Effects of Ethylene Glycol on the Structure and Stability of Myoglobin Using Spectroscopic, Interaction, and In Silico Approaches: Monomer Is Different from Those of Its Polymers

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ABSTRACT: Investigation of changes in thermal stabilities and structures of proteins in the presence of different co-solutes (ligands) is an integral part in the basic research, discovery, and development of drugs. Ethylene glycol (EG) is known to be toxic and causes teratogenic, inducing primarily skeletal and external malformations and other diseases. The effect of EG on the structure and thermal stability of myoglobin (Mb) was studied using various spectroscopic techniques at pH 7.0 and two different temperatures. As revealed by circular dichroism, Trp fluorescence, nano-DSF, and absorption (UV and visible) measurements, EG (i) has no significant effect on secondary and tertiary structures of Mb at 25 °C, and (ii) it decreases the thermal stability of the protein, which increases with increasing concentration of EG. As revealed by ANS (8-anilino-1-naphthalene sulfonic acid) fluorescence measurements, heat-induced denatured protein has newly exposed hydrophobic patches that bind to ANS. Isothermal titration calorimetry revealed that the interaction between EG and Mb is temperature dependent; the preferential interaction of EG is entropy driven at low temperature, 298 K (25 °C), and it is enthalpy driven at higher temperature, 343 K (70 °C). Molecular docking study showed that EG interacts with side chains of amino acid residues of Mb through van der Waals interactions and hydrogen bonding.

1. INTRODUCTION

Significant efforts have been devoted to explore the potential effects of macromolecular crowding on protein folding and crowder–protein interactions. Structural and thermodynamic studies of proteins in crowded and confined conditions in vitro⁷−⁸ have been exploited to describe in vivo protein behavior, which is affected by dominance of various types of interactions occurring in cells.⁸−¹³ In 2008, Zhou et al.⁸ presented a thoughtful argument in their review in which polyethylene glycol (PEG) should be avoided as a crowding agent precisely because of its potential for the favorably interaction with the proteins that it is intended to be crowded. As a result, PEG is now fast-becoming something of an exile in the macromolecular crowding community. Certainly, if one’s intention is to specifically disentangle contributions from excluded-volume effects, then this sideling of PEG makes perfect sense. Interestingly, new information on the nature of PEG–protein interactions has recently been observed by Crowley group, which performed NMR studies to explore the interaction between PEG and the small model protein cytochrome c.¹⁴ Many therapeutic proteins have been coupled to poly(ethylene glycol) to prolong their circulating lifetimes and increase their potencies in vivo.¹⁵−¹⁷ PEGs had been successfully used to enhance activities of enzymes of which l-asparaginase¹⁸ and adenosine deaminase¹⁹ are excellent examples. The 3–5 strands of PEG with molecular mass > 30 kDa were observed more effective than 7–15 strands of low molecular mass PEG (5 kDa) for preserving the biological activity, increasing the plasma persistence, and reducing the antigenicity of bovine and recombinant human super oxidize dismutase (SOD).²⁰ PEG-mediated stabilization of the protein suggested that a conformational change occurred in the protein after the PEG interaction and demonstrated the highest stability of protein at the optimum BSA:PEG molar ratio.²¹ EG may prove to be a reagent, which is relatively inert for most of the proteins.²² It has been observed that the native conformation of γ-globulin and β-lactoglobulin appears to remain stable in the presence of a very high concentration of EG.²² In contrast, there are a few studies where PEG has been observed to destabilize the structures of proteins. EG had been observed to decrease melting temperature by about 7.5 °C in
ribonuclease. Interestingly, it has been shown that polyols stabilize chymotrypsinogen, whereas EG decreases its melting temperature ($T_m$) with increasing concentration.\(^{24,25}\) Ficoll 70 and Dextran 40 and 70 crowding agents are known protein stabilizers, and protein stabilization is due to their preferential exclusion from the protein domain.\(^{26-28}\) On the contrary, these crowding agents employed by Malik et al.\(^{3}\) impart significant destabilization of the native state of Mb. This observation indicates that the effect of macromolecular crowders can be quite protein specific. PEG 8 kDa distorts the Mb structure; however, EG, a monomer of PEG, has no visible effect on the Mb structure.\(^{2}\) Recently, in our laboratory, it was observed that PEGs destabilize Mb and cyt c and yield intermediate states.\(^{5,25,30}\)

In this work, a structural and thermal unfolding study has been performed on Mb in the presence of different concentrations of EG using various spectroscopic techniques and nanodiffraction scanning fluorimetry (nano-DSF). On heating Mb in the presence of EG, the Trp-heme separation increases at higher temperature, as revealed by monitoring thermal denaturation by nano-DSF. Furthermore, to estimate binding parameters of the interaction of Mb with EG, isothermal titration calorimetry (ITC) studies were carried out at 295 K ($25^\circ C$) and 343 K ($70^\circ C$), which showed that EG preferentially interacts strongly with the denatured Mb at high temperature. Molecular docking studies were performed to know the sites on Mb, which are involved in binding with EG.

2. RESULTS

2.1. Absorption Studies. The effect of different concentrations (0, 50, 100, 150, 200, 250, 300, and 350 mg mL$^{-1}$) of EG on the Soret absorption band of Mb is shown in Figure 1A. The inset of this figure shows a plot of $\varepsilon_{409}$ vs [EG] where each point represents the average estimated from triplicate measurements. (B) Near-UV and Soret-absorption spectra of Mb in the presence of different concentrations (0, 300, and 350 mg mL$^{-1}$) of EG at pH 7.0 and 25 °C.
Table 1. Spectral Properties of Myoglobin in the Absence and Presence of EG (300 and 350 mg mL⁻¹) at pH 7.0

| Mb under different solvent conditions | ε₄⁰⁹ (M⁻¹ cm⁻¹) | ε₂₈₀ (M⁻¹ cm⁻¹) | F₃₃₅ | [θ]₂₂₂ (deg cm² dmol⁻¹) | [θ]₂₇₃ (deg cm² dmol⁻¹) | ANS Iₘₕₚ (λₘₜₐₜ nm) |
|---------------------------------------|-----------------|-----------------|------|------------------------|------------------------|------------------------|
| buffer                                | 171,645 (±285)  | 37,559 (±57)    | 2.64 (±0.17) | 182 (±6)               | -26,190 (±218)         | 4.48 (512)             |
| 300 mg mL⁻¹ EG                        | 171,462 (±299)  | 37,425 (±150)   | 2.51 (±0.11) | 181 (±3)               | -27,104 (±150)         | 10.59 (520)            |
| 350 mg mL⁻¹ EG                        | 167,346 (±172)  | 37,641 (±191)   | 2.49 (±0.13) | 183 (±3)               | -27,023 (±118)         | 34.9 (501)             |

A plus–minus sign (±) in each parameter represents the mean error obtained from the triplicate measurements.

2.2. Circular Dichroism (CD) Studies. 2.2.1. Far-UV CD. The CD spectra of Mb (without EG) resemble that of a typical α-helix with negative bands at 208 and 222 nm (see Figure 2A). Figure 2B shows no significant change in the CD spectrum of Mb in the presence of all concentrations of EG. The inset of this figure shows the plot of mean residue ellipticity, [θ]₁₂₂ versus [EG] in milligram per liter (mg mL⁻¹), which depicts no significant change in [θ]₁₂₂ and [θ]₃₃₀ in the structure. The inset of this figure also shows red, green, and black circles, which are data points of triplicate measurements. [θ]₁₂₂ values of the native protein in the presence of EG (300 and 350 mg mL⁻¹) are given in Table 1.

2.2.2. Near-UV CD. To compare observation from the near-UV absorption (Figure 1B) measurements, the near-UV CD was employed to investigate the effect of EG at higher concentrations (300 and 350 mg mL⁻¹) on Mb. Figure 2B shows the near-UV CD of Mb in the absence and presence of EG (300 and 350 mg mL⁻¹). Figure 2B shows that the spectrum of Mb was unaffected in the presence of EG even at higher concentrations. [θ]₁₂₂ values of Mb in the presence of EG (0, 300, and 350 mg mL⁻¹) are given in the Table 1.

2.3. Intrinsic Fluorescence Studies. Figure 3 shows the plot of fluorescence intensity versus wavelength. This figure shows that there is no change in Trp fluorescence on increasing [EG] from 0 to 350 mg mL⁻¹, i.e., the heme and Trp distance in Mb is unperturbed in the presence of EG at all concentrations. The inset of this figure shows a plot of fluorescence intensity at 335 nm (F₃₃₅) versus [EG] where it is seen that fluorescence intensity of Mb remains unchanged with increasing concentration of EG. Red, green, and black circles in the inset of this figure are data points of triplicate measurements. F₃₃₅ values of Mb in the presence of various concentrations of EG (0, 300, and 350 mg mL⁻¹) are given in the Table 1.

2.4. Thermal Denaturation Studies. From the structural studies presented above, it is observed that EG has no effects on the secondary and tertiary structures of Mb (see Figures 1–3). To see the effect of EG on the stability of the protein, heat-induced denaturation of Mb in the presence of various concentrations of EG was carried out using two different properties, Δε₄⁰⁹ (the probe to monitor change in the globin–heme interaction) and [θ]₂₂₂ (the probe to monitor change in the secondary structure).

2.4.1. Thermal Denaturation Studies Using UV–vis Spectroscopy. Thermal denaturation of Mb in the presence of different concentrations of EG was monitored by Δε₄⁰⁹ measurements at pH 7.0 (phosphate buffer). These measurements are shown in Figure 4A. It is seen in this figure that the temperature dependence of y₀ (Δε₄⁰⁹) does not depend on [EG]. On the contrary, the temperature dependence of y₀ depends on the concentration of the crowder, which is more significant at higher concentrations (Figure 4A). Thermal denaturation curve (Δε₄⁰⁹ vs T) of Mb in the presence of each concentration of EG was analyzed according to eq 3 to obtain the values of Tₘ and ΔHₘ that are given in Table 2. It should be noted that heat-induced denaturation of Mb in the absence and presence of EG was >90% reversible.

2.4.2. Thermal Denaturation Studies Using CD Spectroscopy. Thermal denaturation of Mb in the presence of different concentrations (0, 50, 100, 150, 200, 250, 300, and 350) of EG was followed by measuring changes in [θ]₂₇₃ (see Figure 4B) at pH 7.0. It is seen in Figure 4B that the temperature dependencies of y₀ and yₘ are unaffected by the presence of EG. The thermal denaturation curve ([θ]₂₇₃ vs T) of Mb in the presence of a given concentration of EG was analyzed according to eq 3 to obtain the values of Tₘ and ΔHₘ that are given in Table 2. It should be noted that heat-induced denaturation of Mb in the absence and presence of EG was >90% reversible.

2.5. ANS Binding Studies. ANS binding fluorescence has been used as one of the techniques for the characterization of protein folding intermediates (molten and premolten globules). Generally, the native state of a globular protein possesses a tightly packed, solvent inaccessible hydrophobic core that prevents ANS from binding to it. The denatured state,
which is devoid of all elements of the native secondary and tertiary structures, also does not bind ANS due to high polypeptide chain flexibility. Figure 5 shows emission spectra of Mb in the absence of EG at 25 and 70 °C, and values of $\lambda_{\text{max}}$ and intensities at $\lambda_{\text{max}}$ of ANS are given in Table 1. A comparison of the spectrum at 25 °C with that at 70 °C suggests that the emission intensity increases with a blue shift when protein is heated to 70 °C (see also Table 1). Figure 5 also shows the emission spectra of ANS in the presence of protein containing 300 and 350 mg mL$^{-1}$ EG at 25 and 70 °C. These spectra were used to determine values of $\lambda_{\text{max}}$ and intensity at this wavelength under each experimental condition, which are given in Table 1. Furthermore, these spectra show that fluorescence intensity of ANS increases with a blue shift when solution is heated from 25 to 70 °C.

### 2.6. Thermal Unfolding Studies Using Nanodiffraction Scanning Fluorimetry (Nano-DSF).

Figure 6A shows the change in the tryptophan fluorescence ratio ($F_{350}/F_{330}$) of Mb in the presence of different concentrations of EG (0–350 mg mL$^{-1}$) as a function of temperature. Notably, the raw fluorescence data ($F_{350}/F_{330}$) show a reasonable transition from the folded state to the denatured state in the absence and presence of EG (Figure 6A), which could be directly used for $T_m$ analysis from the first derivative of $F_{350}/F_{330}$ with respect to $T$ versus temperature. A peak at the point of maximal slope yields $T_m$ of the protein (see Figure 6B). Values of $T_m$ of Mb under different EG concentrations are given in Table 2.

### 2.7. Binding Studies.

#### 2.7.1. Isothermal Titration Calorimetry Studies.

Figure 7 shows calorimetric titrations for the binding of EG with Mb at pH 7.0 and two different temperatures (298 and 343 K). Figure 7A shows the titration of EG in the cell containing Mb at 298 K (25 °C). In the upper
panel of this figure, each peak in the binding isotherm represents a single injection of EG solution. The integration of the area under each injection peak in the heat profile gives a differential curve shown in the bottom panel of the thermogram where it can be seen that this heat profile does not show any specific binding pattern. However, when the titration was performed at higher temperature (343 K) where the protein hydrophobic patches are exposed to the polar solvent, EG may directly interact with the protein; in the early phase, EG binds to the protein and it quickly saturates the binding sites (see Figure 7B). Table 3 gives the thermodynamic parameters for the binding of EG with Mb at 298 and 343 K.

2.7.2. Molecular Docking Studies of Mb with EG. To know the structural changes or retention of environment of the heme moiety in Mb in the presence of EG, molecular docking studies were carried out. PEG 400 (polymer of EG) had been reported to interact with the heme and polypeptide chain of Mb, which results in heme disruption and the loss of secondary and tertiary structures of the protein.29 However, Figures 1–3 show no significant changes in the heme environment and secondary and tertiary structures of Mb in the presence of EG.

Figure 6. (A) Change in $F_{350}/F_{330}$ of the tryptophan fluorescence decay upon thermal unfolding of Mb in the presence of different concentrations of EG (0–350 mg mL$^{-1}$). (B) First derivative plot ($d(F_{350}/F_{330})/dT$) vs $T$ to determine $T_m$ of Mb in the presence of different concentrations of EG (0–350 mg mL$^{-1}$).

Figure 7. Typical ITC thermograms of Mb (20 μM) with EG (600 μM). (A) The calorimetric response as successive injection of the EG added to the reaction cell (upper panel) and resulting binding isotherm (lower panel) at pH 7.0 and 298 K. (B) The calorimetric response as successive injection of the EG added to the reaction cell (upper panel) and resulting binding isotherm (lower panel) at pH 7.0 and 343 K.
at pH 7.0 and 25 °C. However, the computational analysis showed that the weak interaction exists between EG and Mb with a binding energy of $-2.7 \text{ kcal mol}^{-1}$ under these experimental conditions. Figure 8 shows the surface view of the protein with the pocket-binding site of EG and the hydrogen bond donor–acceptor residues. This figure also shows the 2D-representation of the amino acids showing weak interactions (conventional H-bonding and van der Walls forces) with EG shown in the ball and stick model. EG interacts with Asp109, Glu136, and Arg139 through hydrogen bonding with bond distances of 3.33, 3.12, and 2.97 Å, respectively.

### 3. DISCUSSION

The visible spectrum of met-Mb (black line) has a sharp Soret band at 409 nm, which is characteristics of a six-coordinated high-spin heme with a histidine residue (His-93) and a water molecule bound at the fifth and the sixth coordination positions of the iron atom, respectively. The change in the heme environment leads to disruption of the protein spectrum. EG-treated Mb shows no significant changes in the visible spectrum even at its higher concentrations (Figure 1A). This observation is in agreement with that reported earlier. All proteins display a characteristic ultraviolet (UV) absorption spectrum around 280 nm predominately due to aromatic amino acids tyrosine and tryptophan. This property is exploited from monitoring the change in the environment of aromatic residues (tertiary structure) of proteins. EG even at its higher concentrations (300 and 350 mg mL$^{-1}$) does not perturb the environment of the aromatic residue (Figure 1B), hence the tertiary structure of Mb. This observation is supported by the near-UV CD measurements (Figure 2B). The proximity of the two tryptophan residues (Trp7 and Trp14 on helix A) to the heme moiety in the native Mb results in a partial quenching of the tryptophan fluorescence. Figure 3 shows neither an increase in fluorescence intensity of Mb nor any shift in $\lambda_{\text{max}}$ in the presence of EG, suggesting that the

### Table 3. Binding Parameters of EG with Mb Estimated from ITC Measurements at 298 K (25 °C) and 343 K (70 °C)$^a$

| thermodynamic parameters (units) | $K_a$ (M$^{-1}$) | $\Delta H^0$ (cal mol$^{-1}$) | $\Delta S^0$ (cal mol$^{-1}$ deg$^{-1}$) | $\Delta G^0$ (cal mol$^{-1}$) | $K_d$ (M) |
|----------------------------------|----------------|-----------------------------|---------------------------------|-----------------------------|-----------|
| At 298 K                          |                |                             |                                 |                             |           |
| step 1                            | $177 \times 10^3$ ($\pm 6.3 \times 10^3$) | $-299.1$ ($\pm 6.60$) | 23.0 | $-7.153 \times 10^3$ ($\pm 0.007 \times 10^3$) | $0.056 \times 10^{-4}$ |
| step 2                            | $24.0 \times 10^2$ ($\pm 4.3 \times 10^2$) | $-2274$ ($\pm 405$) | 7.84 | $-4.610 \times 10^3$ ($\pm 0.405 \times 10^3$) | $0.145 \times 10^{-3}$ |
| step 3                            | $23.2 \times 10^2$ ($\pm 4.2 \times 10^2$) | $-2668$ ($\pm 929$) | 6.45 | $-4.590 \times 10^3$ ($\pm 0.929 \times 10^3$) | $0.148 \times 10^{-3}$ |
| At 343 K                          |                |                             |                                 |                             |           |
| step 1                            | $13.4 \times 10^3$ ($\pm 4.2 \times 10^3$) | $-32.20 \times 10^3$ ($\pm 7.21 \times 10^3$) | $-74.9$ | $-6.509 \times 10^3$ ($\pm 7.21 \times 10^3$) | $0.7 \times 10^{-4}$ |
| step 2                            | $6.9 \times 10^3$ ($\pm 2.3 \times 10^3$) | $31.00 \times 10^3$ ($\pm 7.29 \times 10^3$) | 117 | $-9.131 \times 10^3$ ($\pm 7.29 \times 10^3$) | $0.145 \times 10^{-3}$ |
| step 3                            | $6.73 \times 10^3$ ($\pm 1.6 \times 10^3$) | $-19.06 \times 10^3$ ($\pm 1.38 \times 10^3$) | $-38.0$ | $-6.026 \times 10^3$ ($\pm 1.38 \times 10^3$) | $0.148 \times 10^{-5}$ |

$^a$A plus–minus sign ($\pm$) has the same meaning as in Table 1.
environment of Trp is not perturbed on the addition of EG. It is interesting to recall that Mb in the presence of polymers of EG (PEG 400 Da and 10 kDa) loses its tertiary structure as revealed by fluorescence measurements. 2,29

Far-UV CD is a sensitive technique to monitor the change in the secondary of proteins. 40-42 The far-UV CD spectra of Mb in the presence of different concentrations of EG show that there was no significant change in the secondary structure (Figure 2A). The low molecular weight PEG (PEG 400 Da) was found to disrupt both the secondary and tertiary structures of Mb, and it induces the premolten globule state. 29 Another study on Mb in the presence of PEG (PEG 10 kDa) showed that this polymer disrupts the Mb tertiary structure without any significant change in the secondary structure still, suggesting that PEG 10 kDa yields a molten globule structure. 5

Our observation that EG has no effects on its secondary and tertiary structures of Mb (see Figures 1–3) is in agreement with that on other proteins, which shows no or insignificant change in their structures. 25,43 On the contrary, the effect of PEGs on Mb and other proteins in the presence of polymers of EG of various sizes reported earlier is protein specific. Particularly, PEGs (i) affect both secondary and tertiary structures, 1,2,29,44 (ii) affect tertiary structure without any significant change in the secondary structure, 2,5,30 and (iii) do not affect both secondary and tertiary structures. 44,45

The above discussion shows that EG has no effect on the secondary and tertiary structures of Mb at pH 7.0 and 25 °C. A question arises: Does EG affect the thermodynamic stability of the protein? To answer this question, we have measured thermal denaturation of Mb in the absence and presence of different concentrations of EG (see Figures 4 and 6). Monitoring the denaturation by [θ]222 shows that the temperature dependences of the native and denatured protein molecules are independent on [EG] (Figure 4B). This observation suggests that the structural properties of the native and denatured molecules are not perturbed by EG. On the contrary, although temperature dependency of heme and Trp environment in the native Mb is not perturbed on the addition of EG, temperature dependence of this property of the denatured molecule is perturbed in the presence of the crowder (see Figures 4A and 6A). To know whether the denatured protein has exposed hydrophobic patches, we measured ANS binding with the heat-denatured protein in the presence of two highest concentrations of EG (300 and 350 mg mL⁻¹) at 70 °C (Figure 5). It has been observed that the heat-induced-denatured Mb has exposed hydrophobic patches in the presence of EG, which binds to ANS (Figure 5), for there is an increase in emission intensity with a blue shift. 52

Analyses of the heat-induced denaturation curves [ε]409 and [θ]1222 (Figure 4) for thermodynamic parameters (Tm and ΔHm) according to eq 3 gave values of Tm and ΔHm of the protein (Table 2). It is seen in Table 2 that Mb is destabilized in terms of Tm by EG. This observation was checked by nano-DSF measurements (Figure 6), which also show that Tm decreases with increasing concentration of EG (Table 2).

Values of Tm obtained from the analysis of denaturation curves of ε, 409 [θ]1222, and F330/F380 of Mb in the absence of EG are, within experimental errors, identical. This value is in excellent agreement with that obtained from DSC measurements. 46 ΔHm values of Mb in the absence of EG obtained from optical methods (Table 2) are also in agreement with spectroscopic techniques reported earlier. 47,48 These agreements of thermodynamic properties obtained from optical and thermodynamic methods support that the thermal denaturation of Mb is a two-state process. On the contrary, values of Tm and ΔHm of the protein in the presence of a given concentration of EG are not identical. For example, values of Tm and ΔHm of the protein estimated using probe ε, 409 decrease from 81.5 °C (0 mg mL⁻¹ EG) to 62.5 °C (350 mg mL⁻¹ EG) and ΔHm from 120 kcal mol⁻¹ (0 mg mL⁻¹ EG) to 101 kcal mol⁻¹, whereas Tm estimated using probe [θ]222 decreases from 81.6 °C (0 mg mL⁻¹ EG) to 63.7 °C (350 mg mL⁻¹ EG) and ΔHm from 120 kcal mol⁻¹ (0 mg mL⁻¹ EG) to 101 kcal mol⁻¹. This observed noncoincidence suggests that thermal denaturation of Mb in the presence of EG is not a two-state process. Another source of the observed discrepancy could be due to the variation of the characteristics of the denatured state of Mb with a change in [EG] (see Figure 4A).

It is known that preferential binding of an additive with the protein leads to protein destabilization. 49 To confirm the extent of binding and the type of interaction of EG with Mb at low and high temperatures, calorimetric studies using ITC were done. Furthermore, to know the binding pocket on the protein for the ligand, computational studies (molecular docking) were also carried out.

In the upper panel of thermograms (Figure 7), each peak in the binding isotherm represents a single injection of EG solution. The integration of the area under each injection peak in the heat profile gives a differential curve shown in the bottom panel of this figure. The heat profile does not show any specific binding pattern between Mb and EG at 298 K (25 °C), which could be the most probable reason for having no effect of EG on structural properties of Mb at this temperature. However, when the titration was performed at 343 K (70 °C), binding occurred, for at this temperature protein’s hydrophobic patches are exposed to the polar environment (see Figure 5), which would facilitate EG’s early binding to exposed non-polar surfaces of Mb and fast saturation.

The binding affinity, defined in terms of the dissociation constant (Kd) is an experimental measure that determines whether an interaction of the ligand and protein is feasible. The binding affinity is used to measure the bimolecular interactions and rank the order of its strength. 50 Table 3 compares values of thermodynamic binding parameters at two temperatures. It is seen in this table that Kd is smaller at 343 K than at 298 K, suggesting that EG binds more strongly at the higher temperature. 50,51 Besides Kd, the enthalpy change (ΔH°) measures a change in the strength of the interaction between molecules, while the entropy change (−TΔS°) measures a change in the order of the system. It is more difficult, however, to interpret free energy changes, for it depends on both enthalpy and entropy changes. 52,53 The free energy values given in Table 3 show that the interaction is feasible (spontaneous) both at 298 and 343 K; however, the total free energy change (ΔG°) of three-step binding is more negative at 343 K than at 298 K, which shows spontaneity of the bimolecular interaction. The phenomenon of entropy–enthalpy compensation is applicable in our case, for binding will decrease enthalpy whereas a stronger interaction between molecules will also result in a reduction of the configurational freedom of the system and thus a reduction of the entropy. Correspondingly, weaker molecular interactions will produce a looser molecular association and an increase of the entropy. 52 Table 3 shows that the binding between EG and Mb is weak at 298 K and is entropy driven. Most probably, this weak association results in no change in the structure (Figures 1–3).
On the other hand, the bimolecular interaction is relatively stronger at 343 K, and it is overall both entropy and enthalpy driven. This stronger binding of EG with Mb is most probably due to the newly exposed hydrophobic patches on the surface of the protein. Hence, thermal destabilization of Mb in the presence of EG is due to the interaction of the ligand with protein’s exposed hydrophobic patches. Therefore, destabilization of the protein in the presence of EG shown by ITC experiments is due to binding. As it is known fact that hard-core repulsion could result in compaction of unfolded proteins and potentially macromolecular crowders could behave like an entropic crowder. However, the unfolded state is more open, and hence, the residues are more accessible to crowder molecules; results in binding by the soft part of interactions (soft interactions or chemical interactions) will lead the protein in the unfolded state. Therefore, crowder molecules are expected to exhibit different interactive natures at different temperatures leading stabilization or destabilization of the protein.

Molecular docking studies showed that the interaction of EG with Mb is a weak interaction with a low binding energy of −2.7 kcal mol$^{-1}$. Figure 8 shows the surface view of the protein with the pocket-binding site of EG and the hydrogen bond donor-acceptor residues. This figure shows 2D representation of the amino acids showing weak interactions (conventional H-bonding and van der Walls forces) with the ligand (shown in ball and stick model). EG interacts with Asp109, Glu136, and Arg139 through hydrogen bonding with bond distances of 3.33, 3.12, and 2.97 Å, respectively. Also, protein residues involved in van der Waals interactions are Thr132, Ser108, Ile112, and Leu135. The molecular docking studies reported earlier showed that PEG 400 (which is the polymer of EG) interacts with the heme and polypeptide chain of Mb resulting in heme disruption and the loss of secondary and tertiary structures of the protein. However, in the case of its monomer (EG), heme is retained in the presence of EG at all its concentrations (Figure 1A), and there is no significant change in the secondary and tertiary structures of Mb (Figure 2). This study shows that, although EG shows a preferential interaction with Mb (Figures 7A and 8), it has no significant effect in the structure of Mb at 25 °C. Thus, docking study supports the observations from in vitro studies.

4. CONCLUSIONS

The EG has no significant effect on secondary and tertiary structures of Mb at 25 °C. EG decreases the thermal stability ($T_m$) of Mb, which increases with increasing concentration of EG. Heat-induced denatured protein has newly exposed hydrophobic patches that bind to ANS. The interaction between EG and Mb is temperature dependent; the preferential interaction of EG is entropy driven at low temperature, and it is enthalpy-entropy driven at higher temperature. Molecular docking study showed that EG interacts with side chains of amino acid residues of Mb through van der Waals interactions and hydrogen bonding.

5. MATERIALS AND METHODS

5.1. Materials. Commercial lyophilized horse heart myoglobin and ethylene glycol (EG) were purchased from Sigma chemical company (USA). 8-Anilino-1-naphthalene sulfonic acid (ANS), potassium chloride (KCl), sodium hydroxide (NaOH) pellets, and hydrochloric acid (HCl) were bought from Merck (India). Ultrapure guanidinium chloride (GdmCl) was obtained from MP Biomedicals, LLC (France). Disodium hydrogen phosphate anhydrous and sodium phosphate monobasic anhydrous were procured from Himedia (Germany). Dialysis tubing with a 3–8 kDa molecular mass cut off was purchased from Spectrum Medical Industries Inc. (USA). Whatman filter paper was purchased from Whatman Laboratories, England. Filters with a 0.22 μm pore size were obtained from Merck Millipore Corporation Ltd. (Ireland).

5.2. Methods. 5.2.1. Preparation of Solutions of Protein and Reagents. The lyopholized powdered form of Mb of the required amount was dissolved in 50 mM phosphate buffer. The solution was then oxidized by potassium ferricyanide ($K_2Fe(CN)_6$) as reported earlier. To remove excess of potassium ferricyanide in the solution, the protein solution was dialyzed against several changes of 50 mM phosphate buffer solution at pH 7.0 and 4 °C, and the protein solution was filtered through 0.22 μm Millipore filter. Protein solutions were stored at 4 °C for further use. All spectral measurements were taken in triplicates. To determine concentrations of Mb and ANS, values of 171,000$^{fl}$ and 5000$^{fl}$ for molar absorption coefficient (M$^{-1}$ cm$^{-1}$) were used, respectively.

Concentrated solutions of the crowder (EG) and the denaturant (GdmCl) were prepared in phosphate buffer. The pH of the solution was then adjusted to 7.0 using sodium dibasic and monobasic phosphate salts, if needed. These solutions were then filtered through Whatman filter paper no. 1. The concentrations of GdmCl$^{fl}$ and EG$^{fl}$ were estimated by refractive index measurements.

For experiment measurements, each protein solution containing the additive (GdmCl and/or EG) was thoroughly mixed and incubated overnight at room temperature, which was a sufficient time to attain equilibrium. Also, all spectral measurements were taken in triplicates.

5.2.2. Spectral Measurements. 5.2.2.1. Absorption Spectroscopy. Spectral measurements were made in a Jasco V-660 UV–vis spectrophotometer; the temperature of which was controlled by a programmable Peltier type temperature controller (ETCS761). Protein concentrations of 3–4 and 20–25 μM were used for the Soret-absorbance (a wavelength region of 440–340 nm) and near-UV absorbance (a wavelength region of 700–240 nm), respectively, and cuvettes with a path length of 1.0 cm were used. The raw data were converted into molar absorption coefficient using the relation

$$A = εl$$

where $A$ is the absorbance, $ε$ is the molar concentration, $l$ is the path length of the cuvette in centimeter (cm), and $ε$ is the molar absorption coefficient (M$^{-1}$ cm$^{-1}$).

5.2.2.2. Fluorescence Measurements. Fluorescence spectra were recorded in a Jasco FP-6200 Model no. STR-312 spectrofluorimeter at 25 ± 0.1 °C, with both emission and excitation slits fixed at 10 nm. A quartz cell with a 1.0 cm path length was used. The cell temperature was controlled with the help of an external thermostated water bath. Seven micromolar μM protein was used in all the fluorescence experiments. The wavelength of excitation was 280 nm for tryptophan (Trp) fluorescence measurements, and emission spectra were recorded in the wavelength region of 380–400 nm. For ANS fluorescence experiments, emission spectra were recorded in the range of 400–600 nm at 25 and 70 °C, after exciting the solution at 360 nm.
5.2.2.3. Circular Dichroism Measurements. Circular dichroism (CD) measurements were done in a Jasco spectropolarimeter (J-1500 model) attached with a circulating bath (MCB-100) at 20 °C. Far- and near-UV CD spectra were obtained using protein concentrations of 5–7 and 25–29 μM in 0.1 and 1.0 cm path length cuvettes, respectively. The calibration of the machine was consistently done with D-10 camphor sulfonic acid. Each spectrum was corrected for the contribution of the blank. Five scans of each solution were taken to get a better signal-to-noise ratio in all cases together with the base line. N2 at the rate of 5 L min⁻¹ was flushed continuously to minimize the noise level. CD data were transformed to concentration-independent parameter [θ]l, (deg cm² dmol⁻¹), the mean residue ellipticity (MRE), using the relation

\[ [\theta]_l = M_c \theta_c / 10c \]  (2)

where \( \theta_c \) is ellipticity in millidegrees at wavelength \( \lambda \), \( M_c \) is the mean residue weight of the protein, \( c \) is the concentration of the protein in gram per cubic centimeter (g cm⁻³), and \( l \) is the cell path length in centimeter.

Reversibility of Mb in the absence and presence of the crowding molecule at the highest concentration was checked using both UV–vis absorption (Soret) and far-UV CD spectroscopy.

5.2.3. Thermal Denaturation Measurements. 5.2.3.1. UV–vis Spectrophotometer and Circular Dichroism Measurements. Thermal denaturation experiments of Mb were performed in both a Jasco V-660 UV/vis spectrophotometer outfitted with a Peltier-type temperature controller (ETCS-761) and a Jasco spectropolarimeter (J-1500 model) attached with a circulating bath (MCB-100). The change in the absorbance and MRE of the protein with increasing temperature was followed at 409 and 222 nm, respectively. Experiments were performed in the presence of various concentrations of EG (0, 50, 100, 150, 200, 250, 300, and 350 mg mL⁻¹) at pH 7.0. The protein solution was heated from 20 to 100 °C with a heating rate of 2 °C min⁻¹. All the measurements were carried out in triplicate. After denaturation, each protein sample was immediately cooled down to measure the reversibility of the reaction. All solution blanks were subtracted before analysis of the data. The raw absorbance data was converted into change in molar absorption coefficient (Δεl, M⁻¹ cm⁻¹) at a given wavelength, \( \lambda \). Similarly, CD signals (mdeg) were converted to mean residue ellipticity ([θ]l, deg cm² dmol⁻¹), at a given wavelength, \( \lambda \). Each heat-induced transition curve was analyzed for \( T_m \) (midpoint of denaturation) and ΔH_m (enthalpy change at \( T_m \)) using a nonlinear least-squares analysis according to the relation

\[ y(T) = \frac{y_N(T) + y_D(T)\exp\left(-\Delta H_m/R\left(\frac{1}{T-1/T_m}\right)\right)}{1 + \exp\left(-\Delta H_m/R\left(\frac{1}{T-1/T_m}\right)\right)} \]  (3)

where \( y(T) \) is the optical property at temperature \( T \) (K), \( y_N(T) \) and \( y_D(T) \) are the optical properties of the native and denatured molecules of the protein at temperature \( T \) (K), respectively, and \( R \) is the gas constant. In the analysis of a denaturation curve, it was assumed that a parabolic function describes the dependence of the optical properties of the native and denatured protein molecules (i.e., \( y_N(T) = a_N + b_N T + c_N T^2 \) and \( y_D(T) = a_D + b_D T + c_D T^2 \) where \( a_N, b_N, c_N, a_D, b_D, \) and \( c_D \) are temperature-independent coefficients). The \( T_m \) values obtained were converted from Kelvin (K) to degree centigrade (°C).

5.2.3.2. Nano-Diffraction Scanning Fluorimetry (Nano-DSF) Measurements. For thermal unfolding experiments, the protein solution was diluted to a final concentration of 10 μM. For each condition, 10 μL of sample per capillary was prepared. The samples were loaded into UV capillaries (NanoTemper Technologies), and experiments were carried out using Prometheus NT.48. The temperature gradient was set to an increase of 1 °C min⁻¹ in a range from 20 to 100 °C. Protein unfolding was measured by detecting the temperature-dependent change in tryptophan fluorescence at emission wavelengths of 330 and 350 nm in the presence of different concentrations of EG. For analysis, melting temperatures were determined by detecting the maximum of the first derivative of the observed fluorescence ratios (F₃₃₀/F₃₅₀). For this, the 8th order polynomial fit was performed for the transition region. Next, the first derivative of the fit was formed and the temperature at the peak, which is that \( T_m \) was determined.

5.2.4. Binding Measurements. 5.2.4.1. Isothermal Titration Calorimetry Measurements. A VP ITC calorimeter (MicroCal, Northampton, MA) was employed for isothermal titration calorimetry measurements. The concentration of EG titrated into the calorimeter cell containing Mb was in the ratio of 30:1 (EG:Mb). In the syringe, the EG solution was filled, and in every 260 s, and aliquots of 10 μL were injected. Data were normalized and analyzed by software of MicroCal Origin ITC. All experiments were conducted in 50 mM phosphate buffer (pH 7.0) at two different temperatures, 25 °C (298 K) and 70 °C (343 K). Origin 8.0 was used to fit the raw data using the three-step sequential binding model, which in turn gives the parameters such as change in enthalpy (ΔH°), change in entropy (ΔS°), and the association constant (K°). From these key parameters, change in Gibbs free energy (ΔG°) can be calculated from equation

\[ \Delta G^\circ = -RT \ln K_a = \Delta H^\circ - T \Delta S \]  (4)

where \( R \) is the gas constant, and \( T \) is the absolute temperature in Kelvin (K).

5.2.4.2. Computational Studies (In Silico). To dock the small molecule (EG) to a macromolecule (Mb) to find compounds with a desired biological function for virtual molecular screening, PyRx software was used. PyRx software is written in the Python programming language with an intuitive user interface that run on all major operating systems (Linux, Windows, and Mac OS). It is a combination of several softwares such as AutoDockVina, AutoDock 4.2, Mayavi, Open Babel, etc. PyRx uses Vina and AutoDock 4.2 as docking softwares. The input files ligand, EG, and macromolecule, Mb (PDB id: 1ymb) in the .pdb format were changes to .pdbqt files using AutoDock of software. After preparing the files, they were subjected to docking by means of AutoDock 4.2 and Vina. Grid box dimensions were set to be X, Y, and Z conformations equal to 49, 42, and 43, respectively. The grid size was assigned perfectly, which allows selecting search space for the receptor to perform docking with the ligand, normally, at the binding site. The interaction between Mb and the respective EG was interpreted using the Lamarckian genetic algorithm (LGA). Once the Vina calculations were done, results of binding affinity (kcalmol⁻¹) of various conformations of the macromolecule with the ligand were...
provided by the software in the table. Finally, the best docked complexes of protein–ligand chosen were further modified and analyzed using visualize PyMOL.64

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Notes
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