Polyoxometalate Binding to Human Serum Albumin: A Thermodynamic and Spectroscopic Approach

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The molecular recognition of polyoxometalates by human serum albumin is studied using two different polyoxometalates (POMs) at pH 7.5. The results are compared with those obtained at pH 3.5 and 9.0. At pH 7.5, both POMs strongly interact with the protein with different binding behaviors. The Keggin shaped POM, $[H_2W_{12}O_{40}]^{6-}$ (H2W12), specifically binds the protein, forming a complex with a 1:1 stoichiometry with $K_a = 2.9 \times 10^6\ M^{-1}$. The binding constant decreased dramatically with the increase of the ionic strength, thus indicating a mostly electrostatic binding process. Isothermal titration calorimetry (ITC) experiments show that the binding is an enthalpically driven exothermic process. For the wheel shaped POM $[NaP_5W_{30}O_{110}]^{14-}$ (P5W30), there are up to five binding sites on the protein. Increasing the ionic strength changes the binding behavior significantly, leading to a simple exothermic process, with several binding sites. Competitive binding experiments indicate that the two POMs share one common binding site. In addition, they show the existence of another important binding site for P5W30. The two POMs exhibit different binding dependences on the pH. The combination of the experimental results with the knowledge of the surface map of the protein in its N−B conformation transition domain leads to the proposal for the probable binding site of POMs. The present work reveals a protein conformation change upon P5W30 binding, a new feature not explicitly documented in previous studies.

Introduction

Molecular recognition and binding of drugs by the target proteins constitute the first step in the complex mechanism of their biological action. A detailed description of the structural and energetic aspects of these processes is expected to provide a rational basis for the fundamental understanding of the interaction and for the development of efficient therapeutic agents. In the present study, it is shown that the interaction of polyoxometalates with human serum albumin may illustrate this purpose.

Polyoxometalates (POMs) are early transition metal−oxygen anionic clusters which exhibit remarkably rich redox and photochemical properties, ascribed to their ability to act as electron and energy acceptors. These properties have made them attractive for applications in catalysis, materials science, photo- and electrochromism, analytical chemistry, magnetochemistry, and medicine.[1−3] We are interested here in their promising antibacterial, antiviral (particularly anti-HIV), antitumor, and anticancer activities, a field in which a renewal of interest is hoped.[4] Specific POM−protein interaction studies include POM inhibition of HIV reverse transcriptase activity,[4a] POM inhibition of HIV protease,[4b] and ribosomal crystallography with POMs.[4d] Finally, a chemotherapeutic point of view was thoroughly discussed.[4e] POMs may open the way toward new, innovative, and cheap therapeutic strategies for various human diseases. However, the mechanisms of these potential therapeutic effects remain to be described at a molecular level. In this context, the study of the interactions between polyoxometalates and various proteins of biological interest appears as one of the necessary steps toward a fundamental understanding of the biological activity of these chemicals.

Human serum albumin (HSA) is the most abundant protein in the blood plasma (40 mg/mL). It is a relatively large, multidomain protein which, as a major soluble protein constituent of the circulatory system, has many physiological functions. HSA contributes significantly to colloid osmotic blood pressure and to the transport, distribution, and metabolism of many endogenous and exogenous ligands. These ligands include fatty acids, amino acids, metal ions, and numerous hydrophobic and acidic drugs.[5−7] HSA also facilitates the transfer of many ligands across the interface between the circulatory system and organs, such as liver, intestine, kidney, and brain. Because of its abundance and versatile binding properties, HSA is the best studied model to understand the physicochemical basis of drug−protein interactions. Finally, HSA has a dominant role in the drug disposition and efficiency.[8]

In a previous study using the fluorescence quenching of the single tryptophan of HSA,[9] we identified an effective interaction between HSA and two polyoxometalates with different structural characteristics, shown in Figure 1. The same two POMs are studied in the present work. Understanding of molecular recognition processes between ligands and biological macromolecules requires a complete characterization of the binding energetics and correlation of thermodynamic data with the structural properties of the interacting molecules. As a step in this direction, isothermal titration calorimetry (ITC), circular dichroism (CD), and fluorescence spectroscopy are used here

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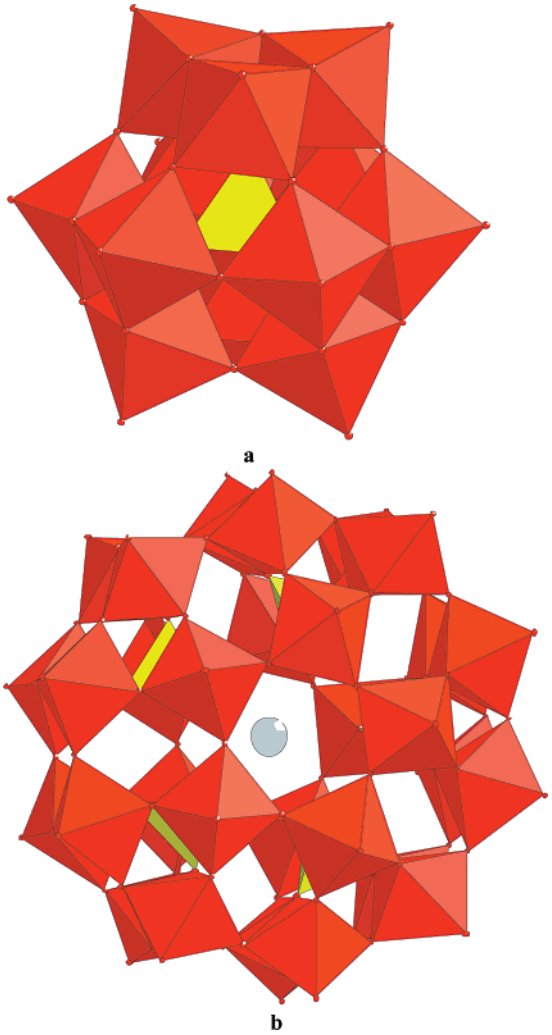

Figure 1. Structure of the polyoxometalates used: (a) the Keggin structure $[H_2W_{12}O_{40}]^{6-}$ (H2W12), (b) the wheel-shaped structure $[NaP_5W_{30}O110]^{14-}$ (P5W30). The relative proportion of molecular sizes was not taken into account, since octahedrals in both structures have the same size.

to obtain a multidisciplinary insight into the molecular and energetic details of the POM−HSA interaction. The data may have a practical interest for the development of new POM-based drugs.

Experimental Section

Materials. Fatty acid free human serum albumin was purchased from Sigma. The samples were dissolved in a buffer solution of pH 7.5 (0.05 M Tris, 0.15 M NaCl), pH 3.5 (10 mM CH_3COOH/CH_3COONa, 0.15 M NaCl), and pH 9.0 (50 mM Tris, 0.15 M NaCl) unless otherwise stated. The concentration of the protein was determined spectrophotometrically using the extinction coefficient $\epsilon_{280} = 36600\ M^{-1}\ cm^{-1}$.[10] All the chemicals were of high-purity grade and were used as purchased without further purification. Ultrapure water with a resistivity of 18.2 MΩ cm was produced by passing through a RiOs 8 unit followed by a Millipore-Q Academic purification set. The two polyoxometalates $[H_2W_{12}O_{40}]^{6-}$ (H2W12) and $[NaP_5W_{30}O110]^{14-}$ (P5W30) were synthesized by the methods in the literature.[11,12] The stability of these POMs in the pH 7.5 medium mainly used in the following was assessed by UV−visible spectroscopy for at least 6 h.

Fluorescence Methods. All fluorescence spectra were recorded on a SPEX-spectrofluorimeter (Jobin-Yvon-Horiba) equipped with a 250 W xenon lamp and a thermostated bath. Quartz cuvettes with 1.0 cm optical path were used. The excitation wavelength was selected at 295 nm to avoid the excitation of tyrosine residues. The UV spectra were recorded at room temperature on a Perkin-Elmer Lamda19 spectrophotometer. All the measurements were performed at room temperature (20 °C) in aqueous solution, unless otherwise stated. Typically, 2 mL of a solution containing an appropriate concentration of HSA was titrated by successive additions of a polyoxometalate solution. Titrations were performed manually by using syringes. At each addition, the fluorescence spectrum was recorded. Raw data were systematically corrected for inner filter effect.[9]

CD Spectroscopy. CD experiments were performed on a Jasco 715 spectropolarimeter equipped with a Peltier temperature control unit. Far-UV spectra were recorded between 200 and 260 nm at 20 °C using 1 mm quartz cells. Samples were dissolved in 10 mM Tris buffer (pH = 7.5). Temperature denaturation curves were recorded between 5 and 95 °C with a temperature increase rate of 1 °C/ min.

Isothermal Titration Calorimetry. ITC experiments were performed using a MicroCal MCS instrument (MicroCal Inc., Northampton, MA). Protein and polyoxometalate solutions were properly degassed prior to the titrations to avoid the formation of bubbles in the calorimeter cell. In a standard experiment, a protein solution (10−30 μM) in the 1.337 mL calorimeter cell was titrated by 40 successive automatic injections of 5−10 μL each of a polyoxometalate solution (generally 10−30 times more concentrated). The first injection of 2 μL was ignored in the final data analysis. Integration of peaks corresponding to each injection and correction for the baseline were carried out using Origin-based software provided by the manufacturer. Fitting the data to various interaction models results in the stoichiometry (n), equilibrium binding constant (K_a), and enthalpy of complex formation (ΔH). The other thermodynamic parameters were calculated according to the formulas

$$\Delta G = \Delta H - T\Delta S \quad (1)$$

and

$$\Delta G = -RT \ln K_a \quad (2)$$

where T is the absolute temperature (in the current experiment $T = 303$ K) and $R = 8.3151\ J\ mol^{-1}\ K^{-1}$.

Results and Discussion

Binding of the Keggin-Type POM, H2W12. We showed in a previous fluorescence study that the Keggin-type polyoxometalate H2W12 can effectively bind to HSA.[9] In the present work, ITC experiments were undertaken in various physicochemical conditions to obtain a detailed thermodynamic characterization of this binding. A representative calorimetric titration profile of 0.30 mM H2W12 with 0.03 mM HSA at pH 7.5 and 30 °C is shown in Figure 2. H2W12 shows a 1:1 binding stoichiometry to HSA with a binding constant of $2.9 \times 10^6\ M^{-1}$, which indicates a strong and specific interaction. This binding constant is several orders of magnitude larger than those obtained in the case of 1:1 complexes between M^+ ($M^+ = Li^+$, Na^+, K^+) and $[SiVW_{11}O_{40}]$.[5−13a] The binding is an exothermic process with a reaction enthalpy of −50.0 kJ/mol. The corresponding standard free energy of binding (ΔG) and the entropy contribution ($T\Delta S$) are −37.8 kJ/mol and − 12.2 kJ/mol, respectively. This means that the binding reaction is enthalpically driven, while the entropy component is unfavorable. We

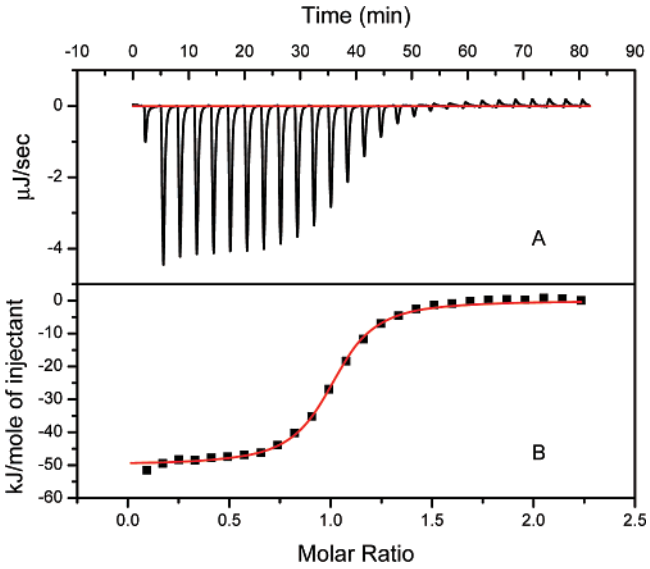

Figure 2. Thermogram and the isotherm corresponding to the H2W12 binding to HSA. Aliquots of a solution of H2W12 (300 μM) were added to 30 μM HSA at 30 °C, in Tris/HCl, 50 mM, pH = 7.5. Each peak in panel A represents a single injection of the POM solution into the protein solution. Panel B shows the plot of the amount of heat liberated per mole of injectant as a function of the molar ratio of the POM to the protein. A standard best nonlinear least-square fit to a single binding site model is shown by the solid red line.

TABLE 1: Binding Parameters of the H2W12 Binding to HSA at pH 7.5 at Different Ionic Strengths

I (M)	n	K_a (10^4 M^{-1})	ΔH (kJ/mol)	$T\Delta S$ (kJ/mol)	ΔG (kJ/mol)
0	1.0 ± 0.1	290 ± 24	−50.0 ± 0.3	−12.2 ± 0.5	−37.8 ± 0.2
0.15	1.1 ± 0.1	8.6 ± 0.8	−70.6 ± 0.5	−42.0 ± 0.7	−28.6 ± 0.2
1.0	1.0 ± 0.1	0.8 ± 0.2	−71.4 ± 0.2	−49.1 ± 0.4	−22.3 ± 0.1

expected a more favorable entropy contribution resulting from the increase of water entropy upon polyanion dehydration concomitant with the POM binding to the protein. However, it must be reminded that plenary Keggin POMs have been shown to be hardly solvated in aqueous media.[13b,c,d] As a consequence, the dehydration entropy change should be minimal.

The strong exothermicity observed in the binding process suggests the involvement of electrostatic interactions, while the unfavorable entropy term suggests that the complexation is accompanied by an increase in the degree of order.

Biological interactions take place in an ion-rich environment, usually constituted by 150 mM Na(K)Cl. To test the biological viability of the polyoxometalate/HSA interactions, titration experiments were carried out at higher ionic strength. In addition, the salt sensitivity of the reaction affinity may shed light on the nature of the forces involved in the binding.[14] Therefore, ITC experiments were performed at three different ionic strengths with H2W12. The results are summarized in Table 1. With the increase of ionic strength, the binding affinity decreased dramatically, as reflected by the decrease of the value of K_a, whereas the binding stoichiometry remains equal to 1. Between 0 and 0.15 M NaCl, the binding affinity decreases more than 30 times, and an additional 10-fold reduction is observed for 1.0 M NaCl. The energetic analysis shows that between 0 and 0.15 M NaCl, the decreased affinity is determined by the unfavorable entropic variation overcoming the favorable variation of the enthalpy. From 0.15 to 1.0 M NaCl, the decrease in affinity is again due to a further decrease of the negative entropy term. The slope of a double logarithmic plot of log(K_a) against log [Na^+] was −1.38, which is generally equivalent to the number of counterions released upon the drug binding.[15] H2W12 has six free negative charges per molecule and thus can strongly interact with the positively charged sites available on the protein. The salt components in the buffer may bind to the charged amino acid residues of the protein, resulting in a decrease of the binding affinity between the POM and the protein. Also, formation of ion pairs between POMs and cations in the buffer might lead to the same result, a rather unlikely possibility in the case of H2W12.[13]

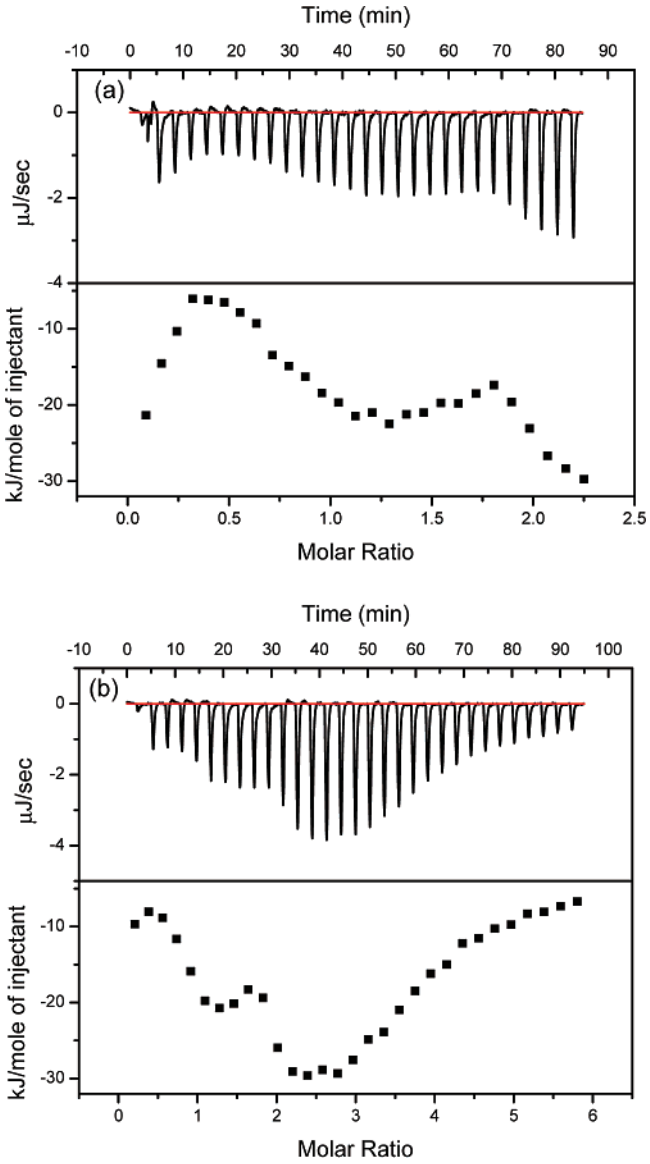

Figure 3. ITC results of HSA titration with P5W30: (a) a solution of 30 μM HSA was titrated with P5W30 (300 μM); (b) a solution of 17 μM HSA was titrated with P5W30 (400 μM).

The results suggest that the binding is almost purely due to an electrostatic interaction.

Binding of the Wheel-Shaped P5W30. The wheel-shaped polyoxometalate P5W30 has been shown, in our previous fluorescence study, to exhibit a quite different binding behavior toward HSA when compared with H2W12.[9] One of the main differences appears in the upward curvature of the Stern−Volmer plot for the fluorescence quenching by P5W30 while a straight line was obtained with H2W12. The ITC analysis of the HSA−P5W30 system gives a further characterization of this difference. Figure 3a shows the result of the titration of HSA (30 μM) with P5W30 (300 μM). The binding of P5W30 is a

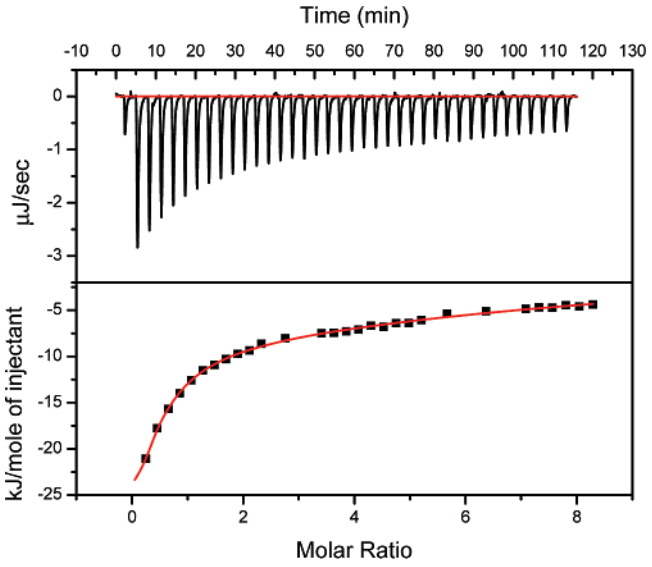

Figure 4. Titration of HSA (18 μM) with P5W30 (600 μM) in 1.0 M NaCl.

multiphase process. At this relatively low polyoxometalate concentration, the protein binding sites are not totally saturated. We have repeated the experiment using a higher polyoxometalate concentration (400 μM) and lower protein concentration (17 μM) (Figure 3b). Two new binding sites are revealed, having a lower affinity and a similar reaction enthalpy. However the binding isotherm is too complex for any accurate quantitative characterization of the thermodynamic properties of each binding site. The different sites have distinct affinity and reaction enthalpies, meaning that individual steps can be distinguished. We noted a superimposed endothermic process during the first steps of the titration.

The ionic strength dependence of the P5W30 binding to HSA is also more complicated than that of H2W12. Figure 4 shows the titration of HSA with P5W30 at an ionic strength of 1.0 M NaCl. The binding process turned out to be a simpler exothermic process. The data can be fitted by a model with several independent binding sites with similar affinity and negative enthalpy changes. Whatever the number of binding sites used for the fitting, the affinity constant is low, and falls on the order of 10^5 M^{-1}. For example, considering two possible binding sites gives values of n1 = 0.98 and n2 = 1.23. The reasons for this undetermined number of binding sites are not clear at present.

Competition between the Two POMs for HSA Binding. The binding of one POM in the presence of the other was studied in order to assess if these species share a common binding site on the protein. The outcome of such experiments is expected to reveal the relative affinity of the POMs for the protein in the presence of each other and to provide information on the nature of the binding sites themselves. Figure 5 shows the ITC profiles obtained for the binding of H2W12 to HSA in the presence of P5W30 and vice versa. The POM−HSA complex in the cell was prepared in a 2:1 molar ratio.

In the presence of P5W30, there is no binding pattern revealing the presence of H2W12, indicating that P5W30 blocks the binding site of H2W12 on the protein (Figure 5a). Several possible rationales exist for the binding inhibition of H2W12 in the present experiments: (i) the same site can also accommodate P5W30. In this case, it can be concluded that the binding affinity of P5W30 for this site should be larger than that of H2W12 or at least on the same order of magnitude; (ii) as indicated by the CD spectra published previously for the HSA−P5W30 system,[9] the binding of P5W30 induces a partial

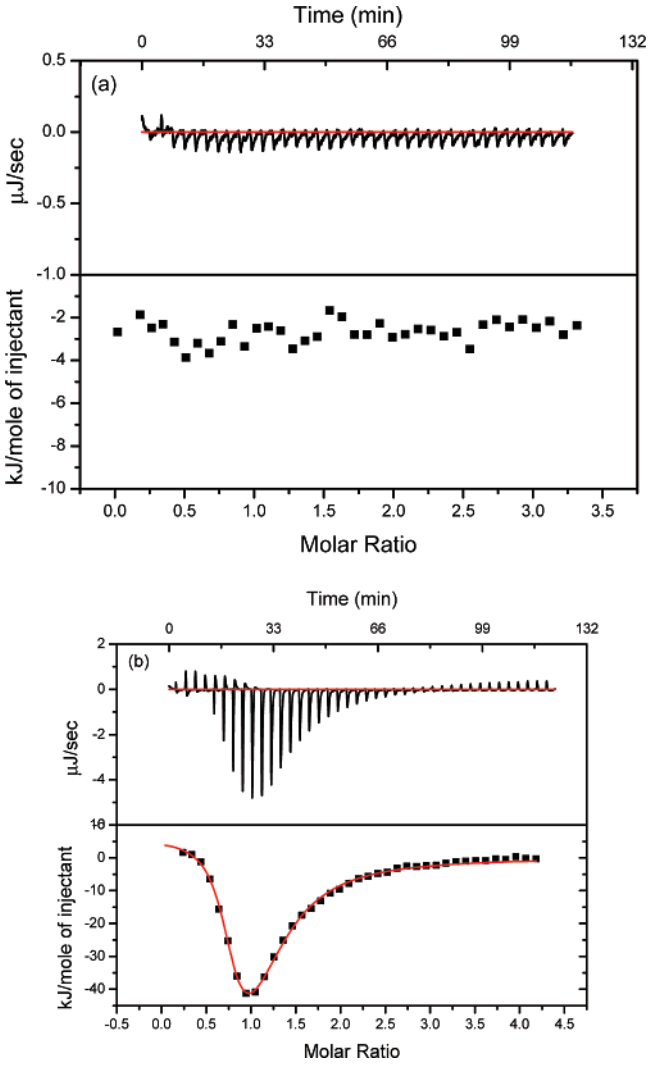

Figure 5. ITC profiles for the titration (a) of 30 μM HSA and 60 μM of P5W30 with 300 μM H2W12; (b) of 20 μM HSA and 40 μM H2W12 with 200 μM P5W30.

denaturation of the protein, resulting in a perturbation of the H2W12 binding site. Separately or taken together, these two effects might prevent H2W12 from binding to HSA in the presence of P5W30; (iii) mere steric hindrance due to the presence of P5W30 might impede the accessibility of H2W12 to its binding site.

The experiment starting with a 2:1 mixture of H2W12:HSA was also performed. In the presence of H2W12, the binding behavior of P5W30 changes significantly (Figure 5b). However, the binding of H2W12 does not entirely block the binding of P5W30 to the protein. At the beginning of the experiment, there is an endothermic process. This behavior agrees with the superimposed endothermic process observed during the binding of P5W30 on its own. We have noted that the background ITC curves of the two POMs in the absence of HSA are constituted by small positive signals, as observed in Figure 2 (at molar ratio > 1.5) and Figure 5 (at molar ratio > 1). These endothermic processes may be due to the enthalpy of diffusion and dilution of the POMs. A two-site fit may by made, one with higher affinity and positive enthalpy and the other with a lower affinity and a negative enthalpy (Figure 5b). However, lack of complementary data impedes any reliable speculation on the significance of the values that could be extracted from this fitting.

In the competition experiments, the thermograms changed, indicating that there exists an obvious overlap for binding sites

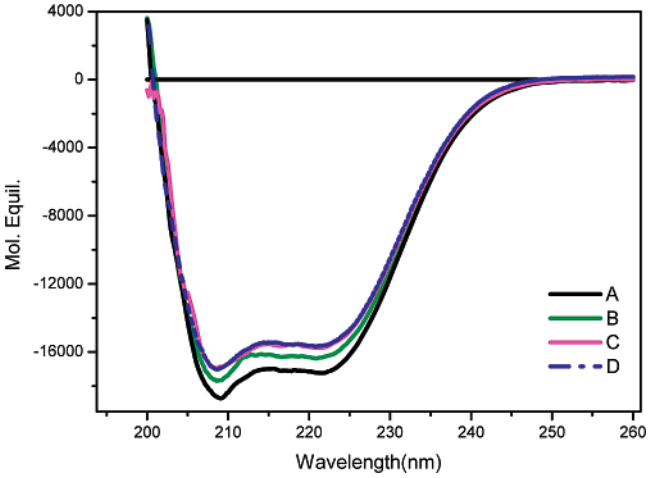

Figure 6. CD spectra of HSA prebound with H2W12 in the absence and in the presence of different concentrations of P5W30: (A) HSA 10 μM, H2W12 20 μM; (B) HSA 10 μM, H2W12 10 μM, P5W30 1 μM; (C) HSA 10 μM, H2W12 10 μM, P5W30 2 μM; (D) HSA 10 μM, H2W12 10 μM, P5W30 10 μM.

of the two POMs. In short, the binding site that accommodates H2W12 can also accommodate P5W30. Since there is only one binding site for H2W12, the initial binding of P5W30 entirely blocks the binding of H2W12. Alternatively, the binding of P5W30 is also affected by the previous binding of H2W12, without being entirely suppressed. At least two binding sites remain. In the absence of prebound H2W12, there are about six binding sites for P5W30. As H2W12 can only occupy one of the sites and that binding does not change the secondary structure of the protein,[9] it might be inferred that five binding sites should remain left for P5W30. However, experimental results do not support this conclusion. Altogether, these observations and their consequences shed some light on the binding processes of POMs to HSA and lead to the following conclusions. Binding of P5W30 partially unfolds the protein and makes other binding sites appear. In the process, the most important site is the H2W12 binding site, which can be termed a "gate site". Occupancy of this site by P5W30 opens the gate for other P5W30 binding sites by unfolding the protein. However, if this site is first occupied by H2W12, the gate is closed since binding of H2W12 does not change the structure of the protein. In support of this conclusion, it should be reminded that Figure 5b begins with an endothermic process which might be assigned to the partial denaturation of the protein. This may lead to a moderate unfolding of the protein upon binding of P5W30 to HSA prebound to H2W12. The results of CD experiments on the binding of P5W30 to HSA prebound to H2W12 are shown in Figure 6 and clearly support this view. In agreement with this hypothesis, the unfolding is small and stops when the molar ratio ([P5W30]/[HSA]) reaches 0.2. Such unfolding is probably induced by the binding of P5W30 to a pre-existing site, as no unfolding is detected in the presence of H2W12. It may be inferred from this observation that there are two main binding sites for P5W30 on HSA in the absence of H2W12, both having close affinities for the POM.

Influence of the pH on the Binding of Polyoxometalates to HSA. HSA is an acidic protein ($pH_i \sim 5.9$) with a significant excess of negatively charged residues at neutral pH. The disruption of salt bridges or the protonation of carboxylate groups (aspartate and glutamate) at lower pH may lead to a significant change in the charge distribution on the protein surface and to a modification of the interaction with charged species.[16,17] We therefore explored the pH effect on the

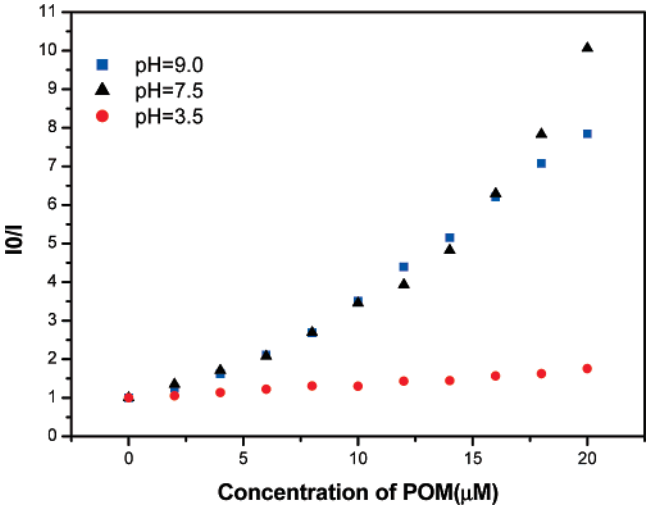

Figure 7. Stern−Volmer plot of fluorescence quenching of HSA (10 μM) by P5W30 at different pH values.

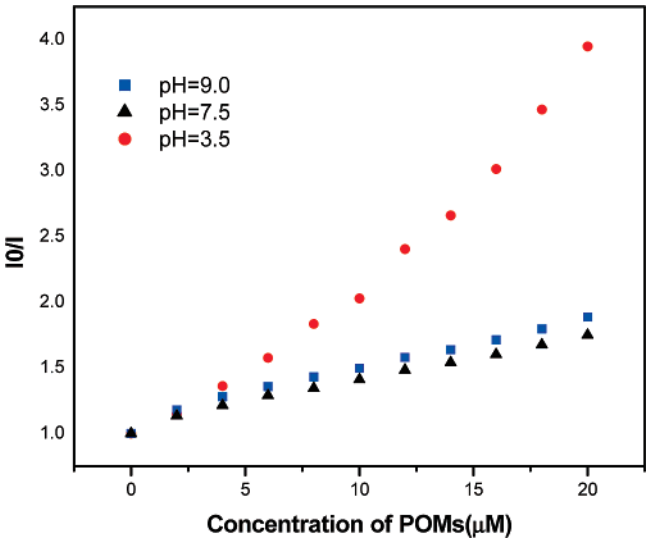

Figure 8. Stern−Volmer plot of fluorescence quenching of HSA (10 μM) by H2W12 at different pH values.

molecular interaction between polyoxometalates and HSA, even though some of the encountered situations may not correspond to real physiological conditions.

HSA has just one tryptophan (Trp) residue which constitutes a useful probe for studying the interaction between a ligand and the protein.[5−7,18,19] In a previous study, we have observed significant Trp fluorescence quenching upon POM binding in native conditions.[9] Therefore, fluorescence quenching is selected as the reference phenomenon to study the pH effect. The Stern−Volmer plots of the quenching process at different pH values are shown in Figures 7and 8 for P5W30 and H2W12, respectively. For both polyoxometalates, the quenching profile is almost unchanged when the pH increases from 7.5 to 9. In contrast, when the pH decreases to 3.5, the two polyoxometalates show totally different behaviors. For P5W30, the quenching constant decreased significantly, while for H2W12 the quenching was greatly enhanced.

The observed difference in the Stern−Volmer plots for the two POMs could be attributed to the difference in their behaviors in the pH 3.5 medium and/or to the behavior of the protein as a function of the pH. The extent of the changes suggests that the observed behavior may be assigned to the protein itself. As a matter of fact, HSA is known to undergo different pH-

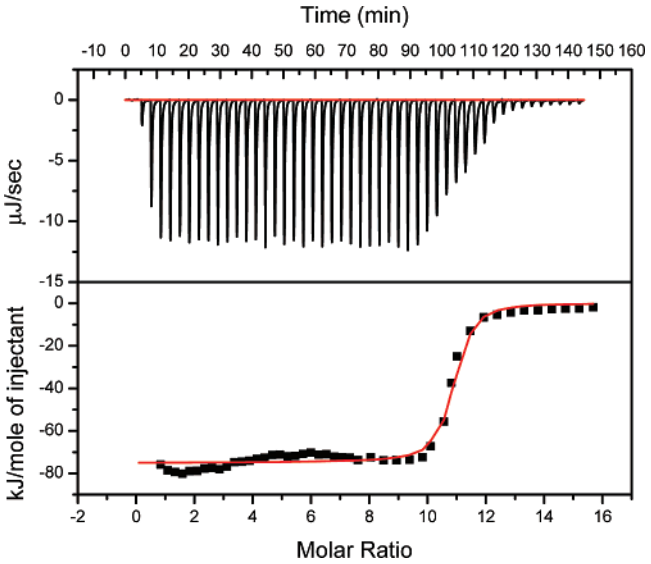

Figure 9. Titration of HSA (12 μM) with H2W12 (800 μM) at pH 3.5.

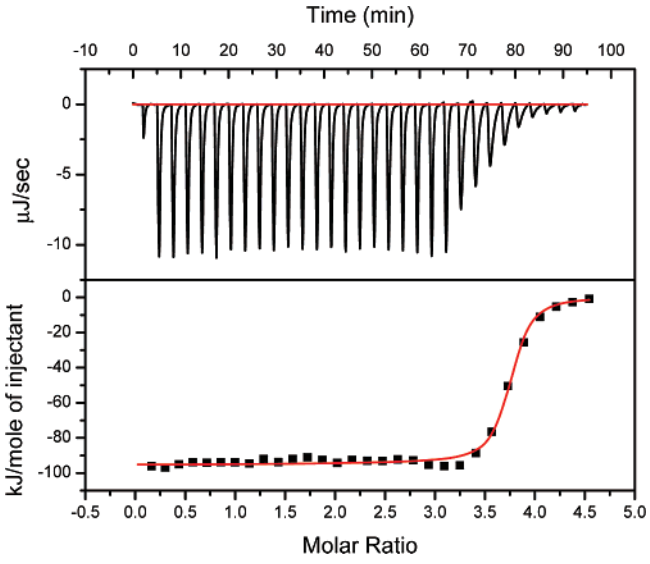

Figure 10. Titration of HSA (20 μM) with P5W30 (360 μM) at pH 3.5.

dependent conformational transitions:[7,20,21] the N−F transition between pH 5.0 and 3.5 and the F−E transition between pH 3.5 and 1.2, both affecting the secondary and tertiary structures. The N−F transition is characterized by α helix → β sheet and α helix → coil transition. Further unfolding is observed in the F−E transition until the molecule is expanded to its full extent. Another conformational change is obtained in the N−B transition between pH 7.0 and 9.0. The quasi-invariance of the Stern−Volmer plots for both POMs between pH 7 and 9 is an expected behavior. In contrast, and of particular interest in the present context, the N−F transition involves unfolding and separation of the (sub)domain III from the rest of the molecule without significantly affecting the remaining part of the molecule.[22,23] Such conformational change may substantially modify the charge distribution across the protein surface and hence lead to the observed phenomena. ITC experiments are expected to shed more light on these behaviors and to support or rule out such conclusion.

At pH 3.5, the binding affinity of H2W12 proved to be much higher than that at pH 7.5, and about 11 binding sites with equivalent affinity were detected, as shown in Figure 9. Unfolding of the protein might make available new binding sites for H2W12. In short, the specificity of the binding site of H2W12 is lost as a consequence of the protein conformation change. In contrast, there were only 4 equiv binding sites available for P5W30 in the same conditions, as shown in Figure 10.

The detailed thermodynamic parameters of the binding processes with the two POMs at pH 3.5 are given in Table 2. It appears that the binding affinity (K_a) for H2W12 increased significantly, whereas the other thermodynamic parameters hardly change. The results are in agreement with fluorescence quenching data. For P5W30, the affinity at this pH value is high (in the range of 10^7 M^{-1}) and the large value of enthalpy change indicates a very strong electrostatic interaction between the POM and the protein. The decrease of the quenching constant of P5W30 at this pH value may be due to an increase in the distance between the bound P5W30 and the Trp214 residue upon unfolding of the protein, thus decreasing the efficiency of energy transfer between P5W30 and the protein.[9]

Mode of Interaction of the POMs with HSA. The results described in this work indicate that the POM−HSA interaction is mainly electrostatic in nature, even though interference of other kinds of forces cannot be discarded. However, the present data may tentatively be interpreted in terms of the large difference in structure, size, and charges of the two compounds. Any influence of the structure must be postponed until a larger number of structurally different POMs have been used in these experiments. Therefore, the following proposal takes mainly into account the size and charge of the two POMs studied so far. The diameter of H2W12, a typical Keggin structure, is about 1 nm and the polyanion carries 6 negative charges. The cluster P5W30 carries 14 negative charges. Its lateral dimensions are 2.4 × 1.8 × 1.8 (nm^3) giving roughly 2.0 nm mean spherical diameter.[24] The size of P5W30 is therefore two times larger than that of H2W12. The negatively charged polyoxometalates must bind on some positively charged areas on the surface of the protein. The surface map of the protein is shown in Figure 11. Upon close examination, it appears that a cavity with a positively charged inner wall exists and is located roughly in the center of the protein. The width of this cavity is measured

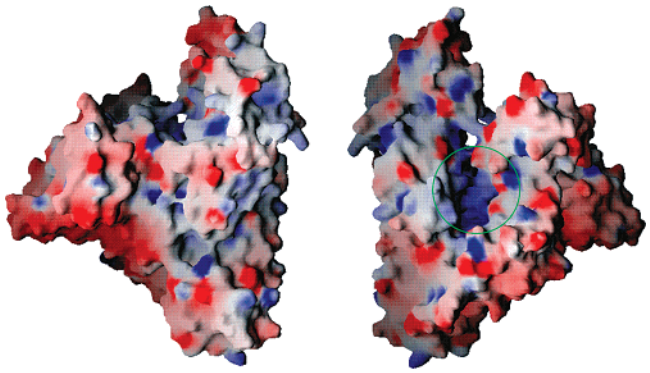

Figure 11. Surface potential of HSA calculated with the GRASP software. The protein is in its N conformation. The two views are related by a 180° rotation about the vertical axis of the sheet. The region in the green circle is the most likely the unique (for H2W12) or initial (for P5W30) binding site for POMs (blue for positively and red for negatively charged areas).

TABLE 2: Thermodynamic Parameters of POM−HSA Binding at pH 3.5

	n	K_a (10^6 M^{-1})	ΔH (kJ/mol)	$T\Delta S$ (kJ/mol)	ΔG (kJ/mol)
P5W30	4.0 ± 0.1	12.8 ± 1.5	−100.0 ± 0.4	−58.8 ± 0.6	−41.2 ± 0.2
H2W12	10.9 ± 01	9.2 ± 0.3	−75.2 ± 0.1	−34.4 ± 0.2	−40.8 ± 0.1

to be 1 nm, which would fit to the diameter of H2W12. Tentatively, it might be hypothesised that this cavity would easily accommodate H2W12. In contrast, any enforced accommodation of P5W30 into this cavity might induce partial unfolding of the protein. Also, as concerns other positively charged domains, there may be some overlap between the cluster and the nearby negatively charged area of the protein, as a consequence of the large size of the cluster. This repulsive interaction might loose and partially unfold the structure of the protein, as observed previously by CD experiments.[9] This conformational change may reveal additional positively charged areas which can bind other P5W30 molecules. As a result, the protein can bind more than five P5W30 clusters. Due to the large size of the cluster, such kind of binding is not expected to be very specific.

These tentative conclusions support the idea that H2W12 binds specifically to an isolated positively charged area of the protein. Such specific binding should not change the structure of the protein since the interaction concerns only a small area of the protein. This behavior can be due to the smaller size of H2W12 and to its smaller negative charge when compared to P5W30.

When the pH value is decreased to 3.5, structural changes of the protein, such as unfolding of domain III and structural rearrangement of domain II, are accompanied by an important modification of its surface charge distribution.[17] There is no negatively charged area on its surface at this pH value. Thus, the negatively charged polyoxometalates can bind on every positively charged area of the protein up to the precipitation of the complex. It is interesting to note that only four binding sites are left for P5W30 on the protein in a pH 3.5 medium. One can argue that, due to the large molecular weight of P5W30, the 4:1 POM−HSA complex is insoluble in the medium, which means that the protein is removed from the solution. This line of reasoning would also support the observation that the protein binds more H2W12 clusters before the precipitation of the HSA−POM complex.

It is worth noting that at pH 3.5, all the protein was precipitated at the end of the titration either with H2W12 or P5W30. In contrast, at neutral pH value, the protein keeps stable in the solution even with six or more bound P5W30. This means that the negatively charged area on the protein may also play a key role in the molecular recognition process.

4. Conclusion

The molecular interaction between HSA and two different polyoxometalates, $[H_2W_{12}O_{40}]^{6-}$ (H2W12) and $[NaP_5W_{30}O_{110}]^{14-}$ (P5W30), was studied by ITC complemented by fluorescence and CD experiments. Both polyoxometalates strongly bind to the protein, mainly by electrostatic interactions. It is suggested that both size and charges of the polyoxometalates play key roles in the molecular interaction process. At neutral pH value, the Keggin type polyoxometalate H2W12 specifically binds on one site of the protein and forms a 1:1 protein−POM complex, whereas the wheel shaped P5W30 binds to more than five sites of the protein and forms a complicated complex with the protein. The binding of H2W12 has almost no effect on the protein structure, in contrast with the binding of P5W30 which destabilizes the protein structure. The binding of either polyoxometalate at an acidic pH value induces the precipitation of the protein.

There is now considerable insight into the bioactivity of POM molecules. The present work reveals a protein conformation change upon POM binding, a new feature not explicitly documented in previous studies.

The results constitute a helpful guide in an attempt to unravel the mechanism of POM−protein interactions. They might also be meaningful in subsequent design of polyoxometalate-based inorganic drugs.

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