GdmCl-induced unfolding studies of human carbonic anhydrase IX: a combined spectroscopic and MD simulation approach

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Communicated by Ramaswamy H. Sarma

(Received 17 March 2016; accepted 13 April 2016)

Carbonic anhydrase IX (CAIX) is a transmembrane glycoprotein, associated with tumor, acidification which leads to the cancer, and is considered as a potential biomarker for hypoxia-induced cancers. The overexpression of CAIX is linked with hypoxia condition which is mediated by the transcription of hypoxia-induced factor (HIF-1). To understand the biophysical properties of CAIX, we have carried out a reversible isothermal denaturation of CAIX-induced by GdmCl at pH 8.0 and 25°C. Three different spectroscopic probes, the far-UV CD at 222 nm ([θ]222), Trp fluorescence emission at 342 nm (F342) and difference molar absorption coefficient at 287 nm (Δε287) were used to estimate stability parameters, ΔG₀ pep (Gibbs free energy change in the absence of GdmCl); Cm (midpoint of the denaturation curve), i.e. molar GdmCl concentration ([GdmCl]) at which ΔG₉₀ = 0; and m, the slope (=εΔG₀/ε[GdmCl])). GdmCl induces a reversible denaturation of CAIX. Coincidence of the normalized transition curves of all optical properties suggests that unfolding/refolding of CAIX is a two-state process. We further performed molecular dynamics simulation of CAIX for 40 ns to see the dynamics of protein structure in different GdmCl concentrations. An excellent agreement was observed between in silico and in vitro studies.

Keywords: human carbonic anhydrase IX; GdmCl-induced denaturation; protein stability; protein folding; molecular dynamics simulation

1. Introduction

Human carbonic anhydrase IX (CAIX) is a transmembrane metallo enzyme that belongs to the α-CA enzyme family. Human CAIX is a multidomain protein comprised 459 amino acid residues, which catalyzes the reversible conversion between carbon dioxide and bicarbonate ion and proton (Alterio et al., 2009). Apart from its main role in the pH regulation, CAIX is also found to be associated with cell proliferation, intercellular communication, cell adhesion and spreading (Hilvo et al., 2008). CAIX is primarily expressed in few of normal tissues, whereas it is overexpressed in large number of solid tumors such as colon, cervical, breast, renal carcinomas and brain (Pastorekova et al., 1997; Saarnio et al., 1998). Expression of CAIX is induced under hypoxic conditions, their activity leads to a pH imbalance in tumor tissues and provides favorable environment for the survival of cancer cells (Hockel & Vaupel, 2001; Ivanov et al., 2001; Wykoff et al., 2000). Moreover, CAIX is also known to contribute in cancer progression by stimulating cancer cell migration and invasion. Under hypoxia condition, CAIX get overexpressed, and it is upregulated by hypoxia-inducible factor (HIF-1). The overexpression of CAIX is associated with tumor formation and it promotes tumor growth and metastasis. Thus, CAIX is considered as an attractive target for anticancer therapy (Leitans et al., 2015).

CAIX protein is produced from the CA9 gene located on 9p12–13 chromosomal locus, which is composed of 11 exons encoding distinct structural domains (Barathova et al., 2008). Exon 1 encode the N-terminal domain homologous to proteoglycans (PG domain), exons 2–8 encode carbonic anhydrase catalytic domain, exons 9–10 encode transmembrane region (TM) and exon 11 encodes the intracellular tail-coding part (Opavsky et al., 1996).

Proteins perform various biological activities and they are functionally active in their specific conformation, called the folded or native state. Thus, the folded or native states of proteins are essential for their biological activity (Uversky, 2002). Protein folding is a matter of great interest because it provides a deep insight into the basic thermodynamics of the process of folding and the general mechanisms used to stabilize proteins and also the factor affecting them (Nick Pace, Scholtz, & Grimsley, 2014). The failure of proteins to fold into native structure generally produce inactive proteins and these are referred as misfolded proteins. To estimate the protein stability parameter, the native structure of...
proteins are perturbed in order to make an unfolded state (Naiyer, Hassan, Islam, Sundd, & Ahmad, 2015; Rahaman et al., 2008, 2015; Rahman, Ali, Islam, Hassan, & Ahmad, 2015; Rehman, Dey, Hassan, Ahmad, & Batra, 2011; Shahid, Ahmad, Hassan, & Islam, 2015; Singh, Hassan, Islam, & Ahmad, 2015). Unfolded state can be achieved by increasing temperature, changing the solvent pH or adding a chemical denaturants such as urea and guanidium chloride (GdmCl) (Alam Khan et al., 2010; Ali, Hassan, Islam, & Ahmad, 2014; Anwer et al., 2014; Anwer et al., 2015; Ubaid-ullah et al., 2014; Alam Khan et al., 2009; Haque, Zaidi et al. 2015).

A large body of information on human CAIX has been documented during the past years (Bracht & de Alencastro, 2016; De Simone & Supuran, 2010; Qiu et al., 2011); however, a limited information is available on its biophysical characterization. Focus of the present study is on structural characterization and measurement of stability of CAIX using GdmCl. Our structural and biophysical analysis suggest that GdmCl-induced denaturation of CAIX is a two state process. We further performed molecular dynamics (MD) simulations of CAIX in water and GdmCl as a cosolvent at 300 K to understand the structural and dynamic stability as we reported for other proteins in different solvent conditions (Khan, Parkash, Islam, Ahmad, & Hassan, 2016; Naz, Singh, Islam, Ahmad, & Imtaiyaz Hassan, 2015; Naz, Shahbaaz, Bisetty et al., 2016).

2. Materials and methods

2.1. Material

Luria-Bertani broth was purchased from HiMedia Laboratories, India. Tris buffer, imidazole and NaCl were purchased from Merck (Darmstadt, Germany). Ampicillin, GdmCl and IPTG were purchased from Sigma (Saint Louis, MO, USA). Plasmid pET-21c used as expression vector was purchased from Novagen, Wisconsin, USA. All chemicals used were of molecular biology research grade.

2.2. Expression and purification of CAIX

The catalytic domain of CAIX (377-amino acid residues) was generated and subeloned in pET21c. Bacterial system *E. coli* BL21 (DE3) was used for expression. We have isolated and purified protein form inclusion bodies followed by Ni-NTA affinity chromatography with slight modifications in our published protocol (Idrees, Kumar et al., 2016). Purified protein of 41 kDa was confirmed by SDS-PAGE and Western blot. The protein concentration was determined by absorbance measurement using molar absorption coefficient (ε) is 35,000 mol⁻¹ cm⁻¹ at 280 nm.

2.3. Sample preparation

To study the chemical denaturation of CAIX, stock solution of GdmCl was prepared in 50 mM Tris buffer, pH 8.0. GdmCl concentration was determined by refractive index measurements (Pace, 1986). For denaturation studies, corresponding concentration of GdmCl (from .25 to 5.0 M) was added in protein and incubated for 5–6 h before spectral measurements. A similar procedure was employed for the renaturation experiments with the exception that the protein was first denatured by adding required (2.0, 4.0 and 5.0 M) concentration of GdmCl and then diluted with the same buffer (Ahmad & Bigelow, 1982).

2.4. Measurements of absorption spectra

Jasco UV–visible spectrophotometer (Jasco V-660) was used for the measurements of absorption spectra of CAIX, which is equipped with peltier-type temperature controller (ETCS-761). Absorption measurements of the protein (.40 mg ml⁻¹) were performed in the wavelength range 340–240 nm at 25 ± .1°C. All experiments were performed at least thrice and their average values are taken for analysis.

2.5. Measurements of fluorescence spectra

Fluorescence spectra of human CAIX in the presence on varying concentrations of GdmCl were measured using Jasco spectrofluorimeter (Model FP-6200) whose temperature was maintained at 25 ± .1°C by an external thermostat water circulator. To measure Trp fluorescence of CAIX which has five intrinsic tryptophan (Trp), the protein was excited at 280 nm, and its emission spectra were recorded in the range of 300–400 nm using 5 mm quartz cuvette. The protein concentration used was .20 mg ml⁻¹. All experiments were performed at least five times and their average were taken for analysis.

2.6. Circular dichroism (CD) measurements

To see the effect of GdmCl on the secondary structure of protein we performed the far-UV CD measurements in Jasco spectropolarimeter (model J-1500) which was equipped with a peltier type of temperature controller (PTC-517). The far-UV CD spectra (250–200 nm) were collected using cuvette of .1 cm path length. The raw CD signal (elipticity) was converted to mean residue ellipticity, [θ]λ using the following relation,

\[ [\theta]_\lambda = \frac{M_o \theta_\lambda}{10lc} \]  

where, \( \theta_\lambda \) represents the ellipticity in millidegrees, \( M_o \) represents the mean residue weight of the protein, \( c \) is the concentration of the protein in mg ml⁻¹ and \( l \) is the path length of cuvete in centimeter.
2.7. Analysis of GdmCl-induced denaturation of CAIX

To obtain protein stability parameters, the entire \((y(u), [u])\) data of each GdmCl-induced transition curves, shown in Figures 1(B), 2(B) and 3(B) were analyzed using a non-linear least-squares method (Santoro & Bolen, 1988):

\[
y(g) = y_N(g) + y_D(g) \times \frac{\exp[-(\Delta G_D^0 + m[g])/RT]}{1 + \exp[-(\Delta G_D^0 + m[g])/RT]} \tag{2}
\]

where \(y(u)\) is the observed optical property at \([u]\); \(y_N(u)\) and \(y_D(u)\) are, respectively, optical properties of the native and denatured protein molecules under the same experimental conditions in which \(y(u)\) was measured; \(\Delta G_D^0\) is the value of Gibbs free energy change \((\Delta G_D)\) in the absence of the denaturant; \(m\) is the slope \((\partial \Delta G_D/\partial [u])\); \(R\) is the universal gas constant; and \(T\) is the temperature in Kelvin. It should be noted that Equation (2) assumes that a plot of \(\Delta G_D\) versus [GdmCl] is linear, and the dependencies of \(y_N\) and \(y_D\) on [GdmCl] are also linear (i.e. \(y_N(u) = a_N + b_N[p]\), and \(y_D(u) = a_D + b_D[p]\), where \(a\) and \(b\) are [p]-independent parameters, and subscripts N and D represent these parameters for the native and denatured protein molecules, respectively). The analysis of denaturation curves of different optical properties according to Equation (2) gave values of \(\Delta G_D^0\), \(m\) and \(C_m(=\Delta G_D^0/m)\).

2.8. Molecular dynamic simulation

Biomolecular simulation package GROMACS (v4.6.5) was used to carry out the MD simulation of CAIX in water and at different concentration of GdmCl. Atomic coordinates of CAIX (PDB ID: 3IAI) was taken from the protein databank (www.rcsb.org) and simulated with...
CHARMM27 force-field (Pronk et al., 2013) and SPC216 as a water model. As control, CAIX was solvated in the pure water, and a MD simulation of 40 ns was carried at 300 K. The final simulation box contained one protein molecule in a centre of cubic box having size nearly 74.5 × 74.5 × 74.5 Å and water molecules were padding around the protein. The system was neutralized by adding Na⁺ and Cl⁻ ions (.150 M) and periodic boundary conditions were applied in all directions. We used Berendsen thermostat and barostatto maintain the temperature and the pressure of the system, with a coupling times of $\tau_T = .1$ ps and $\tau_P = 2$ ps, respectively (Berendsen, Postma, van Gunsteren, DiNola, & Haak, 1984). The coordinates of protein were energy minimized with steepest descent, up to a tolerance of 100 KJ mol⁻¹ to remove bad contacts. Covalent bonds containing hydrogen atoms at their equilibrium distances were restrained by using SHAKE algorithm. An equilibration of system was carried at 300 K in two steps: first a simulation with canonical ensemble, NVT with no pressure coupling and Berendson thermostat, while the second step is isothermal–isobaric (NPT) ensemble with pressure of 1 bar (P). To compute the long-range electrostatic interactions, Particle Mesh Ewald (PME) method was used. A cut-off of 1.4 nm for van der Waals interactions, a 1.0 nm for Coulombic interaction was adopted during the course of simulation.

Dynamic stability of CAIX in GdmCl solution was carried at 2.0–4.0 M. Parameters and topology files for GdmCl were taken from recent publication (Camilloni et al., 2008; Khan et al., 2016). GdmCl molecules were equilibrated with SPC216 water model in a cubic box with the size of 6 nm. Thereafter, protein was placed centrally in the well-equilibrated solution of the denaturant (GdmCl) and overlapping solvent molecules were deleted. Four independent simulations were carried out in different GdmCl solutions (2.0, 2.5, 3.0 and 4.0 M) with a defined method (Camilloni et al., 2008; Khan et al., 2016). The final 2.0 M GdmCl solution contained one protein placed in the centre of cubic box of size nearly 73.34 × 73.34 × 73.34 Å along with 10,298 water, 451 guanidinium ions (Gdm⁺), 479Cl⁻ ions and 37 Na⁺ ions. Leap-frog integrator was used to provide constrains on all bonds (including heavy H-atom). GROMACS utilities: g_rmsd, g_gyrate, g_rmsf and g_sas used to obtain the radius of gyration ($R_g$), root mean square deviation (RMSD), root mean square fluctuation (RMSF) and solvent accessible surface area (SASA) analyze the MD trajectory files. We plotted all analytical graphs with Xmgrace. DSSP (installed in gromacs) and VMD (Time-line) were used to carry the secondary structure analysis (Humphrey, Dalke, & Schulten, 1996).

3. Results

An increase in CAIX expression was generally observed under hypoxia conditions, which may lead to the tumorigenesis. During the stress condition, alteration in the protein structure always takes place. Thus, thermodynamic studies of CAIX in the presence of denaturant may be helpful to understand its structure-function relationships. Here, we have performed both in vitro and in silico studies of CAIX to measure its structure and stability under the increasing concentrations of a chemical denaturant, GdmCl. We have used different spectroscopic probes like $[\theta]_{222}$ (mean residue ellipticity at 222 nm), a probe to measure change in backbone orientation (Ahmad, 1991), $F_{342}$ (Trp-fluorescence emission intensity at 342 nm and $\Delta\varepsilon_{287}$ (difference absorption at 287 nm). We also carried the GdmCl-induced denaturation of CAIX using MD simulations up to 40 ns, at 300 K to determine the structural stability and dynamics progression at the atomic level.
3.1. Expression and purification
We have successfully expressed and purified CAIX protein from inclusion bodies. The purity and yield of fractions of purified CAIX were checked on SDS-PAGE (Supplementary Figure S1).

3.2. CD measurements
To see the effect of GdmCl on the secondary structure of the protein, we carried out the GdmCl-induced denaturation of CAIX in 50 mM Tris-HCl buffer at pH 8.0 and 25°C. A probe \( [\theta]_{222} \) (mean residue ellipticity at 222 nm) was used to measure change in secondary structure and backbone orientation. Figure 1(A) and (B) shows representative CD spectra in the presence of different concentrations of GdmCl and the transition curve of CAIX induced by GdmCl. It is evident from this figure that there is no change in the secondary structure of CAIX up to ~1.75 M GdmCl concentration. Above this GdmCl concentration a remarkable loss of secondary structure occurred and it continued up to 3.0 M GdmCl, after that no further change in the optical property was observed.

3.3. Fluorescence measurements
To understand the unfolding behavior of CAIX by GdmCl, we monitored the change in the environment of the aromatic amino acid Trp, and hence tertiary structure, by using a probe \( F_{342} \) (Trp-fluorescence emission intensity at 342 nm). Figure 2(A) shows representative emission spectra CAIX in the presence of different concentrations of GdmCl at pH 8.0 and 25 ± .1°C. Transition curve shown in Figure 2(B) was constructed by plotting fluorescence emission intensity at 342 nm \( F_{342} \) vs. [GdmCl]. It is seen in this figure that the pretransition base line in the denaturant concentration range 0–1.75 M and the post transition baseline starts from 3.0 M GdmCl. Furthermore, an equilibrium between the native and denatured states of CAIX is observed in the GdmCl concentration range 1.75–3.0 M.

3.4. Absorption measurements
Absorption spectra of CAIX were measured in the presence of increasing concentration of GdmCl at pH 8.0 and 25 ± .1°C. Figure 3(A) shows representative absorption spectra of CAIX in the region of 240–320 nm. \( \Delta \varepsilon_{287} \) (difference in molar absorption coefficient values in the presence and absence of denaturant at 287 nm) were plotted against [GdmCl]. \( \Delta \varepsilon_{287} \) monitors the change in the environment of aromatic amino acids, and hence the tertiary structure.

3.5. MD simulation studies
We further performed MD simulation for 40 ns to gain insight into the structure and dynamics of CAIX in water and different concentrations of GdmCl. The RMSD value was calculated to examine the time evolution conformational changes of CAIX from the X-ray structure (PDB ID: 3IAI) in neat water and denaturant GdmCl. Plot of time versus RMSD for all C\(^\circ\) atoms is shown in Figure 4. Following observations can be made from results shown in this figure. (i) in water RMSD trajectory (indigo) achieved the equilibrium in initial 5 ns, and a stable state is maintained up to 20 ns. Transition of ~.1 nm is seen in RMSD value after 20 ns, which get drops at 30 ns. An additional drift of .05 nm in trajectory is seen at 30–33 ns, which drops quickly and a stable conformation of protein is maintained up to the end of simulation time. (ii) When protein is placed in GdmCl (2.0 M) system, a gradual increase in RMSD value is seen in 0–25 ns. Trajectory shows two sharp drift in RMSD value >.125 nm at 15 and 25 ns, respectively. Similarly, two more consecutive drift of .1 nm is observed in between 20 and 30 ns, which gets dropped after 30 ns. Thereafter, an average fluctuation of .05 nm in RMSD is continued up to 40 ns of simulation, shows the conformational flexibility of protein in GdmCl environment (iii) In GdmCl (2.5 M), three major drift of .1 nm is seen at 7, 10 and 12 ns, respectively, and trajectory shows an increase in RMSD value >.1 at 30 ns, with respect to the native conformation. At 33 ns, a sharp drift of >.2 nm is observed and an unstable system is maintained till the end of simulation. (iv) Trajectory (red) obtained from protein in GdmCl (3.0 M), shows large deviation in RMSD from the beginning of simulation and an average fluctuation of ~.1 nm is observed up to initial 10 ns. As compared to the native conformation, a total increase in RMSD value >.15 is seen at 0–10 ns.

Although it gets dropped at 15 ns, however, an average fluctuation of .05–.1 nm is maintained up to 25 ns. At 26 ns, a sharp drift of >.15 nm represented the major change in structure, and significantly larger fluctuation in structure of CAIX is seen up to 40 ns. (v) Similarly, at higher concentration of GdmCl (4.0 M), instability in structure can be seen from the beginning of simulation, and a continuous fluctuation of .1–.2 nm is recorded up to 15 ns. A drop down in trajectory is seen at 17 ns, however, again RMSD reaches at 3.5 nm in 20–25 ns. Another, sharp drift in RMSD value >.3 nm is observed near 30 ns at which an unstable system is maintained at the end of simulation.

We further measured the radius of gyration \( R_g \) to examine the dynamic stability and compactness of protein in water and GdmCl solutions. A time evolution plot of \( R_g \) (backbone) is shown in Figure 5. Following observations can be made from this figure. (i) Structural
integrity of isozyme CAIX is maintained in water during the entire simulation time (0–40 ns). $R_g$ trajectory (indigo) quickly achieved the equilibrium in initial few nanoseconds and the structural integrity CAIX is maintained at the end of simulation. (ii) In 2.0 M GdmCl (blue), a continuous fluctuation of .03 nm is seen in $R_g$ trajectory from beginning of simulation, and as compared to native structure a constant fluctuation is seen in $R_g$ up to 40 ns. We can see the major loss of structure in the first 15 ns, which is due to several consecutive drifts. Furthermore, drift in $R_g$ value of >.05 nm at 15–20 ns show the significance loss of residual contacts, involve in regular conformation. This notion of structural loss is carried up to 40 ns. (iii) In 2.5 M GdmCl, a continuous rise in $R_g$ value >.05 nm is seen in first 7 ns and we find an average fluctuation of .05 is continued up to 20 ns. Trajectory shows maximum change in $R_g$ value >.08, at 7 and 18 ns, respectively. A continuous increase in radius is seen during 20–40 ns and the simulation ends with the gain in average $R_g$ value 1.79 ± .02 nm. (iv) In GdmCl (3.0 M), $R_g$ trajectory (red) shows sharp transition in the initial 5 ns, which is gradually dropped at 10 ns. A continuous increase in $R_g$ value is seen at 10–25 nm. With the consecutive drifts of .03 nm at 23 and 27 ns, a continuous fluctuation of .03 nm is seen till the simulation ends. (v) In GdmCl (4.0 M), higher fluctuation in $R_g$ trajectory (maroon) is seen from the beginning of simulation and the $R_g$ value reaches to ~1.80 nm. This drift in $R_g$ is maintained up to 17 ns. Thereafter, an instant drop down in trajectory is seen, again a gain in $R_g$ is observed up to 25 ns. A significant change in $R_g$ was seen at 28–35 ns and we noticed the highest $R_g$ value 1.84 ± .03 nm at 35 nm, which showing a massive loss of structure during the simulation period.

To determine the residual fluctuation and dynamic progression CAIX in water and denaturant, we analyzed the time–average RMSF plot of all $C^\alpha$ atoms. From Figure 6, we can see that (i) RMSF plot (indigo) obtained for CAIX in water, shows stable conformation for residues Pro15-Val19, Val131-Ala134, Ser155-Leu161 and Ala220-Thr228 belonging to $\alpha$-helices. We observed stable fluctuations of residues Asp32-Arg34, Ala39-Phe40, Glu48-Leu50, Leu57-Asn61, Val66-Thr69, Glu78-Gly82, Arg86-Trp97, Thr108-Val109, His112-Arg113, Ala116-Ser124, Glu173-Val176, Tyr191-Ser197, Gln205-Phe212, Val216-Ser219, Trp230-Gly231, Ser239-Arg240 and Glu257-258 all of which are represent in the $\beta$-strands of CAIX. We also carried out secondary structure analysis with DSSP installed in GROMACS and results show additional secondary conformation for residues Pro21-Gly25, Pro35-Ala38, Glu165-Ala168 and Ile181-Leu184 and are belonging to 3_{10}-helices.
Residues having an average fluctuation >.08 nm are defined as the part of loop. We observed that residues (Pro21-Gly25 and Pro35-Ala38) belonging to 3_10-helices at N-terminus are also showing a higher fluctuation in the water as compared to 3_10-helices present at the C-terminus of protein. Result shows a maximum residual mobility for free N-terminal loop, whereas, the higher residual fluctuation at C-terminus represented the longest loop CAIX. Addition of GdmCl (2.0 M) to the system shows an increase in overall residual fluctuation, however, N-terminus residues belonging to 3_10-helices showed a stable fluctuation. The obtained sharp peaks for residues Gln175-Pro177 are belonging to loop present at the C-terminus of β_{12}. RMSF plot (green) obtained for CAIX in GdmCl (2.5 M) shows a higher residual flexibility in comparison to the native structure. A major change in the structure are observed for residues of N-terminus loops and β_{9-11}. However, we found a significant increase in the flexibility of residues belonging to stable conformation (α/β), when CAIX is placed in GdmCl (3.0 M). This result shows a clear picture of perturbation in structure which may leads to loss of secondary conformation during the unfolding process. This can be seen in RMSF plot (red) which shows a higher residual fluctuations for residues belongs to the stable conformation. A drastic changes in structure was observed when CAIX is placed in the system prepared for 4.0 M GdmCl. RMSF plot (maroon) shows that all residues of CAIX having an average fluctuation >.1 nm, which provide a clear evidence of loss of secondary structure.

We examined the time-dependent change in the SASA to quantify the GdmCl-induced unfolding process of CAIX. In X-ray structural of CAIX (PDB ID: 3IAI), out of five tryptophan residues (Trp5, 16, 97, 209 and 230), only Trp209 is found to be buried in structure. We plot the time evolution graph of the surface area of residues accessible to solvent as shown in Figure 7. From this SASA plot, we find that buried Trp209 is gradually exposed to solvent in presence of denaturant (GdmCl). Furthermore, the loss of hydrophobic contact provides a clear evidence of unfolding process.

4. Discussion

The functional properties of CAIX were well studied in the last few decades, but the complete understanding has not yet been obtained of the role of the entire protein and its domains in growth, survival, migration and invasion of tumor cells (Imtaiyaz Hassan, Shajee, Waheed,
Ahmad, & Sly, 2013). Careful examination of the existing literature seems to attribute a different roles to each of the CAIX domains (Pastorek, Pastorekova, & Zatovicova, 2008). Earlier, we have reported the effect of pH and urea on the structure and stability of CAVA (Idrees et al. 2016; Idrees et al. 2016). Here, we carried out biophysical study of CAIX using the far-UV CD, fluorescence and absorption spectroscopy.

To understand folding and stability of CAIX, the GdmCl-induced denaturation of protein in 50 mM Tris-HCl buffer (pH 8.0) was carried out at 25 ± .1°C. For this, three different spectroscopic probes were used. The far-UV CD spectra in the range 200–250 nm were measured, which gave a sigmoidal transition curve on plotting $[\theta]_{222}$ (mean residue ellipticity at 222 nm) versus [GdmCl]. Figure 1(A) and (B) shows far-UV CD spectra and denaturation curve of CAIX at increasing concentration of GdmCl. A cooperative unfolding of CAIX was observed in the range from 1.75 to 3.0 M GdmCl concentration. The data were fitted in equation 2 as discussed in the results section in order to obtain the thermodynamic parameters.

Further, the effect of GdmCl on the tertiary structure of CAIX, i.e. environment of buried Trp were also monitored by fluorescence spectroscopy at same condition. Figure 2(A) shows a representative emission spectra CAIX. Transition curve shown in Figure 2(B) was constructed by plotting fluorescence emission intensity at 342 nm ($F_{342}$) versus [GdmCl]. Tertiary structure was also monitored by absorption spectroscopy. A sigmoidal transition curve of $\Delta \varepsilon_{287}$ (difference absorption at 287 nm) versus [GdmCl] was used to monitor the change in the environment of aromatic amino acid and hence tertiary structure. Values of thermodynamic parameters are given in Table 1.

The analysis of transition curves of three different probes (Figure 1(B), 2(B) and 3(B)) for the determination of stability parameters, $\Delta G^0_D$, $m$ and $C_m$, according to Equation (2), we assumes that denaturation of CAIX by GdmCl follows a two-state mechanism. A coincidence of normalized sigmoidal curves of different optical probes were used to test for two-state behavior of protein denaturation (Pace, 1986; Tanford, 1968). Values of $f_D$ as a function of [GdmCl] were determined from denaturation curves of $[\theta]_{222}$, $F_{342}$ and $\Delta \varepsilon_{287}$, shown in Figure 1–3 using the relation (Rahaman et al., 2013; Haque et al., 2015),

$$f_D = \{y(g) - (a_N - b_N[g])\}/\{(a_D - a_N) + (b_D - b_N[g])\}$$

(3)

Figure 8 shows normalized transition curves of GdmCl-induced denaturation of CAIX (i.e. plot of $f_D$, the fraction of the denatured molecule versus [GdmCl]). The observed coincidence of normalized transition curves of different optical properties of CAIX (Figure 8) suggests that GdmCl-induced denaturation of each protein is a two-state process. Furthermore, identical values of $\Delta G^0_D$, $C_m$ and $m$ (Table 1) were obtained from the analysis of denaturation curves of different properties ($[\theta]_{222}$, $F_{342}$ and $\Delta \varepsilon_{287}$) further support our assumption that GdmCl induces a two-state transition (Rahaman et al., 2013; Haque et al. 2015). Thus, our assumption of two-state behavior of GdmCl-induced denaturation of CAIX is correct.

We further investigated our experimental results with MD simulation studies. It provides an important insight about the modulation in structure during the protein-unfolding process in a given environment with respect to time (Haque et al. 2015; Naz et al., 2016). In view of this, MD simulations of 40 ns for CAIX in the water and GdmCl were performed to determine its structural stability. As shown in Figure 4, RMSD trajectory (indigo) obtained for CAIX in water at 300 K, quickly achieved the equilibrium in initial 5 ns, and this is equilibrium is maintained up to 40 ns of simulation. This observation suggests that the native structure of the protein remained intact during the simulation. However, addition of denaturant GdmCl (2.0 M) to the system shows a gradual increase in RMSD value >.1 nm in first 25 ns, showed the loss of tertiary contact of protein. Thereafter, consecutive drift of .1 nm in RMSD trajectory (blue) at 25 and 27 ns, respectively, significantly showed the expansion in

Table 1. Thermodynamic parameters obtained from GdmCl-induced denaturation of CAIX at pH 8.0 and 25 ± .1°C.

| Probes | $\Delta G^0_D$, kcal mol$^{-1}$ | $m$, kcal mol$^{-1}$ M$^{-1}$ | $C_m$, M |
|--------|--------------------------------|-------------------------------|----------|
| $[\theta]_{222}$ | 5.10 ± .15 | 2.40 ± .05 | 2.10 ± .06 |
| $F_{342}$ | 4.90 ± .13 | 2.36 ± .04 | 2.13 ± .05 |
| $\Delta \varepsilon_{287}$ | 5.01 ± .12 | 2.45 ± .05 | 2.05 ± .05 |
| Average | 5.00 | 2.40 | 2.11 |

Figure 8. Normalized GdmCl-induced denaturation curves (plots of $f_D$ of CAIX versus [GdmCl]) of CAIX.
regular fold. These conformational changes are more clearly seen in trajectory (green) obtained for protein placed in higher concentration of GdmCl (2.5 M). Here, we noticed the consecutive drift in RMSD at the beginning of simulation, which is represented the structural disturbance due to effect of the cosolvent. Further, the gradual increase in RMSD at 20–40 ns, indicated the loss of regular contact of residues involved in the secondary conformation. When the protein is placed in a higher concentration of GdmCl (3.0 M), this unfolding process is more clearly seen in initial time scale of simulation. Higher fluctuation of \( >0.15 \) nm in RMSD trajectory (red) at 0–10 ns, clearly indicated the loss of secondary contacts. Although, a drop down in trajectory is observed at 10 and 25 ns, respectively. However, average fluctuation in RMSD >0.05 nm during this period suggested that structure remained unstable. Thereafter, a consecutive sharp drift in RMSD >2 at 27 and 33 ns, indicated the further loss of structure as the simulation end at 40 ns. For more clarity, we placed the protein in 4.0 M GdmCl and observed the higher drift of \( \approx 3.0 \) nm in RMSD at the initial 5 ns. This showed the loss of structure at the beginning time scale of simulation. Furthermore, sharp drift in RMSD value (>3 nm) at 25 and 35 ns, respectively, provide a clear evidence of massive structural loss. Importantly, the efficacy of structural stability and folding rate of a protein largely depend on the packing of amino acid residues. Herein, we measured the structural integrity of CAIX in the water and GdmCl. The time evolution of \( R_g \) against time for all five simulations are shown in Figure 5. Results from this figure show that in water the secondary and tertiary structure of CAIX remained stable during the entire simulation time with an average \( R_g \) value 1.77 ± 0.02 nm. Addition of GdmCl (2.0 M) to the system lead to consecutive drift in average \( R_g \) trajectory at the initial time frame of simulation, which was continued up to 40 ns of simulation. This suggested the unstable behavior of protein in GdmCl. In 2.5 M GdmCl, we noticed the average fluctuation in \( R_g \) trajectory is constantly increased with the progression of time. The significant change in \( R_g \) value >0.05 nm at 20–40 ns showed the expanding of regular conformations. The simulation ended with higher change in \( R_g \) value >0.05 nm compared to the native protein, which suggests a loss of structural contact under the influence of cosolvent. This observation was consistent at higher concentration of GdmCl. \( R_g \) trajectory obtained for protein in 3.0 M GdmCl showed the sharp change in \( R_g \) value in initial few nanoseconds and the process of unfolding is continued up to the end of simulation. A notable change in average \( R_g \) value 1.79 ± 0.03 could be depicted in trajectory at 20–40 ns, which showed the comprehensive loss of structure. However, a remarkable change in \( R_g \) trajectory (maroon) was observed when protein is placed in higher concentration of GdmCl (4.0 M). \( R_g \) trajectory showed average fluctuation >0.05 nm from the beginning of simulation, which is indicated the massive loss of structural due to higher concentration of co-solvent.

Furthermore, we examined the atom-positional RMSFs to demonstrate the dynamic progression in structural adaptability of CAIX in water and GdmCl (Figure 6). We found that in neat water all stable conformation (\( \alpha \)-helices and \( \beta \)-strands) of CAIX having an average residual fluctuation ≤0.06 nm and only residues belonging to loops showed average fluctuation >0.08 nm. However, an increase in average RMSF for all C\( ^\alpha \) atoms is observed, when we placed the protein in system prepared for different concentrations of GdmCl. A gradually increase in average residual fluctuation was noticed with increase in concentration of GdmCl to the system. At the initial concentration of 2.0 M GdmCl, RMSF plot showed major structural changes at N and C-terminus of CAIX. This was probably due to the presence of loops at the both ends. However, effect of GdmCl-induced structural deformation was also observed for residues which are belonging to four \( \beta \)-strands (\( \beta_3, \alpha \)), interconnecting loops (Arg45-Glu75) and \( \alpha_4 \) and \( \beta_{12} \) (Glu150-Asp180). RMSF plot for GdmCl 2.5 M showed that N-terminus residues and regions belonging to Val120-Phe260 having higher residual fluctuation, which was consisted of \( \alpha \) and \( \beta \) regions in native protein. This progression in structural loss was found to be continued in 3.0 M GdmCl. However, a remarkable change in RMSF plot was observed for protein placed in 4.0 M GdmCl. Here, all stable conformations (18 \( \beta \)-strands and 4 \( \alpha \)-helices) and loops, all showed average residual fluctuation >1 nm, which is an evident for the complete loss of secondary conformation during the unfolding process.

We investigated the time evolution plot of the SASA for each residues of CAIX to define the unfolding process (Figure 7). A systematic increase in the SASA provides direct evidence of unfolding of protein (Granata et al., 2015). The purpose of this analysis was to capture the dynamic behavior of hydrophobic residues, which are gradually exposed to the solvent, during the protein unfolding process. Structural analyses suggested the presence of five tryptophan (Trp5, 16, 97, 209 and 230) residues in CAIX (PDB ID: 3IAI). Out of these, four tryptophan residues (Trp5, 16, 97 and 230) are in the loop region and surface exposed. Only Trp209 (\( \beta_3, \alpha \)) is located in the core of CAIX structure (Figure S2). This could be used as a better indicator for protein unfolding. The plot obtained from SASA analysis (Figure 7) clearly showed that buried Trp209 is exposed to solvent in unfolding process. We find MD simulation studies are in excellent agreement with spectroscopic analysis. Our comparative analysis and observations facilitated that structural integrity and stability of protein is maintained in water. And GdmCl-induced denaturation of CAIX is a two-state process.
5. Conclusions
In the present study, human CAIX was expressed and purified successfully. We employed different spectroscopic techniques and MD simulations approaches to understand the structural stability of CAIX in the presence of the denaturant, GdmCl. Analysis of sigmoidal transition curves from the far-UV CD and other spectroscopic results revealed that the cooperative unfolding of CAIX occurs in the range from 1.75 to 3.0 M GdmCl concentration. Within experimental error, identical value of $\Delta G^o$ measured by different probes and coincidence of all three normalized transition curves suggest that unfolding of CAIX by GdmCl is a two-state process. In simulation, buried hydrophobic residue Trp209 is used as indicator for the unfolding process. A good correlation is observed in our in silico and in vitro studies.

Supplementary material
The Supplemental material for this article can be accessed http://dx.doi.org/10.1080/07391102.2016.1179596.

Acknowledgements
DI, AP and MAH are thankful to University Grants Commission (UGC), New Delhi, India, for their fellowship. Authors sincerely thank Jamia Millia Islamia for providing high speed server in the Central Instrumentation Facility. Harvard University plasmid facility is acknowledged providing the CAIX gene. We thank Department of Science and Technology, India for FIST support (SR/FST/LSI-541/2012).

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
This work was supported by the Council of Scientific and Industrial Research, Government of India (Ref. No.: 37(1665)/ 15/EMR-II).

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