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A size dependent folding contour for cytochrome *C*

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Abstract

The paper describes an experimental construct of the folding route of the heme protein cytochrome-*C*. The construct highlights a slowing down near the nose of the folding funnel caused by the multiplicity of the energy traps near the native conformation created as a result of complex heme–peptide interaction. Interestingly the hydrodynamic size, the size heterogeneity and peroxidase activity serve as a triple measure of the distance of this near equilibrium departure from native conformation. Accordingly, the folding process is marked with a gradual and reversible reduction of mean hydrodynamic size, size heterogeneity and peroxidase activity (higher in unfolded state). The Dynamic Light Scattering based straightforward illustration of hydrodynamic size variation may serve as a model to slow folding observed in case of heme proteins, the heme itself serving as a natural facilitator for the native peptide conformation.

Keywords: Cytochrome-*C*; Hydrodynamic size; Size heterogeneity; Dynamic Light Scattering

1. Introduction

An intriguing aspect of the protein-folding pathway is its wide diversity in time scale. The folding events may occur starting from a few nanoseconds to microsecond [1] and may be extended to hours [2]. While traversing through the folding funnel, the initial barrier of folding is crossed within the sub-millisecond order burst phase [3], but the post burst phase barrier is overcome in a longer scale of time. Normally, the slower component of folding manifests itself in the functional regime while the structural components are reportedly restored much faster. This dichotomy of time scale often causes problems in understanding the systems aspect of the process. In this paper we report that there exists a coupled structural and functional dynamics of folding in certain class of proteins. We have previously reported a slow dynamics of folding of hemoglobin and some attenuation of such folding processes as a result of protein modification by glycation [4].

Cytochrome-*C* (Cyt-C) folding has recently come into focus as it has been used as a model system for reporter gold nanoparticles [5]. The binding of gold nanoparticle to this protein, and the resultant color (a function of the nanoparticle size) depends on the folding state(s) of the protein [5]. However, the hydrodynamic meaning of the "unfolded" states needs to be further explored. The question of particular importance is how the statistical nature of the population (having different aggregated size) depends on the distance from the native state; partially when the secondary structure is intact and dynamics is in the close vicinity of the equilibrium that makes the native state. The heme group and its interaction with some key peptide moiety are the deciding factors in the route to folding [6]. Like the reporter gold nano particles as referred above, heme group, having a clear spectroscopic signature, also serves as a "natural" indicator of the folding states [7]. The presence of heme group assists the folding and assembly of the globin chain [8] and for heme proteins this prosthetic group may be considered to be a natural chaperon. In case of in vivo folding of Cyt-C, the heme attachment is coupled with mitochondrial transport. Importantly, these heme binding amino acids (cystine residues with thio-ethar linkage) play a key role in this folding [9]. This heme group is attached to the peptide framework with non-covalent and/or covalent bonding. Cyt-C is a classic example of the combination of both the types of bonding. More importantly, both fast [10,11] and slow kinetics [2] of the folding of this protein has been reported.

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Cyt-C is characterized by the covalent attachment of heme to protein through two thio-ether bonds to a Cys-Xaa-Xaa-Cys-His peptide motif [12]. For the intermediate formation of folding for equine heart Cyt-C, residues Cys13, Cys14, His18 and four other positions are decisive for the formation of key nucleus [13]. Even the heme–peptide moiety is such an important key to the structural integrity that its distortion may lead to amyloid formation [14] in some cases. The unfolding pathway of Cyt-C is however characterized by evolution of different aggregated structures due to His–heme misligation and proline mis-isomerisation [15]. The heme binding motif, mentioned earlier, becomes unstable due to denaturation. These unfolded (or misligated) monomers become prone to form self assembly of varying molecularity that leads to the formation of aggregates of higher order. From 2D–IR correlation analysis it has been shown that this two-step aggregation process is characterized by simultaneous formation of intermolecular bonds and unfolding of the α-helices [16].

It has been reported that the higher energy barrier of refolding for Cyt-C due to His–heme misligation and proline mis-isomerisation slows down the process markedly [2]. This slow pathway is not well characterized although some correlations have been drawn between unfolding and associability (i.e. aggregation [17] of unfolded or native like intermediates [18]). As the refolding pathway has been a function of its initial denatured (or unfolded) state, there exists a high probability of occurrence of parallel folding pathways [18,19], rather than a strict or predetermined route to attain the unique native state. The variations of the initial extent of unfolding cause a distribution of intermediate states. These states differ not only in their position at the energy co-ordinate, but also in their size, aggregation, functionality etc. More denatured or unfolded is the state; more probable it becomes to attain a heterogeneous population [20]. Hence shrinkage of population diversity or narrowing of the population distribution is certainly an indicator of the folding pathway.

The most intriguing aspect of our study is to bring out a significant coherence of ‘size diversity’ or ‘population heterogeneity’ with ‘peroxidase activity’, which are two almost individual parameters. This peroxidase activity is a very sensitive marker for the folding state of the protein because heme–peptide interaction is the key to the enzymatic activity and this heme–peptide moiety is also a major victim of unfolding (i.e. Heme–His misligation) along with the mis-isomerised Proline-25. As it is well known that the folding intermediates may be diverse from both structural and functional viewpoint [19] our study amalgamates functional aspect of the folding process with the conformational dynamics.

2. Materials and methods

2.1. Preparation of protein solution

Horse heart cytochrome-*C* has been purchased from Sigma Aldrich. Protein solution is prepared in phosphate buffer of pH 7.4.

2.2. Preparation of denaturant solution

Guanidine hydrochloride has been purchased from SRL (Sisco Research Laboratory). The stock has been prepared in double distilled water. The stock concentration was 8 M.

2.3. Reagent preparation for peroxidase activity

The substrate *o*-dianisidine has been purchased from Sigma Aldrich. The solution was prepared as 5 mg/ml conc of *o*-dianisidine in methanol/sodium acetate [(0.25 M), pH 5.0, 3 : 7 (*v*/*v*)]. Hydrogen peroxide stock was prepared at a conc. of 0.3% in double distilled water.

2.4. Unfolding assay

Protein solutions with varying concentration (0.1 to 1 mg/ml) and unfolding condition were prepared. The unfolding kinetics was assayed spectrophotometrically by Diode Array spectrophotometer Analytik Jena (spekol 1200). In this study the time kinetics has been studied for 10 min time scan with 5 s cycle length. The spectral data points have been analyzed and the kinetics of wavelength maxima of the soret peak is plotted against time.

2.5. Refolding of the protein samples

Initially the protein solution is denatured in 8 M Gdn–HCl concentration. This highly unfolded state is gradually refolded by half diluting the primary solution to trap different intermediate steps during refolding. Hence unfolding concentration gradually decreases from 8 to 1 M in 4 steps of dilution. For a wider sampling, we have taken a large variety of protein concentration (from a higher range of 1 to 4 mg/ml to a lower range of 0.1 to 0.5 mg/ml). Different assays have been performed in all the sets.

2.6. Activity assay

For the assay of the peroxidase activity for different refolding states of the protein, the *o*-dianisidine assay has been performed with standard protocol. Peroxidase activity of heme can be used for probing heme proteins with high degree of sensitivity. The published method [21–23] was followed. The activity kinetics was measured in the previously described Spectrophotometer and its dynamics was studied in different time intervals of the spectra.

2.7. Dynamic light scattering

The Nano-ZS (Malvern) instrument we have used for our experiment, is equipped with a 4 MW He–Ne Laser ($\lambda = 632$ nm). The sample is poured in a 3 ml glass cuvette (path length 1 cm) with all transparent walls. Prior to the DLS study, protein samples were passed through a 2 μm filter. The operating procedure was programmed (using the DTS software supplied with the instrument) such that there are average 20 runs, each

being averaged for 10 s, and a particular Rh is computed in each case and ultimately the result is presented as the distribution of Rh. In DLS one intends to measure the three dimensional pdf (probability distribution function) for diffusion process P, a general expression is given by

$$P(r,t|0,0) = (4\pi Dt)^{-3/2}\exp\left(-r^2/4Dt\right).$$

Since this function only depends on D, the diffusion constant of the system, this allows us to obtain the value for the Stokes radius Rh$=a$, if the pdf can be measured. The Stokes relation can compute the diameter of the scattering particle,

$$D = F/6\pi\eta a.$$

The link between the pdf and the power spectrum is a consequence of the translation of the relative motion of the scattering particles in to phase differences of the scattered light. So, if $I(t)$ is the intensity of the scattered light, then $C(\tau)$ satisfies the following relation,

$$C(\tau) = \langle I(t)\ I(t+\tau)\rangle_t \approx \exp(-\tau/D)$$

where, $C(\tau)$ is the auto correlation function.

It is then straightforward to measure D from the slope of the $\log C - \tau$ plot, and the hydrodynamic size (dh) follows from the Stoke's relation. A particular dh is evaluated several times and the result is presented in terms of a distribution of the hydration diameter. The instrument provided the size distribution in (a) Intensity mode (b) Number mode. While the first, providing the size distribution of scattered intensity is more sensitive to alteration in size (intensity varying as $\sim r^6$), the second mode provides size distribution of number of particles in the light path. For monitoring the population of aggregates (whose numbers are appreciably high in some cases) the multimode intensity distribution was used.

3. Result

3.1. Spectral study of the unfolding pathway

The native state of Cyt-C shows a Soret peak at 408 nm. As the protein gets unfolded in presence of Gdn–HCl the cumulative heme–peptide interaction, that combines the covalent and co-ordinate bond interactions, decreases. This results in a gradual dissociation of heme from the peptide framework. Consequently, the Soret peak undergoes a blue shift towards the free heme region (i.e. towards 380 nm). In the Fig. 1 the dynamics of the unfolding pathway is demonstrated by the blue shift of the Soret peak. The left panel represents the Soret peak of Cyt-C in 2 M Gdn–HCl concentration which is still 408 nm, that corresponds to the native state of the protein. The right panel at 3 M Gdn–HCl concentration, with λmax at 406 nm, signifies the unfolding state. Between these two extremes, the middle panel (Gdn–HCl concentration 2.25 M) shows a dynamic alteration in the Soret peak. A staircase like dynamics is observed with an intermediate λmax value at 407 nm. Hence at a critical denaturing stress the intermediate

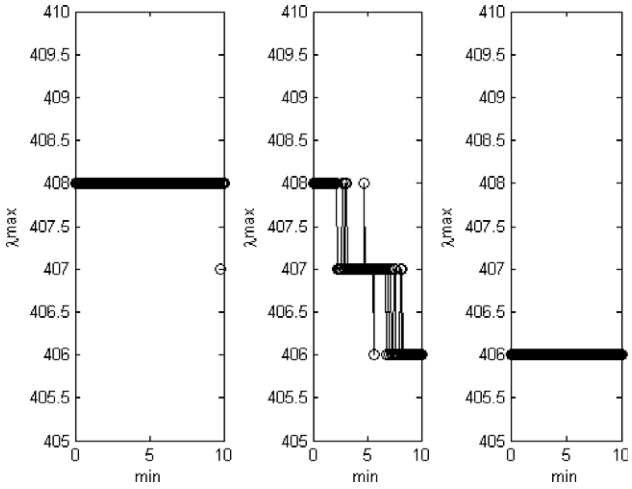

Fig. 1. Dynamics of the wavelength maxima during unfolding—X-axis represents time scale in minutes and Y-axis demonstrates wavelength maxima. Wavelength maxima plotted against time during unfolding. The left and right panel corresponds to the kinetics of wavelength maxima at 2 and 3 (M) Gdn–HCl conc., respectively; whereas the middle panel is the same kinetics at an intermediate critical condition with 2.25 (M) Gdn–HCl concentration.

energy states appear for a time in order of minutes. This indicates the slow nature of unfolding in Cyt-C and other heme proteins (data not shown).

3.2. Folding experiments by dynamic light scattering

3.2.1. Folding intermediates by size dynamics of Cyt-C folding

The nature of the hydrophobic collapse in Cyt-C has been observed by the size dynamics profile. However this collapse includes the occurrence of various molten globule structures in different intermediate unfolded states. In the Fig. 2, the distribution of population of Cyt-C is shown in different refolding states, starting from 8 (M) Gdn–HCl concentration to 1 (M) Gdn–HCl concentration. This distribution reflects that the unfolded population favors the formation of the aggregated forms. There is a dynamic equilibrium between the monomeric native state, oligomeric intermediate state and aggregated unfolded state. However this equilibrium may shift to either of the ways depending upon the unfolding condition. It has clearly been observed that in the higher unfolded states (i.e. 8 (M) Gdn–HCl conc.), the major population of the protein is in the aggregated form (diameter 8.83 ± 3.27 nm). As it undergoes refolding, the size of the protein decreases. The diameter becomes 6.88 ± 3.65 nm at 4 (M) Gdn–HCl conc. The high standard deviation value is reflective of the large variety of the species present. Further refolding reduces the size close to the reported size of native state, 3.1 nm. In 2 (M) and 1 (M) Gdn–HCl concentrations the diameter of Cyt-C was found to be 4.06 ± 1.78 and 4.09 ± 1.22 nm, respectively.

The description of the equilibrium is given in Fig. 3(a), which shows the Bar diagram as well as the Pie chart of the constituent states in the whole Cyt-C population in various unfolding concentrations (shown as: state-1=native state; state-2=oligomeric intermediate; state-3=higher order aggregates). More aggregated population and far less monomeric

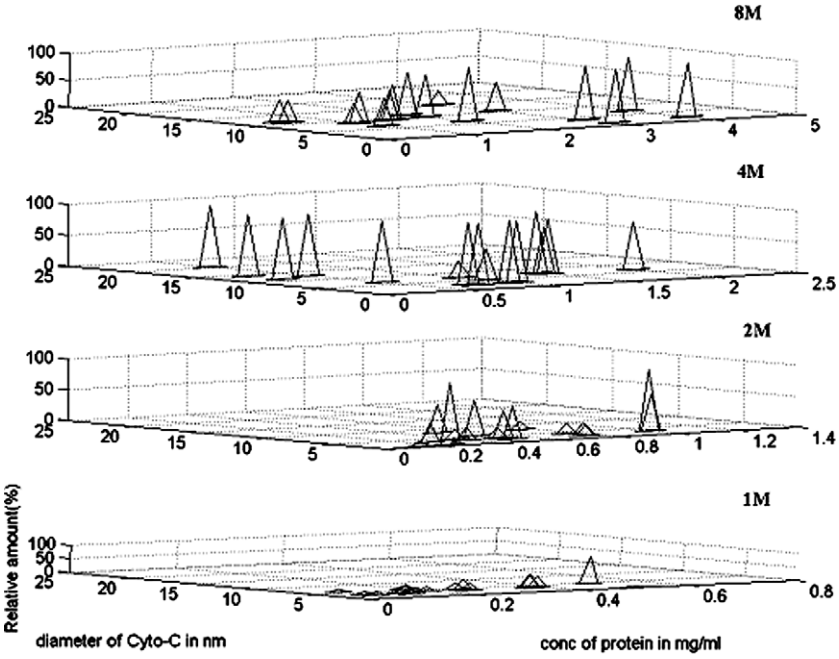

Fig. 2. Visualization of the folding intermediates by size dynamics of Cyt-C during refolding pathway observed in DLS. *x*-axis represents the conc. of protein in mg/ml, *y*-axis shows diameter of the protein in nm and *z*-axis shows the relative intensity, which is representative of the percentage contribution of the species having the corresponding diameter. The four vertical panels represent the size distribution of Cyt-C in 8, 4, 2 and 1 M Gdn–HCl conc., respectively.

native population characterize the unfolded states. More the extent of refolding, more is the participation of native population (i.e. diameter of ~3nm), less is the participation of aggregated forms as well as folding intermediates. The profile of the standard deviation, plotted against denaturant concentration is shown in Fig. 3(b). It is shown that with decrease in denaturant concentration there is a corresponding decrease in the size diversity. The rate of decrease has some dependence on the initial extent of unfolding. The decrease in standard deviation, coupled with increase in coherence, indicate the homogenization of the species due to refolding; since, the majority of the population in refolded condition was found to have the diameter in the 3 nm regions and minor amount of population was found in oligomeric or aggregated form.

3.2.2. Protein size is a function of unfolding concentration and marker points of folding locus

Interestingly, along with the diversity the mean size of the protein also changes along with the folding pathway. A notable aspect of this size dependent folding contour is that it is independent of the concentration of the protein. However the initial extent of unfolding still plays an important role. In Fig. 4 it has been demonstrated that in different unfolding concentrations (during the refolding pathway) but in the same protein concentration (0.5 mg/ml), the protein size decreases from unfolded (11.35 nm in 8 (M) Gdn–HCl conc.) to refolded state (2.91 nm in 1(M) Gdn–HCl conc.). Locus of the mean size of the protein provides hint towards a probable sigmoidal folding pathway. Similar curves have been observed (data not given) in other concentration ranges (both higher and lower) also.

3.3. Spectral study of enzymatic activity of Cyt-C

3.3.1. Decrease of the peroxidase activity during refolding

Fig. 5 demonstrates the activity-profile of Cyt-C in different unfolded states during refolding (Gdn–HCl conc being 8 (M) to 1 (M)). Specific activity being an important variable of the enzymatic activity of any protein, it has been taken as the marker parameter of peroxidase activity. The colored points represent absorbance of oxidized substrate (*o*-dianisidine) in the corresponding maximum wavelength. The profile shows that in the unfolded state (8 (M) Gdn–HCl conc.), the specific activity is the highest, which is $9.24e+03$ unit (mole of *o*-dianisidine oxidized/min/mg of protein). As the protein undergoes refolding the activity starts to decrease significantly and becomes as low as $1.32e+03$ unit in 1 (M) Gdn–HCl conc. This activity profile may be explained in the light of heme–peptide interaction. Cyt-C has two covalent bonds attached between heme and Cys residues. The tightness of Cyt-C heme group due to covalence; coupled with the buried topology—inhibits its activity. In the unfolding condition heme group becomes much more free and accessible to the substrate (*o*-dianisidine) resulting in higher peroxidase activity.

3.3.2. Dispersion of the active state population during refolding

The inset shows the pattern of the population distribution of the active state of Cyt-C. However this inset magnifies the Fig. 5 in a shorter time length (230 to 250 s) for a more precise observation. *X*-axis is the time scale, *Z*-axis is the absorbance and *Y*-axis is the wavelength maxima of the protein i.e. the wavelength(s)which correspond(s) to the maximum absorbance at a single time frame. In highly unfolded state, the peroxidase

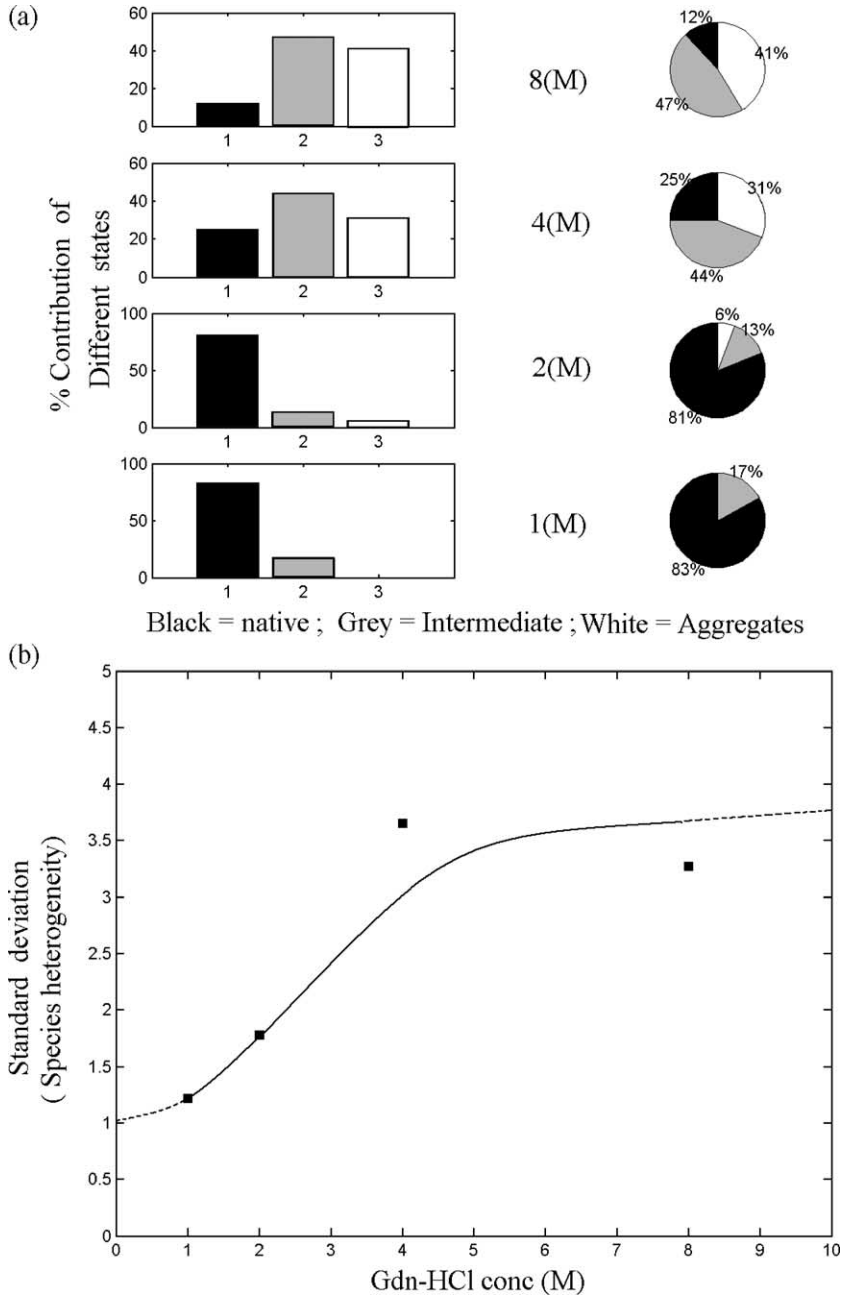

Fig. 3. (a) A parallel representation of bar diagram and pie diagram to show the nature of the different conformational contribution in protein population. The left panel is the bar diagram of different sets and right panel is the corresponding pie diagram. The three species shown in both the diagrams are of the following color code. Black represents native, grey represents oligomeric intermediate states and white is the representative of the aggregates. *Y*-axis of the bar diagram denotes the percentage contribution of different states. (b) Decrease of STD (standard deviation) during refolding. This plot shows the decrease of STD (*Y*-axis) along the folding pathway the *X*-axis representing the molar concentration of Gdn–HCl.

activity is much higher and the peroxidising peak is much sharper. Due to this sharpness, the range of wavelength corresponding to the maximum absorbance is much narrow (440 to 470 nm). More the protein gets refolded, flatter the peak becomes—to result in the broader range of wavelength maxima. The range of this distribution in refolded state becomes much higher (from 420 to 500 nm) by around 50 nm. Actually, with the decrease in the activity, the population density near the substrate utilization region decreases proportionately and gets dispersed in the less active state of the population. In addition to this, the distribution pattern of the reaction dots is much irregular in the unfolded state and gradually becomes regular during refolding.

3.3.3. Dynamics observed in interstate cross-over between two energy states in highly unfolded condition

Another very important observation in the peroxidase reaction has been found in the highly unfolded state of the protein (i.e. in presence of 8 (M) and 4 (M) Gdn–HCl concentration) shown in Fig. 6. There is a simultaneous

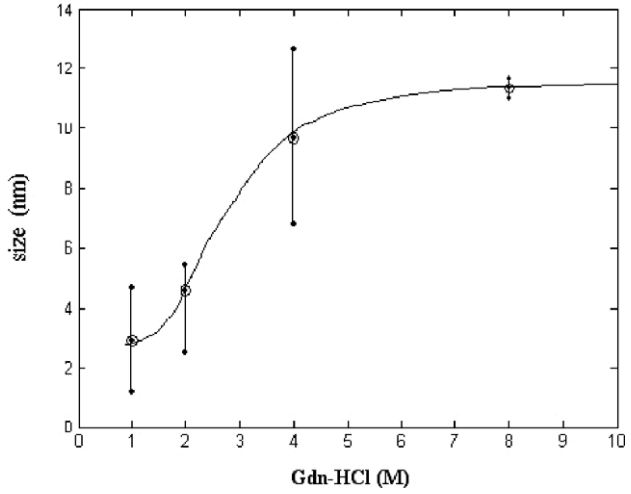

Fig. 4. Protein size is a function of unfolding stress-diameter of the protein (nm) is plotted in the *Y*-axis against the molar conc. of Gdn–HCl as the *X*-axis. The protein conc. in each case is kept same as 0.5 mg/ml.

appearance of a twin peak; first one in the dissociating heme range, i.e. 380 to 400 nm (we will designate it as H peak) and the second one in the usual substrate utilization region in the range of 450 to 500 nm (we will designate it as S peak). However the presence of the H peak is not found in refolded state. Its appearance only in the unfolded state is explained in the light of high amount of heme exposure towards the substrate that results in the formation of this activated heme peak. This H peak appears only while showing peroxidase activity, not in the resting state (data not shown). The most important aspect of the new H peak in 380–400 nm region is dynamics of energy state crossover. This dynamics, observed in Fig. 6, is a snap shot diagram of the spectra in the critical stages of the dynamics, where the two peaks cross talk with each other through an isosbestic point at 420 nm. The detail of this dynamics is characterized by the 4 critical stages as given in the Table 1. The H peak–S peak interaction via an isosbestic point (~420 nm) demands a closer look. Primarily, the occurrence of the isosbestic point is indicative of a two-state process, where two states are represented as the energy states of H peak and S peak. The gradual decrease of the H peak denotes the unstable nature of the corresponding energy state, which is commonplace for any reaction intermediate. Essentially, the energy states corresponding to the H peak and S peak appear to be in the same phase with low energy barrier between them which facilitates the energy transfer from H to the S peak.

4. Discussion

One of the most interesting physiological aspects of Cyt-C is its role in the Electron Transport Chain (ETC) of oxidative phosphorylation. 1 Cyt-C molecule is not capable of transporting 1 electron in one cycle. In the physiological condition, population of Cyt-C resides in the hydrophobic environment of mitochondrial intermembrane space, which is suitable for inducing an unfolding stress. This may lead to the formation of different ordered oligomeric population—capable of transporting one or more electron co-operatively.

In the mitochondrial respiratory chain Cyt-C acts as a linker between Complex-3 (i.e. ubiquinone to cytochrome-*c*) and Complex-4 (i.e. Cyt-C to oxygen). In the Complex-3 cytochrome-bc1 mediated coupling of electron transfer takes place from ubiquinol (QH2) to Cyt-C, which leads to the vectorial transport of proton from the matrix to the intermembrane space. The reaction of this step begins

$$QH_2 + 2Cyt - C(ox) + 2H^+ = Q + 2Cyt - C(red) + 4H^+.$$

Cyt-C being a single electron acceptor (for a single heme group), this soluble protein of the intermembrane space

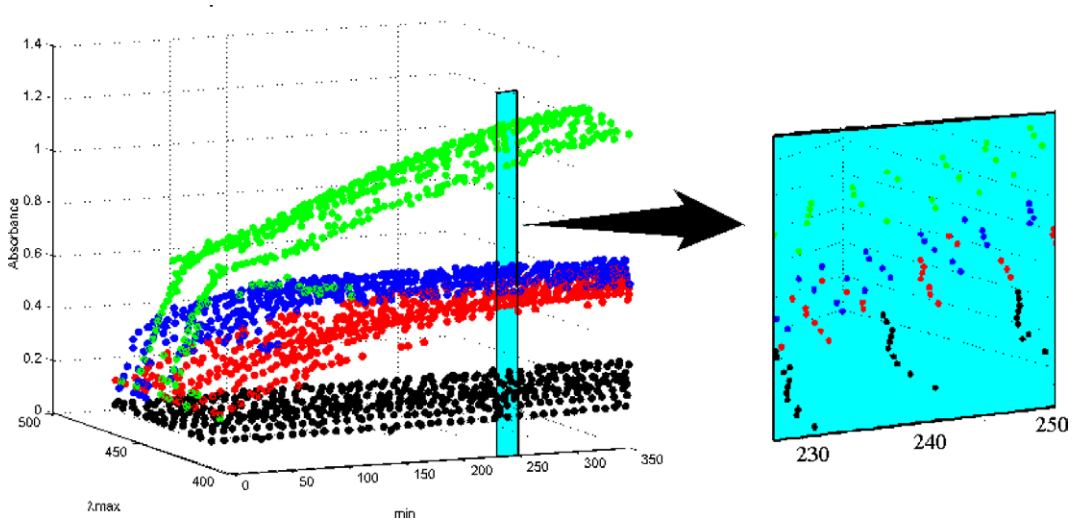

Fig. 5. Peroxidase activity and distribution of their population. The reaction dots of different colors are representative of the population of the active state in different unfolded states during refolding. The *X*-axis is the timescale in second, the *Y*-axis, the wavelength maxima, i.e. at which wavelength (or wavelength range) the protein is giving the maximum absorbance and *Z*-axis, the absorbance at the corresponding wavelength maxima. The color code follows like Green is for 8 M, Blue for 4 M, Red for 2 M and Black for 1 M of Gdn–HCl. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

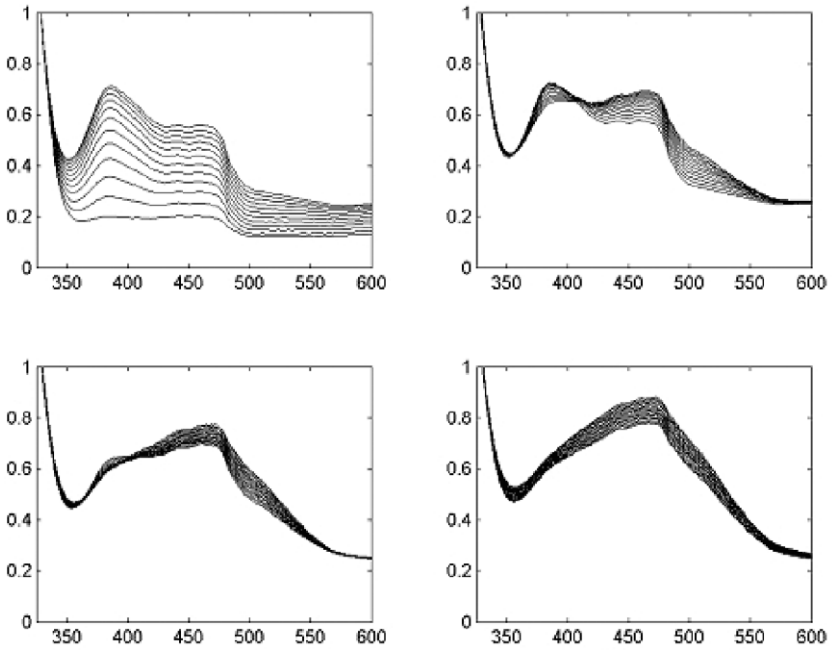

Fig. 6. Spectral dynamics of Cyt-C in enzymatically active state in 8 (M) Gdn–HCl. The spectral kinetics of the peroxidase assay is represented in four critical stages shown in four separate boxes. Here *X*-axis is the wavelength and *Y*-axis is the absorbance. The time frame of each boxes are given as; Box I: 0 to 1 min, Box II: 1 to 2 min; Box III: 2 to 3.5 min and Box III: 3.5 to 5 min.

transports an electron from complex3 to complex 4. This electron is carried to molecule of oxygen by reducing it to H_2O. The chemical reaction being,

$$2H^+ + 1/2O_2 + 2e^- = H_2O \text{ (chemical)}$$

$$4\text{Cyt} - \text{C(red)} + 8H^+ + O_2 = 4\text{Cyt} - \text{C(ox)} + 4H^+ + 2H_2O \text{ (enzymatic)}.$$

Here lies the vital question in the stoichiometry of the reaction. As it is clear that 1 Cyt-C molecule is capable of only transferring one electron, how can there be the production of one water molecule in each cycle remains a question. There cannot be any probability of reacting with atomic oxygen, as in the normal case of ETC.

The most plausible answer to this fundamental question may be given in the light of possible enzymatic co-operativity. It is previously reported that there is an electron transfer induced triggering of Cyt-C(I) folding [24]. Hence there may be a coupling between ETC and folding trajectory of Cyt-C. Presence of a hydrophobic surface (e.g. membrane) is likely to cause ordered exposition of hydrophobic size. A regulated extent of unfolding in turn can induce a controlled molecularity of oligomers. The paradox of Cyt-C and absence of any oligomerity in aqueous environment, may therefore be resolved by postulating a pseudo co-operative behavior of the unfolding induced oligomer state. This oligomeric distribution may be heterogeneous [20] in nature but have proper order (of associability or co-operativity) such that it may carry sufficient electrons for one or more cycles efficiently. The spectral dynamics (between H peak and S peak) via isosbestic point in the peroxidase activity profile under high denaturing stress is also supportive of this interstate crossover between the energy state of various aggregates. In addition to this, this enhancement of peroxidase activity during unfolding and the reverse trend while refolding hints us towards a folding dependent regulation of the enzymatic activity of Cyt-C in the physiological milieu. Hence two more points needed to be clarified—firstly, the nature of this aggregatory equilibrium; secondly, the condition that facilitates the formation of aggregates. From our experimental results, the participation of a huge variety of aggregates during the folding pathway is shown clearly. The instability of the aggregates being a driving force of the entropically favorable folding process and the kinetic barrier [2] due to His–heme misligation and proline mis-isomerisation being the reason of high potential barrier, the overall process becomes much reversible and slow. This reversibility between various aggregates, formed by the unfolded monomers are shown in Fig. 7, on the basis of the

Table 1
Time course of peroxidase activity in presence of 8 M of Gdn–HCl

Period	0 to 1 min	1 to 2 min	2 to 3.5 min	3.5 to 5 min
EVENTS	Increase of both H and S peak simultaneously	Decrease of the H peak and increase of the S peak via an isosbestic point in ~420 nm	Gradual overlaying of the H peak by the S peak	Individuality of the H peak is lost as the S peak has emerged as the sole peak in this spectral range

The table describes the spectral events associated with substrate utilization by peroxidase (S peak) under extreme unfolding conditions and the corresponding structural changes (H peak), the symbols (H and S) being explained in Section 3.3.3.

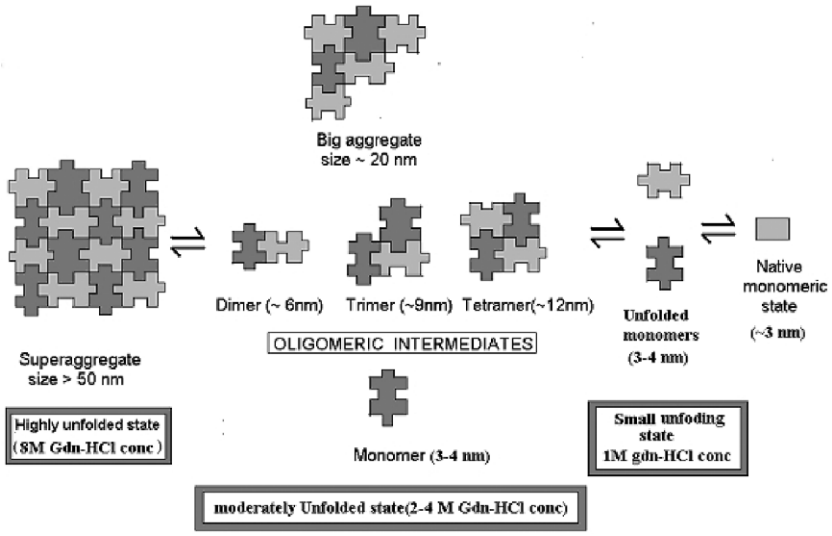

Fig. 7. A description of the reversible de-aggregatory refolding pathway. Aggregates of different sizes are present in the equilibrium. This reversible equilibrium shows de-aggregatory folding pathway and aggregatory unfolding pathway.

experimental results. This shows the gradual formation of big aggregates (size ~20 nm) and then superaggregates (size ~50 nm) during unfolding. The reversible pathway, i.e. the refolding pathway is infested with various aggregates with oligomeric intermediate states having molecularity of 2, 3, 4 etc. Now, let us try to give the answer to the second question. The conventional fast folding funnel with steep inclination does not allow the refolding protein to form intermediates. Hence there comes the concept of rugged landscape [20] of the folding pathway to support the intermediate formation by introducing different energy traps. But what we are showing is that, there exists a rough surface infested with various energy traps in the equilibrium zone with native state, which is at the bottom of the protein-folding funnel in this slow folding process. We named it as the rugged nose of the whole folding body as shown in Fig. 8 that is equivocal with presence of multiple minima near a true minima. In the vicinity of the funnel tip (that is its nose), the hydrodynamic size, size heterogeneity and peroxidase activity forms the distance parameters from the native or the refolded state as we have already noticed that magnitude of these parameters are decreased along the refolding trajectory. The energy traps within this construct are energetically suitable for formation of the oligomeric aggregates due to the less steep landscape than the fast folding funnel. This nose structure is not only embedded with different energy traps, it also has the definite downhill trajectory defined by the decrease in size diversity and peroxidase activity of the folding. The larger aggregates are gradually broken to a smaller size to reach its final destination, i.e. the native state.

5. Conclusion

One forthcoming conclusion in this paper is a co-operative decrease in mean size and its variance along with the folding. The slow dynamics of the folding process is conjectured to

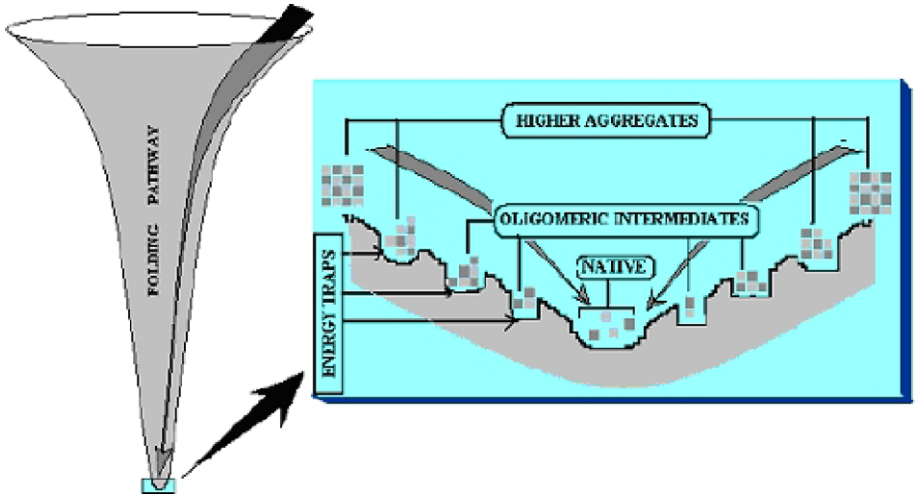

Fig. 8. Elucidation of a rugged nose at the bottom of the folding funnel infested with energy traps to describe the suitable condition for the formation of different aggregates and their probable role in the near equilibrium region with the native state.

have some correlation with the electron transport chain activity of the protein. Although the typical co-operativity as seen in say allosteric interactions for multisubunit proteins, a folding/unfolding assisted cooperative behavior may be important in the regulation of the free radical generation, which is likely to happen unless the electron transport activity is perfect. The question of half oxygen participating in the transport chain activity is also answered if one considers this coherent aggregation de-aggregation process occurring in the vicinity of the membrane surface.

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