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Mixture of Natural Polyamines Exhibit an Additive Effect on Polyamines-Mediated Decrease in Thermal Stability of Horse Myoglobin

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Abstract—*Natural polyamines (PAs) are organic cations having basic character. Natural PAs play a critical role in biological processes. Although the impact of natural PAs on the stability and aggregation of proteins has been previously investigated but, how their mixture affects the thermal stability of horse myoglobin (Mb) has not explored so far. Using various spectroscopy techniques like UV-visible spectroscopy and far-UV circular dichroism (far-UV CD), this paper examined the role of the mixture of natural PAs on the local (heme-globin interaction) and structural (secondary structure) thermal stability of myoglobin (Mb) at physiological pH 7.4. Analysis of thermal melting curves (based on absorbance at 409 nm and ellipticity at 222 nm) of Mb collected in buffer only and in the presence of 50 mM putrescine (PUT), 50 mM spermidine (SPD), and 25 mM PUT + 25 mM SPD provided several important information (i) natural PAs decrease the thermal denaturation mid-point (T_m) (based on absorbance at 409 nm and CD at 222 nm), which suggests that natural PAs decrease the local (heme-globin interactions) and structural (secondary structure) thermal stability of Mb (ii) the natural PAs-mediated decrease in T_m-value is more pronounced for 50 mM SPD than the 50 mM PUT, which suggests that the SPD is more efficient natural PAs to decrease the local and structural) thermal stability of Mb, and (iii) the natural PAs-mediated decrease in T_m for the mixture of natural PAs (25 mM PUT + 25 mM SPD) was found to be average of 50 mM SPD and 50 mM PUT, suggesting that the mixture of natural PAs exhibit an additive effect on PAs-mediated decrease in local and structural thermal stability of Mb.*

INTRODUCTION

Proteins are biological macromolecules found in all living system. Proteins perform various essential roles in the body [1]. Proteins are necessary to maintain and control cells, tissues and organs in all living organisms [2]. For proper functioning, the protein must fold to a correct three-dimensional structure [3, 4]. However, because of cellular stress and changes in environmental conditions such as extreme temperature, pH, salts, crowding environment, etc., the protein folding in the cell is often challenged [5]. Several small molecules such as osmolytes, PAs, and amino acids, are typically present in the body. The presence of these small organic molecules in the body generally alters the thermodynamic stability and folding mechanism of proteins. PAs are small aliphatic compounds that consist of two or more amino groups ($–NH_3^+$) and are found in all eukaryotic and prokaryotic cells [6, 7]. Natural PAs are in-general involved in cell growth, proliferation, differentiation, and apoptosis [8, 9]. At physiological pH conditions (pH ~7.4), these natural PAs are regarded as the positively charged cosolute [10]. Natural PAs can be used as cosolvents in biological and industrial applications. Due to their cationic nature, these natural PAs can easily make non-covalent interactions with the negatively charged group of macromolecules (i.e., nucleic acid, DNA, RNA, proteins, phospholipids etc). Formation of such interactions between the natural PAs and biomacromolecules could alter the structure and function of these macromolecules. [11]. The biological activity of these natural PAs is anticipated due to the positively charged amino groups linked with each PA. While studies on the effect of natural PAs on protein stability and aggregation have been already conducted [12-14], the role of their mixture on the thermodynamic or thermal stability of proteins is not been explored yet. The current work investigates the effect of the mixture of natural PAs (PUT & SPD) on local (heme-globin interactions) and structural (secondary structures) thermal stability of Mb at pH 7.4.

Mb is a monomeric oxygen-binding protein with 153 amino acids [15]. It is a heme protein found in the heart and skeletal muscles. The primary role of Mb is to provide oxygen to the muscles whenever needed [16]. The heme-globin interaction in Mb is very sensitive to environmental fluctuations, so it becomes more interesting to study the effect of the mixture of PAs on this interaction.

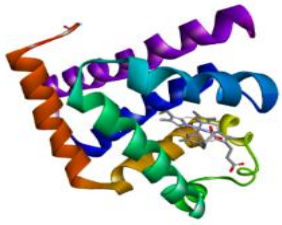

Fig. 1 PDB structure of Mb (1YMB)

MATERIALS AND METHODS

Myoglobin from horse heart, SPD, and salts of buffer (monobasic and dibasic sodium phosphate) were acquired from Sigma-Merck. PUT was obtained from TCI. Every experiment was conducted in 50 mM sodium phosphate buffer at pH 7.4. All thermal melting data were analyzed using SigmaPlot (v.9) software.

Measurement of visible absorption and far-UV CD spectra of Mb

To determine the effect of the mixture of natural PAs on heme-globin interaction and secondary structures of Mb, the visible absorption and far- UV CD spectra of Mb were collected in buffer only and in presence of 50 mM PUT, 50 mM SPD, and mixture of PUT and SPD (25 mM PUT +25 mM SPD) at pH 7.4 on Shimadzu UV- visible spectrophotometer (UV-2600) and Jasco J-815 CD spectropolarimeter respectively. The visible (380 nm-700 nm) and far-UV CD (200 nm-250 nm) spectra were recorded in 10 mm and 1 mm path length cuvette, respectively. Protein concentration of ~ 8 μM was used for spectral measurements.

Measurements of thermal melting of Mb

To determine the effect of mixture of PAs on local (heme-globin interactions) and structural (secondary structure) thermal unfolding of Mb, the thermal melting curves (based on absorbance (409 nm) and CD (222 nm)) of Mb were performed in buffer only and in presence of 50 mM SPD, of 50 mM PUT, and mixture of SPD and PUT (25 mM SPD +25 mM PUT) at pH 7.4. Visible absorption thermal melting curves were performed with 1°C/min heating rate on a Shimadzu UV 2600 spectrophotometer that was linked with a S-1700 temperature controller accessory. The CD-based melting curves were performed with 1°C/min heating rate on a Jasco 810 CD spectropolarimeter coupled with a PTC-510. The Mb concentration used in these experiments was ~ 8 μM. The thermal melting data was analyzed using the transformed form of van't Hoff equation [17],

$$S^* = \frac{(S_N + M_N T) + (S_U + M_U T) exp(\frac{\Delta H_m}{R}(\frac{1}{T} - \frac{1}{T_m}))}{1 + \exp(\frac{\Delta H_m}{R}(\frac{1}{T} - \frac{1}{T_m}))} \quad 1)$$

Here, the terms S_N and M_N; and S_U and M_U described the intercepts and slopes of native-folded state and denatured-state, respectively. The term ΔH_m describes the change in van't Hoff enthalpy at melting temperature, T_m.

RESULTS AND DISCUSSION

3.1 Natural PAs and their mixture slightly alter the visible absorption and far-UV CD spectra of Mb

The native Mb at pH 7.4, 25 °C shows the maxima at 409 nm in visible absorption spectra and two negative peaks at 208 nm and 222 nm in far-UV CD spectra, which reflect the heme-globin interaction and helical content of native Mb, respectively [18, 19]. Fig. 2a and 2b display the visible absorption and far-UV CD spectra of Mb in buffer only and with 50 mM PUT, 50 mM SPD, and mixture of PUT and SPD (25 mM PUT +25 mM SPD) at 25 °C, pH 7.4. Data in Fig. 2a and Fig. 2b show that the natural PAs and their mixture slightly alter the heme globin interaction and secondary structure of native Mb at pH 7.4, 25 °C.

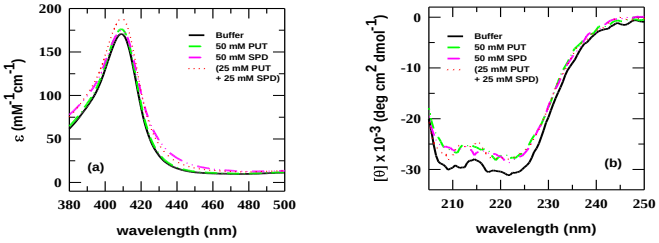

Fig. 2: Panel (a) and (b) depict the visible absorbance and far-UV CD spectra of Mb, respectively: Buffer only (black solid line); 50 mM PUT (green dash dot line); 50 mM SPD (pink dash dot dot line); the mixture of PAs (25 mM PUT + 25 mM SPD) (red dotted line) at 25 °C, pH 7.4.

3.2 Mixture of natural PAs exhibits additive effect on PAs-mediated decrease in local and structural thermal stability

Fig. 3a and 3b represent the visible absorption and far-UV CD spectra of Mb collected in 50 mM sodium phosphate buffer at 298.15 K and 373.15 K, pH 7.4. At 373.15 K, pH 7.4, the significant decrease of intensity of Soret band in visible absorption spectra of Mb with a blue shift at 380 nm (Fig. 3a) suggests the loss of heme-globin interaction of Mb due to thermal denaturation of protein [20]. At 373.15 K, pH 7.4, the two negative peaks at 208 nm and 222 nm in far-UV CD spectra of Mb also loss, which suggests thermal induced disruption of secondary structure of protein. These findings warrant an investigation of natural PAs and their mixture impact on local (heme-globin interaction, based on absorbance at 409 nm) and structural (secondary structure, based on CD at 222 nm) thermal stability of Mb at pH 7.4.

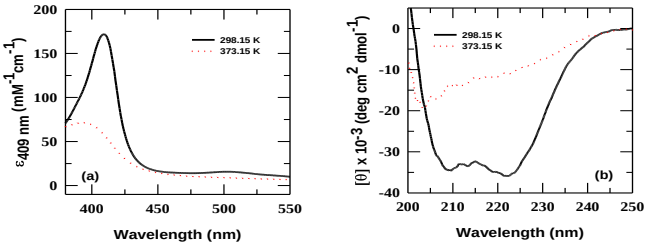

Fig. 3: Represents the spectra of Mb at 298.15 K and 373.15 K at pH 7.4. Panel (a) and (b) represent the visible and far UV CD spectra of Mb, respectively: 298.15 K (black solid line) and 373.15 K (red dotted line).

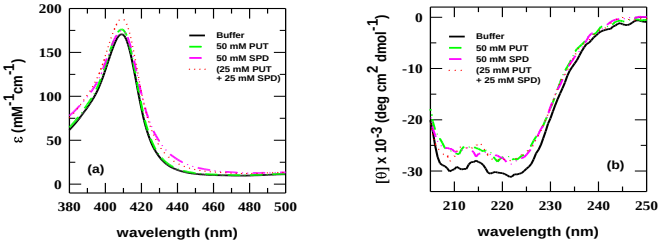

Fig. 4a and 4b show the variations in molar absorptivity at 409 nm and ellipticity at 222 nm with temperature of Mb,

respectively collected in buffer only and in 50 mM PUT, 50 mM SPD, and the mixture of PAs (25 mM PUT + 25 mM SPD) at pH 7.4 respectively. For optimal presentation of thermal unfolding curves, the temperature range from 330 K to 365 K are shown in fgure, although the experiments were carried out from 298.15 K to 373.15 K. Fig. 4c and 4d depict the normalized heating curves of Mb at $\Delta\varepsilon_{409}$ and $[\theta]_{222}$ with 50 mM PUT, 50 mM SPD, and mixture of PUT and SPD (25 mM PUT +25 mM SPD) at pH 7.4, respectively. The fraction of unfolded (F_u), determined using equation (2)[17],

$$Fraction\ unfolded\ (Fu) = \frac{(Y - Y_N)}{(Y_U - Y_N)}\ 2)$$

where the term, Y represents the measured extinction coefficient value (409 nm) or ellipticity value (222 nm) at a given temperature.

The data in Fig. 4a, b and Fig. 4c, d reveal that PAs and their mixture shift the thermal unfolding curve of Mb towards a lower temperature. Furthermore, the PAs-mediated shift towards lower temperature is more for SPD than PUT and it lies between PUT and SPD for mixture (Fig. 4a, b and Fig. 4c, d). The thermal unfolding curves were analysed by the transformed form of van't Hoff equation (1) to get the values of thermal denaturation mid-point (T_m) and enthalpy change (ΔH_m) at T_m. The calculated values of ΔH_m and T_m measured using equation (1) are given in Table 1.

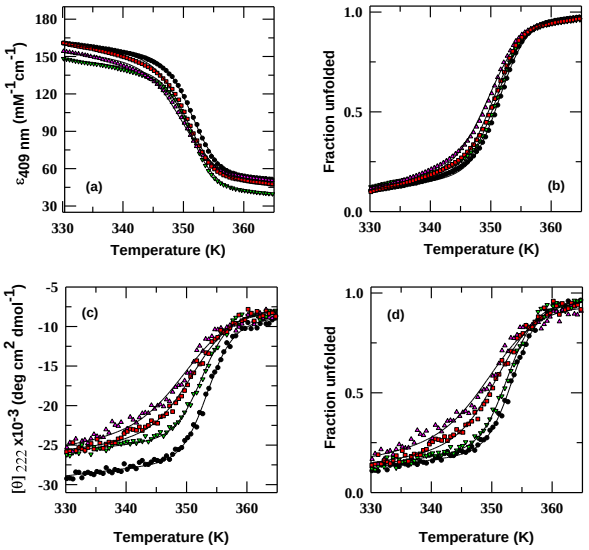

Fig. 4: Panel (a) and (b) characterize the change in the molar extinction coefficient ($\Delta\varepsilon_{409}$) and ellipticity value ($[\theta]_{222}$) of Mb, respectively, in buffer only (●) and with 50 mM PUT (▼), 50 mM SPD (▲) and the mixture of PAs (25 mM PUT + 25 mM SPD) (■). Panel (c) (absorbance 409 nm) and (d) (CD 222 nm) are corresponding fraction unfolded as a function of temperature determined through equation (2). The black solid lines in each panel represent the fitting of thermal denaturation curves to the equation (1).

Data in Table (1) provided several important information (i) the natural PAs and their mixture decrease the magnitude of T_m, suggesting PAs decrease the local (heme-globin interaction) and structural (secondary structure) thermal stability of Mb, (ii) PAs-mediated decrease in T_m-value is more pronounced for SPD than PUT, which suggests that the PAs-mediated decrease in the local and structural thermal stability of Mb might be related to the number of amino groups present in PA, and (iii) the PAs-mediated decrease in T_m-value for mixture of PAs (25 mM PUT + 25 mM SPD) is nearly the average of the PAs-mediated decrease in T_m-value for 50 mM SPD and 50 mM PUT, which suggests that mixture of natural PAs exhibits additive effect on PAs-mediated decrease in local and structural thermal stability of Mb at pH 7.4. The ΔH_m value of Mb also decreases in the presence of PAs and their mixture, suggesting that the electrostatic effect might contribute to PAs-mediated decrease in local and structural thermal stability of Mb.

Table 1: Effects of PAs and their mixture on thermally-induced denaturation parameters (T_m and ΔH_m) of Mb at pH 7.4, 25 °C.

	Visible spectroscopy (at 409 nm)		**Far-UV CD (at 222 nm)**	
PAs	T_m **(K)**	ΔH_m **(kcal mol^{-1})**	T_m **(K)**	ΔH_m **(kcal mol^{-1})**
Buffer	351.6	108.4	353.5	118.9
50 mM PUT	351.1	113.7	352.7	109.5
50 mM SPD	349.6	82.7	350.6	59.1
(25 mM PUT+ 25 mM SPD)	350.6	94.6	351.4	71.6

CONCLUSION

The natural PAs, as well as their mixture decrease the local and structural thermal stability of Mb. The PAs-mediated decrease in local and structural thermal stability of Mb was estimated larger for SPD than the PUT, which reveals that the PAs-mediated decrease in the local and structural thermal stability of Mb is affected by the number of amino groups present in PA. Most remarkably, the PAs-mediated decrease in T_m-value for mixture of PAs (25 mM PUT + 25 mM SPD) was found average of the PAs-mediated decrease in T_m-value for 50 mM SPD and 50 mM PUT, which demonstrates that the mixture of natural PAs shows additive effect on PAs-mediated decrease in local and structural thermal stability of Mb at pH 7.4.

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AUTHOR CONTRIBUTIONS

Manisha Yadav and Rajesh Kumar designed the research. Manisha Yadav carryout the research experiments. Manisha Yadav, Jayanti Rawat, and Shabnam have been analysed the experimental data. Rajesh Kumar, Manisha Yadav, Jayanti Rawat, and Shabnam composed and wrote the manuscript.

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