Modification of the Thermal Unfolding Pathways of Myoglobin upon Drug Interaction in Different Aqueous Media

Mohammad Arif Cheema,[†,‡] Pablo Taboada,[*,†] Silvia Barbosa,[†] Emilio Castro,[†] Mohammad Siddiq,[§] and Víctor Mosquera[†]

Grupo de Física de Coloides y Polímeros, Departamento de Física de la Materia Condensada, Facultad de Física, Universidad de Santiago de Compostela, E-15782, Santiago de Compostela, Spain, and Department of Chemistry, Quaid-i-Azam University, Islamabad 45320, Pakistan

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In this work, we have analyzed the influence of two structurally related phenothiazine drugs, promazine and triflupromazine hydrochlorides, when bound to myoglobin, a model protein, and how the drug concentration and solution conditions may affect the denaturation process of this protein. In this manner, we derive the thermodynamic quantities of the unfolding process by using a spectroscopic technique such as UV−vis spectroscopy at different drugs concentrations and at pH 2.5, 5.5, and 9.0. To do this, a thermodynamic model was used which included experimental data corresponding to the pre- and post-transition into the observable transition. It has been found that both drugs play a destabilizing role for the protein, at least at low concentrations. In addition, at acidic pH and higher drug concentrations, a stabilizing effect can be observed, which may be related to the formation of some type of protein refolding, subsequent aggregation, or both. The reason for this behavior has been suggested to be the different protein conformations at acidic pH, the increase of solvent-exposed hydrophobic and hydrophilic residues after denaturation and/or binding, and the different strength of drug−protein interactions when changing the solution conditions. For this reason, thermodynamic quantities such as Gibbs energies, ΔG, and entropies of unfolding, ΔS_m, increase as the solution pH increases provided that additional solvent-exposed hydrophobic residues are present, which were previously buried at room temperature. Moreover, the larger binding affinity at pH 9.0 due to enhanced electrostatic interactions between protein and drug molecules (drug and protein differ in their net electrical charge) additionally collaborates to this residue exposition to solvent as a consequence of the alteration of protein conformation as due to drug binding. Comparison of thermodynamic data between promazine and triflupromazine hydrochlorides also shows that drug−protein affinity and hydrophobicity also affect the thermodynamic denaturation parameters.

Introduction

The wide variety of highly specific structures that results from protein folding and that brings key functional groups to proximity has enabled living systems to develop diversity and selectivity in their underlying chemical processes. In addition, to generate biological activity, it is known that folding is coupled to many other biological processes, including the circulation of molecules to specific cellular locations and the regulation of cellular growth and differentiation.[1] Thus, only correctly folded proteins have long-term stability in crowded biological environments and are able to interact selectively with their natural partners.

The marginal stability of the native protein conformation is a delicate balance of various interactions (van der Waals, electrostatic, hydrogen bonds, hydrophobic, and disulfide bridges)[2] and is affected by pH, solvent, temperature, and/or the addition of small molecules such as substrates, coenzymes, inhibitors, and activators that bind especially to the native state and alter this fragile equilibrium. In fact, failure of proteins to fold correctly or to remain correctly folded is the origin of a wide variety of pathological conditions. Therefore, a perfect knowledge of the mechanisms underlying protein folding−unfolding pathways is necessary in order to elaborate adequate strategies to prevent and/or treat these pathologies.

Thus, in the present study, we have examined the nature of the interaction of two selected structurally related drugs, the phenothiazines promazine and triflupromazine hydrochlorides (see Figure 1 for structures), with a model globular protein, myoglobin, by means of optical methods, which are less invasive. We have pursued the aim of analyzing how drug interactions affect the thermal stability of the protein, their influence on the thermodynamic quantities characterizing the unfolding process in the light of an adequate theoretical modelization, and the role of the different forces in the protein stabilization/destabilization upon drug binding when changing the solution conditions. In this way, a simple model describing the protein denaturation process as a transition between two macroscopic states,[3,4] the native state and a denaturated state, was used. This allows us to use valuable thermodynamic information of the temperature-induced denaturation process, as the Gibbs, ΔG, enthalpy, ΔH_m, and entropy, ΔS_m, of unfolding, and the temperature at the midpoint of the transition, T_m.

* To whom correspondence should be addressed. E-mail: fmpablo@usc.es.
† Universidad de Santiago de Compostela.
‡ Permanent address: Quaid-i-Azam University.
§ Quaid-i-Azam University.

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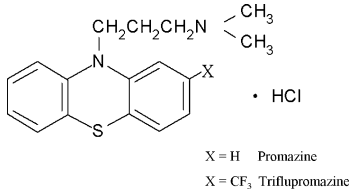

Figure 1. Structure of phenothiazine drugs.

The interest on phenothiazine drugs has being increased in the past years since, apart from their use as tranquillizer blocking dopamine receptors,[5] they have being proved useful to fight against multidrug-resistant bacteria,[6] in the treatment of Creutzfeldt−Jacob disease,[7] as inhibitors of HIV-1 viral replication cycle,[8] as modulators of anticancer drug transport,[9] and as RNA scaffolds.[10] It is also known that these drugs exert their activity by interaction with biological membranes. In addition, the self-aggregation process and the physicochemical properties of these two drugs in solution have been characterized very recently.[11−14] On the other hand, myoglobin is one of the most used proteins as a model to check binding capacity and affinity and to reveal possible structural changes in its native conformation due to its peculiar structure and function. It is a monomeric heme protein found mainly in muscle tissue where it serves as an intracellular storage site for oxygen.[15] It possesses an unusual secondary structure containing a high proportion of α helix (75%), with the heme prosthetic group inserted into a hydrophobic cleft in the protein.

Experimental Section

Materials. Horse skeletal muscle myoglobin (100684-32-0; 0.30% iron content), promazine (*N*,*N*-dimethyl-3-(10H-phenothiazine-10-yl)propan-1-amine HCl), and triflupromazine (*N*,*N*-dimethyl-3-[2-(trifluoromethyl)-10H-phenothiazine-10-yl]-propan-1-amine HCl) hydrochlorides were obtained from Sigma Chemical Co. The protein was used after a further purification by size exclusion chromatography using a Superdex 75 column equilibrated with 0.01 M phosphate buffer at pH 7. Experiments were carried out using double distilled, deionized, and degassed water. The buffer solutions used were glycine + HCl ($I = 0.01$ M) for pH 2.5, sodium monophosphate-sodium diphosphate for pH 5.5 ($I = 0.01$ M), and glycine + NaOH for pH 9.0 ($I = 0.01$ M), respectively. Horse myoglobin was dissolved in each buffer solution and dialyzed extensively against proper buffer. Protein concentration was determined spectrophotometrically, using a molar absorption coefficient of 1.71×10^5 M^{-1} cm^{-1}.[16]

Difference Spectroscopy. Difference spectra were measured by using a Beckman spectrophotometer (Model DU 640), with six microcuvettes, which operates in the UV−visible region of the electromagnetic spectrum wavelength. All measurements were made using myoglobin solution with a fixed concentration of 0.5 g dm^{-3} in carefully matched quartz cells (50 μL capacity). For absorbance difference spectra, just two cells were used. The first microcuvette contained only protein in the corresponding medium and was used as the blank reference. Meanwhile, the other cuvette was filled with the corresponding protein−drug solution. The microcuvettes were filled and placed in the same orientation for all tests. Measurements were made after protein and drug had been incubated for 1 h, after which the difference spectra did not change. For absorbance measurements with temperature variation, a Beckman Du Series Peltier temperature controller was used in the range 20−100 °C. On the other hand, difference spectra were taken in the Soret band maximum (approximately 409 nm), which results mainly from the interaction of the heme moiety with a well-defined tertiary structure and, thus, can be used to monitor protein unfolding.[17] In this region, the drug absorption can be considered negligible in the concentration range analyzed.

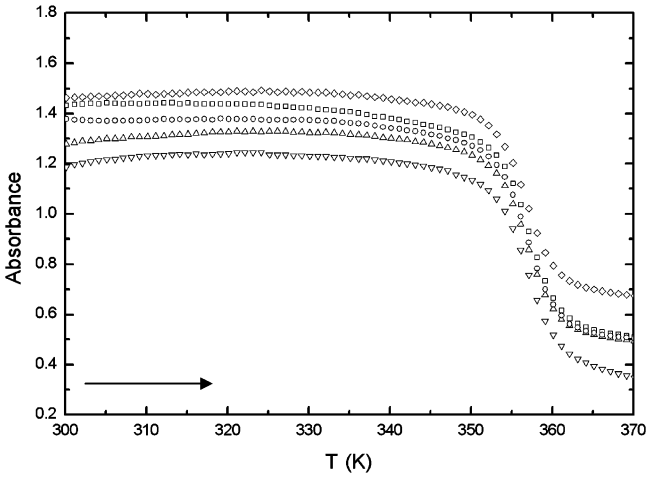

Figure 2. Thermal unfolding curves for 0.5 g dm^{-3} of myoglobin using the differences in absorbance at 409 nm at pH 9.0 in the presence of promazine concentrations of (◇) 1.0×10^{-5} M; (□) 7.5×10^{-5} M; (○) 3.5×10^{-4}, (△) 1.0×10^{-3}; and (▽) 5.0×10^{-3} M. The arrow indicates the direction of heating.

Results and Discussion

Plots of the absorbance of horse myoglobin at 409 nm as a function of temperature in the presence of different promazine concentrations at pH 9.0 are shown in Figure 2. Similar plots have been obtained for the rest of concentrations, drug, and pH solutions. The data show that there is a transition region over which the absorbance changes with temperature. Thus, for our systems under study, a two-state mechanism was assumed in order to evaluate the thermodynamic parameters obtained from spectroscopic techniques since reversibility of the transition can be ensured if final temperature is not excessively high.[18] In this regard, after thermal denaturation, the protein solution was cooled to room temperature to measure the absorbance and to compare with that of the unheated sample. The concordance of absorbance values was taken as a measure of the reversibility of the heat-induced denaturation. The thermal transitions were reversible and hence amenable to thermodynamic analysis, although at acidic pH, since the protein is already partially destabilized; as commented below, some variation in absorbance values was detected.

From a thermodynamic point of view, the protein denaturation process can be described as a transition between two macroscopic states,[3,4] that is, from the native state (N) to a denaturated state (D)

$$N \Leftrightarrow D \tag{1}$$

The stability of a globular protein is usually quantified with Gibbs energy values, ΔG, since ΔG is the work required for disruption of the native protein structure. For that reason, the difference in Gibbs energy at a given temperature can be expressed by the Gibbs−Helmholtz equation:

$$\Delta G(T) = \Delta H_m(1 - T/T_m) - \Delta C_p(T_m - T + T\ln(T/T_m)) \tag{2}$$

where T_m refers to the temperature at the midpoint of the transition ($T_m = T$ at $\Delta G = 0$), ΔH_m is the enthalpy of denaturation at T_m, and ΔC_p is the heat capacity change between the folded and the unfolded conformations.

The evaluation of thermodynamic parameters obtained from spectroscopic techniques is based on the equilibrium constant K for a transition between the native and the denaturated states. The equilibrium constant was deduced from the equation

$$K = \frac{[\text{unfolded}]}{[\text{native}]} \tag{3}$$

or as a function of spectroscopic parameters:

$$K = \frac{A_N - A_{OBS}}{A_{OBS} - A_D} \tag{4}$$

where A_N is the absorbance corresponding to the pure native state, A_D the absorbance corresponding to the pure denaturated state, and A_{OBS} the absorbance at any point of the observable transition at a certain temperature.

To avoid large errors in the estimation of thermodynamic parameters, the procedure created by Kaushik et al. was followed.[18] We have used the experimental data points and fitted them to the equation in the following way:

$$A_{OBS} = \frac{A_N + KA_D}{1 + K} \tag{5}$$

The equilibrium constant can be expressed by a Gibbs energy function:

$$K = \exp(-\Delta G/RT) \tag{6}$$

where R is the gas constant and T is the running temperature. By substituting the value of K in eq 5, the following equation is obtained:

$$A_{OBS} = \frac{A_N + A_D\, e^{-\Delta G/RT}}{1 + e^{-\Delta G/RT}} \tag{7}$$

Finally, substituting the $\Delta G(T)$ expression for eq 2, we derive:

$$A_{OBS} \times \frac{A_N + A_D \exp\left[-\frac{1}{R}\left(\Delta H_m\left(\frac{1}{T} - \frac{1}{T_m}\right) - \Delta C_p\left(\frac{T_m}{T} - 1 + \ln\left(\frac{T}{T_m}\right)\right)\right)\right]}{1 + \exp\left[-\frac{1}{R}\left(\Delta H_m\left(\frac{1}{T} - \frac{1}{T_m}\right) - \Delta C_p\left(\frac{T}{T_m} - 1 + \ln\left(\frac{T}{T_m}\right)\right)\right)\right]} \tag{8}$$

Pre- and post-transition baselines were extrapolated to the observable transition zone, and the relative concentrations of native and denaturated protein were determined from the distances between observed and extrapolated spectral values. For proper evaluation of fitting errors, terms for baselines should be included in any equation used to fit the spectroscopic data.[4] Since A_N and A_D have been found to be linear functions of temperature, they can be written as

$$A_N = a_N + m_N T \tag{9}$$

$$A_D = a_D + m_D T \tag{10}$$

Finally, the difference in Gibbs free energy between folded and unfolded conformations, ΔG, depends mainly on the first term at temperatures near T_m. The second term becomes important at temperatures below T_m.[19] By plotting ΔG against temperature, the temperature of maximum stability can be obtained by means of

$$T_S = T_m \exp(-\Delta H_m/T_m \Delta C_p) \tag{11}$$

Figure 3 shows the dependence of the unfolding temperature, T_m, of horse myoglobin as a function of drug concentration under the different solution conditions studied. T_m was calculated by fitting the experimental absorbance difference to eq 8. The numerical analysis of the data was made by means of the Levenberg−Marquardt least-squares fitting algorithm.

For promazine, unfolding temperature exhibits different patterns depending on solution pH since both the protein conformational state and the drug binding affinity for the protein change with solution conditions[20] (isolectric point of myoglobin 7.2).[21] In acidic and alkaline media, myoglobin undergoes a transition to a denaturated form, occurring in the pH range 4.0−4.5 for acidic media and at pH 11.5−12 in the alkaline range,[22−23] and both characterized by distortions of tertiary and secondary structures and certain dissociation of the heme group into the solution.[16,17] In addition, a recently discovered destabilized and extended form of myoglobin (called E state) was observed around pH 3.0, with a distinctive organized core.[25] For this state, heme dissociation is not full, with the possibility of establishing nonspecific interactions between the heme and the different folded and unfolded states of the apoprotein[25] or a four-coordinate heme remaining associated to the globular protein in spite of the loss of the iron−histidine linkage,[22] with the regions losing secondary structure not associated with the heme group.[23] Despite these facts, it is clear that the population of myoglobin with disordered and dissociated hemes is larger at acidic pH as a consequence of the titrations of the proximal and distal histidine residues inside the heme pocket[26] and the stability of the holoprotein being determined by heme affinity.[27]

In the light of the above comments, it is not surprising that the profile at pH 2.5 is the most different since the original state of the protein prior to thermal destabilization is not the native state but the E-extended one. As a consequence of the already disturbed protein structure, the unfolding temperatures reached the lowest values at acidic pH, with a decreasing profile up to drug concentrations around 1 mM. This indicates that the drug is acting as a further structure destabilizer and needs less thermal energy to additionally break the protein structure. However, at higher drug concentrations, T_m starts to increase, which points out that, in this case, temperature is acting as a structure stabilizer. This fact can be related to the early formation of little protein−drug complex clusters at the largest drug concentrations used in the present study,[20,28] which can display a gain in ordered protein structure[29] and also result in a protection against additional protein thermal denaturation. At the two highest drug concentrations analyzed (0.0075 and 0.01 M), thermodynamic analysis was not performed since reversibility of the unfolding process was not reached. Moreover, it is necessary to bear in mind that at acidic pH both the protein and the drug are positively charged, so the protein−drug interaction should be predominantly hydrophobic since ionic sites should be, at least, partially neutralized.[20]

On the other hand, myoglobin is in its native state at pH 5.5, which involves an increase in the absolute T_m values. At this pH, the protein and drug still possess the same net electrical charge; then, hydrophobic interactions should also be predominant.[20] The profile of T_m values with drug concentration, in this case, points out a certain reduction of the denaturation temperature at low drug concentrations (destabilizing effect), and T_m remains almost constant at higher drug concentrations. A similar profile has been also found for pH 9.0. At this pH, protein and phenothiazine differ in their net electric charge, so electrostatic

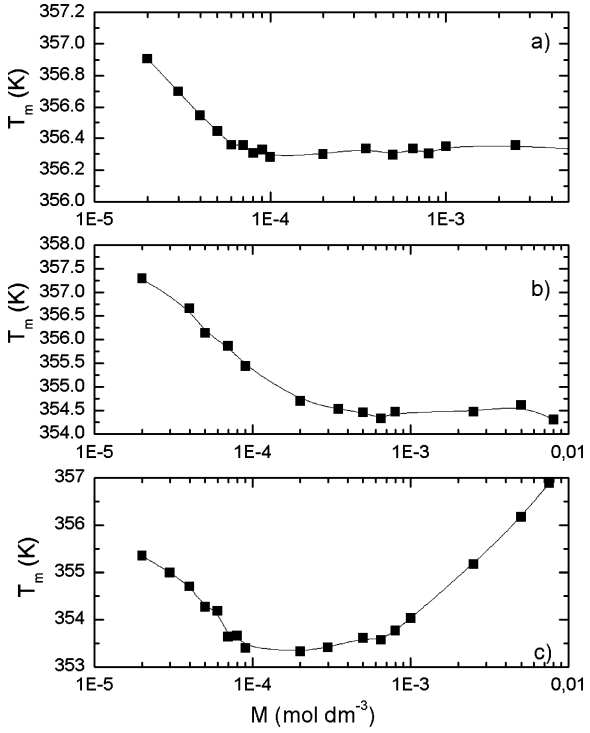

Figure 3. Myoglobin unfolding temperature, T_m, as a function of promazine concentration at pH (a) 9.0, (b) 5.5, and (c) 2.5. Estimated uncertainties in $T_m \pm 0.3$ K.

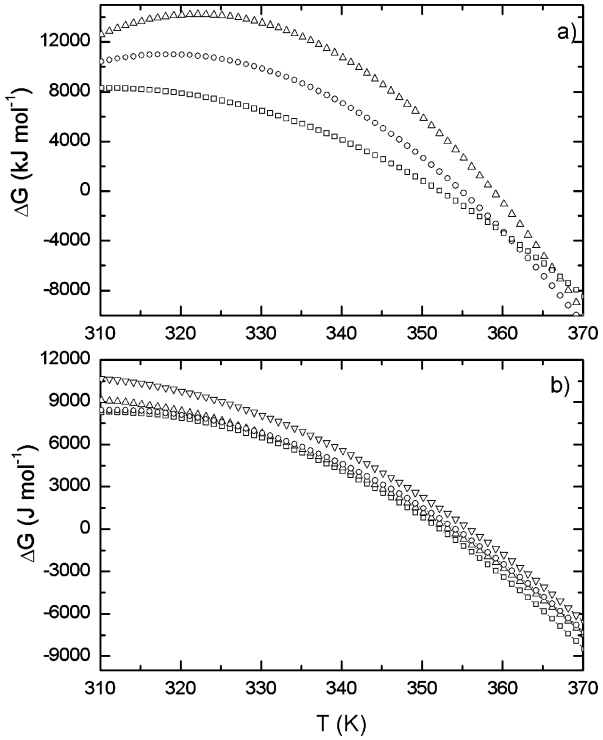

Figure 4. Gibbs energy of unfolding (ΔG) of myoglobin (0.5 g dm^{-3}) as a function of temperature in the presence of (a) 1.0×10^{-4} M of promazine at (□) pH 2.5, (○) pH 5.5, and (△) pH 9.0; (b) of promazine concentrations of (□) 1.0×10^{-4}, (○) 5.0×10^{-4}, (△) 1.0×10^{-3}, and (▽) 5.0×10^{-3} M at pH 2.5. Estimated uncertainties ±10%.

interactions should be stronger. In this regard, it has been pointed out that electrostatic interactions between an amphiphilic molecule and a globular protein probably should be predominant, at least, at very low drug concentrations,[30] and the electric shielding effect on protein surface by amphiphile binding would stabilize the protein as a result of a cross-linking function of the amphiphile ion between a group of nonpolar residues and a charged residue located on different loops of the protein.[31,32] However, such stabilizing effect at very low drug concentrations is not observed at this pH, although the observed destabilizing effect is lower than at pH 5.5. In this regard, it should be also bear in mind that hydrophobic interactions are also present even at very low ligand concentrations[33] and play a key role.[20] Thus, this behavior at pH 9.0 might arise from the important hydrophobicity of phenothiazine drugs, the rigidity of their tricyclic ring system to be accommodated in the protein, and to exert the cross-linking action. In this regard, hydrophobicity has also been claimed as the key factor in determining the destabilization of myoglobin by the fluorinated surfactant perfluorooctanoate, in contrast with its hydrogen counterpart sodium octanoate, which stabilizes the protein at low concentrations.[34] On the other hand, an additional factor which also influences this is the close pK_a of this class of drugs to the solution pH (9.4 and 9.2 for promazine and trifluprom azine, respectively),[35] which leads to all phenothiazine molecules not being in their ionized state. Then, part of the drug molecules are in their neutral form and cannot interact with the protein via electrostatic interactions, with the subsequent decrease of electrostatic binding, as observed in previous reports, which also allows hydrophobic interactions to play the predominant role at this pH,[20,36] as commented previously.

Comparison of promazine with trifluprom azine at pH 5.5 shows that, with the latter drug, slightly lower T_m values are derived. This can be a consequence of the larger hydrophobicity of trifluprom azine due to the presence of an extra bulky CF_3 in the hydrophobic ring system if compared with promazine, which seems to enhance its affinity for the protein.[20] In addition, this CF_3 group also involves a restriction in the number of conformations that molecules can adopt, which implies the necessity of a larger surface area in order to accommodate the drug molecules at the protein interface.[14]

Regarding thermodynamic parameters, Gibbs energies of unfolding, $\Delta G(T)$, have been calculated from equilibrium constants. Plots of Gibbs energies as a function of temperature for various drug concentrations and different pH are shown in Figure 4. In this figure, a certain parabollic type behavior can be observed, mainly at pH 9.0, with a certain increase in $\Delta G(T)$ at low temperatures, going through a maximum and followed by a nonlinear decrease as temperature rises. For the other pH, the region where $\Delta G(T)$ increases is not displayed since it is present at lower temperatures than the scale showed in the graph. It is worth mentioning that $\Delta G(T)$ values increase with drug concentration and pH.

On the other hand, it has been demonstrated that denaturant-induced unfolding and temperature-induced unfolding pathways show different conformational distributions at unfolded and transition states. The Gibbs free energy of unfolded states far from the native one under temperature-induced unfolding is lower than that for the denaturant-induced unfolding. In contrast, the free energy of the unfolded state close to the native state is higher under temperature-induced unfolding than under a denaturant-induced one. In addition, the latter also shows a wider conformational distribution provided that denaturant molecules bound to a protein molecule break contacts between amino acid residues, resulting in a higher conformational entropy.[37]

Figure 5 shows the experimental Gibbs energies of unfolding of myoglobin in the presence of promazine at pH 2.5 and 9.0 along with theoretical curves calculated by fitting the experimental points to eq 2. Similar plots have been obtained for the rest of promazine concentrations and for trifluprom azine. These

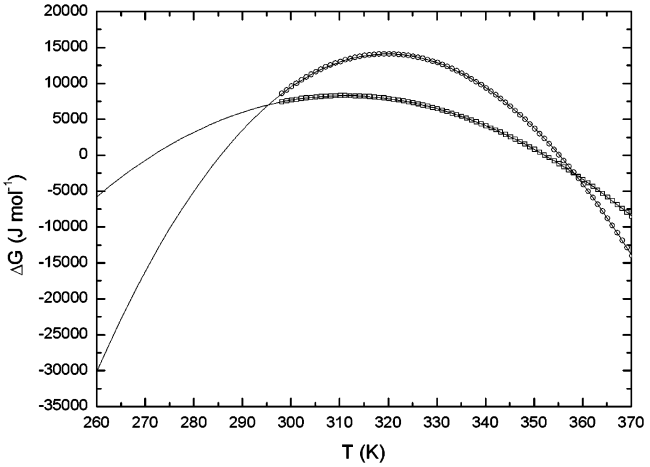

Figure 5. Energy of unfolding (ΔG) of myoglobin (0.5 g dm^{-3}) as a function of temperature in the presence of 1.0×10^{-4} M of promazine (□) pH 2.5 and (○) pH 9.0. The solid lines represent fit of experimental points to eq 2.

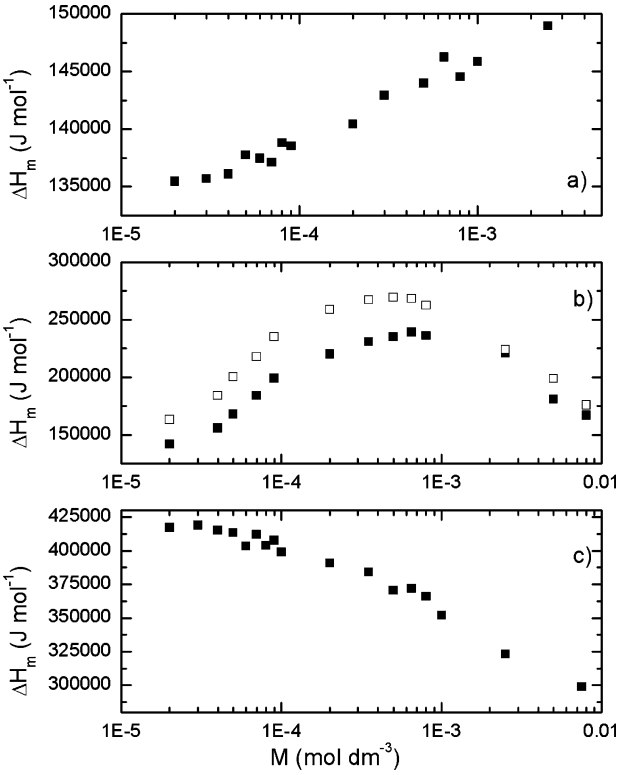

Figure 6. Enthalpies of denaturation (ΔH_m) as a function of drug concentration at pH (a) 2.5, (b) 5.5, and (c) 9.0. Closed symbols denote promazine, and open symbols denote trifluprom azine. Estimated uncertainties ±10%.

types of plots are known as protein stability curves[38] and show that the temperature of maximum stability, T_s, where $\Delta S = 0$, can be calculated by using eq 11. T_s values of different proteins generally fall between 263 K, for a relatively hydrophilic protein such as RNase A, and 308 K, for a relatively hydrophobic one such as β-lactoglobulin,[19] for example. The calculated values of T_s for the curves in Figure 5 are 310.5 and 319 K, respectively, whereas for the other concentrations and solution conditions fall also in a similar range.

On the other hand, values derived for the enthalpies of denaturation of myoglobin in the presence of both phenothiazine drugs are shown in Figure 6. ΔH_m values are positive; that is, unfolding is endothermic. In the case of promazine, it can be observed that the absolute values of ΔH_m, increase as the solution pH rises, which agrees with the increase in the denaturation temperature at pH 9.0 and 5.5, where the protein is in its native state (not as in the case of pH 2.5, as previously referred to). This emphasizes the importance of the stronger drug binding affinity at higher pH, where stronger electrostatic interactions are established. In this way, at acidic pH, ΔH_m (see Figure 6) continuously increases with phenothiazine concentration whereas, at pH 5.5, ΔH_m first increases, goes through a maximum, and then decreases; at pH 9.0, almost a continuous decrease occurs as drug concentration is raised. These different profiles might be related to changes in protein hydrophobicity as the charges appear and disappear on the protein surface (for example, at low pH groups such as COO^- get protonated, leading to an increase in protein hydrophobicity[39]) and to the different proportion of previously buried polar and apolar parts which becomes exposed to solvent after denaturation.

On the other hand, comparison of promazine and trifluprom azine shows lower enthalpy values for the former drug, which enhances the importance of drug hydrophobicity with regards to binding, that is, involving a larger binding affinity.[20] Similar behavior has also been found for the interaction of anionic surfactants with bovine serum albumin or cationic surfactants with catalase.[40,41]

Table 1 summarizes the values obtained for the entropy and heat capacity of myoglobin unfolding for the different systems. Since phenothiazine molecules are bound to a flexible biomacrolecule, it seems feasible that a protein expansion should occur to accommodate the drug molecules, as reflected by DLS measurements.[20] As entropy is a measure of disorder in the system, the positive denaturation entropy values (ΔS_m) point out that the denaturated state is more disordered than the native one. This behavior is related to the fact that, at room temperature, the entropies of hydration of both apolar and polar residues are negative, which indicate a more ordered aqueous environment. However, these entropies differ with respect to how they change with increasing temperature. The entropy of hydration of nonpolar residues increases through zero with increasing temperature, pointing out a reduction of ordered water molecules at higher temperatures, contributing to disorder by interfering with the water hydrogen-bonded network and allowing an easier rotation of water molecules. Furthermore, there is an entropy gain from the greater freedom of the apolar groups when the protein is unfolded. In contrast, the entropy of hydration of polar groups decreases, becoming more negative with increasing temperature as they may create ordered hydration shells even at higher temperatures.

Thus, we can derive from the larger ΔS_m values at pH 9.0 that temperature induces additional solvent-exposed hydrophobic residues previously buried at room temperature. The larger affinity of drug binding at this pH additionally collaborates this residue exposition to solvent, if compared with values at pH 5.5. For the latter pH, the protein is also in its native state but a lower drug binding affinity takes place.[20] In contrast, at acidic pH, since the protein is already partially denaturated, additional exposition of residues is much lower, as reflected in the entropy values. Moreover, the behavior of ΔS_m with pH may arise from the balance between hydration of hydrophilic and hydrophobic residues upon denaturation due to the different pathways of the process. In this regard, it has been suggested that upon thermal unfolding different components of the helical conformation of myoglobin might change asynchronously because of the different environments and different responses to perturbations.[42,43] This local unfolding has also been claimed as the key factor triggering protein aggregation in a cooperative manner.[43] In

TABLE 1: Thermodynamic Quantities Characterizing the Thermal Unfolding of Myoglobin-Phenothiazine Interaction with Estimated Uncertainties ±10%

concentration (mol dm^{-3})	ΔS_m (J mol^{-1} K^{-1})	ΔC_p (J mol^{-1} K^{-1})
	Promazine	
	pH 2.5	
0	375	2752
2×10^{-5}	381	2956
3×10^{-5}	382	2428
4×10^{-5}	384	2168
5×10^{-5}	389	2961
6×10^{-5}	388	3046
7×10^{-5}	388	2684
8×10^{-5}	392	3170
9×10^{-5}	392	2686
2×10^{-4}	397	4217
3×10^{-4}	404	1788
5×10^{-4}	407	3378
6.5×10^{-4}	414	4127
8×10^{-4}	409	2884
1×10^{-3}	412	3130
2.5×10^{-3}	419	2648
5×10^{-3}	433	3372
	pH 5.5	
0	395	3330
2×10^{-5}	397	3345
4×10^{-5}	437	4121
5×10^{-5}	472	2987
7×10^{-5}	517	4845
8×10^{-5}	560	5025
9×10^{-5}	620	5789
2×10^{-4}	652	6025
3.5×10^{-4}	663	5102
5×10^{-4}	675	6231
6.5×10^{-4}	666	4569
8×10^{-4}	623	5785
2.5×10^{-3}	510	5121
5×10^{-3}	471	4823
8×10^{-3}	397	4456
	pH 9.0	
0	1095	7750
2×10^{-5}	1169	9340
3×10^{-5}	1175	11553
4×10^{-5}	1165	4503
5×10^{-5}	1160	12186
6×10^{-5}	1132	10226
7×10^{-5}	1156	11946
8×10^{-5}	1134	7424
9×10^{-5}	1144	10810
1×10^{-4}	1120	11681
2×10^{-4}	1097	10732
3.5×10^{-4}	1078	5474
5×10^{-4}	1040	12641
6.5×10^{-4}	1044	10063
8×10^{-4}	1027	10640
1×10^{-3}	988	8785
2.5×10^{-3}	907	11440
7.5×10^{-3}	839	5623
	Triflupromazine	
	pH 5.5	
2×10^{-5}	457	4206
4×10^{-5}	516	2553
5×10^{-5}	562	2590
7×10^{-5}	612	5711
8×10^{-5}	661	5160
9×10^{-5}	730	3464
2×10^{-4}	754	7119
3.5×10^{-4}	760	7896
5×10^{-4}	757	7393
6.5×10^{-4}	740	7323
8×10^{-4}	632	5434
2.5×10^{-3}	560	7032
5×10^{-3}	457	2846
8×10^{-3}	516	4741

direct relation to entropy values, it has been determined experimentally that for apolar groups or surfaces the hydration contribution to the heat capacity is positive, whereas it becomes negative for polar surfaces. Thus, a variation in heat capacity upon unfolding of proteins is also related to the hydration change of proteins and to the change of solvent accessible and nonaccessible surface area.[44,45] We have previously indicated that the unfolding process of the protein has different pathways depending on the solution conditions, drugs, and their concentrations. ΔC_p also seems to corroborate this fact (see Table 1) despite that it is not easy to extract conclusions due to the dispersion for the points. However, a general feature can be inferred. The heat capacity values at the highest pH are the largest which would indicate a major exposure of the hydrophobic protein surfaces to solvent. The same trend is observed when comparing promazine and trifluprom azine at pH 5.5 as a consequence of the major affinity of the latter drug to bind to the protein[20] involving a little higher destabilization of protein structure.

Summary

Spectroscopic techniques have been demonstrated to be useful to determine the thermodynamics of protein unfolding in the presence of different drugs. One of the main advantages of these techniques is that much less protein is needed in the experiments. It has been demonstrated that both drugs play a destabilizing role for protein, at least at low concentrations. In addition, at acidic pH and higher drug concentrations, a stabilizing effect can be observed, which may be related to the formation of some type of protein refolding and/or subsequent protein aggregation. The reason for this difference has been suggested to be the different protein conformations at acidic pH, the extent of solvent-exposed hydrophobic and hydrophilic residues after denaturation and binding, and the different binding affinity when changing solution conditions. The lowest unfolding temperature, T_m, also occurs at acidic pH, where the protein is already partially destabilized. Various pathways of unfolding due to differences in solution conditions have been suggested as a result of different components of the helical conformation of myoglobin to change asynchronously because of the different environments and different responses to perturbations. Since drugs bound to the protein break contacts between amino acid residues, higher values of conformational entropy are obtained. In this regard, ΔS_m increases as the solution pH increases provided that temperature induces additional solvent-exposed hydrophobic residues which were previously buried at room temperature. Moreover, the major affinity of drug binding at this pH due to enhanced electrostatic interactions additionally collaborates to this residue exposition to solvent.

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