A versatile tool in controlling aggregation and Ag nanoparticles assisted in vitro folding of thermally denatured zDHFR

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A B S T R A C T

Background: Proteins have tendency to form inactive aggregates at higher temperatures due to thermal instability. Maintenance of thermal stability is essential to gain the protein in sufficient quantity and biologically active form during their commercial production.
Methods: BL21-DE3 Rosetta *E. coli* cells which contains plasmid pET43.1a vector was used for producing zDHFR protein commercially. The purification of N-terminal Histidine tagged zDHFR was performed by Immobilized Metal Ion chromatography (IMAC). Investigations were performed in existence and non existence of Silver nanoparticles (AgNPs). The inactivation kinetics of zDHFR in existence and non existence of AgNPs were monitored over a range of 40–80 °C as monitored by UV–Visible absorption spectroscopy.
Results: The protein completely lost its activity at 55 °C. Kinetics of inactivated zDHFR follows first order model in presence and absence of AgNPs. Decrease in rate constant (k) values at respective temperatures depicts that AgNPs contribute in the thermostability of the protein. AgNPs also assists in regaining the activity of zDHFR protein.
Conclusions: AgNPs helps in maintaining thermostability and reducing the aggregation propensity of zDHFR protein.
General significance: Result explains that AgNPs are recommended as a valuable system in enhancing the industrial production of biologically active zDHFR protein which is an important component in folate cycle and essential for survival of cells and prevents the protein from being aggregated.

1. Introduction

Recombinant proteins in *E. coli* systems is always inhibited by the formation of insoluble aggregates which causes the protein to get misfolded and aggregated inside the cell and troubling the process of purification of correctly folded protein during their commercial production [1]. Aggregation can cause loss of product, reduce potency and change the biological activity of medically important proteins [2]. It is a dominating issue to acquire protein stability for the commercial production of therapeutically important recombinant proteins. Recombinant proteins are frequently produced in *E. coli* by homologous and heterologous overexpression commercially [3]. The protein must be folded into a three dimensional structure to be functionally active. The protein's native structure can be influenced by pH variation, thermal treatments, chemical denaturation such as chaotropic agents, alcohols, detergents etc., which induces protein unfolding and at last make it unstable.

Proteins have many types of responses to thermal treatment, some are thermophilic [4–6] and some can't combat elevated temperatures while many proteins shows positive response to reversible folding mechanism during thermal unfolding [7,8]. There is great interest in developing methods to get back refolded protein in sufficient quantity and in biologically active (native) form.

Proteins and peptides helps in determining the activity of nano particles when they form interaction with them due to its structural-functional characteristics. Protein and peptides in the conjugate system contribute the biological and physical support to the nano particle based technology. Different studies have been executed in the scenario

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of nanomedicine and nanoscience but the information on the impact of nano particles on the structure and function of proteins and peptides is still lacking. Many biomolecules may cause unwanted changes in their activities which may lead to faulty biological responses. Proteins form conjugates with the nano particles and help the conjugated system to become stable but still many interaction studies still need to be examined.

Dihydrofolate reductase (DHFR, EC 1.5.1.3) is a crucial enzyme which catalyzes the reaction that involves the conversion of dihydrofolate into tetrahydrofolate using NADPH as an electron donor (Fig. 1). The de novo formation of purines, thymidylic acid and certain amino acids is supported by this methyl group shuttle. DHFR has become very important nowadays as an anticancer drug target. Inspite of being a pharmacological target, protein chemists has develop great interest in DHFR which becomes a model protein for the research on the structure/functional relationships because of its small size [9]. The knowledge of the structure/functional relationship is advantageous in the designing of inhibitors of the protein [10]. zDHFR is a monomer of 190 amino acids with a disulfide bond and 3 tryptophan residues and contains many parallel and anti-parallel β sheets which are embedded by α helices. It is an emerging model protein for research on drug discovery and human diseases. zDHFR and hDHFR has high sequence similarity due to this it is highly advantageous to investigate mammalian species as an alternate [11].

Silver nano particles (AgNPs) shows admiring anti-microbial and cyto-toxic activities. Due to these features, AgNPs becomes the most useful nanomaterials. The small size of these nano particles make them efficient enough to get inside the human body and thus helping in distribution of various therapeutic agents. Many characteristics of nano-conjugated system with protein are still not clear. Ability of nano-conjugated protein system in refolding experiments and achieving protein stability under thermal inactivation process is still not clear.

In our research, we have monitored the unfolding process of zDHFR in existence and non existence of silver nanoparticles (AgNPs) using biochemical tools. Kinetic and thermodynamic parameters of zDHFR are determined in presence and absence of AgNPs which depicts that the stability of inactivated protein is retained with the help of AgNPs and thereby protect the protein from being aggregated. Due to high Gibb's free energy AgNPs get conjugated to protein with increased adsorption capacities [12]. AgNPs function as synthetic chaperones which interact with unfolded protein through their hydrophobic binding sites and help them to refold [13].

2. Materials & methods

2.1. Materials

Recombinant zDHFR protein were expressed and purified by BL21-DE3 Rosetta *E. coli* cells. Plasmid pET43.1a, which was carrying zDHFR gene obtained from Dr. Tzu-Fun, Taiwan. Isopropyl-ᴅ-1-thio-galactopyranoside (IPTG), Dihydrofolic acid (DHF), Nicotinamide Adenine Di-nucleotide Phosphate (NADPH), Sodium citrate ($Na_3C_6H_5O_7$), Tannic acid ($C_{76}H_{52}O_{46}$), Silver Nitrate ($AgNO_3$), Oxidized (GSSG) and reduced (GSH) form of glutathione, high purity grade Imidazole, Magnesium chloride ($MgCl_2$), Lysozyme, Phenyl-methylsulfonyl fluoride (PMSF), Sodium phosphate buffer, pH 7.4 (Sodium dihydrogen Phosphate (NaH_2PO_4), Disodium Phosphate (Na_2HPO_4)), Tris Hydrochloride (Tris HCl), Potassium chloride (KCl), 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB), Sodium chloride (NaCl), Dialysis membrane and Coomassie Blue R-250 were purchased from HiMedia, India. The buffer for unfolding experiments contains 20 mM Tris, 25 mM KCl, 1 mM reduced glutathione (GSH), 0.1 mM oxidized glutathione (GSSG), 10% Glycerol, pH 7.4. Refolding of zDHFR was performed in 20 mM Tris, 25 mM KCl, 10% Glycerol, 1 mM oxidized glutathione (GSSG), pH 7.4. Tris KCl buffer (20 mM Tris, 25 mM KCl, pH 7.4). All other reagents were of analytical grade. Double distilled water or Milli-Q (Merck Millipore) was used for experimentation.

2.2. Methods

2.2.1. Expression and Purification of zDHFR

The transformation of BL21-DE3 cells was carried out by recombinant vector for the overexpression of zDHFR protein. LB media containing ampicillin (100 μg/ml) was used for growing the cells at 37 °C. When the O.D. reached ~0.6 then there was addition of 1 mM IPTG for the induction of histidine tagged protein at 25 °C [14]. Centrifugation was performed at 6000 rpm for 30 min and then the lysis buffer (0.2 M sodium phosphate pH 7.4, 500 mM NaCl, 0.2 M $MgCl_2$, 0.1 mg/ml Lysozyme and 1 mM PMSF) is used for the resuspension of these cells. The lysis of cells was done by Ultrasonication at 4 °C after 30 min of
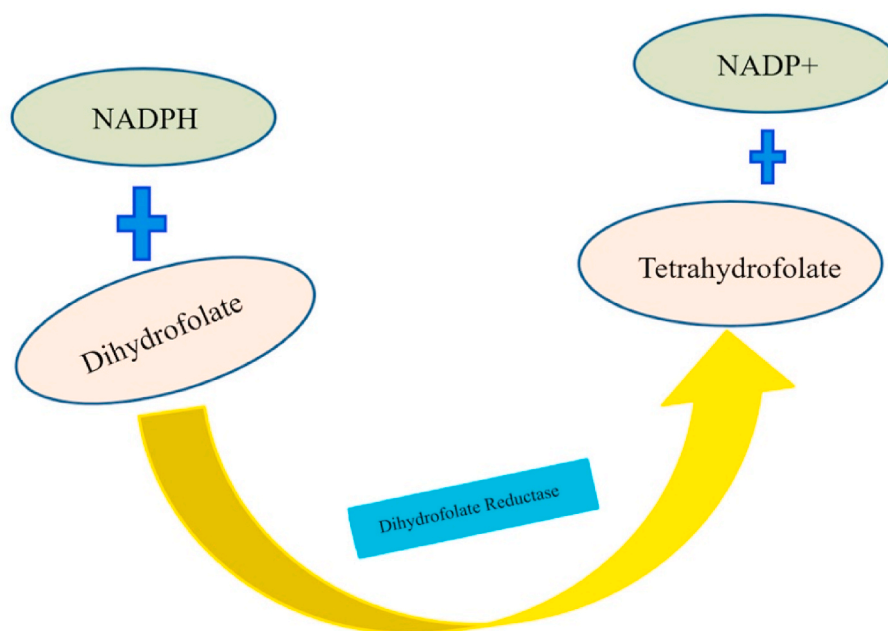

Fig. 1. Enzyme reaction catalyzed by DHFR.

incubation. Supernatant was filtered by 0.22 μm millipore filter. The process of purification was done by Immobilized Metal Ion Affinity Chromatography (IMAC) using Ni^{2+} as chelating agent. Histidine tagged protein was eluted by a gradient of 0–500 mM Imidazole. The diverse fractions of zDHFR were identified by SDS-PAGE and the fraction with greater than 99% of purity was pooled. The dialysis was carried out for these pooled fractions against 20 mM Tris buffer, pH 7.4, 100 mM NaCl, 10% Glycerol. The estimation of concentration of dialyzed protein was executed by Amicon tubes (Millipore, U.S.A.) which contain a cut off membrane with 10 kDa molecular weight. An extinction coefficient of 24,075 M^{-1} cm^{-1} was used in the estimation of the concentration of purified protein. The computation of extinction coefficient of zDHFR was performed by ProtParam tool of ExPASy (http://web.expasy.org/protparam/).

2.2.2. Synthesis of AgNPs

Sodium citrate and Tannic acid were mixed to form an aqueous solution. The solution was heated with a heating mantle for 15 minutes under vigorous stirring. To this solution, $AgNO_3$ was added after boiling. The solution appears bright yellow immediately. Now the purification of AgNPs was done by centrifugation (10000 g) to discard excess tannic acid and further it was redispersed in milli-Q-water before sample characterization [15].

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

Ellman's assay is a colorimetric reaction which involves protein's free thiols and Ellman's reagent [DTNB, 5, 5-dithiobis (2-nitrobenzoic acid)] [14]. Quantity of free thiols has been monitored by measuring the release of 5-thio-2-nitrobenzoate (TNB^-) from DTNB at 412 nm after incubation for 1 min 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 at 412 nm after incubating all the samples. Calculated absorbance was then averaged and the resulting value was divided by extinction coefficient of the reagent (13600 M^{-1} cm^{-1}) to get the molarity of the solution. The assays were performed in triplicate. As zDHFR contains 3-cysteine residues, the number of disulphide bonds was calculated to be (given in Eq 1.0),

$$(3.0-1.0)/2 = 1 \quad\quad\quad\quad 1.0$$

Thus, because of the presence of one disulphide bond in zDHFR, glutathione was added in the buffer composition.

2.2.4. Activity assay of zDHFR protein

DHFR catalyzes the reaction which converted dihydrofolate into tetrahydrofolate using NADPH as cofactor. The reaction was monitored by U-5100 HITACHI Spectrophotometer (Tokyo, Japan) at 25 °C with the decrement in absorbance of NADPH at 340 nm. Each assay mixture comprised of Tris KCl buffer (pH 7.4), 100 μM DHF and 140 μM NADPH and 0.2 μM zDHFR protein. All the components were formed fresh to avoid their degradation and all experiments were done in triplicate [16].

2.2.5. Interaction of AgNPs with zDHFR protein

Conjugates of AgNPs with zDHFR protein were prepared by mixing the zDHFR protein with AgNPs in TrisKCl buffer, pH 7.4. The incubation for the mixture was 1 h at 4 °C in test tubes. Experiments were executed in various ratios in which concentration of AgNPs (5 nM) remains constant while the concentration of protein was kept different (0–500 nM). We have observed the UV Visible spectra of different zDHFR+AgNPs conjugates at different wavelengths (300–1100 nm). The errors of the background were eliminated by Tris KCl buffer which was used as blank.

2.2.6. Effect of temperatures on the stability of zDHFR protein

The thermal inactivation of zDHFR and zDHFR+AgNPs were studied for 100 minutes in the temperature range (40 °C–80 °C). zDHFR protein

samples were kept at predetermined temperatures in water bath. A mercuric glass thermometer was used to adjust the temperature of water bath. For the completion of inactivation process, samples were then cool down to 25 °C after the incubation process and after that enzyme activity was monitored at 340 nm. All the experiments were conducted in triplicate.

2.2.7. Evaluation of kinetic parameters

The first-order equation was used to elucidate the enzyme inactivation of zDHFR protein and revealed algebraically by the following equation:

$$\ln C/C_0 = -kt \ldots \quad\quad\quad\quad (i)$$

where C represents the enzyme activity value at time t, C_0 is the initial value at time zero; k represents the rate constant at precise temperature. The slope of semi-logarithmic plot of residual activity against treatment time was used to attained inactivation rate constant (k).

The half-life ($t_{1/2}$) was estimated by the following equation:

$$t_{1/2} = \ln(2)/k \ldots \quad\quad\quad\quad (ii)$$

The time in which the initial activity of enzyme depletes by 90% is known as D-value. D-value and inactivation rate (k) shows an alliance that can be elucidated mathematically by following relationship [17]:

$$D = \ln (10)/k \ldots \quad\quad\quad\quad (iii)$$

2.2.8. Thermodynamic study

Arrhenius law elucidates that the temperature depends on the rate constant i.e. k-value which is algebraically explained by:

$$\ln (k) = -E_a/R. \ 1/T + \ln C$$

where the activation energy is symbolized by E_a (kJ/mol) and C is the Arrhenius constant, T (K) represents the absolute temperature and R (8.31J/molK) indicates the universal gas constant. E_a is expressed as the slope of linear regression analysis of natural logarithm (ln) of k-values against the inverse of the absolute temperature.

The values of the activation energy (E_a) and Arrhenius constant (C) were used to estimate the values of different thermodynamic parameters:

The activation enthalpy change ΔH* for specific temperatures were calibrated by:

$$\Delta H^* = E_a \text{-} RT \ldots \quad\quad\quad\quad (v)$$

The variation in the free energy of inactivation (ΔG*) can be calculated as:

$$(\Delta G^*) = \text{-} R.T. \ln (k.h /k_B. T) \text{ ..} \quad\quad\quad\quad (vi)$$

where h is the Plank's constant (6.6262×10^{-34} Js), k_B is the Boltzmann's constant (1.3806×10^{-23} J/K), and k (s^{-1}) is denoted as the inactivation rate constant at selected temperatures.

Equation (v) and (vi) were used in the calibration of activation entropy (ΔS*):

$$\Delta S^* = (\Delta H^* \text{-} \Delta G^*)/T. \quad\quad\quad\quad (vii)$$

2.2.9. Data analysis by statistical parameters

The Sigma Plot 10.0 was applicable for statistical analysis. Boltzmann sigmoidal fit function was executed for the formation of 'Thermal melt' curves. k-values were obtained and compared with the inactivation kinetics of zDHFR in existence and non existence of AgNPs. Values of $p < 0.05$ were obtained and observed as statistically important.

2.2.10. Thermal inactivation analysis

The decrement in enzymatic activity was observed at 340 nm to perform thermal inactivation kinetics by thermal treatment of zDHFR for prolonged duration (up to 100 min) at 40 °C, 50 °C, 60 °C, 70 °C, 80 °C. Protein was mixed after the pre-equilibration of buffer for each temperature. 10 sec was the dead time for mixing reaction.

Arrhenius plot was retrieved by the graph plotted between ln (k) and $1/T$. Activation energy (E_a) for inactivation was evaluated by Arrhenius equation,

$$\ln (k) = -E_a/R. \; 1/T + \ln C \textbf{ from eq.} \qquad (iv)$$

where T represents the absolute temperature and R represents the universal gas constant. The rate of heat inactivation and activation energy of zDHFR has also been measured in existence of AgNPs. The protein stability was monitored in presence and absence of AgNPs by determining thermodynamic parameters.

2.2.11. Thermal induced in vitro unfolding studies

The unfolding of zDHFR was estimated by monitoring the reduction in enzymatic activity at 340 nm in presence and absence of AgNPs at higher temperatures. zDHFR and conjugates of zDHFR+AgNPs were exposed to different temperatures *i.e.* 40 °C, 50 °C, 60 °C, 70 °C, 80 °C for 15min and then analysed at 25 °C for zDHFR activity.

2.2.12. Refolding studies of thermally inactivated zDHFR

The samples were denatured thermally at 50 °C, 60 °C, 70 °C, 80 °C and then the temperature was decreased to 25 °C for a time period of 5 min and measurement was taken only after equilibration was done for 10 minutes. The equilibration time was selected as it was suitable to give constant results on additional remaining time at the same temperature. The regaining of enzyme activity was monitored in existence and non existence of AgNPs at 340 nm in UV–Visible Spectrophotometer.

3. Results

3.1. Expression and Purification of zDHFR

Fig. 2A shows the expression of recombinant zDHFR which was certified by 12% SDS-PAGE analysis. Molecular mass of over-expressed protein was found to be about 21.6 kDa. Fig. 2B shows the purified fraction of zDHFR obtained by Immobilized metal ion affinity chromatography (IMAC).

3.2. Characterization of disulphide bond

Redox environment is extremely essential for disulphide or free sulphydryl groups present in the active sites of proteins. So, free cysteine residues are very important in the reduced form for the functionality of zDHFR protein. zDHFR protein contains 3-cysteine residues, the number

of disulphide bonds were calculated using Ellman's test and was estimated to be $(3.0–1.0)/2 = 1.0$. So, zDHFR contains a free cysteine residue along with a disulphide bond.

3.3. Synthesis of AgNPs

The characterization of synthesized AgNPs was done by using transmission electron microscopy (TEM) and UV–visible absorbance spectroscopy and of ∼20 nm in size (Fig. 3A and B). AgNPs shows a single peak with absorbance maxima at 404 nm.

3.4. Interaction of AgNPs with zDHFR

AgNPs interacts with zDHFR protein which has been investigated by UV–Visible spectroscopy. The absorption spectrum of AgNPs (5 nM) with varying conc. of zDHFR (0–500 nM) depicts that the solutions which contain zDHFR protein at lower concentration (0nM–200nM) indicates a sharp drop in the absorption band intensity which reveals the presence of special indicators of aggregation [18] while 250 nM concentration of protein shows a highest peak at 400 nm which shows that it provides stability to AgNPs and shows maximum interaction (Fig. 4A). However, further increase in the protein concentration up to 500 nM causes the broadening of the bands which indicates the presence of aggregates in the solutions [18]. It can be depicted that AgNPs form aggregates which may be due to the association between various nanoparticles (NPs) and various binding sites on protein and form crosslinks together [19].

Fig. 4B shows that the colour of AgNPs without zDHFR changes from yellow to dark brown while the zDHFR+AgNPs conjugate retain its yellow colour up to 1 hr. The same solution when incubated for 2 hrs shows that the AgNPs changes from dark brown to grey (aggregated stage) while the zDHFR+AgNPs conjugate does not show any change in its colour (yellow). This shows that zDHFR helps in maintaining the stability of AgNPs. The experiment was conducted at 4 °C.

3.5. Impact of temperatures on stability of conjugated and non-conjugated zDHFR

Thermal unfolding studies of zDHFR in presence (conjugated) and absence (non-conjugated) of AgNPs were investigated using UV- spectroscopy at 340 nm. Residual enzymatic activity of zDHFR and zDHFR+AgNPs were monitored against temperature from 25 to 80 °C (Fig. 5). It was observed that at 25 °C both zDHFR and zDHFR+AgNPs shows 100% activity but the activity of zDHFR decreases with increase in temperature and gets completely unfold at 55 °C. zDHFR indicates ∼10% activity at 50 °C whereas zDHFR+AgNPs retains about 42% of its activity at the same temperature.
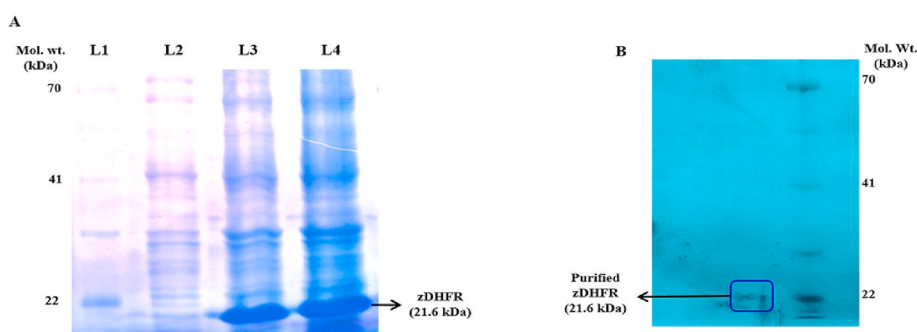

Fig. 2. Expression and Purification profile of recombinant zDHFR analysed by 12% SDS PAGE. A) L1- Molecular Marker, L2- Uninduced cells, L3, L4 - Induced with 100 µM IPTG. B) Purified zDHFR obtained by Immobilized Metal Ion Affinity Chromatography (IMAC).
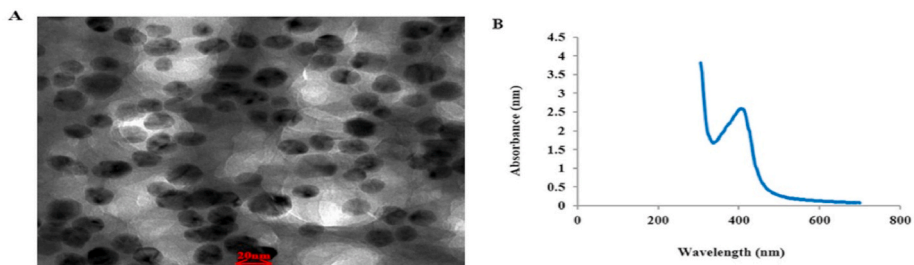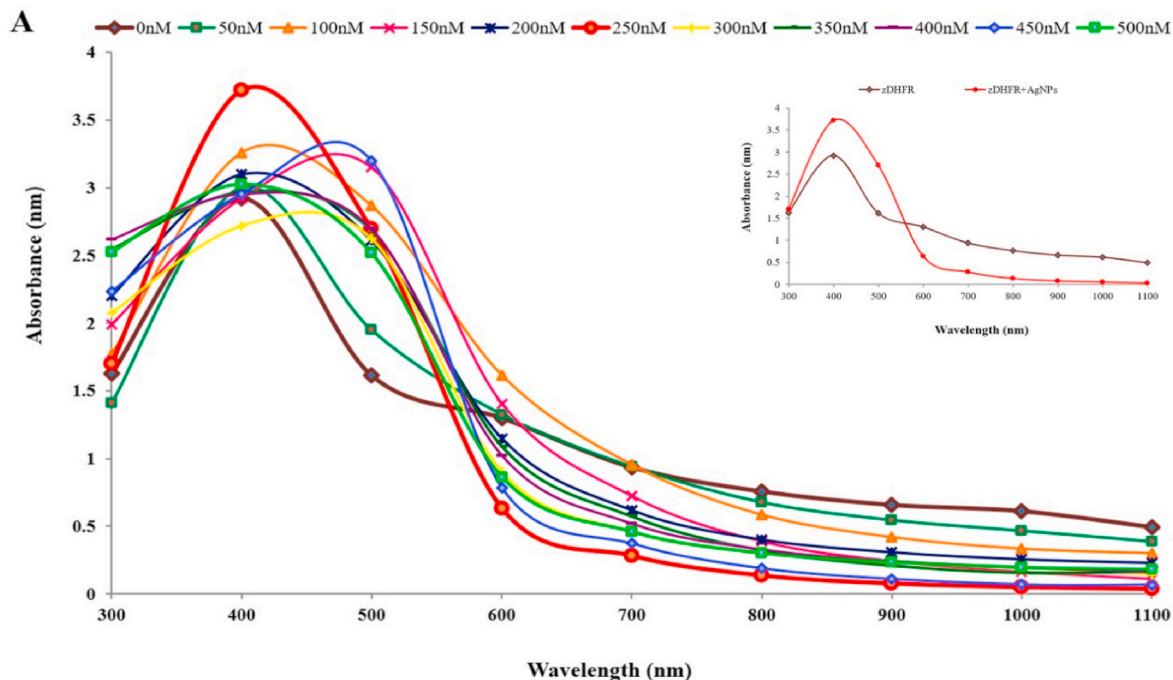
Fig. 3. Synthesis of AgNPs. A) TEM image showing uniform size distribution of synthesized AgNPs B) UV–visible absorbance spectra of AgNPs.
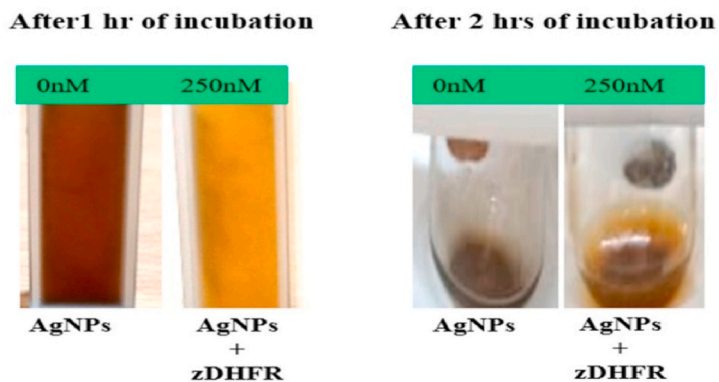

Fig. 4. Interaction of AgNPs with zDHFR. A) UV–Visible spectra of AgNPs (5 nM) and zDHFR+AgNPs (0–500 nM) in presence of Tris KCl buffer. Inset in figure represents the UV–Visible spectra of AgNPs (5 nM) with and without zDHFR (250 nM), Tris KCl buffer, pH 7.4. B) AgNPs in absence and presence of zDHFR in Tris KCl buffer after incubation of 1 hr & 2 hrs at 4 °C.

3.6. Kinetics of thermally inactivated zDHFR and zDHFR+AgNPs

Residual enzyme activity was monitored to perform inactivation kinetics at different intervals of time with the temperature range 40–80 °C. The effects of thermal inactivation procedures on zDHFR and zDHFR+AgNPs have been shown in Fig. 6A and C, respectively for
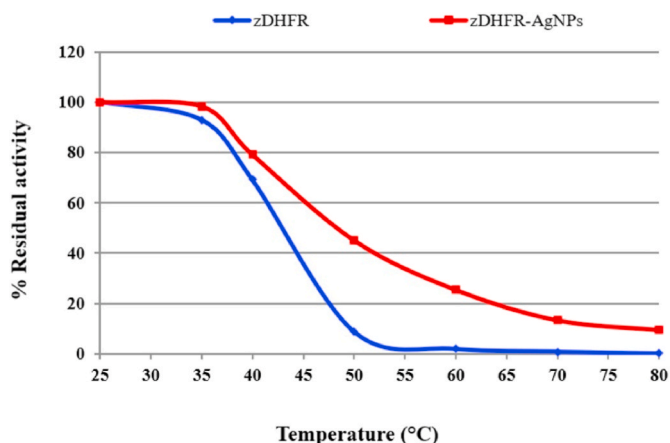
Fig. 5. Unfolding study of thermally exposed zDHFR protein in presence and absence of AgNPs at temperature range 25–80 °C as monitored at 340 nm by UV–Visible Spectrophotometer.

various temperatures which shows that AgNPs found to rise the thermal inactivation time of zDHFR. The measurement of enzyme activity was done at diverse intervals of time in the temperature ranges of 40–80 °C to achieve inactivation kinetics. After the time period of 100 min at

different temperatures, the activity of zDHFR becomes zero.

Single exponential decay was shown by the plot between residual enzyme activity and time. The exponential fit of residual enzyme activity against time shows the inactivation rate (k). The value of rate constant (k) increases at elevated temperatures which indicate the low thermostability of protein [20,21]. Our results explain that the values of k were reduced in case of zDHFR+AgNPs for a specific temperature. Calibrated kinetic parameters for zDHFR and zDHFR+AgNPs are mentioned in Table 1.

The semi log plots were accessed by putting residual activity versus treatment time for zDHFR (Fig. 6B) and zDHFR+AgNPs (Fig. 6D). The plots for both (zDHFR and zDHFR+AgNPs) were linear at all temperatures which depicts that the thermally inactivated zDHFR protein and zDHFR+AgNPs follows a first order monophasic kinetic model.

Results explain that the rate for thermal inactivation was retarded significantly in the existence of AgNPs which depicts that it helps in increasing the thermostability of zDHFR and reduces the protein's aggregation propensity. The decreased rate constant in the presence and absence of AgNPs is depicted in Table 1.

The inactivation rate constants (k) was achieved by the process of thermal inactivation of zDHFR which follows Arrhenius law (Fig. 7) and we can calculate the activation energy, E_a using the Arrhenius equation by executing linear regression analysis. For native zDHFR, the activation energy E_a was 33.98 kJ/mol and the equation of regression was
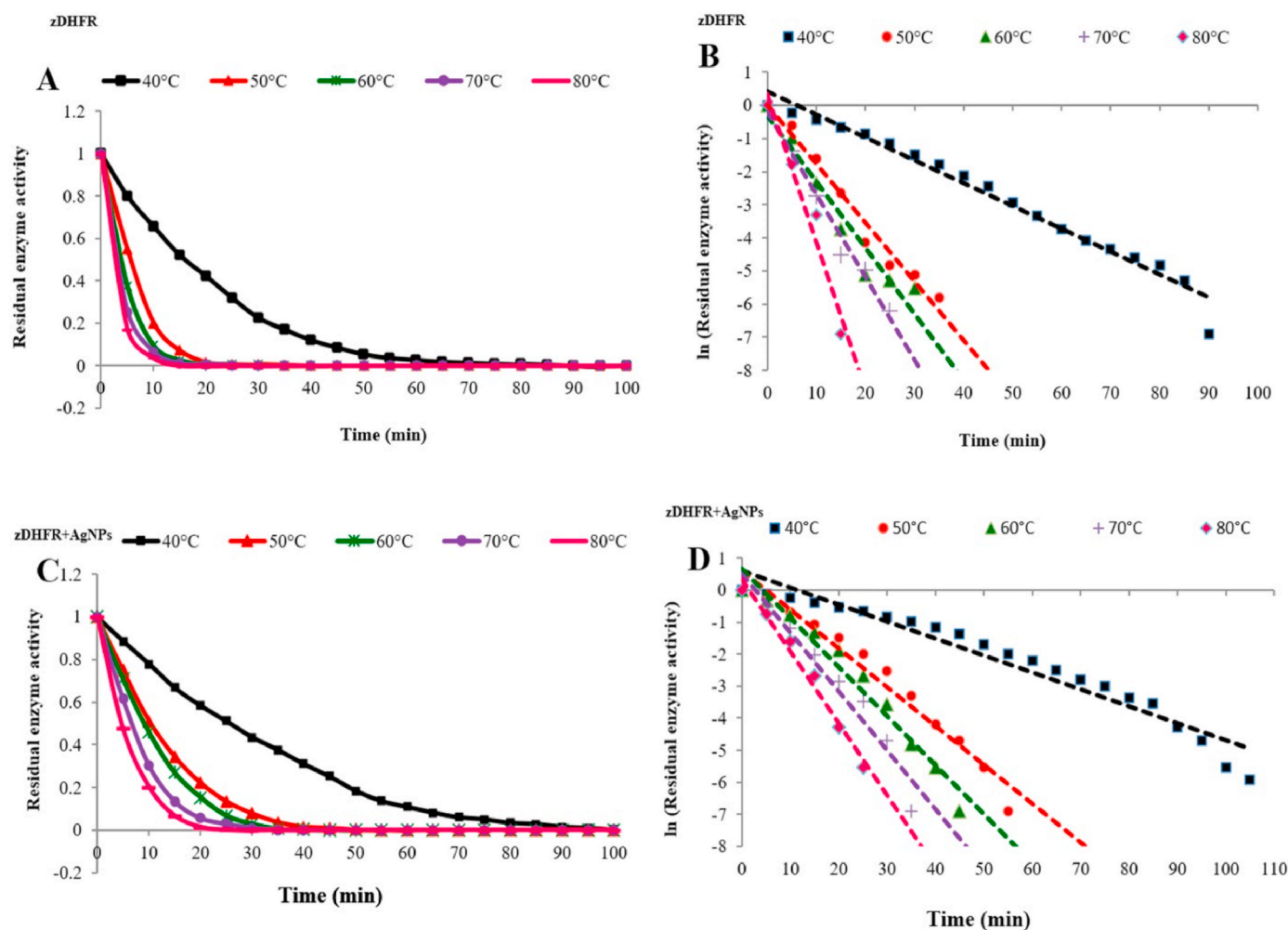
Fig. 6. Kinetics of thermally inactivated zDHFR and zDHFR+AgNPs as monitored by UV–Visible Spectrophotometer at 340 nm. A) Residual enzyme activity of zDHFR vs time when subjected to various temperatures. B) Effect of thermal inactivation of zDHFR represented as the natural logarithm (ln) of residual activity of zDHFR for temperature range 40–80 °C. C) Residual enzyme activity of zDHFR+AgNPs vs time when subjected to different temperatures. D) Effect of thermal inactivation of zDHFR+AgNPs represented as the natural logarithm (ln) of residual activity of zDHFR for temperature range 40–80 °C.

Table 1

Kinetic parameters of thermally inactivated zDHFR in the existence and non existence of AgNPs.

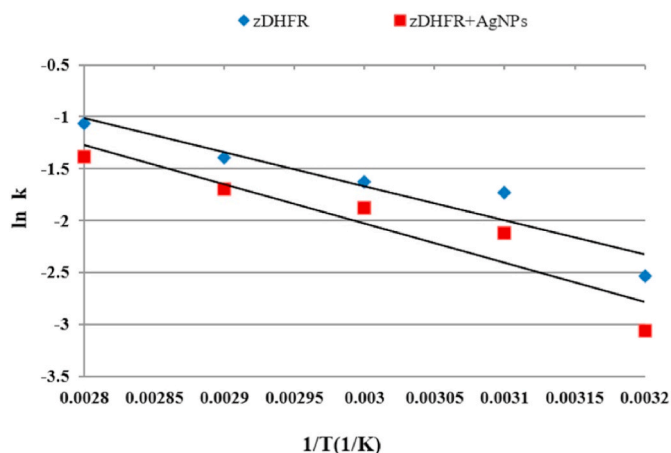|	Temp (°C)	k (min^{-1})	R^2	$t_{1/2}$ (min)	D (min)
zDHFR	40	0.079	0.973	8.77	29.15
	50	0.177	0.976	3.92	13.01
	60	0.196	0.990	3.54	11.75
	70	0.249	0.983	2.78	9.25
	80	0.344	0.956	2.02	6.67
zDHFR+AgNPs	40	0.047	0.943	14.74	49
	50	0.120	0.965	5.78	19.19
	60	0.153	0.965	4.53	15.05
	70	0.183	0.949	3.8	12.6
	80	0.25	0.982	2.77	9.21

Fig. 7. Arrhenius plot of thermally inactivated zDHFR in presence and absence of AgNPs.

calculated as ln $(k) = -3284x + 8.180/T$, $(R^2 = 0.898)$ and for zDHFR+AgNPs was 38.61 kJ/mol and the regression equation was calculated as ln $(k) = -3766x + 9.270/T$, $(R^2 = 0.879)$. The value of E_a from our study suggest that zDHFR+AgNPs has higher thermal stability as compared to zDHFR.

Equation (iii) helps in the estimation of decimal reduction time (D value) which provides us information about enzyme stability and is explained as the time needed for 90% decrement in the initial activity of enzyme. The values of D for zDHFR+AgNPs have been increased in comparison with zDHFR which indicates the AgNPs helps in increasing the stability of the protein and protect it from being aggregated.

Thermostability of the protein is defined by Dvalue and half-life time [22,23]. The values of D and $t_{1/2}$ of zDHFR and zDHFR+AgNPs depicts that the conjugated zDHFR is more stable than zDHFR at selected temperatures. This recommends that AgNPs increases the stability of zDHFR and thus impedes its aggregation. Full information about the stability of the protein is furnished thoroughly by the thermodynamic parameters.

To determine the activation energy (E_a), thermal inactivation experiments were conducted in order to study and correlate the stability of zDHFR and AgNPs conjugated zDHFR protein.

ΔH^* is the enthalpy of activation energy which is calculated according to the eq.:

$$Ea = \Delta H^* + RT \ldots \text{(eq. v)}$$

Thus, calculations of all the thermodynamic parameters, i.e. enthalpy change of activation (ΔH^*), the change in Gibbs free energy for activation (ΔG^*), and the activation entropy change (ΔS^*) were calculated using eq. (v), (vi) and (vii). The parameters are summarized below in Table 2.

In protein unfolding process, the two main reasons which are

responsible for change in enthalpy are the interruption in internal interactions of the protein and the exposure of the hydration groups present within the protein molecule. Higher values of ΔH^* represents lower aggregation propensity of protein and thereby increases the stability of the protein [27].

The conversion of protein's state from folded to unfolded determines the variation in the free energy of activation. Higher value of ΔG^* of AgNPs conjugated zDHFR shows higher stability of the protein which indicates that AgNPs protects the protein from aggregation [28].

The entropy of a system is described as an estimation of randomness or disorder. ΔS^* of a system is higher at higher temperature due to greater randomness. Thus, the higher value of ΔS^* in non-conjugated zDHFR shows that AgNPs assures stability of the protein and preserve it from being aggregated [29].

3.7. Refolding studies of thermally inactivated zDHFR in presence and absence of AgNPs

zDHFR shows thermal denaturation at different temperatures (Fig. 5). It is essential to determine the restoration profile of zDHFR protein. We have monitored the spontaneous refolding of zDHFR when cooled at 25 °C which gives recovery of about 6.2%, 5%, 4.4% and 3.9% at 50 °C, 60 °C, 70 °C and 80 °C respectively (Fig. 8). Further, refolding was done at same temperatures using AgNPs which shows that approximately 39.2%, 34.8%, 32.4%, 32.0% of activity was recovered. Thus, AgNPs helps in enhancing the refolding yield which indicates less aggregation of thermally inactivated zDHFR to an appreciable level.

4. Discussion

Our study elaborates that thermal unfolding of zDHFR protein was monitored by enzyme kinetics and estimating thermodynamic parameters which give information about structural/functional relationship of the protein with its stability and aggregation against temperature.

zDHFR protein contains three cysteine residues from which two cysteine residues forms a disulphide bond and one cysteine residue remains free. Therefore, the authenticity of disulphide bond in the protein has been characterized precisely to assure correct disulphide bond formation. A redox buffer system (GSG:GSSG, 10:1) have been chosen for unfolding of zDHFR to give convenient reducing surroundings for protein unfolding, while we have used oxidized glutathione (GSSG) to provide oxidizing surrounding for the formation of disulphide bond [29].

Silver nanoparticles (AgNPs) show growing functions in biological systems such as antimicrobial agents and potential candidates for control drug release systems. In all these applications, there is interaction of silver nanoparticles with proteins and other biomolecules. So, the study of such interactions is of major importance.

For the conjugation studies, we have taken fixed concentration of AgNPs (5 nM) and varying concentration of zDHFR (0–500 nM) in Tris

Table 2

Thermodynamic parameters of the thermal unfolding of zDHFR in existence and non existence of AgNPs.

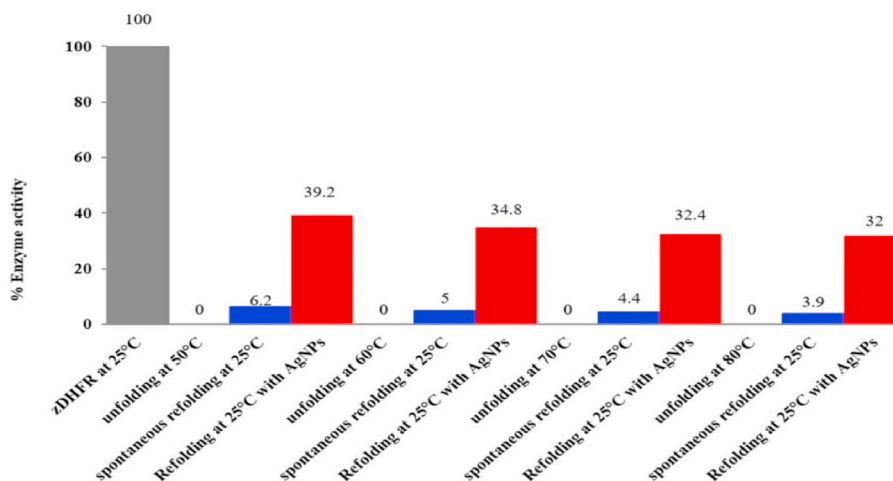|	E_a (KJ/mol)	T (K)	ΔH^* (KJ/mol)	ΔG^* (KJ/mol)	ΔS^* (J/mol)
zDHFR	33.98	313	31.38	94.05	−200.2
		323	31.29	94.96	−197.12
		333	31.21	97.7	−199.66
		343	31.13	100.04	−200.9
		353	31.05	102.1	−201.2
AgNPs+zDHFR	38.61	313	36.01	95.39	−189.97
		323	35.92	96	−186.01
		333	35.84	98.39	−187.83
		343	35.76	100.92	−189.97
		353	35.68	103.04	−190.82

Fig. 8. Refolding of thermally inactivated zDHFR in presence and absence of AgNPs at different temperatures (50 °C, 60 °C, 70 °C and 80 °C) monitored at 340 nm in UV–Visible Spectrophotometer.

KCl buffer (pH 7.4). AgNPs shows a narrow peak in presence & absence of zDHFR (250 nM) in Tris KCl at 400 nm. It was also confirmed with the colour of zDHFR+AgNPs conjugate which retain its yellow colour (Fig. 4).

The unfolding of zDHFR protein was observed by measuring enzyme activity at 340 nm in the temperature range (40°C–80 °C) for up to 100 minutes and the results (Fig. 6A and C) shows that first-order reaction kinetics was followed by zDHFR. It shows faster inactivation at higher temperatures due to deviation in *k*-value. The linear plot between natural logarithm of residual activity vs. time (Fig. 6B and D) also proves that the thermal unfolding of the protein is a single step denaturation process elucidated by first-order exponential decay. We have made an effort to investigate the role of silver nano particles in maintaining thermostability and thereby eradication of zDHFR protein which is a valuable component in folate cycle and necessary for cell survival. Table 1 shows all the kinetic parameters (D-value and half-life) and explains that AgNPs provide stability and protect it from being aggregated during the inactivation of zDHFR.

The unfolding kinetics of zDHFR in presence and absence of AgNPs follows Arrhenius law as shown in Fig. 7 with activation energies listed in Table 2 which also depicts that higher activation energy in the presence of AgNPs clearly linked with the decreased aggregation propensity and as a result increase in stability of the protein.

In thermal inactivation process of zDHFR, the thermodynamic parameters were calibrated for the temperature range 40°C–80 °C based on equations (v)-(vii) and listed in Table 2. During the thermal inactivation of zDHFR, the enthalpy (ΔH^*) was higher in presence of AgNPs; this shows that extra energy is required to break the intramolecular forces like hydrogen bonds, ionic interactions etc. during the conversion from folded to unfolded form. Gibb's free energy (ΔG^*) is an energy barrier for inactivated enzymes which measures the spontaneity of the inactivation process. ΔG^* directly indicates the protein stability . The entropy change (ΔS^*) explains the randomness and solvent disorder. Results indicate that zDHFR+AgNPs conjugate is more stable than zDHFR and shows decreased aggregation propensity as compare to zDHFR protein. Thus, AgNPs stabilizes zDHFR to a significant level during its thermal inactivation process.

We have monitored the refolding of thermally unfolded zDHFR in presence and absence of AgNPs. zDHFR shows less spontaneous recovery as compare to zDHFR+AgNPs (Fig. 8). This increase in activity recommends that AgNPs was identified as a valuable system for zDHFR stabilization.

5. Conclusions

Our experimental data shows the interaction of zDHFR protein with AgNPs. The enzyme inactivation studies were then performed for zDHFR in presence and absence of AgNPs. We have observed that protein is completely unfolded at 55 °C. Kinetic parameter shows that inactivated zDHFR follows first order model in presence and absence of AgNPs. Decrease in rate constant (*k*) values at respective temperatures depicts that AgNPs contribute in the protein's thermal stability. From our thermodynamics data it was proved that AgNPs increases the thermal stability of zDHFR and thus decreasing its susceptibility to aggregation. It was also analysed from our data that AgNPs helps in improving the refolding yield of the protein. Thus, from the above observations suggest that nano-conjugated protein system provide support and stability to thermally inactive proteins and also increase their refolding ability. These are important points towards various biological applications of AgNPs-conjugated protein. It is also suggested that AgNPs could be a model system in achieving the adequate amount of protein in functional form for commercial uses.

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