Polyols, increasing global stability of cytochrome c, destabilize the thermal unfolding intermediate

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ABSTRACT
Studies on the intermediate states of proteins provide essential information on folding pathway and energy landscape of proteins. Osmolytes, known to alter the stability of proteins, might also affect the structure and energy states of folding intermediates. This was examined using cytochrome c (Cyt) as a model protein which forms a spectroscopically detectable intermediate during thermal denaturation transition. Most of the secondary structure and the native heme-ligation were intact in the intermediate state of the protein. Denaturants, urea and guanidinium hydrochloride, and ionic salt destabilizes the intermediate and drive the protein to follow two-state transition. The effect of polyol class of osmolytes, glycol, glycerol, erythritol, xylitol and sorbitol (with OH-groups two to six), on the intermediate was studied using Soret absorbance and far-UV circular dichroism. With the increasing concentration of any of the polyols, the transition-midpoint temperature ($T_m$) and the enthalpy change ($\Delta H$) for native to intermediate transition were decreased. This indicated that the intermediate was destabilized by the polyols. However, the polyols increased the overall stability of the protein by increasing $T_m$ and $\Delta H$ for intermediate to unfolded transition, except for glycol which destabilized the protein. These results show that the polyols could alter the energy state of the intermediate, and the effect of lower and higher polyols might be different on the stability and folding pathway of the protein.

KEYWORDS
Thermal unfolding intermediate; cytochrome c; ethylene glycol; polyols; protein stability

1. Introduction
Small globular proteins tend to exhibit an ‘all or none’ two-state transition between their native to unfolded states upon chemical, thermal and mechanical perturbations (Ferreon & Bolen, 2004; Ramprakash et al., 2008; Schönfelder et al., 2016; Zhou et al., 1999). The two-state behaviour could be attributed to weak non-covalent interactions stabilizing the native conformation against unfolded conformations (Freire & Murphy, 1991). These transitions could be modulated by change in the solvent conditions such as pH or by the addition of salts or small organic molecules (Anumalla & Prabhu, 2018; Devaraneni et al., 2012; Hamada et al., 1994; Khurana et al., 1995; Kumar et al., 2006). At some instances, intermediate states could not be recognized due to the limitations of the probe to distinguish the intermediate states from the native or denatured conformations. However, using multiple spectroscopic probes, intermediates have been identified and characterized for small globular proteins that were earlier known to exhibit a two-state behaviour (Bhuyan & Udgaonkar, 1999; Mayne & Englander, 2000; Radford et al., 1992). Identification of such intermediates is essential to understand the folding pathway and the energetics involved. One of the well-characterized intermediate state in many small globular proteins is a molten globule (MG) state (Ptitsyn et al., 1990). MG state is an expanded state as compared to the native state and it retains most of the native secondary structures, but lacks a tight packaging of the side-chains (Chamberlain & Marqusee, 2000; Ptitsyn et al., 1990). MG intermediate is identified under equilibrium conditions and it is also found transiently during the kinetics of refolding reactions (Fujiwara et al., 1999; Judy & Kishore, 2019).

Apart from the MG intermediates, there are other intermediate structures such as dry MGs (DMGs). DMG is an expanded form of the native state in which the hydrophobic core is not exposed to the solvent, but lacks the close packing found in the native structure (Khorasanizadeh et al., 1996). For instance, ribonuclease A, dihydrofolate reductase, monellin and villin headpiece show DMG during their unfolding transitions (Hoeltzli & Frieden, 1995; Jha & Udgaonkar, 2009; Kiefhaber et al., 1995; Reiner et al., 2010). Moreover, a close structural proximity has been established between the burst-phase kinetic intermediates and the equilibrium intermediates. For example, hydrogen exchange pulse labelling...
experiments on apomyoglobin shows that the kinetic intermediate formed within 5 ms has a structure similar to that of equilibrium MG (Jennings & Wright, 1993). Such reports are available for other proteins like α-lactalbumin, ribonuclease H and β-lactoglobulin as well (Fujiwara et al., 1999; Ikeguchi et al., 1986; Raschke & Marqusee, 1997). Further, the kinetic intermediates may be an on-pathway (U to I transition leads the formation of N, U → I ↔ N) or an off-pathway (formation of I requires to attain again U state for proper refolding, I ↔ U ↔ N) (Baldwin, 1996; Dasgupta & Udgaonkar, 2012; Ivarsson et al., 2007; Khorasanizadeh et al., 1996). On-pathway species are known to increases the rate of folding while the off-pathway intermediates slower down the folding rate (Baldwin, 1996).

Small organic molecules accumulated by living organisms to counter various environmental stresses are known as osmolytes (Yancey, 2005). Osmolytes are also widely used in biotechnology industries to suppress protein aggregation, improve the refolding yield and to protect proteins during storage (Franks & Hatley, 1991; Hasan et al., 2019; Ruddon & Bedows, 1997; Rudolph & Lilie, 1996; Samuel et al., 2000; Wang, 2000). Polyols are a group of organic molecules and many of them serve as osmolytes in various living organisms (Burg & Ferraris, 2008). They impart stability to proteins and stabilize certain intermediate structures as well (Mishra et al., 2007). For example, during the folding of L-amino acid oxidase, glycerol stabilizes the intermediate that allows the enzyme to fold correctly to the active conformation (Raibekas & Massey, 1996). In case of cytochrome c (Cyt) and yeast hexokinase A, the acid unfolded structures (A-states) could be folded into MG states by the addition of polyols (Devaraneni et al., 2012; Kamiyama et al., 1999).

Cytochrome c (Figure 1) is a small heme-protein (~12.4 kDa) which serves as a model to understand various aspects of protein folding (Hu et al., 2016; Jones et al., 1993; Sosnick et al., 1994). Cyt contains a central heme, covalently linked by thioether linkages to Cys14 and Cys17. It is a sensitive spectroscopic probe to follow unfolding transitions of the protein. The iron of the porphyrin is coordinated with His18 and Met80 of the protein main chain (Bushnell et al., 1990). The stability and folding kinetics of Cyt varies depending on the oxidation state of the central iron (Trewhella et al., 1988). The reduced ferro-state (Fe²⁺) is relatively stable to pH variations (Bhuyan & Udgaonkar, 2001) whereas the oxidized ferri-state (Fe³⁺) exhibits a large variation in the conformation depending on the solution pH (Soffer & Schweitzer-Stenner, 2014). The oxidized Cyt forms an unfolded state at acidic pH conditions (Uacid) which under low ionic strength is converted into a MG conformation (A-state). Similarly, at alkaline pH, Cyt forms MG-like states (B-state) which varies in conformation with pH and ionic strength of the solution (Bhuyan, 2010; Hagarman et al., 2008; Kumar et al., 2006). Addition of ionic salts and polyols induces formation of intermediates similar to A-state or B-state (Bongiovanni et al., 2002; Haldar & Chattopadhyay, 2012; Kim et al., 2003) and a quasi-native state is also identified in the presence of glycerol (Joshii & Bhuyan, 2020). Denaturants, guanidinium hydrochloride and urea also induces three-state conformational transitions with a detectable unfolding intermediate at pH 5 which resembles the alkaline-denatured state of Cyt (Latypov et al., 2006). Urea and arginine are also found to induce early folding intermediates in yeast Cyt (Haldar et al., 2010). Spectroscopically detectable intermediates have been identified for Cyt during temperature-induced denaturation as well. Though the thermal-intermediate state observed at neutral pH is similar to the alkaline-denatured state, at lower pHs, it is different in terms of heme ligation and secondary structure content (Hagarman et al., 2008; Varhac et al., 2009).

In spite of the fact that polyol class of osmolytes is well-known for altering the stability of globular proteins (Devaraneni et al., 2012; Haque et al., 2006; Joshi & Bhuyan, 2020; Naidu et al., 2020; Tiwari & Bhat, 2006), their effect on folding intermediates has not been fully understood. In this study, we examined the effect of polyols, with OH-groups ranging from two to six, on the equilibrium intermediate identified during thermal denaturation of Cyt at pH 5 using absorption and circular dichroism spectroscopic methods. The effect of denaturants and an ionic salt on the stability of the intermediate was also studied to characterize the intermediate state. The results suggest that the intermediate state is destabilized by all the polyols. The overall stability of the protein is increased by the higher polyols (with OH-groups ≥ 3) whereas the diol decreases the stability.

2. Materials and methods

2.1. Materials

Horse heart Cyt c and polyols were purchased from Sigma-Aldrich, St. Louis, MO and acetic acid was from Merck, India. Sodium acetate, guanidine hydrochloride (Gdm), urea and sodium chloride (NaCl) were purchased from SRL, Mumbai, India.
2.2. Spectroscopic measurements

All the experiments were carried out in 10 mM of acetate buffer at pH 5. The protein concentration was calculated from the absorbance value at 410 nm using the molar extinction coefficient value of 101.6 mM$^{-1}$ cm$^{-1}$. Absorbance spectra were recorded with the protein concentration of 10 µM in Cary 100 UV-Vis spectrophotometer equipped with a Peltier for temperature control. All the thermal denaturation transitions in presence of Gdm, urea, NaCl and polyols were measured at 395 nm with a scan rate of 1 °C/min. Ellipticity changes at far- and near-UV regions were measured in Jasco J-1500 spectropolarimeter equipped with a Peltier and supported by a circulating water bath. Far-UV CD measurements were performed using 2 mm path length cuvette with 15 µM of the protein while near UV-CD measurements were performed using 10 mm path length cuvette with 80 µM of the protein. The experiments were performed at least twice to confirm the reproducibility.

2.3. Data analysis

Thermal denaturation curves following a three-state transition (N→I→U) were analysed using the following equation (Anumalla & Prabhu, 2018; Beermann et al., 2007)

$$Y(T) = \frac{y_f + m_f T}{1 + e^{-\frac{H_{m2} - H_{m1}}{RT}}} + \left(\frac{y_i + m_i T}{1 + e^{-\frac{H_{m1} - H_{u}}{RT}}} \right)$$

where

$$\Delta G_1 = \Delta H_{m1} \left\{ 1 - \left(\frac{T}{T_{m1}}\right) - \Delta C_p (T - T_{m1} - \ln \left(\frac{T}{T_{m1}}\right)) \right\}$$

$$\Delta G_2 = \Delta H_{m2} \left\{ 1 - \left(\frac{T}{T_{m2}}\right) - \Delta C_p (T - T_{m2} - \ln \left(\frac{T}{T_{m2}}\right)) \right\}$$

In Equation (1), $Y$ is the normalized spectroscopic signal, $y_f$, $y_i$, and $y_u$ are the baselines corresponding to the native, intermediate and the unfolding transitions and $m_f$, $m_i$, and $m_u$ are the corresponding slopes. $T_{m1}$ and $T_{m2}$ are the transition midpoints, $\Delta H_{m1}$ and $\Delta H_{m2}$ are the enthalpy changes, and $\Delta C_p$ are the change in the heat capacities associated with N→I and I→U transitions, respectively.

The thermal unfolding transitions that follow a two-state transitions (N→U) were analysed using the following equation (Agashe & Udgaonkar, 1995),

$$Y(T) = \frac{y_f + m_f T}{1 + e^{-\frac{H_{m1} - H_{m2} + \Delta C_p (T - T_{m1} - \ln \left(\frac{T}{T_{m1}}\right))}{RT}}} + \left(\frac{y_i + m_i T}{1 + e^{-\frac{H_{m1} - H_{u} + \Delta C_p (T - T_{m1} - \ln \left(\frac{T}{T_{m1}}\right))}{RT}}} \right)$$

where $Y$ is the normalized spectroscopic signal, $y_f$ and $y_i$ are the intercepts of the native and unfolding transition, while $m_f$ and $m_i$ are the corresponding slopes. $T_{m}$, $\Delta H_{m}$ and $\Delta C_p$ are the transition midpoint temperature, change in enthalpy and change in heat capacity of the unfolding transition, respectively.

2.4. Global analysis

Global analysis was performed to obtain a single set of thermodynamic parameters for the three-state transition of native Cyt. The independent parameters employed were the baselines and the slopes ($y_f$, $y_i$, $y_u$ and $m_f$, $m_i$, $m_u$), while the common parameters were the thermodynamic parameters, $T_{m}$, $\Delta H_{m}$ and $\Delta C_p$.

3. Results

3.1. Thermal unfolding of cytochrome c

Conformational change in Cyt with increasing temperature was initially monitored by two different probes, Soret absorbance (350–450 nm) which is sensitive to the heme-environment (Latypov et al., 2006) and far-UV CD corresponding to the secondary structural content of the protein (Rodger, 2013). With an increase in temperature, Soret absorbance showed a hypsochromic shift along with an increase in absorbance (Figure 2(A)) and the ellipticity values drastically decreased in far-UV region of CD (Figure 2(B)) suggested the unfolding of Cyt. The spectral changes, however, did not show any noticeable isosbestic or isochoric points. In the Soret region, a difference spectrum was calculated by subtracting the absorbance spectra at 293 K against 363 K which showed a minimum at 395 nm that was used to follow the unfolding transition of the protein. From far-UV CD, the ellipticity changes at 222 nm specific to the α-helix was used to monitor the thermal denaturation of the protein. The shape of the unfolding transition curves (Figure 2(C)) showed a non-sigmoidal behaviour suggesting a deviation from the two-state (N→U) process. Therefore, the data were fitted for a three-state model (N→I→U) using Equation (1). Comparison of the residuals obtained from two-state and three-state models (Figure S1) indicated that curve fit with the three-state assumption had less deviation from the experimental values, thus three-state model was considered. The thermodynamic parameters obtained from the data analysis are presented in Table 1 which is comparable to the earlier report (Varhač et al., 2015).

Further, to obtain a single set of thermodynamic parameters by combining the probes sensitive to different conformational changes, simultaneous fit (global analysis) of the data sets were performed (Figure 2(D)). From the resultant parameters, the fractions of each states, native, intermediate and unfolded were calculated (Figure 2(E)). The intermediate population was found to be largest at 339 K. The far-UV CD spectrum of Cyt collected at 338 K (65 °C) showed that the ellipticity of intermediate state is slightly reduced at 222 nm compared to the native state (Figure 2(F)). Further, the absorption and near-UV CD spectra of Cyt were also measured at 338 K (Figure S2). Soret absorbance showed an increase in intensity without any significant hypsochromic shift generally observed for the unfolded states. The Q-band and charge transfer band collected at 338 K were similar to that of native state of the protein. The near-UV CD spectrum of Cyt also did not show significant changes between native and intermediates species. These spectral changes suggested
that the intermediate conformation retained the heme-ligation state as in the native conformation and had a slight loss of secondary structural content without significant change in its tertiary packing.

### 3.2. Effect of denaturants and ionic salt on the intermediate

To investigate the interactions that might stabilize the equilibrium intermediate, thermal denaturation studies were carried out in presence of urea (non-ionic denaturant), NaCl (ionic salt) and Gdm (ionic denaturant). The concentrations of the denaturants were chosen such that they were within the pre-transition baseline of the respective chemical denaturation curves (Latypov et al., 2006). In the presence of low concentrations of urea (≤ 2 M urea), the intermediate was destabilized and at 3 M of urea the transition was found to be two-state (Figure 3(A1–A2)). Similarly, in presence of 0.5 M NaCl, the intermediate was destabilized and upon increasing the concentration to 1 M, the transition was shifted to two-state (Figure 3(B1–B2)). When Gdm was added, the equilibrium-intermediate was completely destabilized and a two-state transition was observed even with a low concentration of Gdm, 0.6 M (Figure 3(C1–C2)).

Thermodynamic parameters obtained from the respective data analysis are presented in Table 2 and plotted in Figure 4. It was observed that Gdm destabilizes the intermediate more effectively and the effect of urea and NaCl are similar. Further analysis on the fraction of intermediate against temperature (Figure 5) shows that the fraction of intermediate was reduced and the temperature at maximum fraction of intermediate ($T_{max-inter}$) was shifted to lower temperature upon addition of the denaturants or NaCl. The far-UV CD spectra of Cyt at $T_{max-inter}$ were measured to analyse the structure of the intermediate. In the presence of both the denaturants and NaCl (Figure S3), the intermediate showed only a slight structural loss similar to that observed in the absence of any cosolvent.

### 3.3. Effect of polyols on the intermediate

The effect of polyols with varying number of -OH groups, ethylene glycol (EG, two -OH groups), glycerol (Glc, three -OH groups), erythritol (Ery, four -OH groups), xylitol (Xyl, five...
-OH groups) and sorbitol (Sor, six -OH groups), on the stability of the intermediate formed by Cyt was examined. Thermal denaturation studies of Cyt in presence of varying concentrations of these polyols were performed by following the change in Soret absorbance at 395 nm (Figure 6(A1–E1)) and far-UV ellipticity at 222 nm (Figure 6(A2–E2)). The addition of EG shifted the thermal denaturation curves of Cyt towards lower temperature for both Soret absorbance (Figure 6(A1)) and far UV-CD (Figure 6(A2)) suggesting destabilization of the protein upon addition of EG. Also, the early-transition region (temperature ranging from 295 to 340 K) showed significant changes in the presence of EG. The addition of Glc did not show significant shift in the transition curve, though there were slight changes in the early-transition phase (Figure 6(B1,B2)). The polyols, erythritol, xylitol and sorbitol shifted the transition curves toward higher temperature suggesting increase in the overall stability of Cyt. They also significantly altered the early-transition region of the thermal unfolding curves (Figure 6(C1–E2)).

All the thermal transitions were analysed using Equation (1) for three-state transition except for the far-UV CD data of 30% and 40% (v/v) EG. In these two conditions, data fit with two-state assumption (Equation (2)) had the residual values similar to three-state fit (Figure S4), therefore, two-state transition model was considered for further analysis. The transition midpoints, $T_{m1}$ and $T_{m2}$ and enthalpies of unfolding,

| Cosolvent | Probe | $T_{m1}$ (K) | $\Delta H_{m1}$ (kcal/mol) | $\Delta C_p$ (kcal/mol/K) | $T_{m2}$ (K) | $\Delta H_{m2}$ (kcal/mol) | $\Delta C_p$ (kcal/mol/K) |
|-----------|-------|-------------|-----------------------------|--------------------------|-------------|-----------------------------|--------------------------|
| 0.3 M Gdm | 395 nm| 305 ± 0.5   | 13 ± 1.2                    | 0.24 ± 0.02              | 346.7 ± 0.3 | 74.0 ± 1.7                  | 2.1 ± 0.6                |
|           | 222 nm| 308 ± 0.25  | 23.5 ± 0.25                 | 0.2 ± 0.06               | 344.2 ± 0.3 | 80.9 ± 0.3                  | 1.5 ± 0.4                |
| 0.6 M Gdm | 395 nm| 308 ± 0.1   | 23.5 ± 0.25                 | 0.2 ± 0.06               | 349.0 ± 0.1 | 66.1 ± 0.8                  | 2.1 ± 0.1                |
|           | 222 nm| –           | –                           | –                        | 339.0 ± 0.13 | 76.0 ± 2.4                  | 2.0 ± 1.0                |
| 0.9 M Gdm | 395 nm| –           | –                           | –                        | 336.3 ± 0.06 | 62.5 ± 0.6                  | 2.1 ± 0.1                |
|           | 222 nm| –           | –                           | –                        | 334.9 ± 0.26 | 73.2 ± 4.1                  | 2.0 ± 0.8                |
| 1 M urea  | 395 nm| 306.5 ± 1.2 | 19 ± 0.5                    | 0.1 ± 0.02               | 347.5 ± 1.2 | 77.0 ± 3.0                  | 2.3 ± 0.3                |
|           | 222 nm| 308 ± 0.1   | 20 ± 0.23                   | 0.20 ± 0.07              | 347.3 ± 1.3 | 82.2 ± 6.0                  | 1.7 ± 0.6                |
| 2 M urea  | 395 nm| 304 ± 0.5   | 15 ± 0.1                    | 0.1 ± 0.1               | 341.6 ± 0.9 | 72.5 ± 7.0                  | 2.4 ± 1.1                |
|           | 222 nm| 304 ± 0.4   | 14 ± 0.32                   | 0.3 ± 0.06              | 342.6 ± 1.9 | 71.0 ± 1.5                  | 2.3 ± 1.1                |
| 3 M urea  | 395 nm| –           | –                           | –                        | 336.7 ± 0.06 | 69.5 ± 1.1                  | 2.4 ± 0.1                |
|           | 222 nm| –           | –                           | –                        | 336.0 ± 0.3 | 70.2 ± 4.0                  | 2.5 ± 0.1                |
| 0.5 M NaCl| 395 nm| 309 ± 0.3   | 20 ± 0.4                    | 0.4 ± 0.06              | 348.8 ± 0.3 | 74.7 ± 3.5                  | 2.2 ± 0.9                |
|           | 222 nm| 309 ± 0.2   | 20 ± 0.6                    | 0.4 ± 0.06              | 348.2 ± 0.6 | 85.5 ± 0.4                  | 1.8 ± 0.5                |
| 1.0 M NaCl| 395 nm| –           | –                           | –                        | 346.0 ± 0.14 | 69.0 ± 2.2                  | 2.5 ± 0.1                |
|           | 222 nm| –           | –                           | –                        | 347.5 ± 0.6 | 84.0 ± 3.8                  | 2.7 ± 0.3                |

*two-state transition.
$\Delta H_{m1}$ and $\Delta H_{m2}$ obtained from the data-fit are presented in Figure 7. The $T_{m1}$ values correspond to the midpoint of N→I equilibrium decreases with the addition of any of the polyol (Figure 7(A1,B1)). This suggests that the transition is shifted to lower temperature and intermediate is destabilized by the addition of the polyols. This is complemented with the constant decrease in the value of $\Delta H_{m1}$ with the increasing concentration of any of the polyols (Figure 7(A3,B3)). Among the added polyols, EG showed the largest decrease in $T_{m1}$ value and in $\Delta H_{m1}$ as well. The effect of Xyl was found to be the least when probed with Soret absorption whereas Sor showed the least effect when probed with far-UV CD.

The temperature midpoint, $T_{m2}$ correspond to the transition I→U suggested that EG significantly reduced the transition midpoint temperature (Figure 7(A2,B2)); however, Glc did not show significant changes in the $T_{m2}$ values. Ery, Xyl and Sor increased the unfolding midpoint indicating stabilization of the protein against denaturation. Though Xyl displayed a slightly more increment in $T_{m2}$ values compared to Ery and Sor when probed with Soret absorption, the increase in $T_{m2}$ values were almost same when measured with far-UV CD. The changes observed in $\Delta H_{m2}$ (Figure 7(A4,B4)) also indicated that EG destabilized the protein whereas Xyl, Ery and Sor stabilized the protein by increasing the enthalpy of unfolding.

For further analysis, the fraction of intermediate population was calculated in the presence of varying concentrations of (A) urea, (B) NaCl and (C) Gdm.

Figure 4. Transition midpoint temperature ($T_{m1}$) and enthalpy of unfolding ($\Delta H_{m1}$) of Cyt in different concentrations of urea (green), NaCl (cyan) and Gdm (black) calculated from the thermal transitions measured by absorbance (upper panels) and ellipticity changes (lower panels) that are shown in Figure 3. It may be noted that for some of the data points error bars are smaller than the symbol size.

Figure 5. Fraction of native (solid lines), intermediate (dashed lines) and unfolded (dotted lines) states of Cyt as a function of temperature in the presence of varying concentrations of (A) urea, (B) NaCl and (C) Gdm.
Figure 6. Thermal denaturation transitions of Cyt followed by absorption changes at 395 nm (upper panels) and ellipticity changes at 222 nm (lower panels) in the presence of varying concentrations of (A1 and A2) EG, (B1 and B2) Glc, (C1 and C2) Ery, (D1 and D2) Xyl, and (E1 and E2) Sor. The solid lines represent the curve-fit using Equation (1) for three-state transitions or Equation (2) for two-state transitions. The parameters obtained from the curve-fit are presented in Figure 7.

Figure 7. (A1–B2) Transition midpoint temperature, $T_m$, and (A3-B4) enthalpy of unfolding, $\Delta H_m$, for $N \rightarrow I$ and $I \rightarrow U$ transitions of Cyt in different concentrations of EG (black), Glc (red), Ery (green), Xyl (cyan) and Sor (ink) calculated from the thermal denaturation transitions measured by absorbance (upper panels) and ellipticity changes (lower panels) that are shown in Figure 6. It may be noted that for some of the data points error bars are smaller than the symbol size.

Figure 8. Fraction of intermediate (upper panels), native, and unfolded states (solid and dashed lines in lower panels, respectively) of Cyt as a function of temperature in the presence of varying concentrations of (A) EG, (B) Glc, (C) Ery, (D) Xyl, and (E) Sor.
gradually moved to lower temperature (Figure 9(B)). The addition of Glc slightly reduced the population of the intermediate and $T_{\text{max-inter}}$ was slightly moved to lower temperature at lower concentration of Glc ($\leq 30\%$). In case of higher polyols, the population of intermediate was not significantly altered. $T_{\text{max-inter}}$ value shifted towards higher temperature. Structural content of the intermediate in the presence of the polyols was analysed by measuring far-UV CD spectra of Cyt at the respective $T_{\text{max-inter}}$ values (Figure S5). The spectra indicated that there was a minor structural loss compared to the native condition.

4. Discussion
Identifying and characterizing the intermediate states of proteins is essential not alone to decipher the folding pathways, they also find relevance in explaining biological functions of the proteins. For instance, Cyt is proposed to adapt different conformational states during electron transport, peroxidase activity and apoptosis pathway (Hannibal et al., 2016; McClelland et al., 2014; Ow et al., 2008). Most of these conformational changes are related to the heme-ligation and conformational changes in $\Omega$-loops of the protein (Hannibal et al., 2016). These intermediates are similar to the states obtained in vitro conditions by altering pH, salt and temperature (Hamada et al., 1994; Kumar et al., 2006, 2014; Naem & Khan, 2004). Though the effect of osmolytes on the native state of Cyt has been well studied (Anjum et al., 2000; Haque et al., 2006; Kaushik & Bhat, 1998; Taneja & Ahmad, 1994), their effect on structural intermediates are less understood. We investigated the effect of polyol class of osmolytes on the intermediate state of Cyt identified during thermal unfolding of the protein and on the stability of the intermediate.

4.1. Characteristics of thermal denaturation

intermediate of cytochrome c

Cyt, undergoes a three-state unfolding transition at pH 5. This is evident from both absorbance and far-UV CD spectra recorded as a function of temperature that clearly lacking an isosbestic point and an isodichroic point, respectively. The intermediate population was maximum at 339 K and the spectral analysis of the protein near to this temperature suggests that the low-spin state of the heme-iron with Fe$^{3+}$-M80 ligation (Q- and CT-band, Figure S1(B,C)) and the globular packing (near UV-CD, Figure S1(D)) could be similar to the native conformation of the protein. However, there is a slight loss of secondary structure of the protein (far UV-CD, Figure 1(F)) and a hyperchromic shift in Soret absorption as well. This could be attributed to the conformational changes around the $\Omega$-loop of the protein particularly the residues between 70 and 85. The weakening of Met80 ligation might increase the Soret absorption without significant shift in wavelength maximum. The disruption of this $\Omega$-loop is also reported to be an early stage of unfolding of Cyt (Russell et al., 2000; Xu et al., 1998). Therefore, we could conclude that M80 is still intact though the conformational states around the loop is altered at 339 K. Similar intermediate conformation with weakened M80 ligation is observed at 345 K during thermal denaturation of Cyt at pH 7 (Hagarman et al., 2008). This intermediate, labelled as III-h state also shows a loss of secondary structural content in the protein and well distinguished from the alkaline-transition state in which heme misligation is observed.

Increase in temperature above 339 K reduces the population of the intermediate (Figure 2(E)) and the unfolded state’s population starts increasing. Spectral analysis of the unfolded state at 363 K, where the unfolded population reaches its maximum, suggests that Cyt lost its secondary structural content (Figure 2(F)) and tertiary packing (Figure S2(D)) as well. Though the loss of 695 nm band indicates that heme-M80 ligation is disrupted, the absence of any band at 620 nm (Figure S2(D)) suggests that the heme-iron still possesses a low-spin state. This could be probably due to the replacement of M80 by other strong field ligands such as H26 and H33 (Russell et al., 2000). In earlier studies as well, a six-coordinated low-spin unfolded state of Cyt with bis-histidine ligation has been observed in both thermal and denaturant-induced denaturation processes (Russell & Bren, 2002; Varhač et al., 2015).
4.2. Interactions that stabilizing the intermediate state

The major factors that contribute to the stabilization of the intermediate could be analyzed by studying the effect of ionic and non-ionic additives on the stability of the intermediate (Anumalla & Prabhu, 2018; Apetri & Sureswicz, 2003; Zhang et al., 2011). Addition of urea destabilizes the intermediate which is evident from the reduction of enthalpy of unfolding for $N \rightarrow I$ transition, $\Delta H_m$ (Table 2). At higher concentrations, the intermediate is not detectable and the protein follows a two-state transition. The globular stability of the protein is also reduced by urea as expected for a denaturant (Figure 4). Similarly, the addition of NaCl also reduces the stability of the intermediate and the protein adapts a two-state transition when the concentration of NaCl is above 0.5 M. Urea is known to destabilize native-folded structure by disrupting the intra-molecular and protein-water hydrogen bonding interactions, and weakening the hydrophobic effect (Camilloni et al., 2008; Canchi & García, 2013; Das & Mukhopadhyay, 2009; Makhatadze & Privalov, 1992). On the other hand, the destabilization effect of salt would arise from charge-charge interactions. The destabilization of intermediate by both urea and NaCl indicates that hydrogen bonding and Columbic interactions are the major factors stabilizing the structure.

The addition of Gdm, even at a low concentration (0.6 M), destabilizes the formation of intermediate and the thermal unfolding transition shows a two-state process (Figure 3(C1-C2)). Gdm disrupts both hydrogen bonding ionic interactions, thus, acting as a more effective denaturant than urea (Canchi & García, 2013; Makhatadze & Privalov, 1992). Gdm is found to be nearly two-times more effective on globular proteins and it can act as effectively as the equimolar mixture of urea and NaCl (Dempsey et al., 2005; Smith & Scholtz, 1996). These observations support the fact that the globular structure of the protein is still intact in the intermediate state which could be destabilized by the addition of denaturants or NaCl.

4.3. Polyols alter the energy landscape of folding intermediates

Polyols having hydroxyl groups 3 or more are found to stabilize many globular proteins (Devaraneni et al., 2012; Haque et al., 2006; Joshi & Bhuyan, 2020; Tiwari & Bhat, 2006) whereas EG destabilizes proteins (Liu et al., 2010; Naidu et al., 2020; Parray et al., 2020). Polyols, EG, Gly, Ery, Xyl and Sor also show a similar trend on the globular stability of Cyt during thermal denaturation (Figures 6 and 7). However, closer analysis on the individual transitions, $N \rightarrow I$ and $I \rightarrow U$ shows that the intermediate is destabilized by all the polyols. This is evident from a decrease in $T_m$ and $\Delta H_m$ (Figure 7(A3,B3)) for the transition $N \rightarrow I$. The enthalpy of unfolding for $I \rightarrow U$ is reduced by EG whereas it is increased in other polyols. It is interesting to note that the fraction of intermediate detectable and $T_{max\text{inter}}$ is also reduced in the presence of EG. These changes suggest that EG destabilizes both the native and intermediate states and Gly has little effect on both the states. Higher order polyols destabilize the intermediate state, but the extent of destabilization is lesser that that of EG. On the other hand, they increase the overall stability of the protein by increasing the unfolding enthalpy of intermediate state. Based on these observations, the probable energy states are depicted in Figure 10. It proposes that the addition of EG destabilizes the native state more than the unfolded state as generally suggested for denaturants (Timasheff, 1993, 2002). The higher order polyols, however, destabilize the unfolded state more than the native state. All the polyols might destabilize the intermediate state and the effect of EG could be slightly higher than the other polyols.

Structurally, the intermediate state retains most of the secondary structural contents (Figure 55) and the heme-M80 ligation of the native state even in the presence of polyols. The conformational changes might occur around the $\Omega$-loops covering the residues 70–85. Earlier studies have suggested that polyols can induce formation of MG state from the unfolded conformation of proteins, except in the presence of EG (Devaraneni et al., 2012; Kamiyama et al., 1999). The proteins might attain a non-native state which is less stable than native conformation but more stable than unfolded conformations (Cremades & Sancho, 2008). In case of Gly, a quasi-native state has also been proposed which is suggested to follow an alternative folding pathway (Joshi & Bhuyan, 2020). The polyols generally increase the stability of proteins by increasing hydrophobic interactions of non-polar amino acids, since the free energy of transfer from water to polyols is positive for them (Bolen & Baskakov, 2001; Gekko, 1981; Kamiyama et al., 1999). Though such preferential exclusion of polyols from the surface of proteins has been suggested (Liu et al., 2010; Sinibaldi et al., 2007; Sudrik et al., 2019), existence of weak binding interactions are also reported between proteins and polyols (Arakawa, 2018; Schellman, 2003; Timasheff, 1998). EG, an exception case, shows preferential interaction with the proteins (Naidu et al., 2020; Tejaswi Naidu & Prakash Prabhu, 2020; Timasheff &…
Inoue, 1968) and destabilizes. These observations indicate that preferential exclusion of polyols might provide a global stability to the protein, however, their weak interactions with the native-like structural intermediate (analogous to late-folding intermediates) might destabilize the formation of intermediate. Thus, polyols could alter the energy states of different conformations of the protein.

5. Conclusion

Cyt forms an intermediate during thermal denaturation at pH 5. The intermediate retains most of the secondary structure; however, the heme-M80 ligand might be slightly weaken due to conformational changes in $\Omega$-loop. It is stabilized by both hydrogen bonding and electrostatic interactions. Addition of polyols destabilize the intermediate state and the destabilizing effect is reduced with the increasing number of OH-groups. The population of intermediate and $T_{\text{max-inter}}$ is significantly reduced by EG whereas only slightly reduced in Glc. $T_{\text{max-inter}}$ values is increased by higher order polyols; however, they do not affect the population of intermediate. Moreover, EG reduces the overall stability of the protein and behaves like a mild-denaturant whereas other polyols increase the overall stability of the protein.

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Disclosure statement

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