

Spectroscopic Investigations of Interactions of Heteropolyacids with α -Lactalbumin Complexes

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The interaction between α -lactalbumin with heteropoly acids occurred. The best denaturation of α -lactalbumin with Preyssler heteropoly acid occurred in comparison with heteropoly acids including Keggin and Dawson structures. Based on calculated thermodynamics parameters, it is shown that during denaturation of protein, preyssler heteropoly acid has the highest $\Delta G(H_2O)$, m value and lowest C_m at the shortest time compared with other polyoxometalates (POMs) with Keggin and Dawson structures. The electrostatic interaction of protein with various heteropoly acids is important on a molecular level for the interpretation and development of model compounds with selective affinity for particular proteins.

Key Words: Heteropoly acid, Polyoxometalate, Preyssler, α -Lactalbumin, Stability, Protein denaturation.

INTRODUCTION

Bovine α -lactalbumin (α -LA) is a globular protein, containing 123 amino acid residues with a molecular weight of 14,200. It is in the whey of mammalian milk and acts as a regulatory subunit of the lactose synthesis system^{1,2}. The structure of this protein is shown in Fig. 1.

α -Lactalbumin is often chosen as a model for protein folding studies. The main reason for such a choice is that this protein exhibits an molten globule (MG) folding intermediate which is stably populated at equilibrium under various mildly denaturing conditions at acidic pH and at an intermediate concentration of a strong denaturant (*e.g.*, Gdn HCl) at neutral pH³⁻⁵. The study on the interaction of protein with some small molecules is of significance for biochemistry, clinical chemistry and analytical chemistry of protein. However, most works used dyes and some organic small molecules such as porphyrins^{6,7}, triphenylmethane dyes⁸⁻¹², xanthene dyes^{13,14}, azo dyes^{15,19} and some medicine²⁰ for this purpose. The reaction of protein with inorganic ions and their analytical application have not been reported so far.

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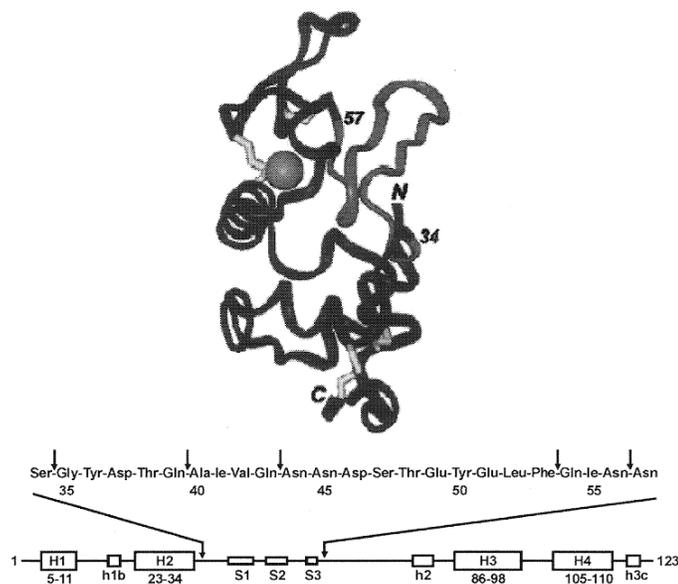


Fig. 1. Structure of protein α -lactalbumin

One of the largest inorganic anions is polyoxometalates (POMs), which form a large and distinctive class of molecular inorganic compounds of unrivaled electronic versatility and structural variation, which impacts many fields of science and technology. These substances continue to attract increasing attention worldwide.

Many polyoxometalates have been shown to be biologically active. Two types of activity, antiviral and antitumoral, have dominated the medicinal chemistry of POMs up to date²¹. Effectiveness of these polyoxometalates against several viruses such as vesicular stomatitis, polio, rubella, rauscher leukemia, rabies, *etc.* has been studied²². The reasons for biological activity of polyoxometalates may include ionic size and charge, electron-transfer properties, behaviour of polyanions in extremely dilute solutions at physiological pH, *etc.*²³.

Because polyoxometalates are potent inhibitors for reverse transcriptase and other related enzymes²⁴, understanding of the nature of the interaction of these compounds with biologically active compounds such as proteins has also been an active focus of research. The antiviral activity of this kind of polyoxometalates has recently been associated with their ability to bind to the viral cell envelope, not with their ability to inhibit viral reverse transcriptase as thought before^{25,26}. The potent biological properties of these heteropoly acids (HPAs) is the result of their interactions with viral enzymes or with proteins in the viral cell envelope. The understanding of these interactions on a molecular level is important to the interpretation and development of future designed compounds with selective affinity for particular proteins.

Recently, we have developed a series of heteropoly acids catalyzed reactions²⁷⁻³⁶. In our research works, we were interested in developing of application of Preyssler in other areas because of its exclusive structure and properties. Therefore, it was great interest to know for us, whether the Preyssler's anion is interacted with proteins. To best of our knowledge, there is no report on the behaviour of this anion in the presence of proteins. In this paper we wish to report our investigation on the interaction of α -lactalbumin with Preyssler heteropolyacid and the results were compared with other heteropoly acids including Keggin and Dawson structures. Finally, thermodynamics parameters were calculated for these interactions.

EXPERIMENTAL

Bovine α -lactalbumin was purchased from Sigma. Polyoxometalates were synthesized and purified according to the procedures published elsewhere³⁷. Preyssler heteropoly acid was prepared *via* the method published³⁸ and characterized by spectroscopic method.

Solution preparation: Stock solutions of protein were prepared by dissolving commercially purchased protein in HCl. Heteropoly acids were also dissolved in HCl. If the initial concentration and volume of the protein solution are $[P]_0$ and V_0 , respectively and the stock heteropoly acid concentration is $[L]_0$, then the total concentration of protein ($[P]_t$) and HPA ($[L]_t$) can be obtained by accounting for the total volume of the aliquot (V_c) added during the titration experiment³⁹:

$$[P]_t = [P]_0 V_0 / (V_0 + V_c), [L]_t = [L]_0 V_0 / (V_0 + V_c)$$

Aliquots of heteropoly acids were injected into the protein solution at 5 min intervals to allow equilibration.

Soret absorption spectra of protein were obtained with a spectrophotometer, model Shimadzu-1650 PC. The protein concentration was determined spectrophotometrically. The spectrum of protein α -lactalbumin in HCl was taken as a reference. Fluorescence measurements were made on Jasco FP-6200 spectrofluorometer in a 10 mm path length quartz cell. Samples containing different concentrations of Preyssler, Keggin and Dawson were equilibrated at room temperature for 5 min before recording for tryptophan fluorescence measurements. The excitation wavelength was 280 nm and the emission was recorded from 300 to 500 nm. The final protein concentration was 10 μ M for all experiments. Infrared spectrum was recorded on a Bruker 500 scientific spectrometer.

RESULTS AND DISCUSSION

Interaction of α -lactalbumin with Preyssler's anion and other heteropoly acids including $H_3PMO_{12}O_{40}$, $H_3PW_{12}O_{40}$, $H_4SiMO_{12}O_{40}$, $Na_2HPW_{12}O_{40}$, $Na_3PMO_{12}O_{40}$, $Na_3PW_{12}O_{40}$, $H_4[PMO_{11}VO_{40}]$, $H_5[PMO_{10}V_2O_{40}]$, $H_6[PMO_9V_3O_{40}]$ and $H_6P_2W_{18}O_{62}$, were studied, using spectral techniques such as UV spectroscopy and fluorescence methods.

Soret absorption spectra: The UV spectrum was obtained in the range of 190–400 nm. The native protein shows a maximum at 280 nm. For example, the results of the changes in the soret absorption spectra of α -lactalbumin are shown in Fig. 2 (panels A, B, C) in the presence of 3 heteropoly acids, including Dawson, Keggin and Preyssler. Other UV spectra are not tabulated.

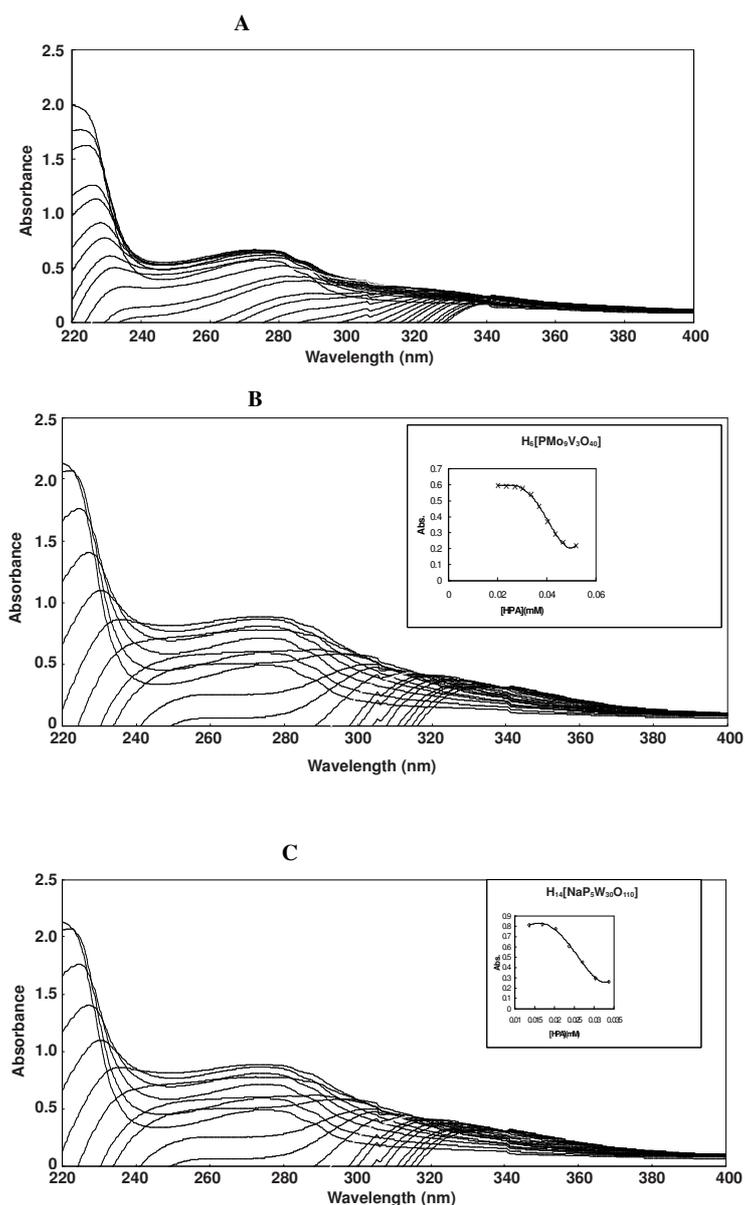


Fig. 2. Soret absorption spectra of α -lactalbumin as a function of heteropoly acids concentration {[A] $H_6P_2W_{18}O_{62}$, [B] $H_6[PMo_9V_3O_{40}]$, [C] $H_{14}[NaP_5W_{30}O_{110}]$ } and plots of absorption *versus* total concentration of heteropoly acids at HCl (20 mM, pH = 3)

As it can be seen that the addition of low concentrations of all of the heteropoly acids (3 μ L), to the α -lactalbumin decreases the absorption intensities and denature it. However, the overall denaturation of α -lactalbumin is obtained after 10 step addition of Preyessler (only 30 μ L) while denaturation occurs after 26 (78 μ L), 19 (57 μ L), 17 (51 μ L), 18 (54 μ L), 21 (63 μ L), 15 (45 μ L), 22 (66 μ L), 25 (75 μ L), 29 (87 μ L), 16 (48 μ L) step addition for $H_3PW_{12}O_{40}$, $H_3PMO_{12}O_{40}$, $H_4SiMO_{12}O_{40}$, $H_5[PMO_{10}V_2O_{40}]$, $H_4[PMO_{11}VO_{40}]$, $H_6P_2W_{18}O_{62}$, $Na_3PW_{12}O_{40}$, $Na_2HPW_{12}O_{40}$, $Na_3PMO_{12}O_{40}$ and $H_6[PMO_9V_3O_{40}]$, respectively. It means that the Preyessler's anion decrease the absorption intensity of α -lactalbumin with lower concentration and denatures α -lactalbumin at a shorter time.

It is proposed that under the acidic conditions, proteins are macromolecules with many positive charges. At the remaining groups of amino acids on their peptide chains, their cationic groups are mainly $-NH_3^+$, $-C(NH_2)NH_2^+$ and $[-C_3H_4N_2]^+$. Under the same conditions, heteropoly compounds exist as inorganic anions. When proteins and some heteropoly compounds coexist, proteins can combine with heteropoly anions through the electrostatic forces and hydrophobic force. Results show that among the different structures of heteropoly acids, Keggin: $[XM_{12-y}M'_yO_{40}]^{p-}$ ($X = P, Si, M = Mo, W, M' = V, y = 0, 1, 2, 3, p = 3, 4, 5, 6$), Dawson: $[P_2W_{18}O_{62}]^{6-}$ and Preyessler: $[NaP_5W_{30}O_{110}]^{14-}$, the rate of denaturation of α -lactalbumin increases according to the number of minutes charges (or H^+), suggesting denaturation occurs *via* electrostatic forces. It is assumed that the interactions through hydrogen bonding are existed between the molecules of α -lactalbumin and the anion. The conformation of protein is determined by the balance of charge repulsion between positive groups at acidic condition. When heteropoly anions are added to the protein in acidic condition, the shielding of intramolecular electrostatic repulsive forces by the heteropoly anions binding reflects the intrinsic forces that favour the formation of the new state.

Fluorescence spectra: Fig. 3 (panels A, B, C) shows the effect of interaction of heteropoly acids with α -lactalbumin on the fluorescence spectra for three heteropoly acids. Other spectra are not tabulated. According to the Fig. 3, the addition of low concentrations (3 μ L) of heteropoly acids to α -lactalbumin causes a decrease in the fluorescence intensity. This result is accordance with the UV observation. Present studies show that all of the used heteropoly acids (or polyoxometalates) in this study can denature the structure of protein, but this effect is best for Preyessler's anion. The reasons are similar to reasons given for UV.

Sigmoidal curves: Figs. 2 (panels A, B, C) and 3 (panels A, B, C) show the profile transitions of the nature state to the denaturation of α -lactalbumin induced by various concentrations (C_m) of heteropoly acids, as measured by the change in emission at 351 nm and absorption at 280 nm. Table-1 shows that the lowest C_m is belong to the Preyessler's anion, $H_{14}[NaP_5W_{30}O_{110}]$ and the highest C_m is belong to the sodium salt of Keggin, $Na_3[PMO_{12}O_{40}]$. It means that the used concentration of the Preyessler's anion for denaturation of the α -lactalbumin is lower than the other

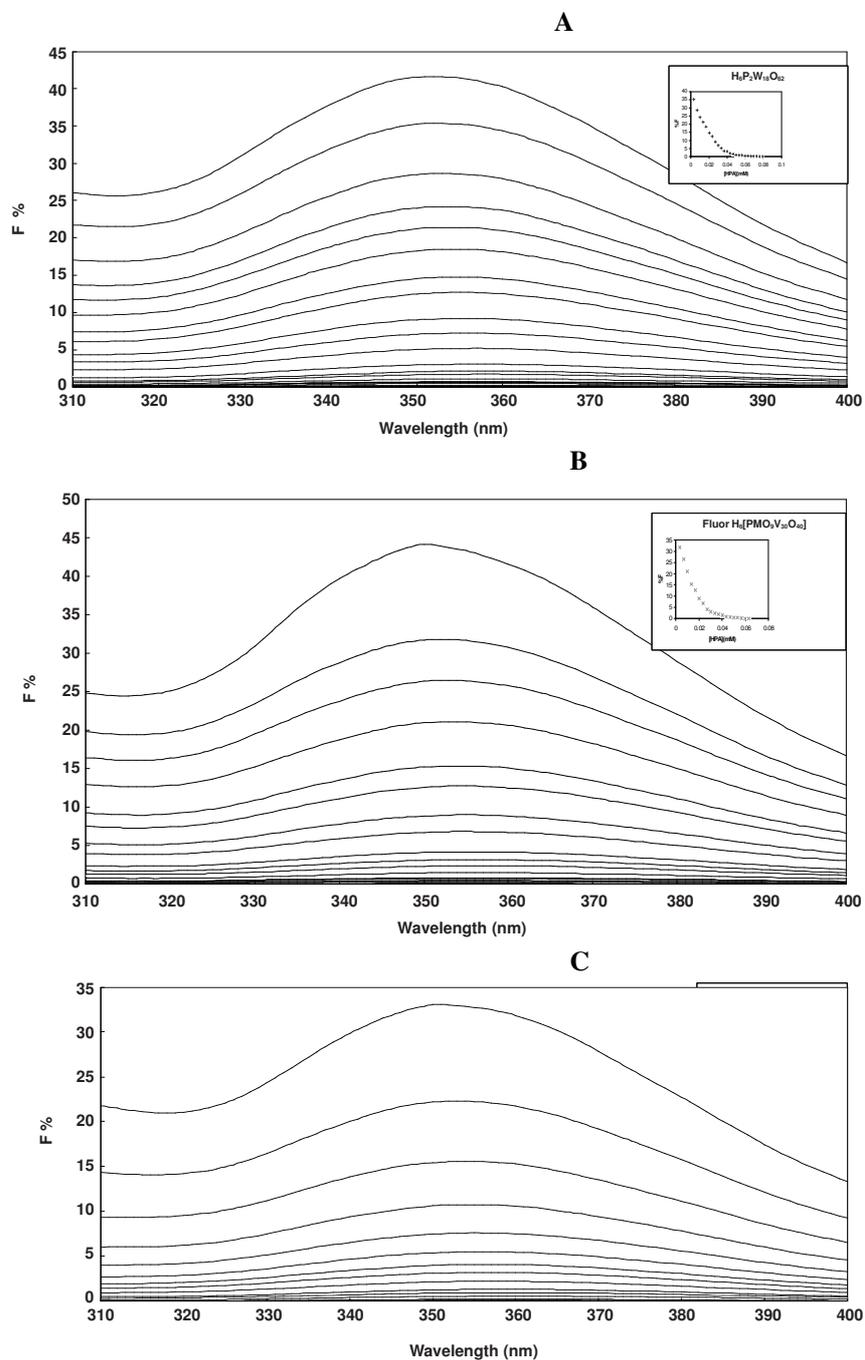


Fig. 3. Fluorescence spectra of α -lactalbumin as a function of heteropoly acids concentration ($\lambda_{\text{Excitation}} = 280$ nm and $\lambda_{\text{Emission}} = 351$ nm) and plots of fluorescence vs. total concentration of heteropoly acids {(A) $H_6P_2W_{18}O_{62}$, (B) $H_6[PMO_9V_3O_{40}]$, (C) $H_{14}[NaP_5W_{30}O_{110}]$ }, at HCl (20 mM, pH = 3)

TABLE-1
CALCULATED THERMODYNAMICS PARAMETERS FOR INTERACTION BETWEEN
 α -LACTALBUMIN (α -LA) WITH HETEROPOLY ACIDS (HPAs)

HPAs	$\Delta G(\text{H}_2\text{O})$ (kJ/mol)	m-value (kJ/mol M)	C_m (mM)
$\text{H}_4\text{SiMo}_{12}\text{O}_{40}$	15.585	486.925	0.030
$\text{H}_3\text{PW}_{12}\text{O}_{40}$	17.577	282.807	0.056
$\text{H}_5[\text{PMo}_{10}\text{V}_2\text{O}_{40}]$	18.537	486.530	0.034
$\text{Na}_3\text{PMo}_{12}\text{O}_{40}$	19.674	276.754	0.070
$\text{Na}_3\text{PW}_{12}\text{O}_{40}$	19.927	378.546	0.048
$\text{H}_4[\text{PMo}_{11}\text{VO}_{40}]$	20.172	388.916	0.048
$\text{H}_6\text{P}_2\text{W}_{18}\text{O}_{62}$	20.567	543.684	0.036
$\text{Na}_2\text{HPW}_{12}\text{O}_{40}$	21.189	331.960	0.060
$\text{H}_3\text{PMo}_{12}\text{O}_{40}$	21.847	371.003	0.056
$\text{H}_6[\text{PMo}_9\text{V}_3\text{O}_{40}]$	26.857	680.994	0.038
$\text{H}_{14}[\text{NaP}_5\text{W}_{30}\text{O}_{110}]$	28.532	1E+06	0.024

heteropoly acids. It is attributed to the structure and exclusive properties of this anion. $[\text{NaP}_5\text{W}_{30}\text{O}_{110}]^{14-}$, so-called Preyssler's anion has formed by five PW_6O_{22} units arranged in a crown (Fig. 5). The Preyssler's anion, is one of the largest known polyanions and so far there is no report on the behaviour of Preyssler's anion in the presence of proteins. This heteropolyacid is remarkable because of the following advantages: (1) strong Bronsted acidity with 14 acidic protons, (2) high thermal stability, (3) high hydrolytic stability (pH 0-12), (4) reusability, (5) safety, (6) quantity of waste, (7) separability, (8) corrosiveness, (9) high oxidation potential and (10) greenness. The oval shape of this anion and 14 acidic protons is responsible for this behaviour, rather than the spiral and lower acidic protons of other heteropoly acids.

Thermodynamic analysis of α -lactalbumin: Figs. 2 (A, B, C) and 3 (A, B, C) show the sigmoidal curves (drawn by a numerical analysis method, called cubic-spline in the MATLAB program, version 6.1) for the denaturated α -lactalbumin state (U) upon the addition of heteropoly acids. Therefore, a two-state analysis based on the pace theory was performed³⁹. It is now possible to obtain equilibrium constants (K) for the U- α -lactalbumin states and to calculate the corresponding Gibb's free energy changes, ΔG , as follows:

$$\Delta G = -RT \ln K = -RT \ln(A_{\text{obs}} - A_U) / (A_N - A_{\text{obs}}) \quad (1)$$

where R is the gas constant, T is the absolute temperature, A_U , A_N and A_{obs} are the physical parameters of absorption of U (denaturated), N (native) and any observed states, respectively. Fig. 4 shows the plot of ΔG against total polyoxometalates (POMs) concentration ($[\text{POM}]_{\text{total}} = [\text{POM}]_{\text{free}} + [\text{POM}]_{\text{bound}}$). The free energies of α -lactalbumin in the absence of polyoxometalates, $\Delta G(\text{H}_2\text{O})$, were calculated by the least-squares method from the following equation:

$$\Delta G = \Delta G(\text{H}_2\text{O}) - m [\text{POM}] \quad (2)$$

where m is the slope of linear curve reflecting the cooperativity and also hydropho-

bicity of the transition state. The m -value correlates very strongly with the amount of protein surface exposed to the solvent upon unfolding³⁹. The $\Delta G(\text{H}_2\text{O})$ and m -values are tabulated in Table-1.

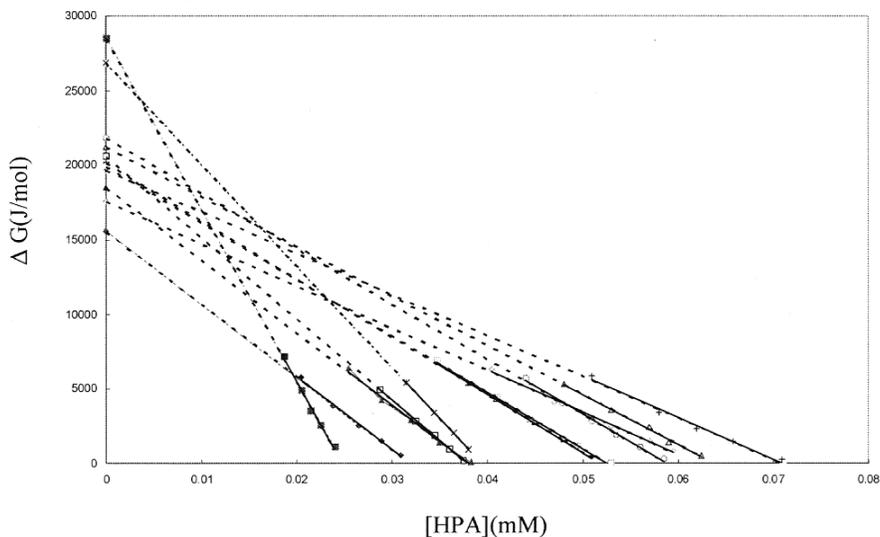


Fig. 4. Free energies values (ΔG) vs. of heteropoly acids (HPAs) including (\square : $\text{H}_6\text{P}_2\text{W}_{18}\text{O}_{62}$) and (\times : $\text{H}_6[\text{PMo}_9\text{V}_3\text{O}_{40}]$), (\blacksquare : $\text{H}_{14}[\text{NaP}_5\text{W}_{30}\text{O}_{110}]$), (\diamond (grey): $\text{H}_3\text{PW}_{12}\text{O}_{40}$), (\blacklozenge : $\text{H}_4\text{SiMo}_{12}\text{O}_{40}$), (\blacktriangle : $\text{H}_5[\text{PMo}_{10}\text{V}_2\text{O}_{40}]$), (\circ : $\text{H}_3\text{PMo}_{12}\text{O}_{40}$), (\square (grey): $\text{Na}_3\text{PW}_{12}\text{O}_{40}$), ($+$: $\text{Na}_3\text{PMo}_{12}\text{O}_{40}$), ($-$: $\text{H}_4[\text{PMo}_{11}\text{VO}_{40}]$), (\triangle : $\text{Na}_2\text{HPW}_{12}\text{O}_{40}$)

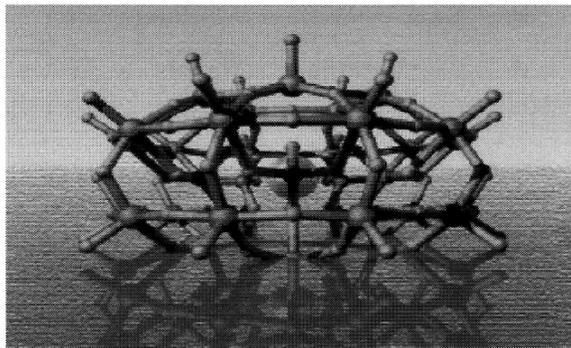


Fig. 5. Structure of Preyssler heteropoly acid

It is apparent from Table-1 that the values of the midpoint concentration (C_m) and m -values of the conformational transitions induced by polyoxometalates are not identical; as the $\Delta G(\text{H}_2\text{O})$ increases, the values of C_m and m decrease and increase,

respectively. It has been reported that anionic surfactants are most commonly used denaturants for proteins at high concentration; and they are responsible of the folded state of some proteins at low concentration^{40,41}. The Preyessler acid shows largest $\Delta G(H_2O)$ and m-value and also lowest C_m (Table-1). Because one of the best criteria for determining of protein stability is $\Delta G(H_2O)$ and m value, this result show that α -lactalbumin is more stable in the presence of Preyessler's anion, while it can be denature with the lowest concentration of this anion at the shortest time.

Conclusion

The results described in this work strongly indicate that the heteropolyacids as ligand play an important role in interaction with proteins. Detailed analysis of the conformational features of protein with interaction of heteropoly acid will be of special importance for understanding the interplay between local and non-local interactions during protein denaturation. Therefore, present results show a direct role of electrostatic interaction to the stability of protein in the presence of various kinds of heteropoly acids.

ACKNOWLEDGEMENTS

The financial supports from the Research Council of the Azad University of Mashhad and Azzahra University are gratefully acknowledged.

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(Received: 27 February 2008;

Accepted: 5 February 2009)

AJC-7204

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