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Lazy dynamics of unfolding and ligand interaction – signatures in hemoglobin and its glycated form

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Abstract : The dynamics of unfolding of hemoglobin (Hb) and its glycated form (GHb) have been studied. The temporal profile of the wavelength maxima in the *Q* band of Soret region (408–420 nm) showed a characteristic periodic dynamics. Typically, an irregular periodic behavior, a staircase like profile or a combination of the two was observed. The time scale associated with unfolding was of the order of seconds. Multiple possible liganded states of heme and heme dependent incremental alterations in the quaternary structure are proposed to cause such sluggish periodic behavior. The mechanism of protein unfolding, unlike the folding process is an important cellular event in protein transport, but has got lesser attention in literature. Further exploration in the dynamical features of unfolding, as described in the present work, can lead to deeper understanding of the regulation of unfolding and it can also provide an indirect insight of the reversible steps in the folding process.

Keywords : Protein unfolding, glycation, hemoglobin, dynamics.

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1. Introduction

Considerable attention has been given to relaxational folding problems of proteins both by chemists and physicists. We have considered here the reverse process, namely the dynamics of unfolding of hemoglobin and its glycated form. Hemoglobin (Hb) molecule has inspired the physicists, chemists and the biologists, ever since its pseudo symmetric crystal structure was reported by Perutz [1]. Hemoglobin has four chains of amino acids known as globin chains and an iron-containing heme group attached to each. Each tiny red cell in the blood contains about 280 million molecules of hemoglobin. Sensitivity of the globin chains to point mutations (one such mutation causing sickle cell anemia [2]), to relative concentrations of chains (α and β), a misbalance of which cause thalassemia [3], have been discussed in literature extensively. The literature includes reports on several important mutations of hemoglobin, some being silent, and some affecting the allosteric equilibrium (that dictates the oxygen carrying capacity), some, shifting the tetramer to dimer equilibrium of this protein [4], the latter being an important thermodynamic parameter that determines the sensitivity of hemoglobin to attack by malarial parasite [5].

In this paper, we consider a class of Hb variant that is neither a globin mutant, nor associated with differential mole fractions of α and β chains. The structural alteration in this case is due to a post-translational event called 'glycation', a non-enzymatic process leading to association of hemoglobin with glucose. This modified form of hemoglobin is seen in abundance in diabetic patients who

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suffer from an exposure to high glucose concentration. This modified Hb form is known as GHb (glycated hemoglobin alternatively called HbA1) [6]. The relative percentage of GHb is well correlated with the overall glucose level in the serum and is often taken as a robust measure of the state of diabetes. To our knowledge, till today, the crystal structure of glycated hemoglobin has not been solved and accordingly, the state of knowledge on its structure is insufficient. Some theoretical simulation study however indicates that glycation causes a push of the *R-T* (relaxed-tense) equilibrium, towards the *R* state [7]. It is also known that the alteration caused by the association of glucose affects the geometry of the allosteric pocket adjacent to β 93 cysteine and one can thus expect an altered geometry of heme. While the modification of the allosteric region is likely to affect the overall structural integrity of this multimeric protein the exact role the thermodynamic bias towards *R* state on the folding or unfolding behavior is unknown. The paper presents some spectacular difference in the dynamics exhibited by Hb and GHb under differential extent of denaturing stress and in presence of binding to different ligands using time lapse absorbtion spectra in the *Q* band region of hemoglobin (Hb) and its glycated form (GHb).

2. Methods

Preparations :

Hb (adult hemoglobin)

Blood samples were collected in EDTA vials from normal persons. The whole blood was centrifuged at 3000 g for 10 min. and washed 3 times in normal saline. Hemolysis was performed by mixing 100 μl of packed RBC in 500 μl of lysis buffer (2.5 M NaCl, 100 mM EDTA-Na salt, 1% triton X-100 and 10% DMSO). The solution was applied to a Sephadex G-50 column equilibrated with 0.1 M phosphate buffer pH 7.0. Elution was carried out with 0.1 M phosphate buffer pH 7.0 at a flow rate of 25 ml/h.

GHb (glycated hemoglobin)

Cation exchange column (Trans Asia) was used to separate the GHb fraction. For normoxic native state, there are very little difference between the spectrum of Hb and GHb. The spectral difference becomes conspicuous in presence of stress (oxidative or nitrosative). The extent of glycation of Hb was identified using the periodate assay method [8] in which formaldehyde was formed and was detected by formaldehyde detecting reagent. A calibration curve with glucose was drawn to determine the level of glycation in different samples. The glucose level of the glycated fraction was much higher (~10 mM). The total Hb on the other hand had a background glucose concentration (~1 mM). The percentage of glycated hemoglobin for a typical normal sample was of the order of 4–5%. The purity of the GHb isolated through cation exchange was further checked by HPLC (Waters) using Chromo System Kit. The HPLC based measurement was carried out for total Hb as well as GHb isolated using the previously mentioned cation exchange column. The sole occurrence of the HbA1 peaks in the cation exchange column separated fraction confirmed the purity of the GHb sample. The percentage of GHb measured using the area ratio in these two respective HPLC runs matched well with the cation exchange measurements (*i.e.* 4–5%).

Unfolding and ligand binding :

Time lapse kinetics

Diode array spectrophotometer (Analytik Jena, Spekol 1200) was used to take snap shot spectrum at different intervals of time. Absorbance maxima λ^{max} (nm) were determined from the analysis of the diode array generated files at different intervals of time. Typically a single kinetic profile contains about 200 wavelength scans. The profile describes the time course of the position of the absorbance maxima in the ±10nm range around 415 nm, the diode array model providing wavelength accuracy of 0.5 nm. The noise interference in the diode array data was checked by sample spectral data comparison to other spectrophotometer models (Perkin Elmer, Model Lamda 35) at slit width 0.5nm.

Denaturant and ligand interaction study

Guanidium hydrochloride (GdnHCl) was used as a denaturant at concentrations 1 M, 2 M and 3 M. The protein concentration in each case was kept such that for Hb and GHb the absorbance at 415 nm remains identical. (~0.15) The ligand interactions were studied using reduced glutathione, hydroxyurea and sodium nitroprusside, each at concentration range 1–3 μM. The concentration dependence was studied in each case but is shown in case of hydroxyurea and glutathione (as transition from one steady state to another was more obvious in these cases).

3. Results

Firstly, we report the response of hemoglobin and its glycated form to varying concentrations (1–3 M) of the denaturant GdnHCl (Figure 1). It appears that the characteristic dynamics of Hb and GHb are distinguishable

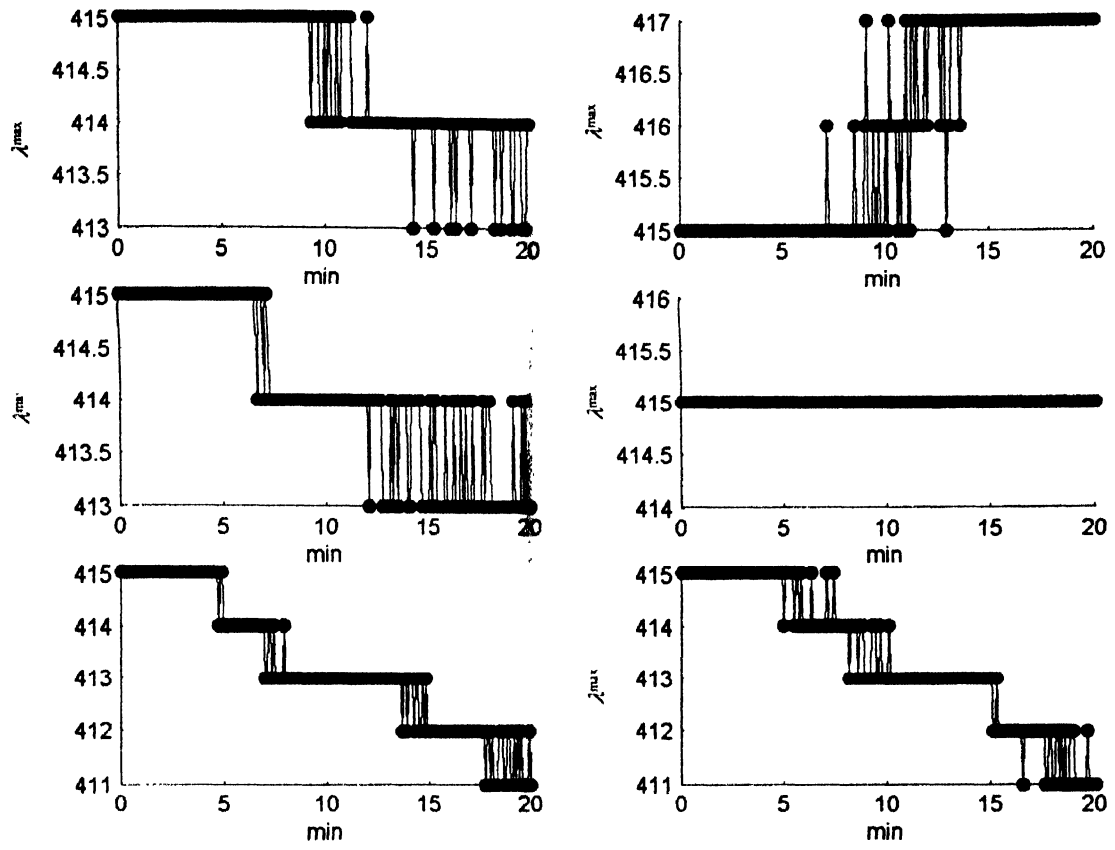

Figure 1. The three horizontal panels respectively corresponds to time course of the λ^{max} around the Soret Q band of hemoglobin and its glycated form. The three successive horizontal pannels (from the top) in the left (Hb) and right (GHb) vertical panels respectively represents the temporal profiles of λ^{max} at concentrations 1 M, 2 M and 3 M of GdnHCl.

during mild unfolding *i.e.* at 1 M and 2 M of GdnHCl (the two horizontal panels from the top of Figure 1). At 2 M of GdnHCl, Hb retains the essential dynamical feature, but there is significant change of unfolding dynamics of GHb (see the middle horizontal panel of Figure 1). As the denaturant concentration is raised to a higher value (3 M), the response of Hb and GHb becomes similar. Since, hemoglobin is a multimeric protein, it is likely that under higher concentrations of denaturants the dissociation effect dominate over the unfolding, the former destroying the quaternary structure. The similar temporal dynamics of Hb and GHb at high concentration of the denaturant may be the result of such dissociation effects (see lower most horizontal panel of Figure 1). The observation noteworthy in Figure 1 is, while hemoglobin has a tendency to approach wavelength lesser than 415 nm (*i.e.* towards the methemoglobin [9]) the glycated hemoglobin tends to shift to the 418 nm range characteristic of the nitrosylated state of hemoglobin [10]. Interestingly, it is known in literature that glycated hemoglobin is more prone to nitrosylation [11]. Both Hb and GHb in absence of any denaturing agent showed no change in respective λ^{max} position even within one hour span of time.

Apart from the GHb specific nitrosylation and the rich dynamics, the point that is revealing, is the characteristic signatures of the unfolding of Hb and GHb. The observation implied existence of a number of intermediate steady states, which were 'unstable' in the broad dynamic sense, and gradually relaxed back to a relatively stable state depending on the local environment (as offered by the protein structure). To have an overall idea of the stability of the different steady states the dynamics represented by Figure 1 is re-plotted in a flow diagram in Figure 2. In this figure $\lambda_{i+1}{}^{max}$ is plotted against $\lambda_i{}^{max}$, i representing the order in which data is taken (the smallest possible sampling interval being second). The line segments headed by arrows (in Figure 2 and subsequent flow diagrams), illustrate the hopping between different possible states. The steady states in this diagram at which i^{-th} and $(i + 1)^{-th}$ values of λ^{max} are equal, are represented by nodes clustered around the 45° angle with abscissa. Interestingly, none of these steady states are strictly stable as there is at least one arrow pointing away such nodes; however there seems to be an order or hierarchy of stability de-

pending on the number of inward arrows (*i.e.* arrows points towards the state).

We plan the next part of our experiments using a working hypothesis that hemoglobin can lend it self to a

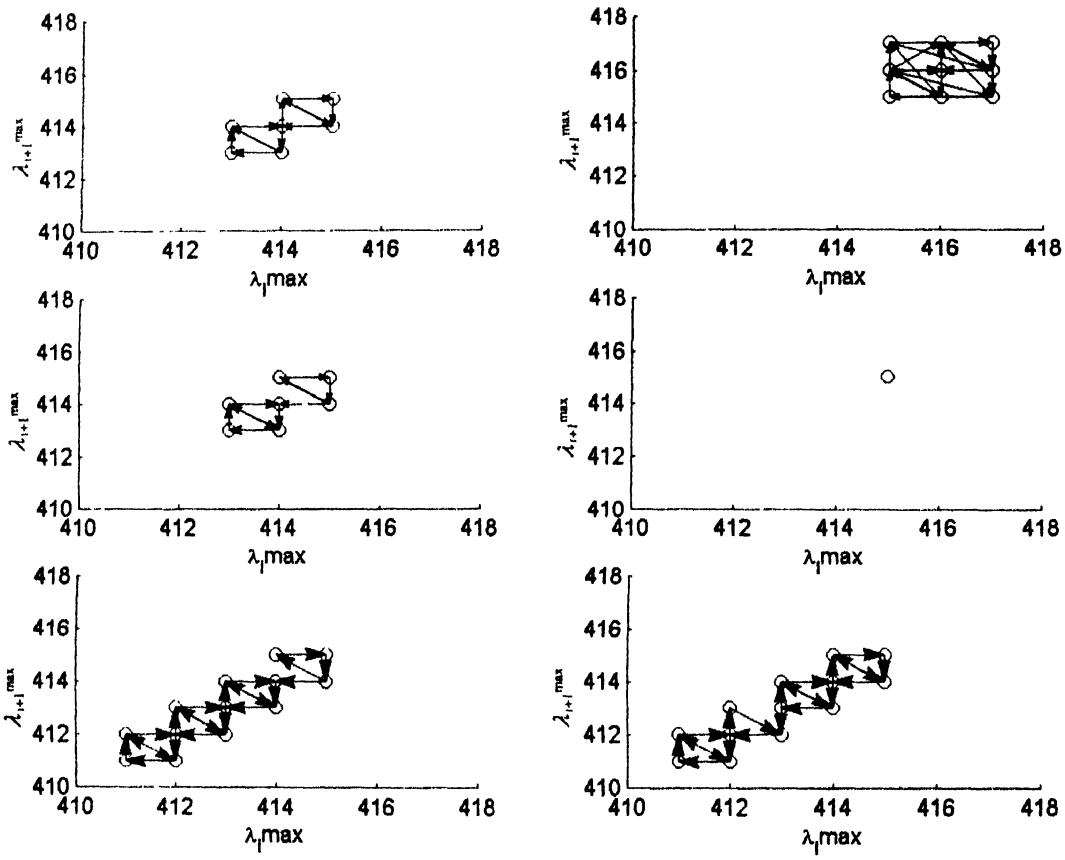

Figure 2. The result of Figure 1 is plotted using a diagram that maps one maxima against the successive maxima observed (in the limit of the sampling time ~sec). The ordinate and abscissa thus represent the absorbance maxima λ_{i+1}^{max} and λ_i^{max} respectively taken at $i + 1$ and i-th time instants. The line segments in the figure terminated by arrows represents the direction of hopping between different possible states, each point in the plot representing a state. It may be seen that for lower denaturant concentration GHb is confined to the upper wavelength range (415–419 nm), whereas the hemoglobin remains at value lower than 415 nm.

It may be emphasized at this stage that the oscillation observed here is obviously not the oscillation in 'concentration' of any particular species, but an oscillation of 'state' represented by the time variation of spectrum. The problem in this conjecture lies in the fact that a shift of 3–5 nm would correspond to a change in a time scale which two decade lower than a femto second. The dynamics on the other hand is observed in the time scale of seconds, an observation not uncommon (but contrasting explanations) in protein folding literature [12]. The aspect that is important from our observation is how mild unfolding, that non specifically breaks the hydrogen bonds and salt bridges, leads to oscillatory transition, the oscillation being dependent on the variant of hemoglobin.

limited number of liganded states under undissociated conditions. In Figuer 3, we describe the interaction of Hb and GHb with reduced glutathione, an agent that shows reactivity to thiol groups [13]. Since the allosteric pocket of hemoglobin is adjacent to a cysteine residue containing such thiol group, it is expected the ligand would act specifically on that region. In fact in Figure 3, one can see a gradual step like oscillation in presence of glutathione (akin to mild denaturing conditions), the steps gradually going up from 415 nm in case of GHb and going down towards the bluer regime in case of Hb. Figure 4 illustrates the flow diagram corresponding to Figure 3. Figure 4, like Figure 2 confirms the existence of a number of unstable intermediate steady states, interestingly in different characteristic wavelength domains for Hb and GHb respectively.

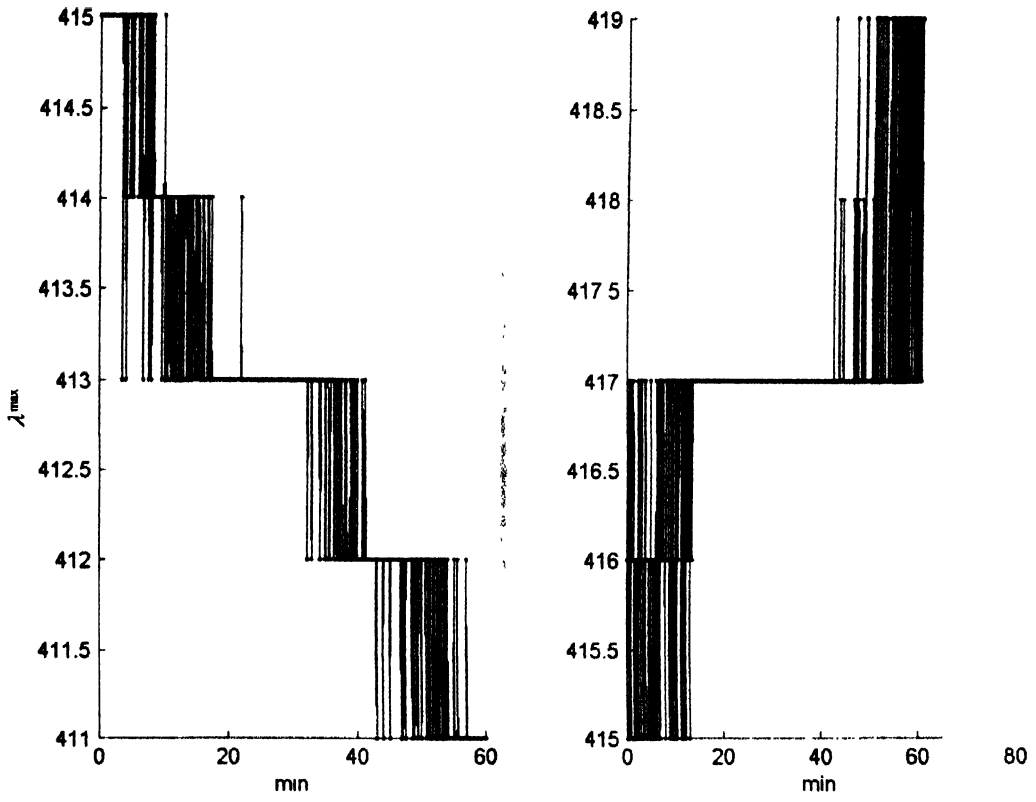

Figure 3. Effect of glutathione (GSH), a thiol reactive reagent, known to act near the cysteine residue near the allosteric site is studied (concentration 1 mM). The abscissa and the ordinate are similar to those described in Figure 1. In one case there is an oscillatory fall to 411 nm and in the other case there is an oscillatory rise to 418 nm.

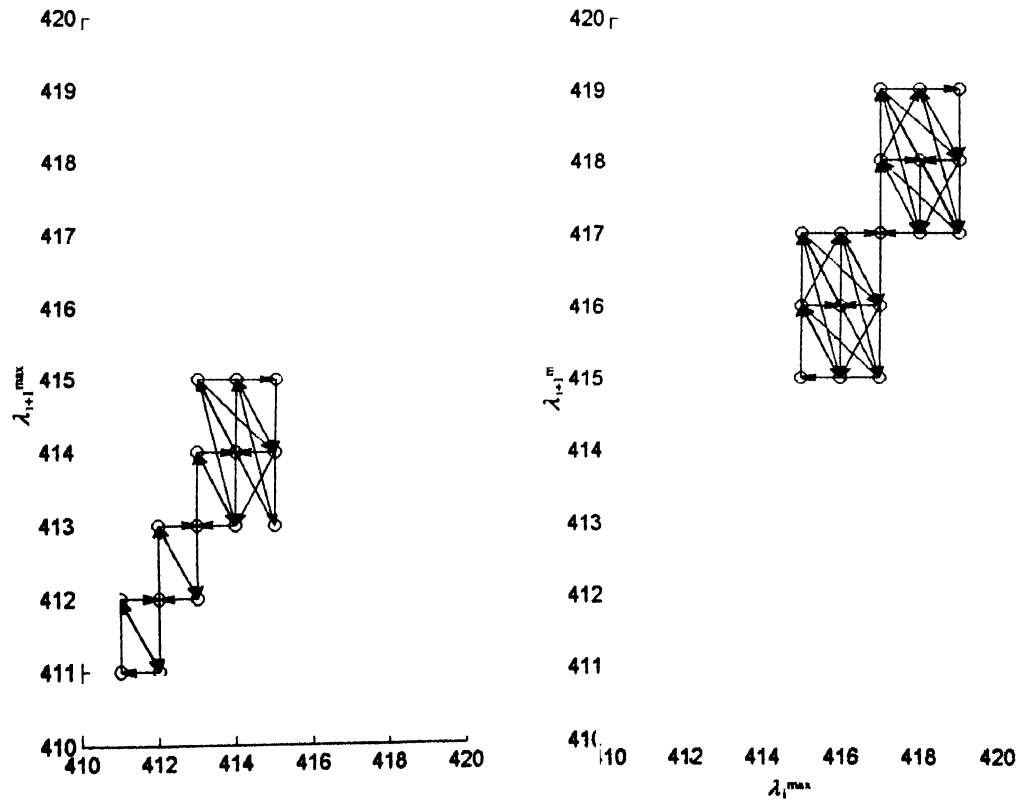

Figure 4. Map representation of the GSH effect.

Figures 5 and 6, demonstrate the sensitivity of hemoglobin and its glycated form to hydroxyurea, a drug [14] that is administered to sickle cell anemia and thalassemia patients. Among its other functionalities, one feature that is important in this drug is that it is a nitric oxide (NO) donor and hemoglobin has well known reactivity with NO, (Figures 5, 6) The feature that is revealed in this experiment is at least in glycated hemoglobin there is a relationship of the concentration of the ligand with the temporal behavior of the spectra. There are two steady states in the

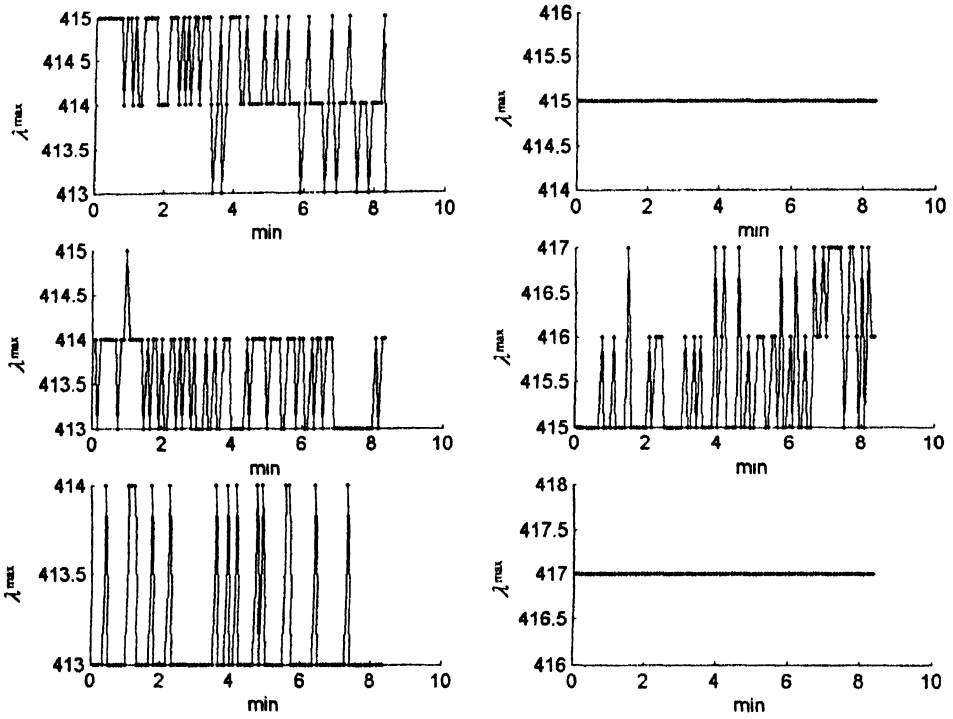

Figure 5. The effect of hydroxyurea, a drug that is useful in treating thalassemia and sickle cell anemia. The concentration used in the horizontal panels are 1, 2 and 3 mM respectively. The figure illustrates how in GHb, there is a critical transition from one steady state to another.

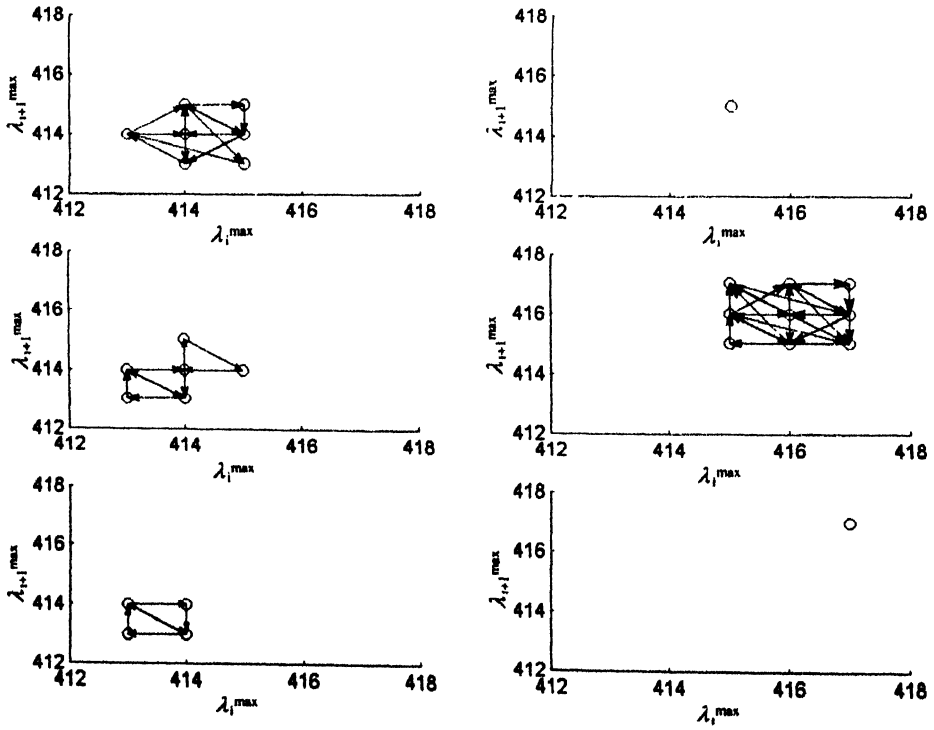

Figure 6. Map representation of hydroxyurea effect.

lower and higher hydroxyurea concentration, but there is an intermediate concentration at which the temporal fluctuation is most predominant.

To check whether NO binding obeys the consumption or storage rule [15,16] (the controversy on whether NO is removed by hemoglobin by the reaction : oxyhemoglobin

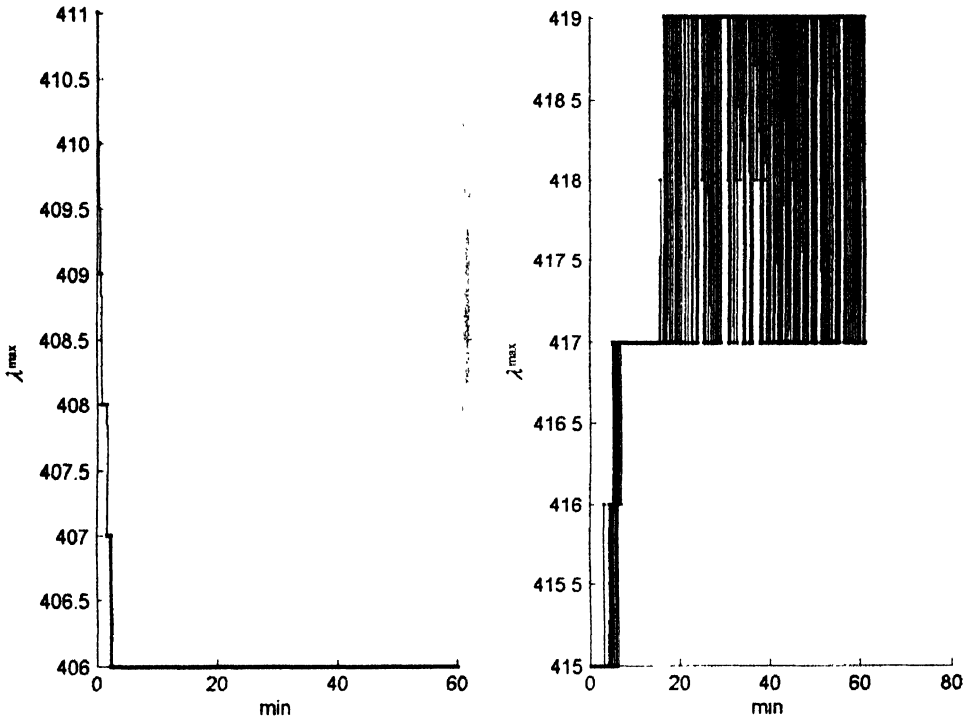

Figure 7. The effect of sodium nitroprusside (an NO donor) at a concentration of 1 mM. The GHb shows sustained oscillation after a delay whereas Hb shows an immediate conversion to methemoglobin (the 408 nm region).

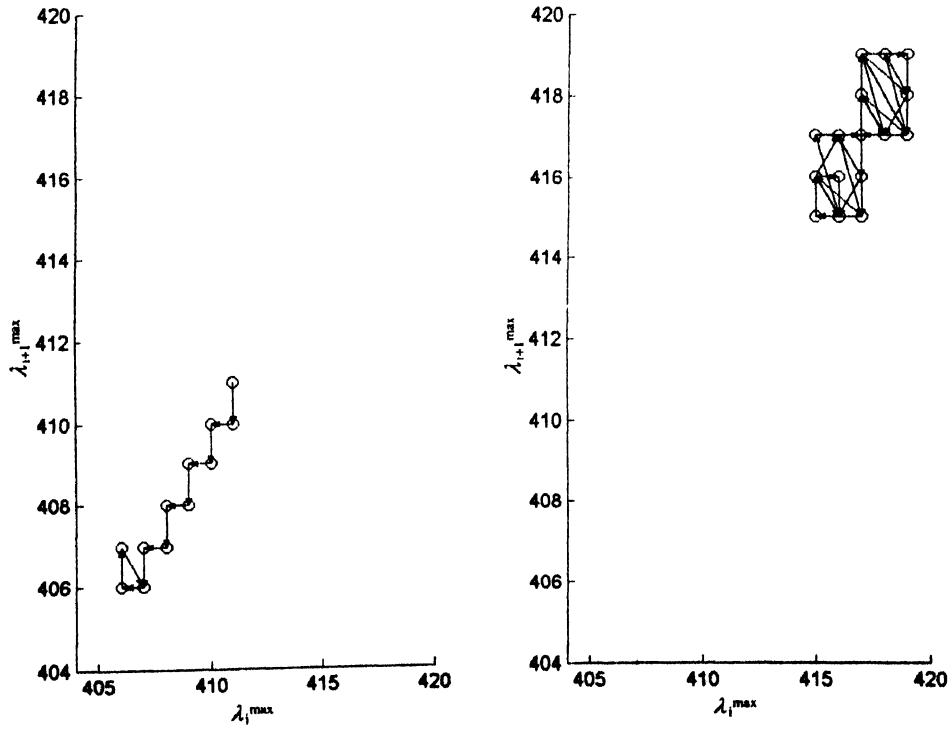

Figure 8. Map representation of the sodium nitroprusside effect.

+ NO → methemoglobin + NO_2 or it is primarily stored in s-nitrosylated form) we chose a more efficient NO donor, sodium nitroprusside. The dynamics for Hb in this case (Figures 7, 8) was an abrupt transition to the methemoglobin (metHb) state as evidenced by the rapid spectral shift towards 408 nm. The glycated hemoglobin on the other hand proceeded towards 418 nm, the rapid transition replaced by a time delay profile that drove the system towards the nitrosylated hemoglobin (HbNO).

The spectral dynamics was ample evidence for presence of intermediates. Figure 9 presents an alternative but direct evidence of the postulated formation of metHb and HbNO in case of Hb and GHb respectively. Figure 9A by the difference spectra [15]. In case of Hb a typical difference spectra of metHb whereas, for a GHb typical difference spectra of HbNO are observed. A direct spectral information is presented in Figure 9C where a few scans from different intervals of time are superposed (nitroprusside data). As described earlier the metHb formation by Hb (see the hump near 630 nm region) is rather clear. The GHb has different isosbestic points in this region.

4. Discussion

The fact that the proteins under consideration exhibit a characteristic robust temporal behavior irrespective of the nature of stress, and they remain confined within a certain

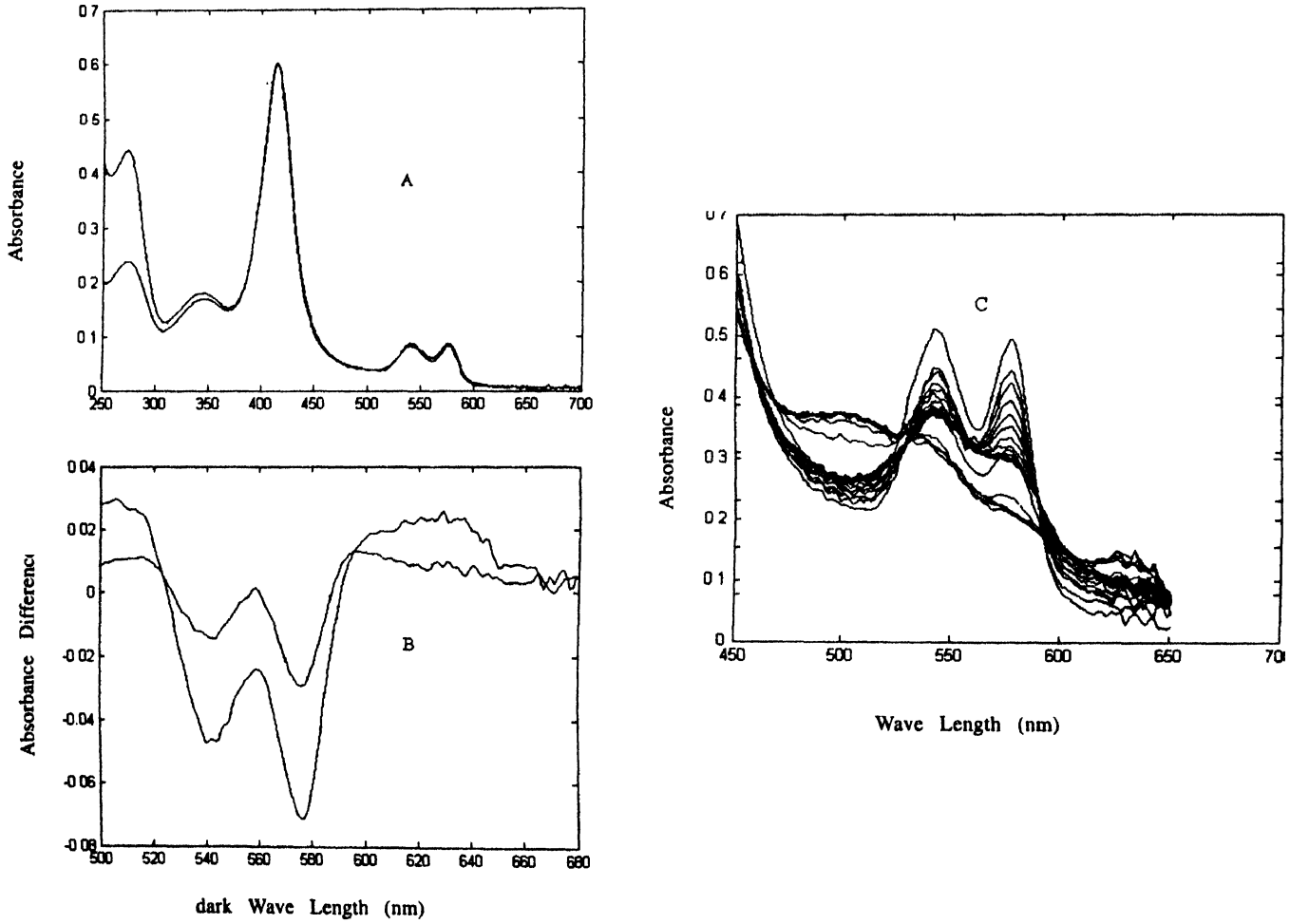

Figure 9. The light and dark lines represent the Hb and GHb spectral data respectively. (A)The figure describes the spectra of native Hb and GHb. (B) Difference spectra (final as compared to initial) of Hb and GHb respectively each in presence of 1 mM nitroprusside. (C) Superposed wavelength scan collected for a period of 10' (taken at 1' interval) in presence of 1mM nitropr usside.

provides a comparative account of a wavelength scan of native forms of Hb and GHb respectively. It is obvious that with exception of the UV region, Hb and GHb have almost identical spectral behavior at their native state. The metHb and HbNO formation (Figure 9B) are best illustrated niche or cluster of steady states, indicate that the unfolding mediated and the ligand induced conformational deformations may progress though similar energy routes. Along with this, it may also be noted that the response to the stress, is dependent on the protein and is somewhat

independent of the nature of the stress. The hemoglobin and its conformational variant (GHb) thus responsd differently to the stress, hemoglobin being thermodynamically biased towards methemoglobin side (~408 nm), glycated hemoglobin to higher wavelength range (~418 nm) the unperturbed state of each starting with the well known Soret *Q* regime (415 nm) [10].

The additional point that is noteworthy is that the two extremes of the absorbance maxima (corresponding to the folded and unfolded states) are separated by multiple possible steady states. While the sluggish response may be because of the innumerable hopping of Hb and GHb in the possible steady states (as illustrated by the flow diagrams) in their corresponding niche, the observation may be a first ever direct spectroscopic evidence of the presence of a sluggish and sticky passage from folded to unfolded state though multiple intermediates. The content that is slightly unrelated, but of some interest is that the method presented here may be scaled up to a tool kit to diagnose glycated Hb in diabetic patients.

In conclusion, the respective energy state dynamics of Hb and GHb observed during ligand binding (*e.g.* the NO donors) and the unfolding route (as seen for example by the GdnHCl mediated dynamics) has a similarity as far as the overall shift direction of the absorbance maxima. The step function like behavior however is typical for the unfolding process. It may be noted at this juncture that though extensive literature exists in connection with the folding process [17], very little experimental or theoretical work has been done on unfolding mechanism [18,19]. It is however understandable that a detailed knowledge on unfolding, may help in understanding regulation of a number of important cellular events, *e.g.* the transport of proteins across the mitochondrial membrane [19].

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