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Investigation of folding unfolding process of a new variant of dihydrofolate reductase protein from *Zebrafish*

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ABSTRACT

The folding and unfolding mechanisms of a small monomeric protein, Dihydrofolate reductase **(1.5.1.3.)** from a new variant, *Zebrafish* (zDHFR) has been studied through GdnHCl denaturation, followed by its refolding through dilution of the denaturant. Intrinsic and extrinsic fluorescence, far-UV CD and enzyme activity were employed to monitor structural and functional changes due to chemical denaturation. The unfolding transitions monitored by intrinsic fluorescence showed that GdnHCl based denaturation of zDHFR is reversible. At low concentration of the denaturant, zDHFR forms intermediate species as reflected by increased fluorescence intensity compared to the native and fully unfolded form. Equilibrium unfolding transition study of zDHFR induced by GdnHCl exhibited three- state process. The non-coincidence of fluorescence and far-UVCD based transitions curves support the establishment of three state model of zDHFR protein which involves native, intermediate and unfolded forms. Analysis of the equilibrium unfolding transition suggests the presence of non- native intermediate species. A comparative study of various species of DHFR shows that zDHFR has comparable thermodynamic stability with human counterpart and thus proved to be a good *in vitro* model system for structure- function relationship studies. Understanding various conformational states during the folding unfolding process of the zDHFR protein may provide important clues towards designing inhibitors against this important protein involved in cell cycle regulation.

1. Introduction

Protein folding is the process by which the amino acid sequence of a protein determines the three-dimensional conformation of the functional protein [1]. Identification and characterization of intermediates is a crucial aspect for studying the mechanisms of protein folding as well as to develop an understanding of many disease-related processes and biotechnological applications [2]. The challenging mechanism of protein folding has been unresolved for a very long time. By virtue of forces such as hydrophobic forces and inter chain hydrogen bonding, the larger proteins usually tend to refold in an inefficient manner. As a result of which they form partially folded intermediates which aggregate to form complexes that are disordered.

Denaturation of native protein leads to changes in the protein conformation by disrupting non-covalent interactions which can be achieved by adding chemical denaturants (urea or GdnHCl), pH induced or thermal denaturation [3]. During unfolding there is a population shift from the native state to the denatured state through a series of different conformations. Misfolding or the production of non-functional protein occurs when a protein fails to attain correct 3D conformation, leading to many fatal neurodegenerative diseases. Exploring the structure and dynamics of such experimentally induced non-functional proteins provide us information about its reversibility as functional and active protein [4]. For analysing the protein conformation and stability studies, GdnHCl is the most commonly used chemical denaturant [5–7].

Optimization of *in vitro* renaturation conditions is highly crucial for efficient refolding of the protein. Refolding buffers containing redox agents is an important factor for the correct refolding of proteins containing free cysteine and disulphide bonds. Most commonly used redox agents are oxidised and reduced glutathione [8]. Refolding of proteins to correct 3D conformation is a complex

Abbreviations: ANS, 8-anilino-1-naphthalene-sulfonic acid; CD, circular dichroism; DHF, dihydrofolic acid; GdnHCl, guanidine hydrochloride; GSH, reduced glutathione; GSSG, oxidised glutathione; IPTG, isopropyl- β- D-1-thiogalactopyranoside; NADPH, nicotinamide adenine dinucleotide phosphate; THF, tetrahydrofolate.

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process and is poorly understood. By controlling the renaturation process, prevention of aggregation may favours the correct refolding of protein leading to higher yield of the functional form of protein. The process of refolding of sulfhydryl containing proteins can be optimized by use of appropriate redox systems in the renaturation buffers along with stabilizers like glycerol [9].

Unfolding of small globular proteins are usually explained by a two-state transition, which does not involve intermediate species during the unfolding process [10,11]. However, there are many studies reporting the existence of intermediates during unfolding/refolding of small proteins as well [12–14]. It has also been observed that environmental condition plays a critical role in unfolding behaviour of a protein molecule. Under different set of conditions various states may exist that may be different from the native or completely unfolded state. When different spectroscopic probes were used to analyse unfolding transitions, the presence of intermediate states have been reported for many proteins like malate synthase G [15], guanidino kinases [16] and a mutant form of human growth hormone [17]. It has also been reported that the intermediate occurring between native and unfolded states are known to be in the molten globule state [18,19]. Such intermediate species exist for many proteins in low denaturing conditions and could be utilised as a powerful tool for designing a new drug entity.

Dihydrofolate reductase, or DHFR (EC 1.5.1.3.), is an enzyme that reduces dihydrofolic acid to tetrahydrofolic acid using NADPH as an electron donor. DHFR is an important pharmacologic target and is a very good model for the study of enzyme structure/function relationships because of its small size, availability of purified enzyme and well developed functional assay [20]. Here we are reporting biophysical characterization of an unexplored variant *i.e. Zebrafish* DHFR (zDHFR). It can be used in research as an alternative to the mammalian species. It has high sequence similarity with its human counterpart [21]. There is no report of equilibrium unfolding of zDHFR available in the literature so far. An equilibrium unfolding study and investigation of the refolding process of zDHFR would provide added information on DHFR folding and comparative information of folding/unfolding behaviour on various species of DHFR proteins.

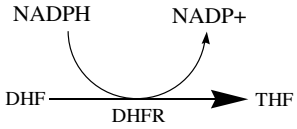
2. Materials and methods

2.1. Materials

BL21 (DE3) Rosetta *Escherichia coli* strains were used for over-expression and purification of *Zebrafish* DHFR. The plasmid, pET 43.1a vector bearing zDHFR gene was obtained from Dr. Tzu-Fun, Taiwan. Isopropyl β-D-1-thiogalactopyranoside (IPTG), 8-anilino-1-naphthalene-sulfonic acid [22], Dihydrofolate (DHF), Nicotinamide Adenine Di-nucleotide Phosphate (NADPH), guanidine hydrochloride (GdnHCl), oxidised (GSSG) and reduced (GSH) form of glutathione were obtained from Sigma Chemical Company (USA). High purity grade imidazole, Tris HCl, potassium chloride and sodium chloride were obtained from Merck, India. All other reagents used were of analytical grade. Milli-Q (Merck Millipore) or double distilled water was used during experimentation.

2.2. Stock solutions

GdnHCl (8 M) stock was prepared as described previously [23] in the refolding buffer, pH 7.4 and filtered (0.22 μm pore) prior to use. The concentration of GdnHCl stock solution was calculated by weight and refractive index [23], and solution was used only if the difference between these two values was not more than 1%. 10 mM GSH and 10 mM GSSG stocks were prepared and pH adjusted to 7.4 prior to use. All the experiments were carried out using *Zebrafish* DHFR unfolding/refolding buffer composed of 20 mMTris, 25 mMKCl, 1 mM reduced glutathione, 0.1 mM oxidised glutathione, 10% glycerol, pH 7.4 at 25 °C.



Scheme 1. Reaction catalysed by zDHFR. Where, DHF, THF and DHFR denote dihydrofolic acid, tetrahydrofolic acid and Dihydrofolate reductase enzyme respectively.

2.3. Expression and purification of Zebrafish DHFR

Recombinant *Zebrafish* DHFR protein was expressed and purified from BL21 (DE3) Rosetta *E. coli* strain, bearing desired plasmid, zDHFR-His/pET43.1a that encodes the DHFR gene having six histidine codons, under Lac Z promoter. A single step purification was performed by immobilized metal ion affinity chromatography using Ni^{+2} as chelating ion, HiTrap HP (New Jersey, USA) in AKTA FPLC purification system [21]. His-tagged zDHFR was eluted around 150 mM imidazole by a linear gradient of 0–500 mM imidazole. Protein aliquots with purity higher than 95% were collected and dialysis was performed using refolding buffer and further concentrated proteins were obtained by using Amicon tubes (Millipore, U.S.A.) with cut off membrane of 10 kDa MW. Expression and purification of zDHFR was confirmed by 12% SDS-PAGE [24]. The verification of MW of purified zDHFR was done by MALDI TOF mass spectrometry (Bruker) (Supplimentary Fig. S1). Concentration of purified protein was calculated using an extinction coefficient of 24,075 M^{-1} cm^{-1} at 280 nm as computed using the ProtParam tool of ExPASy (http://web.expasy.org/protparam/).

2.4. Enzyme assay of zDHFR

Enzyme assay of zDHFR is based on the Scheme 1. zDHFR catalysed conversion of DHF to THF was recorded on a UV spectrophotometer as the decrease in absorbance of NADPH at 340 nm. Each assay mixture comprises of the components similar to refolding buffer along with 140 μM NADPH, 100 μM DHF and 0.2 μM *Zebrafish* DHFR, pH 7.4 in a total volume of 500 μl [25,26]. All enzyme assays were done in triplicate and to minimize the degradation of substrate and cofactor, NADPH and DHF were prepared fresh, incubated in ice and consumed within 2 h of experimentation.

2.5. Ellman's test for determination of free thiols in zDHFR using DTNB

Ellman's assay is a colorimetric reaction involving Ellman's reagent [DTNB, 5,5′-dithiobis (2-nitrobenzoic acid)] and the free thiols present in the proteins [27]. Quantity of free thiols has been monitored by measuring the release of 5-thio-2-nitrobenzoate (TNB^-) from DTNB at 412 nm after 1 min incubation at room temperature. The reduction of DTNB to TNB gives a spectroscopic signal at 412 nm that was purported to interpret the presence of one free thiol out of three cysteine residues in zDHFR. Absorbance was taken for all incubated samples at 412 nm. Calculated absorbance was then averaged and the result was divided by extinction coefficient of the reagent (13600 M^{-1} cm^{-1}) to get the molarity of the solution. The assays were carried out in triplicate. As zDHFR contains 3- cysteine residues, the number of disulphide bonds was calculated to be, $(3.0 - 1.0)/2 = 1.0$.