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Ethanol and Acetic Acid Induces Conformational Changes in Zebrafish Dihydrofolate Reductase Protein

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An equilibrium unfolding research of the recombinant zDHFR would provide information on the conformational changes of protein. In present work, the conformation of recombinant zDHFR was investigated in presence of ethanol and acetic acid. Equilibrium unfolding of recombinant zDHFR was monitored by enzymatic assay after denaturation by ethanol and acetic acid at 340 nm. Changes in secondary and tertiary structure of zDHFR with increasing ethanol and acetic acid concentrations were investigated in far-UV circular dichroism (CD) (190 to 250 nm) and fluorescence spectroscopy (emission spectra from 300 to 400 nm with an excitation wavelength of 295 nm) methods. It has been observed that in case of acetic acid-induced denaturation of zDHFR, the shift from native to denatured state occurs in a single step, whereas intermediates or non-native states are found at low concentrations of ethanol. The recent findings have significant implications for understanding how ethanol and acetic acid affect protein structure.

Keywords: Acetic acid, CD spectroscopy, Ethanol, Intrinsic fluorescence, Protein unfolding, zDHFR.

INTRODUCTION

Proteins have the ability to revert to their native three-dimensional structure, which is functionally active. The genetic code of proteins solely determines its fundamental structure, which is the linear sequence of amino acids in polypeptide backbone. This transition from a one-dimensional fundamental structure to a three-dimensional biologically active form is poorly under-stood, which we refer to as the protein folding challenge. Protein production mechanism is widely understood, but its folding seems to be less. Although protein folding issue is predomi-nantly of academic interest, advances in protein engineering and the ability to generate any protein, often in an insoluble, inactive, unfolded and useless form, have made it of great practical significance, particularly referring to bio-technological applications.

To discuss the protein folding problem characterization of unfolded states and structure-function investigations are two very important approaches,which have been utilized in an attempt. Explanation of forces responsible for folded shape of protein as well as stability requires an analysis of changes in functional and structural properties because of external variables. The three-dimensional protein structure is stabilized by several intramolecular interactions such as van der Waals, hydrophobic, electrostatic and hydrogen-bond interactions [1-4]. As a result, a precise evaluation of conformational stability under varied situations is important for comprehending physical interactions that keep a protein's functional structure stable. Understanding protein stability, function and folding requires a thorough analysis of folded and partially folded intermediate states. Characterization of the structure and identification of intermediate states occupied during folding process of several proteins has garnered considerable attention and a great deal of effort has invested into it. Knowing thermodynamic and structural features of these intermediates may shed light on factors that drives the folding process.

Unfolding of small and compact proteins is well character-ized by a two-state transformation and in such circumstances, probability of all the intermediates amid denatured and native states is quite low [5]. However, it is widely known that unfold-ing/refolding of different proteins involves numerous phases, *i.e.* different states have been discovered under various settings

that do not appear to be native or fully unfolded [6]. In case of several proteins, such as human carbonic anhydrase, β-lactamase [7,8] bovine growth hormone [9] and also a chemically modi-fied type of human growth hormone [10], intermediate states were discovered when transitions were examined using several spectroscopic probes [11,12].

The importance of non-native protein conformations has been highlighted in several publications [13]. It's worth noting that many misfolding disorders start when a folded protein encounters incomplete denaturation, allowing it to self-assemble into amyloid [14-17]. Using an organic solvent as a co-solvent, various non-native states of proteins and polypeptides can be easily generated. Alcohols, for example, are often utilized as a co-solvent that modifies the protein structure. Using circular dichroism (CD) and fluorescence techniques, the current study scrutinize effects of ethanol (polar protic solvent) and acetic acid on conformation of zDHFR in its native state. It has been observed that ethanol can alter natural proteins structure [18].

Dihydrofolate reductase (EC 1.5.1.3.) is an enzyme, which uses NADPH as an electron donor to convert dihydrofolate to tetrahydrofolate. Because of its small size, availability of purified enzyme and well-developed functional assay, DHFR is a significant pharmaceutical target and fantastic model for studying the enzyme structure/function interactions. A biophysical characterization of a previously unstudied variation, zebrafish DHFR (zDHFR) is investigated. It can be utilized as a substitute to mammalian species in research. It shares a lot of DNA with its human counterpart [19]. So far, no report of zDHFR equilibrium unfolding by ethanol and acetic acid has been found in the literature. An equilibrium unfolding research of the zDHFR would provide additional information on DHFR unfolding as well as comparative information on unfolding behaviour on distinct DHFR species.

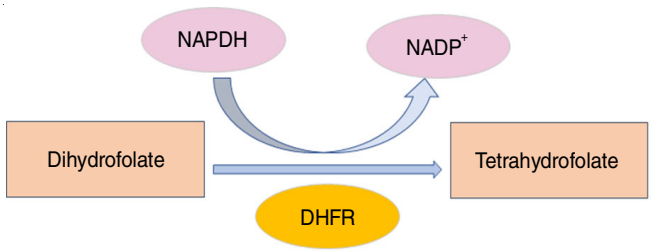
EXPERIMENTAL

The plasmid zDHFR in the pET 43.1a vector containing (His)6 was obtained from Dr. Tzu-Fun, Taiwan. We have used BL21 (DE3) Rosetta *E. coli* strains for expression as well as purification of zDHFR. Isopropyl-D-1-thiogalactopyranoside (IPTG), dihydrofolate (DHF), nicotinamide adenine dinucleotide phosphate (NADPH), Coomassie Blue R-250 were acquired from Sigma-Aldrich. Ethanol and acetic acid were acquired from Changshu Hongsheng Fine Chemical Co., Ltd. and Sisco Research Laboratories Pvt. Ltd., respectively. High purity grade imidazole and sodium chloride were purchased from Merck, India. The entire reagents were of analytical grade. Milli-Q (Merck Millipore) or double-distilled water was used during experimentation.

General procedure

Purification of zDHFR: The BL21 (DE3) Rosetta *E. coli* cells containing the desired plasmid, zDHFR-His/pET43.1a were grown in LB medium containing 100 μg/mL ampicillin at 37 ºC and 250 rpm [19]. Cells were stimulated with 100 μM isopropyl-β-D thiogalactopyranoside (IPTG) and incubated for 6 h at 25 ºC with 250 rpm shaking when the OD at 600 nm approached 0.8 to 1.0. Centrifugation at 6000 rpm for 30 min was used to extract the cells. SDS-PAGE analysis validated the protein synthesis. Cell pellet was resuspended in lysis buffer and sonicated to lyse it. Centrifugation at 13,000 rpm for 40 min at 4 ºC was carried out to remove the cell debris. A 0.22 μm Millipore syringe filter was used to filter the resulting supernatant. Immobilized metal ion affinity chromatography (IMAC) was used to purify N-terminal Histidine tagged zDHFR utilizing Ni^{2+} as a chelating agent.

Enzyme activity assay of recombinant zDHFR protein: As demonstrated in **Scheme-I**, DHFR is responsible for irreversible conversion of dihydrofolate and NADPH to tetrahydrofolate and $NADP^+$. The decrease in NADPH concentration led in a decrease in absorbance measured using a UV-visible spectrophotometer at 340 nm [19]. Based on a molar extinction coefficient of 12,300 M^{-1} cm^{-1} at 340 nm [20], one unit of DHFR activity was defined as 1 μmol of dihydrofolate per min. Assay solution was made up of 25 mM Tris-HCl, 20 mM KCl (pH 7.4), 100 μM DHF, 140 μM NADPH and 0.2 μM DHFR.



Scheme-I: Enzymatic reaction catalyzed by DHFR

Intrinsic fluorescence measurements: Fluorescence emission spectra for zebrafish DHFR in presence of denaturants (ethanol and acetic acid) were recorded from 300 to 400 nm with an excitation wavelength of 295 nm and a 0.5 nm interval and 5 nm slit-width. Each spectrum is the average of three scans. Tris KCl buffer was utilized as a fluorescence emission blank and subtracted to correct the fluorescence background.

Circular dichroism measurements: Far-UV CD spectra of zDHFR in ethanol and acetic acid were recorded on a JASCO model J-815 Circular Dichroism spectrometer. The CD spectra were obtained between 190 nm to 250 at 1 nm interval. Five spectra were obtained for every sample and averaged after subtraction the signal of ethanol and acetic acid under same condition. CD measurements were carried-out in 25 mM Tris-HCl and 20 mM KCl (pH 7.4). The CD (mdeg) was plotted against the wavelength for all denatured states of proteins (0-60% ethanol and acetic acid). The θ at 222 nm was plotted against denaturant concentration, since it can be used to compare the percentage of secondary structure retained during unfolding.

RESULTS AND DISCUSSION

Structural and functional characteristics of zDHFR were studied in the presence of ethanol and acetic acid. Under all denaturant conditions, the maximum incubation time necessary to achieve equilibrium was standardized, with changes occurring in as short as 2 h and no further changes occurring for up to 12 h. A conformational perturbation spectroscopic analysis of zDHFR is presented to know protein folding mechanism, with

an emphasis on UV-visible, fluorescence and circular dichroism spectroscopies. Because of its sensitivity to the environment and higher quantum yield, tryptophan fluorescence was used extensively in the current protein folding study. The maximum fluorescence intensity was determined at 329 nm using the tryptophan fluorescence spectrum of zDHFR, which has three tryptophan residues.

Functional Inactivation of zDHFR: The sigmoidal dependence of zDHFR's residual enzymatic activity on ethanol (0-60%) and acetic acid (0-60%) are displayed in Fig. 1. At the lowest concentration (1%) of ethanol and acetic acid, no influence on residual enzymatic activity of zDHFR was found. At a concentration of 10%, residual enzymatic activity of zDHFR was observed, with ethanol showing 90% activity and acetic acid showing 40% activity. Again, at 20% concentration, residual activity in presence of ethanol was over 60%, but residual activity in acetic acid was just 20%. At 40% ethanol, the activity is nearly negligible and protein is completely inactivated, whereas at 50% acetic acid, total unfolding is detected.

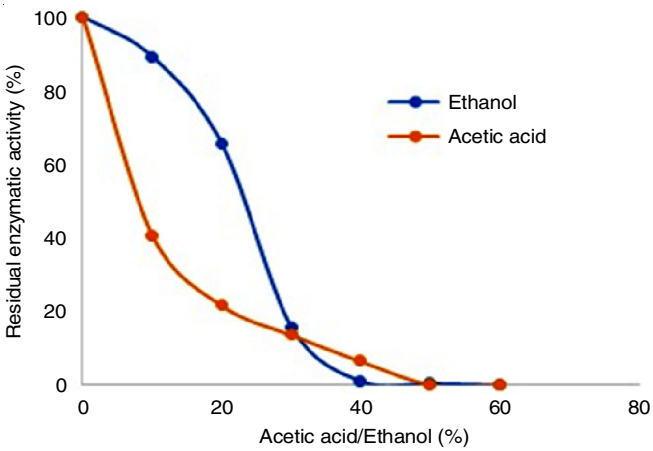


Fig. 1. zDHFR was analyzed for the decrease in enzymatic activity through enzymatic assay. ethanol and acetic acid residual enzymatic activity of zDHFR protein. Ethanol and acetic acid were used to incubate recombinant zDHFR protein at increasing concentrations (0-60%) at 25 °C

Fluorescence spectra of zDHFR with increasing ethanol and acetic acid concentrations: As the electrical and dynamic features of the chromophore environment affect spectral aspects of fluorescence emission spectra such as shape, position and intensity, fluorescence has been widely employed to obtain understanding into structural and dynamic properties of proteins [21]. By tracking changes in intensity and wavelength of emission maxima (λ_{max}) with relation to the concentration of denaturant, the influence of denaturants on microenvironment of zDHFR aromatic residues has been investigated.

Intrinsic fluorescence analysis of zDHFR(2 μM) in presence of varied amounts of ethanol and acetic acid was performed. zDHFR was preincubated in unfolding buffer (pH 7.4) containing the indicated denaturant concentrations for 2 h at 25 ºC. Fluorescence was monitored with a slit width of 5 nm in the emission band (300-400 nm) and an excitation wavelength of 295 nm (Fig. 2a and 2b).

Fig. 2a and 2b show changes in zDHFR fluorescence as denaturant concentration rises, whereas the transition curves are shown in Fig. 2c for two denaturants, ethanol and acetic acid, respectively.

For zDHFR, the emission maximum (λ_{max}) was 329 nm as reported earlier [22] suggesting that aromatic residues are submerged in the protein's hydrophobic core. Fig. 2a and c displays that up to 20% ethanol quenching of fluorescence is observed with decrease in the maximum intensity and no change in λ_{max}. This change may be because there was internal quenching of protein fluorescence and lower concentration of denaturant is quenching the fluorophores. At 30% ethanol, there is a rise in fluorescence intensity further (Fig. 2a) and there was a gradual red shift in λ_{max}; while in the range 40-50% ethanol, fluorescence intensity was almost constant.

The transition curve of acetic acid varies from that of ethanol (Fig. 2c). There is decline in the fluorescence in case of acetic acid as shown in Fig. 2b where red shift in λ_{max} was observed (20-60%)

Secondary structure of zDHFR in ethanol and acetic acid: Far UV-CD analysis on ethanol and acetic acid induced

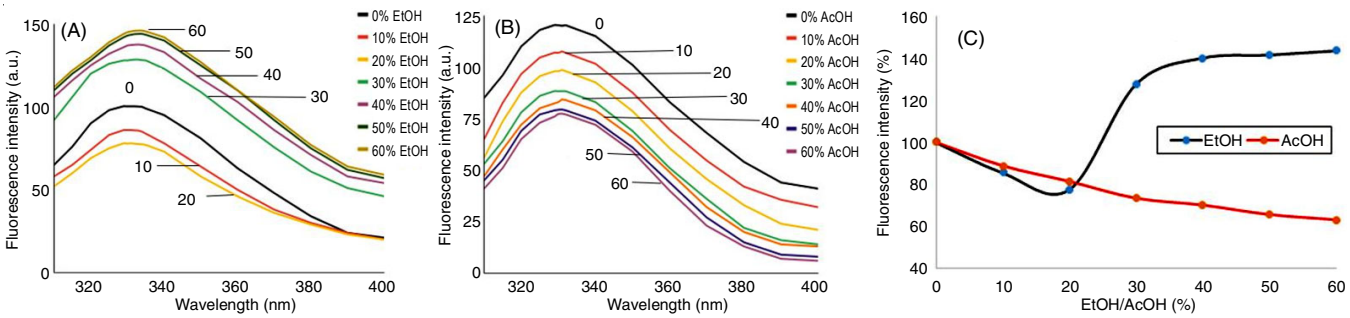


Fig. 2. Intrinsic fluorescence analysis of zDHFR in presence of varied amounts of ethanol and acetic acid. The zDHFR concentration was 2 μM. zDHFR was pre-incubated in unfolding buffer (pH 7.4) containing the indicated denaturant concentrations for 2 h at 25 °C. Fluorescence was monitored with a slit width of 5 nm in the emission band (300-400 nm) and an excitation wavelength of 295 nm. (A) In varied concentrations (0-60%) of ethanol, the fluorescence spectra of zDHFR were measured. (B) In varied concentrations of acetic acid, the fluorescence spectra of zDHFR were measured (0-60%). Spectra of 0-60% ethanol concentrations from bottom to top are displayed, with the lowest trace being 0% and the top one being 60%, as well as spectra of 0-60% acetic acid concentrations from top to bottom are shown, with the lowest trace being 60% and the top one being 0%. (C) Maximum fluorescence intensity of zDHFR transition curves for ethanol and acetic acid denaturation are displayed (with the peak value of native zDHFR taken as 100). (—•—) Values for ethanol in 0–60% range and (—•—) for acetic acid in 0-60% range are shown

unfolding of zDHFR were carried out to look into the impact of these denaturants on secondary structure of protein. In the far-UV spectral region (200-250 nm) CD spectrum of recombinant zDHFR displayed that the enzyme belonged to α and β group of proteins, which was confirmed by analyzing the CD spectra using 'self-consistent' method (selcon 3) as has been reported earlier [22]. Analysis of secondary structural elements suggested that zDHFR contains substantial amount of both α-helix and β-sheet secondary structures.

Effects of increasing the concentration of ethanol and acetic acid on ellipticity of native protein are shown in (Fig. 3a and 3b), where zDHFR was preincubated in unfolding buffer (pH 7.4) containing the indicated denaturant concentrations for two hours at 25 ºC

There is a rise in ellipticity in the region of 10-20% ethanol compared to native protein, but a significant decrease in ellipticity from 30 to 50% ethanol concentration (Fig. 3c). At 60% ethanol concentration, the protein exhibits a random coil configuration. To disclose the influence of acetic acid on secondary structures of recombinant zDHFR, far-UV circular dichroism (CD) spectra of zDHFR in presence of 0-60% acetic acid was obtained. The result displayed the ellipticity of 222 nm (θ_{222}) increased with increasing acetic acid concentration from 0 to 20% (Fig. 3c), indicating that acetic acid influences the formation of zDHFR α-helical structure. The θ_{222} increased about 20% in presence of 50% acetic acid (Fig. 3c), indicating that acetic acid induced a significant conformational transition of zDHFR, thus resulted in the increase of zDHFR α-helical contents.

Ethanol and acetic acid are the chemical denaturants, used for probing the protein conformation, stability and unfolding studies, however, they act in different ways. Protein denaturation is widely acknowledged to be a highly cooperative process that may be represented by a two-state model for small protein molecules, with no substantial intermediates present throughout the shift from native to denatured states. However, new findings reveal that some intermediates occur during protein unfolding. In some circumstances, the intermediate states between native and unfolded states have been characterized as molten globules [23].

In mild denaturing conditions, such intermediates have been discovered for numerous proteins. In molten globule form, the protein molecule is practically as compact as it is in native state, with a loosely packed non-polar core. It is commonly distinguished by far-UV CD spectra, implying the presence of significant secondary structure [24]. When NMR data is merged with hydrodynamic and small angle scattering data for multiple proteins, a finding reveals that the protein chains are rather compact under low denaturing conditions, producing intermediates.

Proteins at high denaturant concentrations are projected to behave like random coils and the dimensions of unfolded states in low denaturant concentrations are particularly interesting [25]. Different response towards denaturants has been observed for various proteins. Ethanol has two different impacts on the zDHFR unfolding pattern. At low denaturant concentrations (10-20%), the protein exhibits higher ellipticity compared to the native state, as well as fluorescence quenching in this concentration range compared to zero denaturant concentration. This decline in fluorescence intensity seen up to 20% ethanol could be imputed to tertiary structural reorganization involving aromatic residues or enhanced mobility of the aromatic residues' surrounding environment. It could also be due to aggregation, which is common in non-native states or a combination of these reasons. Higher concentrations of ethanol and acetic acid has resulted in unmasking the buried residues that are present in the native protein.

Treatment of zDHFR with higher concentration of ethanol and acetic acid has resulted in exposure of buried residues present in the native structure to the solvent as maximum in case of ethanol, 4 nm red shifts and the 3 nm red shift of acetic acid suggests that these denaturants cause zDHFR to unfold. The residual enzyme activity of zDHFR at 30% ethanol declined to less than 20% suggesting protein conformation has changed from its native conformation which is in its fully active functional form. The higher ellipticity at lower ethanol concentrations could be attributable to the change of the native form to an intermediate state that is different from both native and fully denatured states. There are many possible causes for intermediates development. These can be caused by microenvironmental alterations in the protein's aromatic region, minor local native state rearrangements [26] or the stabilizing actions of ethanol at low concentrations.

Ethanol and acetic acid have been shown to provide varied estimates for a protein's structural stability [27-29]. The unfolding of zDHFR suggests different unfolding pathways and

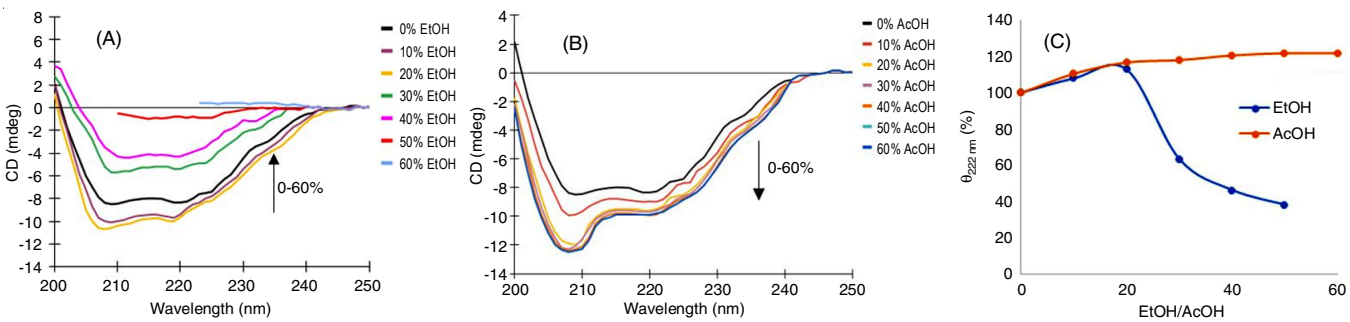


Fig. 3. Effects of increasing the concentration of ethanol and acetic acid on ellipticity of native protein are shown in A and B. In terms of ellipticity values at 222 nm (θ_{222} nm), (C) depicts the respective transition curves for two denaturants. At this wavelength, value of zDHFR protein in absence of ethanol and acetic acid were set to 100

mechanisms for the two denaturants. There are two possible unfolding pathways in ethanol and acetic acid solutions for zebrafish dihydrofolate reductase.

The acetic acid unfolding corresponds to a two-state mechanism where N→D transition takes place in a single step and no intermediate states are observed. The observed results could be the consequence of the combined effect of all the above elements. Understanding the structural alterations that different treatments have on a protein could be important in development of drug and understanding cellular organization at the molecular level. Due to its involvement in subsequent metabolic events such as thymidylate and purine nucleotide production, dihydrofolate reductase plays a crucial role in normal body activities and it is critical that their conformation be stable for maximal functional performance. Thus, the preceding observations have thrown some light on the structural modifications and loss of function that can occur as a result of denaturant exposure, affecting the protein's normal activity.

Conclusion

In present study, the activity and conformational stability of recombinant zDHFR denatured by ethanol and acetic acid was studied using fluorescence and far-UV CD spectroscopy. In case of fluorescence measurement, the intensity quenched up to 20% ethanol concentration relative to native form, however at 30% concentration, intensity surged and emission maxima had red shift with complete unfolding in 40% ethanol. The transition's midpoint was between 10% to 30% of ethanol. In case of acetic acid, intensity of fluorescence gradually diminished progressively with increasing denaturant concentration. According to CD spectroscopy, ellipticity of zDHFR has increased compared to native up to 20% of ethanol concentration, then gradually decreases from 30% to 50% concentration. The protein's coil shape was random at 60% ethanol. Whereas, in case of acetic acid significant conformational transitions of zDHFR occurred in the α-helical structure at 50% concentration. So, it can be concluded from these results that by ethanol denaturation intermediate or non-native states are formed whereas acetic acid denaturation produced no intermediates during denaturation pathway.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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