX-M-15}} \) form \( \text{E. cloacae} \) clinical strain isolated from Aligarh hospital (Genbank accession No.: JN860195.1) and purified the protein to homogeneity (Faheem, Rehman, Danishuddin, & Khan, 2013). In the present study, acid denaturation of CTX-M-15 was studied by monitoring far-UV circular dichroism (CD) at 222 nm, Trp fluorescence at 333 nm, and ANS fluorescence at 480 nm. Moreover, the folding intermediate states of CTX-M-15 were characterized by far-UV and near-UV CD spectra, ANS fluorescence, Trp fluorescence, acrylamide quenching, 3-D fluorescence, thermal denaturation, and dynamic light scattering (DLS). We have identified and characterized MG- and a PMG-like intermediate states of CTX-M-15 in its folding pathway so as gain an insight in to the mechanism by which it attains its native conformation.

2. Experimental procedures

2.1. Materials

1-Anilino-8-naphthalenesulfonic acid (ANS), mono- and di-sodium hydrogen phosphate, sodium acetate, glycine, and sodium chloride were purchased from Sigma Chemical Company (St. Louis, MO, USA). Acrylamide was purchased from Qualigen Fine Chemicals (India). These and other chemicals, which were of analytical grade, were used without further purification.

2.2. Solution and sample preparation

CTX-M-15 was cloned, expressed, and purified as described previously (Faheem et al., 2013). The concentration of CTX-M-15 was determined on a double beam Shimadzu UV–vis spectrophotometer (UV-1800), using a molar extinction coefficient of 25,440 M\(^{-1}\)cm\(^{-1}\) at 278 nm (Rehman, Faheem, & Khan, 2013). Stock solution of GdmCl was prepared and its concentration was determined as described previously (Pace, Shirley, & Thomson, 1989). ANS was dissolved in water and a molar extinction coefficient of 5000 M\(^{-1}\)cm\(^{-1}\) at 350 nm was used to determine the concentration of stock solution (Mulqueen & Kronman, 1982). pH measurements were made at 25°C on a Mettler-Toledo pH meter after calibrating it with standard buffers.

Protein solutions for spectroscopic measurements were prepared in 20 mM buffer of different pH values (5.5–7.0) containing 300 mM NaCl. The buffers used for different pH values were Gly-HCl buffer (pH 5.0–2.50), sodium acetate buffer (pH 2.75–5.00), and sodium phosphate buffer (pH 6.00–7.00).

2.3. Acid denaturation

Samples for acid denaturation studies were prepared by adding 100 µl of protein to 900 µl buffer of desired pH value and incubating it for 12 h at 25°C. The pH values of the protein samples were checked both before and after CD and fluorescence measurements and found to lie within ±1 pH unit. For ANS fluorescence, a 50-fold molar excess of ANS to protein concentration was added to the samples. The concentrations of the protein were 2 µM for fluorescence measurements and 5–15 µM for CD spectral analysis.

2.4. Fluorescence spectroscopy

Fluorescence measurements were performed on a Shimadzu spectrofluorometer (RF-5301 PC) equipped with a thermostatically controlled cell holder and attached to a water bath to maintain constant temperature at 25°C. For all the fluorescence measurements, both excitation and emission slit width were set at 5 nm and the spectra were recorded at fast scanning mode.

For tryptophan fluorescence measurements, 2 µM of protein was used in 1 cm path length cuvette and the spectra were measured in 300–450 nm range upon excitation at 295 nm (Omidvar et al., 2011). For ANS fluorescence measurements, the excitation wavelength was set at 380 nm and the emission spectra were recorded in the 400–600 nm range. For three-dimensional fluorescence measurements, a wavelength range of 220–500 nm was used for emission spectra, by exciting the protein sample at 220 nm with an increment of 10 nm, and 19 spectra were recorded (Pasban Ziyarat, Asoodeh, Sharif Barfeh, Pirouzi, & Chamani, 2014).

2.5. Acrylamide quenching

In the acrylamide quenching experiments, increasing volumes (in 10 µl multiples and up to 150 µl) of the stock acrylamide solution (5.0 M) were added to a constant volume (3 ml) of the 2 µM of protein solution at different pH. The excitation wavelength was set at 295 nm and the emission spectra were recorded in the 300–400 nm range. The excitation and emission slit widths were set at 5 nm. The decrease in fluorescence intensity at emission maximum was analyzed according to the...
Stem–Volmer equation after correcting the fluorescence intensities for inner filter and dilution effects (Eftink & Ghiron, 1981).

\[
\frac{F_0}{F} = 1 + K_{SV} [Q]
\]

(1)

where \(F_0\) and \(F\) are the fluorescence intensities at \(\lambda_{max}\) in the absence and the presence of acrylamide, respectively; \(K_{SV}\) is the Stem–Volmer constant, and \([Q]\) is the concentration of the acrylamide.

2.6. CD spectroscopy

CD measurements were carried out in a Jasco Spectropolarimeter J-815 equipped with a Peltier-type temperature controller (PTC-423S/15) and attached to a water bath. The instrument was calibrated with (+)-10-camphorsulfonic acid. All the experiments were performed at 25 °C using a scan speed of 100 nm/min and response time of 1 s. A protein concentration of 5 μM and 1 mm path length was used for far-UV CD measurements, while 15 μM of protein and 10 mm path length was used for near-UV CD measurements. Each spectrum was an average of five scans and was corrected for the buffer (blank) spectrum. The observed ellipticity is converted to mean residual ellipticity (MRE) in deg cm\(^2\) dmol\(^{-1}\) using the following equation (Rehman, Dey, Hassan, Ahmad, & Batra, 2011).

\[
[\theta]_{MRE} = \frac{\theta_{obs}}{10 \cdot n \cdot c \cdot l}
\]

(2)

where \(\theta_{obs}\) is the observed ellipticity in mdeg; \(n\) is the total number of amino acid residues (291 in CTX-M-15); \(c\) is the molar concentration of the protein, and \(l\) is the path length (cm). The α-helical content of the protein in different conditions was calculated using the following formula (Chen, Yang, & Martinez, 1972).

\[
\%\text{Helix} = \left( \frac{[\theta]_{MRE} - 2340}{30,300} \right) \times 100
\]

(3)

2.7. Thermal denaturation

Heat-induced denaturation studies of CTX-M-15 in its native and pH-induced intermediate states were carried out on Jasco J-815 spectropolarimeter equipped with a Peltier-type temperature controller (PTC-423S/15) and a water bath. The samples were heated at 1 °C per minute, which was adequate for the equilibrium to reach, and the change in \([\theta]_{222 \text{ nm}}\) was measured in the temperature range 30–65 °C. Above 65 °C, CTX-M-15 was found to precipitate. The reversibility of the denaturation process was checked by immediately cooling down the sample to 25 °C and measuring its far-UV CD spectra. It was observed that the data from the renaturation experiments fell on the denaturation curve. The thermal unfolding curve of the native CTX-M-15 was analyzed using the two-state model as described previously (Rehman et al., 2011). All the readings were corrected for the appropriate buffer blank. Assuming that CTX-M-15 followed two-state transition model, fraction denatured \((f_D)\) was calculated using the relation:

\[
f_D = \frac{[y(T) - y_N(T)]}{[y_D(T) - y_N(T)]}
\]

(4)

where \(y(T)\) is the observed optical property at temperature \(T\) and \(y_N(T)\) and \(y_D(T)\) are the optical properties of the native and denatured molecules at temperature \(T\).

The mid-point temperature \((T_m)\) was determined from the \(\Delta G\) vs. \(T\) plot \((T_m\) is the temperature at which \(\Delta G = 0\) as well as from the \(f_D\) vs. \(T\) plot \((T_m\) is the temperature at which \(f_D = .5\)). The change in enthalpy (\(\Delta H\)) was obtained from the plot of \(\Delta G\) vs. \(T\) as described earlier (Pace et al., 1989). The values of \(T_m\) and \(\Delta H\) were used to estimate \(\Delta G^0\) (\(\Delta G\) at 25 °C) with the help of the following Gibbs–Helmholtz equation, assuming the heat capacity change \((\Delta C_p)\) to be zero (Iida et al., 2008; Zaroog & Tayyab, 2012).

\[
\Delta G(T) = \Delta H_m \left( \frac{T_m - T}{T_m} \right) - \Delta C_p \left( \ln \left( \frac{T}{T_m} \right) \right)
\]

(5)

2.8. Dynamic light scattering

DLS measurements were carried out using a RiNA Laser Spectroscatter-201 to obtain hydrodynamic radii of different states of CTX-M-15 at 25 °C. Before measurement, all the solutions (2 mg/ml protein at different pH) were spun at 10,000 rpm for 10 min and filtered through Whatman microfilter having a pore size of .22 μm directly into a 12 μl quartz cuvette. Measurements were made at a fixed angle of 90° using an incident laser beam of 689 nm. Ten measurements of each sample with an acquisition time of 30 s and a sensitivity of 10% were made. The data were analyzed for hydrodynamic radii by Stokes–Einstein equation.

\[
R_h = \frac{kT}{6\pi\eta D}
\]

(6)

where \(R_h\) is the hydrodynamic radius; \(k\) is the Boltzmann’s constant; \(\eta\) is the viscosity of water; and \(D\) is the translational diffusion coefficient.
3. Results

Predominantly, globular proteins exist in four major conformational states: native (N) state, molten globule (MG) state, pre-molten globule (PMG) state, and unfolded/denatured (D) state. Moreover, the intermediate states of a protein have been trapped under different experimental conditions, such as in the presence of low concentrations of urea and GdmCl, extremes of pH, high temperature and pressure (Fink, Calciano, Goto, Nishimura, & Swedberg, 1993; Kristinsson & Hultin, 2003; Nakamura, Seki, Katoh, & Kidokoro, 2011; Wang, Lascu, & Giartosio, 1998; Yang, Dunker, Powers, Clark, & Swanson, 2001).

To gain an insight into the folding mechanism of CTX-M-15, we have studied acid denaturation (pH 5–7.0) of the protein by following changes in Trp fluorescence (333 nm), CD spectra (222 nm) and ANS fluorescence (480 nm) at 25 °C, and characterized the intermediate states by various spectroscopic techniques. Figure 1 represents the X-ray structure of CTX-M-15 (PDB Id: 4HBT) depicting the positions of three Tryptophan residues (Trp210, Trp229, and Trp250) and a neighboring negatively charged Asp residue (Asp253) residue (Lahiri et al., 2013).

3.1. Evidence for intermediate states (acid denaturation)

3.1.1. Trp fluorescence

In a native protein, Trp residues that are buried in the non-polar environment of protein interior can significantly contribute to intrinsic (Trp) fluorescence. Upon excitation at 295 nm, Trp fluorescence of the protein gives characteristic fluorescence spectra in 300–400 nm wavelength range. Quenching in Trp fluorescence intensity is generally observed due to conformational change in the protein that either brings charged residue near the Trp residue(s) or that changes the hydrophobicity around the Trp residue(s). Moreover, change in the wavelength maxima of fluorescence (λmax) is sensitive only to the change in the hydrophobicity of the Trp micro-environment (Khanna, Tokuda, & Waisman, 1986).

Figure 2 shows pH-induced denaturation of CTX-M-15 as monitored by measuring the Trp fluorescence intensity and the shift in λmax. The pH-induced denaturation of CTX-M-15 was a two step process, involving at least three stable states: native (N), intermediate (named as X1), and denatured (D) states. The native protein at pH 7.0 showed maximum fluorescence intensity (222 AU) at 333 nm. The fluorescence intensity as well as λmax was not significantly changed in the pH range 7.0–4.0, suggesting that the overall conformation and stability of the protein was unaltered in this pH range. However, when the pH was lowered from 4.0 to 2.75 (representing the first transition state), a sharp decrease in the Trp fluorescence intensity (from 232 to 156) was observed along with unaltered λmax. The above observation clearly shows that at pH 2.75, conformational changes in CTX-M-15 occur in such a way that a charged group (Asp253) has moved in the proximity of Trp250 and quenched its fluorescence. On further lowering the pH from 2.75 to 2.25, λmax was 7 nm red shifted (from 333 to 340 nm), while the Trp fluorescence intensity was not changed significantly, implying that the polarity of Trp residue(s) micro-environment was (were) drastically increased due to exposure to the polar solvent. It is clear that 70% of the tertiary structure of the protein was lost at pH 2.5 (Table 1). Moreover, on further lowering the pH from 2.25 to 1.5 (representing the second transition state), not only the Trp fluorescence intensity was considerably reduced from 146 to 108 but also the λmax was red-shifted by 5 nm (from 340 to 345 nm). It implied that the tertiary structure of CTX-M-15 was completely lost and all the Trp residues were exposed to the polar solvent. A slight increase in the Trp fluorescence intensity below pH 1.0 and a blue shift of 2 nm in λmax was an indication of anion-induced formation of the tertiary structure of the protein (Goto, Calciano et al., 1990).

3.1.2. Far-UV CD spectra

The CD spectrum of a protein in far-UV region (250–190 nm) characterizes the conformation of the peptide backbone, and hence is useful to determine the secondary structure (α-helices and β-sheets) of the protein. Hence, we measured the far-UV CD spectra of
CTX-M-15 to monitor the effect of different pH on its secondary structure.

Figure 3 represents the far-UV CD measurement at 222 nm of CTX-M-15 at different pH values. We observed a two-step denaturation profile of CTX-M-15 upon acid denaturation, involving at least three stable states: native (N), intermediate (named as XII), and denatured (D)-states. The MRE222 nm of CTX-M-15 in the native condition (pH 7.0) was measured as −14,009 ± 297 deg cm² dmol⁻¹ (Table 1) and it remained unchanged within the pH range of 7.0–2.0, indicating that the secondary structure of CTX-M-15 was stable in this pH range. A drastic reduction in MRE222 nm was observed on further lowering the pH below 2.0, which was evidence for the formation of an intermediate-like state in the pH range of 1.75–1.25. The first transition, which was started below pH 2.0 and completed at pH 1.75, is represented as N→XII transition process, where N represents protein in native state and XII represents protein in intermediate state. The MRE222 nm of intermediate state (XII) at pH 1.5 was found to be −7864 ± 367 deg cm² dmol⁻¹, which was 56% of the native state MRE222 nm at pH 7.0 (Table 1). Moreover, the second transition, which started below pH 1.25 and completed at pH 1.75, is represented as XII→D transition process (Figure 3). Anion-induced stabilization of the protein found at pH < .75 was evident from the marginal increase of MRE222 nm (Goto, Calciano et al., 1990).

3.1.3. ANS fluorescence

ANS is a hydrophobic dye widely used to characterize the hydrophobic regions of a protein that have been exposed as a result of protein unfolding. The fluorescence intensity of ANS (measured at 480 nm) increases dramatically along with a blue shift of the emission maxima once ANS is bound to the hydrophobic patches of the protein.

The pH-induced denaturation of CTX-M-15 as monitored by an increase in ANS fluorescence intensity at 480 nm is shown in Figure 4. Native CTX-M-15 did not bind ANS significantly, suggesting a tightly packed hydrophobic core at pH 7.0. The absence of any prominent change in the ANS fluorescence intensity within the pH range 7.0–4.0 indicated that the protein’s hydrophobic clusters were remained buried in the interior of the protein and were inaccessible to ANS. On further lowering the pH from 4.0 to 2.5, a marked increase in ANS fluorescence intensity was observed, which suggested the loss in the tertiary structure of the protein and, hence, the exposure of the hydrophobic patches to the polar solvent. The maximum increase in ANS fluorescence intensity (333 AU) was measured at pH 2.5 (Table 1). Moreover, when the pH was again lowered from 2.5 to .5, a decrease in ANS fluorescence intensity was noticed up to pH 2.0. At pH 1.5, another peak in the ANS fluorescence (225 AU) was observed and below pH 1.0, anion-induced protein stabilization was evident from the decreased ANS fluorescence intensity. It should be noted that the intensity of ANS fluorescence at pH 1.5 was considerably lower (32.4%) than that at pH 2.5.

The results from Trp fluorescence, far-UV CD, and ANS fluorescence suggested the formation of a MG- (XI state) and PMG-(XII state) like intermediate states at pH 2.5 and pH 1.5, respectively.

3.2. Characterization of intermediate states

Trp fluorescence, far-UV CD, and ANS fluorescence measurements indicated the presence of XI and XII intermediate states at pH 2.5 and pH 1.5, respectively (Figures 2–4). In order to characterize these intermediate states, we performed a detailed structural and thermodynamic investigation to see whether they had all the characteristics of MG and PMG states.

3.2.1. Far-UV CD spectral analysis

Figure 5(a) shows the far-UV CD spectra of CTX-M-15 in its N state and acid induced X1, X11, and D states. CTX-M-15 in the N state (pH 7.0) displayed a spectrum that is the characteristic of a protein predominantly rich in α-helix with minima at 208 and 222 nm. The far-UV CD spectra of CTX-M-15 closely resembled that of the CTX-M-1 (Perez-Llarena et al., 2011). The values of MRE at 208 and 222 nm were −14,082 ± 415 and −14,009 ± 297 deg cm² dmol⁻¹, respectively (Table 1). The overall α-helical content of the native protein was 38.5 ± 2.6% as calculated from Equation (3) and is in good agreement with the value reported (44%) in the crystal structure of CTX-M-15 (Lahiri et al., 2013).
Moreover, the far-UV CD spectrum of CTX-M-15 in XI state (pH 2.5) was similar to that of the N state, suggesting that the secondary structure of the protein was not perturbed at pH 2.5. The MRE208 nm and MRE222 nm of CTX-M-15 in XI state were $-13,363 \pm 281$ and $-13,822 \pm 363$ deg cm$^2$ dmol$^{-1}$, respectively (Table 1), and the corresponding $\alpha$-helical content was 37.9 $\pm$ 2.0%. Further, the XII state of CTX-M-15 at pH 1.5 was characterized by having only 18.2 $\pm$ 1.4% $\alpha$-helix and a considerably reduced MRE208 nm and MRE222 nm values of $-7946 \pm 323$ and $-7864 \pm 367$ deg cm$^2$ dmol$^{-1}$, respectively (Figure 5(a) and Table 1). On the other hand, CTX-M-15 in D state (at pH 5.0) showed complete loss of secondary structural characteristics and represented a random coil-like conformation (Figure 5(a) and Table 1).

### 3.2.2. Near-UV CD spectral analysis

The near-UV CD spectra of CTX-M-15 in its N state and acid induced XI, XII, and D states are represented in Figure 5(b). CTX-M-15 in the N state (at pH 7.0) showed the presence of a prominent negative peak at 278 nm (MRE$_{278}$ nm value of $-22.4 \pm 2.2$ deg cm$^2$ dmol$^{-1}$), indicating the tight packing of aromatic side chains in the interior of the protein (Table 1). A comparison of the near-UV CD spectrum of XI state (at pH 2.5) with that of N state showed that the characteristic band of native CTX-M-15 at 278 nm was partially lost in the XI state (MRE$_{278}$ nm value of $-6.9 \pm 1.7$ deg cm$^2$ dmol$^{-1}$) and it retained only 30% of the tertiary structure. On the other hand, a complete loss of spectral signal throughout the near-UV range was observed in XII and D states (MRE$_{278}$ nm were $-2.7 \pm .8$ and $-1.9 \pm .6$ deg cm$^2$ dmol$^{-1}$, respectively) indicated a complete loss of the tertiary structure (Figure 5b and Table 1).

### 3.2.3. Trp fluorescence

Trp fluorescence is a very sensitive probe for studying the overall conformation of proteins as it is highly sensitive to changes in the microenvironment around the fluorophore. In the case of CTX-M-15, the Trp fluorescence at 333 nm was measured as a function of pH. The fluorescence intensity at 333 nm decreased as pH increased from 5.0 to 7.0, indicating a significant loss of the tertiary structure. The pH-induced denaturation of CTX-M-15 was monitored by following the change in far-UV CD signal (MRE$_{222}$ nm) at 25°C. The fluorescence intensity at 333 nm was plotted against pH (Figure 2), showing a three-state denaturation profile in which the first transition is represented as N $\leftrightarrow$ XI, while the second transition is represented as XI $\leftrightarrow$ D. Protein samples (5 $\mu$M) were prepared in different buffers (20 mM) containing 300 mM NaCl. The buffers used were Gly-HCl buffer (pH 2.75–5.00), sodium acetate buffer (pH 6.00–7.00), and sodium phosphate buffer (pH 6.00–7.00).
susceptible to the polarity of its surrounding environment (Eftink, 1994). The crystal structure of CTX-M-15 showed the presence of three Trp residues that are uniformly distributed all over the protein molecule (Lahiri et al., 2013). It is also clear from Figure 1 that Trp 229 was fully buried in the interior of the protein molecule, while Trp250 was fully exposed to the solvent. Also, another Trp residue at 210th position was partially exposed to the polar solvent.

Figure 6 depicts the Trp fluorescence spectra of CTX-M-15 in its N state and acid induced X1, X11, and D states. The fluorescence spectrum of CTX-M-15 in N state (at pH 7.0) was characterized by the presence of an emission maximum ($\lambda_{\text{max}}$) at 333 nm, which gets shifted to 353 nm in D state (20 nm red shift), indicating the exposure of the Trp residue(s) to the solvent upon denaturation. The $\lambda_{\text{max}}$ is uniquely sensitive to the polarity of the Trp micro-environment and even a small change in the conformation of protein can bring fairly large changes in its position. In X1 state at pH 2.5, a marked decrease (32%) in the fluorescence intensity along with 4 nm red shift was observed indicating that the overall polarity of Trp residues was increased. Also, a red shift of only 4 nm in X11 state as compared to 20 nm as observed in D state indicated the presence of residual tertiary structure at pH 2.5 (Figure 6, Table 1).

The fluorescence intensity of CTX-M-15 in X11 state (at pH 1.5) was considerably reduced by 51% along with a 12 nm red shift, suggesting that the tertiary structure was completely lost and it behaved like a random coil as in the D state (45% reduced fluorescence intensity with 20 nm red shift).

3.2.4. ANS fluorescence

ANS preferentially binds to the hydrophobic patches of a protein that are exposed during denaturation process and, hence, has been widely used to detect MG- and PMG-like states of the protein (Engelhard & Evans, 1995).

The ANS fluorescence spectra of CTX-M-15 in its N state and acid-induced X1, X11, and D states are shown in Figure 7. It was evident that CTX-M-15 in its N state (at pH 7.0) produced a weak ANS fluorescence spectrum with an emission maximum at 489 nm, suggesting the burial of hydrophobic patches in the protein interior. Several proteins have been shown to produce a weak ANS fluorescence in their N states (Muzammil, Kumar, & Tayyab, 1999; Tatsumi & Hirose, 1997). The ANS fluorescence of CTX-M-15 in D state also displayed a weak signal with emission maxima at 506 nm, indicating the lack of available hydrophobic patches for ANS binding. An explicitly enhanced ANS fluorescence (333 AU at 477 nm) along with 12 nm blue shift was observed in X1 state (at pH 2.5), which indicated that a large extent of the hydrophobic patches were exposed to the polar solvent. It was also observed that in X11 state at pH 1.5, the hydrophobic patches were significantly exposed to the solvent as evident from a considerable increase in ANS fluorescence intensity (225 AU) and a blue shift of 8 nm (Figure 7 and Table 1). The ANS fluorescence intensity of CTX-M-15 in X11 state was, however, considerably lower (32.5%) than that observed in X1 state.

3.2.5. Acrylamide quenching

Acrylamide is a neutral quencher which has been widely used to study the exposure of Trp residues of a protein to the solvent. Here, acrylamide quenching was employed to monitor the micro-environment of Trp residues in N state and acid-induced X1, X11, and D states of CTX-M-15.

The Stern–Volmer plots of the Trp fluorescence quenching studies of CTX-M-15 in different states and the corresponding Stern–Volmer constants ($K_{SV}$) are represented in Figure 8 and Table 2, respectively. The exposure of Trp residues to the solvent as a result of conformational changes in the protein was generally characterized by an increase in $K_{SV}$ value (Chakraborty et al., 2001). As shown in Figure 8 and Table 2, the $K_{SV}$ values of CTX-M-15 in X1 state (4.9 ± .5 M$^{-1}$) and X11 state (8.2 ± .7 M$^{-1}$) were relatively higher than that observed in the native state (4.2 ± .6 M$^{-1}$). However, these values were considerably lower than that of D state (9.6 ± 1.0 M$^{-1}$) of CTX-M-15. These results confirmed that the Trp residues of CTX-M-15 were relatively more exposed to the solvent in X11 state than in X1 state, and were least exposed in the native state.

3.2.6. Three-dimensional fluorescence

Recently, three-dimensional fluorescence spectroscopy has been widely used as an additional tool to study the conformational changes in the protein (Li, Zhang, Xu, & Ji, 2011). We used three-dimensional fluorescence spectroscopy to support the results of far- and near-UV CD in characterizing the secondary and tertiary structural changes in CTX-M-15 in N state and acid-induced X1, X11, and D states (Figure 9). The characteristics of three-dimensional fluorescence spectra of CTX-M-15 in terms

<table>
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<tr>
<th>Different states of CTX-M-15</th>
<th>$K_{SV}$ (M$^{-1}$)</th>
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<tr>
<td>N state (pH 7.0)</td>
<td>4.2 ± .6</td>
</tr>
<tr>
<td>X1 state (pH 2.5)</td>
<td>4.9 ± .5</td>
</tr>
<tr>
<td>X11 state (pH 1.5)</td>
<td>8.2 ± .7</td>
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<tr>
<td>D state (pH .5)</td>
<td>9.6 ± 1.0</td>
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of the intensity of the peak and its position under different experimental conditions are given in Table 3. The peaks $a$ and $b$ were common in all spectra, representing the Rayleigh scattering peak ($\lambda_{ex} = \lambda_{em}$) and second-order scattering peak ($\lambda_{em} = 2\lambda_{ex}$), respectively (Zaroog & Tayyab, 2012). On the other hand, peaks 1 and 2 represented the fluorescence spectral characteristics of Trp/Tyr residues and polypeptide backbone, respectively (Glazer & Smith, 1961; Kang et al., 2004). The peak 1 was originated due to $\pi \rightarrow \pi^*$ transition, representing the change in the tertiary structure of the protein (Kang et al., 2004), while peak 2 was a probe for the secondary structural change, representing $n \rightarrow \pi^*$ transition (Glazer & Smith, 1961).

It is evident from Figure 9 and Table 3 that CTX-M-15 in XI state (at pH 2.5) showed a red shift of 2 nm (from 336 to 338 nm) with a 17% decrease in fluorescence intensity of peak 1 when excited at 280 nm, representing the partial loss in the tertiary structure. Moreover, when the XI state was excited at 230 nm, fluorescence intensity was decreased by 25% with no change in the position of peak 2, indicating that the secondary structure of the protein remained unaltered. It was noticed that the fluorescence intensity was decreased as a result of quenching in Trp fluorescence due to conformational changes that brought charged residue (Asp253) in the proximity of Trp250 residue. CTX-M-15 in D state did not show peak 2, while the fluorescence intensity of peak 1 was reduced by 32% with a prominent red shift of 16 nm, which indicated that the secondary as well as tertiary structure was completely lost and the protein was in random coil state.

In the X11 state at pH 1.5, the fluorescence intensity of peak 1 was decreased considerably by 38% and its position was red shifted by 5 nm (when excited at 280 nm), indicating a prominent reduction in the tertiary structure of the protein. Further, upon excitation at 230 nm, peak 2 retained 52% fluorescence intensity and displayed a red shift of 8 nm, indicating that the X11 state retained some secondary structure.

3.2.7. DLS

The X1 and X11 states of CTX-M-15 were further characterized by measuring its hydrodynamic volume ($4/3\pi R_h^3$) from the experimentally determined hydrodynamic radius ($R_h$) and comparing it with that of the CTX-M-15 in its N and D states (Table 4). The hydrodynamic volume of the CTX-M-15 in its N, X1, X11, and D states were found to be 72,200 ± 410, 123,584 ± 784, 235,334 ± 1143, and 693,116 ± 1736, respectively. The observed hydrodynamic volume of X1 state was 1.7 times larger than that of native CTX-M-15 (Table 4). Moreover, the hydrodynamic volume of X11 state was found to be 3.3 times larger than that of the native CTX-M-15.

3.2.8. Thermal denaturation

Heat-induced denaturation was used to monitor the relative stability of CTX-M-15 in the native, X1 and X11 states. MRE$_{222}$ nm was followed as a function of temperature (Figure 10) and the thermodynamic parameters obtained are presented in Table 5.

Figure 10(a) shows the variation in MRE$_{222}$ nm as a function of temperature at different pH values. On the other hand, Figure 10(b) shows the normalized thermal unfolding plot ($f_D$ vs. $T$) of CTX-M-15 at different pH.

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<th>Table 3. Three dimensional fluorescence spectral features of CTX-M-15 under different conditions.</th>
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<tr>
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<tr>
<td>XI state (pH 2.5)</td>
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<td>X11 state (pH 1.5)</td>
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<td>D state (pH .5)</td>
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<th>Table 4. Hydrodynamic radii of CTX-M-15 by DLS under different conditions.</th>
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<th>Table 5. Thermodynamic parameters for the heat-induced denaturation of CTX-M-15 obtained by monitoring change in MRE$_{222}$ nm.</th>
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values and the variation in Gibbs free energy change upon denaturation ($\Delta G_D$) with temperature $T$ (Figure 10(b) inset). The thermal denaturation curves of CTX-M-15 in N state (at pH 7.0) and XI state (at pH 2.5) comprised a pre-transition phase in the temperature range of 25–36°C, a transition phase from 40–51°C, where a marked decrease in ellipticity at 222 nm was observed, and a post-transition phase characterizing complete denaturation beyond 52°C. Samples were precipitated on heating above 60°C at pH 7.0, and pH 2.5, hence data was collected only up to 60°C. It was also observed that the thermal transitions of CTX-M-15 were

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**Figure 5.** Far-UV CD spectra (panel a) and near-UV CD (panel b) spectra of CTX-M-15 in N state (---) at pH 7.0, XI state (-----) at pH 2.5, XII state (- - -) at pH 1.5, D state (----) at pH .5, and ANS only (••••) at 25°C. The protein concentrations used for far-UV and near-UV CD spectra were 5 μM and 15 μM, respectively.

**Figure 6.** Intrinsic (Trp) fluorescence spectra of CTX-M-15 in N state (▲) at pH 7.0, XI state (●) at pH 2.5, XII state (■) at pH 1.5, and D state (▼) at pH .5 at 25°C. The protein concentration used was 2 μM and the excitation and emission slits were set at 5 nm.

**Figure 7.** ANS fluorescence spectra of CTX-M-15 in N state (▲) at pH 7.0, XI state (●) at pH 2.5, XII state (■) at pH 1.5, D state (▼) at pH .5, and ANS only (••••) at 25°C. The concentration of protein used was 2 μM, while ANS was used in 50-fold molar excess ratio.

**Figure 8.** Acrylamide quenching of CTX-M-15 in its N state (▲) at pH 7.0, XI state (●) at pH 2.5, XII state (■) at pH 1.5, and D state (▼) at pH .5 at 25°C. The protein concentration used was 2 μM.
cooperative processes in N and X₁ states, while no cooperativity was observed in the thermal transition of CTX-M-15 in X₁ state at pH 1.5 (Figure 10(a)). The midpoint temperature ($T_m$) value of CTX-M-15 in N and X₁ states were 47.0 ± .3 and 45.9 ± .7 °C, respectively. The observed $T_m$ of CTX-M-15 at pH 7.0 was in agreement with that of other CTX-M variants (Chen, Delmas, Sirot, Shoichet, & Bonnet, 2005; Perez-Llarena et al., 2011). The change in enthalpy at $T_m$ (i.e. $\Delta H_m$) was calculated by multiplying $T_m$ (in K) with the slope of $\Delta G_D$ vs. $T$ plot, and was found to be 89.6 ± 1.7 and 90.0 ± 2.4 kcal mol$^{-1}$ at pH 7.0 and pH 2.5, respectively. The free energy change at 25 °C ($\Delta G_{D_{14}}$) of CTX-M-15 was obtained using Equation (5) after assuming that the change in heat capacity at constant pressure (i.e. $\Delta C_p$) was zero (Zaroog & Tayyab, 2012). The corresponding values were found to be 6.16 ± .41 and 5.89 ± .34 kcal mol$^{-1}$ for N and X₁ states, respectively (Table 5). Thus, the overall stability and the folding pattern of CTX-M-15 in its X₁ and N states were found to be similar.

4. Discussion

4.1. Characterization of X₁ state

The general structural features of MG state (Arai & Kuwajima, 2000; Hamada et al., 1996; Ohgushi & Wada, 1983) can be summarized as (1) intact secondary structure, (2) loss of most of the tertiary structure formed by

![Figure 9. Three-dimensional spectra of CTX-M-15 in its N state at pH 7.0 (panel a), X₁ state at pH 2.5 (panel b), X₁ state at pH 1.5 (panel c), and D states at pH 0.5 (panel d). The protein concentration used was 2 μM and the excitation and emission slits were set at 5 nm. The peaks A and B represent Rayleigh scattering peak ($\lambda_{em}=\lambda_{ex}$) and second-order scattering peak ($\lambda_{em}=2\lambda_{ex}$), respectively. The peaks 1 and 2 represent fluorescence spectral characteristics of Trp/Tyr residues and polypeptide backbone, respectively.](image-url)
the tight packing of amino acid side chains, (3) an increased affinity towards hydrophobic fluorescent dyes such as the ANS. This is the characteristic property generally considered as finger print to identify MG state, (4) 1.5–1.7 times increase in the hydrodynamic volume, and (5) native-like folding pattern.

The Trp and ANS fluorescence studies of CTX-M-15 at different pH values indicated the formation of an intermediate-like state at pH 2.5 that we referred as the XI state (Figures 2, 4). A comparison of CD spectra of CTX-M-15 in N and XI states revealed that 98.4% of the native secondary structure was retained in XI state, while 69% of the tertiary structure was lost (Figure 5, Table 1). The above observation was also well supported by Trp fluorescence whereby the fluorescence intensity of CTX-M-15 in XI state, as compared to N state, was decreased by 32% along with a red shift of 4 nm in $\lambda_{max}$ (Figure 6, Table 1). Moreover, the Stern–Volmer plots and the corresponding $K_{SV}$ values of fluorescence quenching by acrylamide showed that the Trp residues in XI state were more exposed to the solvent as compared to the N state and, hence, more accessible to acrylamide (Figure 8, Table 2). Three-dimensional fluorescence spectra also supported the above observations and showed that the secondary structure of CTX-M-15 remained intact, while most of the tertiary structure was lost in XI state (Figure 9, Table 3). By comparing these spectral properties of CTX-M-15 in XI state with that in D state, we found that the tertiary interactions was only partially lost in XI state. These observations were in harmony with the reports that suggested a remarkable structural heterogeneity among MG states of a protein induced by different conditions (Alam et al., 2009; Dill & Chan, 1997) and the presence of substantial native-like tertiary packing in the MG state of some proteins (Dill & Chan, 1997). Thus, we can clearly say that two of the structural characteristics of the MG state, i.e. characteristics (1) and (2) as mentioned above, were satisfied by the XI state of CTX-M-15.

To further characterize the XI state of CTX-M-15, we measured the far-UV CD spectra in the presence and absence of ANS and found that the secondary structure of CTX-M-15 in XI state was not altered by ANS (data not shown).

To further characterize the XI state of CTX-M-15, we measured its hydrodynamic radius ($R_h$) by DLS and computed the corresponding hydrodynamic volume. As compared to the N state, the hydrodynamic volume of CTX-M-15 in XI state was found to be 1.7 times larger (Table 4). This observation was well supported by previous work on $\beta$-lactamases which showed that the hydrodynamic volume of MG state was 1.4 times larger than that in the native state (Goto & Fink, 1989; Goto, Takahashi et al., 1990). Thus, another criterion of MG state was satisfied by XI state of CTX-M-15.

Thermal denaturation of CTX-M-15 in N and XI states was followed by measuring MRE at 222 nm (Figure 10, Table 5). The results showed that the unfolding of CTX-M-15 in both N and XI states was cooperative in nature. Moreover, the thermodynamic parameters ($T_m$, $\Delta H_m$ and $\Delta G_m$) of CTX-M-15 in XI state were similar to that of the N state. These results suggest that the XI state of CTX-M-15 had native-like folding pattern.

Figure 10. Panel a shows the variation in $[\text{MRE}]_{222\text{ nm}}$ as a function of temperature (°C). Panel b shows fraction denaturation ($f_D$) versus temperature (°C) plot of CTX-M-15 in its N state (●) at pH 7.0, XI state (▲) at pH 2.5, and X_{II} state (▼) at pH 1.5. Inset in panel B shows the variation in free energy change (Δ$G_{D}$) with respect to temperature (K).
and thus fulfilled another characteristic of MG state. These measurements and the optical characterizations led us to conclude that the XI state of CTX-M-15 was indeed an MG state.

4.2. Characterization of XII state

The characteristic structural features of PMG state (Khan, Rahaman, & Ahmad, 2011) are as follows (1) up to 50% loss in the secondary structure (2) complete loss of the tertiary structure (3) an increase in ANS fluorescence as compared to native state, but weaker than that in the MG state (4) considerably less compact structure than MG and native state, but still more compact than the random coil.

Acid denaturation of CTX-M-15 as followed by far-UV CD and ANS fluorescence revealed the formation of an intermediate state at pH 1.5, which we referred as XII state (Figures 3, 4). In order to characterize the XII state, we measured far- and near-UV CD spectra of CTX-M-15 in N and XII states and found that the secondary structure in XII state was reduced by 52.7%, while its tertiary structure was completely lost (Table 1). These results were further confirmed by Trp fluorescence studies which showed that in XII state, the Trp fluorescence intensity was decreased by 51% along with a 12 nm red shift in \( \lambda_{\text{max}} \) (Table 1). On comparing Trp fluorescence of XII with that of the N and D states, it was clear that XII state retained some of the tertiary structure, while most of it was lost. Moreover, acrylamide quenching experiments showed that the \( K_{SV} \) value of CTX-M-15 in XII state was considerably higher than that in N and XI states, but significantly lower than that of the D state. Thus, it was evident that the Trp residues of CTX-M-15 in XII state were more exposed to the solvent as compared to that in the N and XI states but less exposed than the D state. Further, we compared three-dimensional fluorescence spectra of CTX-M-15 in XII state with that in N and D states, and found that the tertiary structure was completely lost while some of the secondary structure was retained. On the basis of these results, we conclude that the characteristics of PMG (1) and (2), as stated above, was successfully fulfilled by the XII state of CTX-M-15.

To further characterize the XII state, we measured ANS fluorescence and compared it with that of the N and XI states. We found that the ANS fluorescence of XII state was significantly increased by 12.5 times along with a blue shift of 8 nm in \( \lambda_{\text{max}} \). However, the ANS fluorescence intensity of XII state was considerably lower (32.5%) than that of the XI state, which had 18.5 times increased fluorescence intensity as compared to the N state. Thus, the most significant structural feature of PMG state was also satisfied by XII state of CTX-M-15.

One of the key structural characteristics of PMG state is that its hydrodynamic volume is approximately three times larger than that of the N state. Thus, we measured the hydrodynamic radius of CTX-M-15 and calculated the corresponding hydrodynamic volume. As shown in Table 4, the hydrodynamic volume of XII state was found to be significantly larger than that of the N and XI states (3.3 and 1.9 times larger, respectively), but considerably lesser than that of the D state (2.9 times lesser). Thus, the structural characteristics of the XII state of CTX-M-15 and its comparison with those of N, XI, and D states led us to conclude that the XII state existed as PMG state.

5. Conclusion

At present, there is a growing interest in the biophysical and structural characterization of \( \beta \)-lactamases involved in antibiotic resistance. This study provides a timely insight into the folding mechanism of CTX-M-15 type \( \beta \)-lactamase, which cleaves \( \beta \)-lactam antibiotics and confers resistance to pathogens in different parts of the world. The three-dimensional structure of CTX-M-15 showed that it maintained a \( \alpha \beta \alpha \)-sandwich fold, which is a characteristic feature of class A \( \beta \)-lactamases (Lahiri et al., 2013). On the basis of the results obtained in this study, we propose that different folds of CTX-M-15 were first separated at pH 2.5 (XI state) and then unfolded independently at pH 1.5 (XII state). The description is further explained by the schematic representation of acid-induced denaturation of CTX-M-15 (Figure 11). Thus, on the basis of the results obtained in this study, we conclude that (1) two intermediates (XI and XII) accumulate transiently during the folding of CTX-M-15.
to its native state, (2) $X_I$ and $X_{II}$ states of CTX-M-15 have all the characteristics of MG and PMG state, respectively, and (3) formation of secondary structure of the protein earlier in the folding pathway is responsible for the stabilization of PMG state.

**Author contribution**

The experiments were designed by AUK and MTR, and performed by MTR. CTX-M-15 was purified by MF. Data were analyzed by MTR and AUK. Manuscript was written by MTR and AUK.

**Conflict of interest**

Authors have no conflict of interest to declare.

**Abbreviations**

- CTX-M-15: Cefotaxime hydrolyzing β-lactamase (discovered in Munich, Germany)
- ESBL: Extended spectrum β-lactamase
- ANS: 1-anilino-8-naphthalene sulfonic acid
- CD: Circular dichroism
- DLS: Dynamic light scattering
- MRE: Mean residual ellipticity
- MG: Molten globule
- PMG: Pre-molten globule

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**References**


