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Effect of prototypic drugs ibuprofen and warfarin on global chaotropic unfolding of human serum heme-albumin: A fast-field-cycling ^{1}H-NMR relaxometric study

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Abstract

Human serum albumin (HSA) is the most prominent protein in plasma, but it is also found in tissues and secretions throughout the body. The three-domain design of HSA provides a variety of binding sites for many ligands, including heme and drugs. HSA has been used as a model multidomain protein to investigate how interdomain interactions affect the global folding/unfolding process. Here, we report on the reversible chemical denaturation of heme-HSA involving three different conformational states (F, N, and B, occurring at pH 4.0, 7.0, and 9.0, respectively) and on the effect of prototypic drugs ibuprofen and warfarin on thermodynamics of the reversible unfolding process. Chaotropic unfolding of heme-HSA in the F, N, and B conformations is governed by different thermodynamic regimes, with the B form showing an entropic stabilization of the structure that compensates an enthalpic destabilization, and the F form easily unfolding under entropic control. Warfarin and ibuprofen binding stabilizes heme-HSA in both N and B states.

Keywords: Human serum heme-albumin; ^{1}H NMR relaxometry; Unfolding; Ibuprofen; Warfarin

1. Introduction

Human serum albumin (HSA) is the most prominent protein in plasma, but it is also found in tissues and secretions throughout the body. HSA is a non-glycosylated 65 kDa protein consisting of 585 amino acids. The amino acid sequence of HSA shows the occurrence of three homologous domains, probably arising from divergent evolution of a degenerated ancestral gene followed by a fusion event. Terminal regions of sequential domains contribute to the formation of flexible interdomain helices linking domain I to II, and II to III, respectively. Each domain is known to consist of two separate sub-domains (named A and B), connected by a random coil [1–7].

HSA abundance (concentration of 45 mg/ml in the serum of human adults) contributes significantly to colloid-osmotic blood pressure and its three-domain design provides a variety of binding sites helping in the transport, distribution and metabolism of many endogenous and exogenous ligands [6–10]. These ligands represent a spectrum of chemically different molecules, including fatty acids, heme, amino acids (notably tryptophan and cysteine), steroids, and metal ions such as calcium, copper, and zinc. Moreover, HSA is an important determinant of the pharmacokinetic behavior of many drugs [2,3,5–7,9–11]. HSA also accounts for most of the antioxidant capacity of human plasma, either directly or by binding and carrying radical scavengers, or by sequestering transition metal ions with pro-oxidant activity. HSA

Abbreviations: HSA, human serum albumin.

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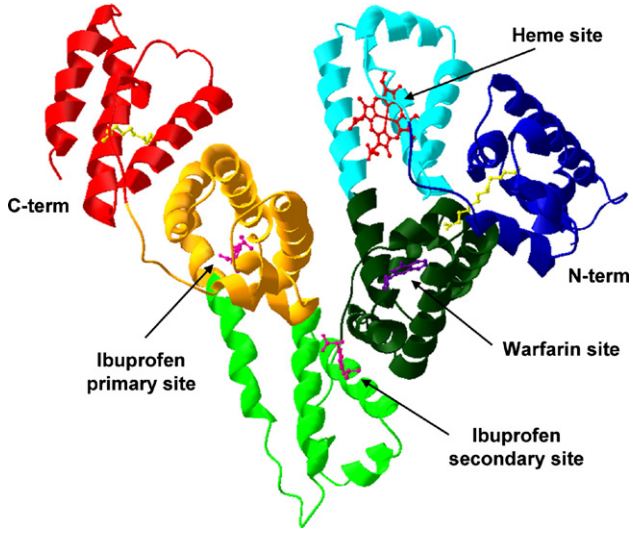
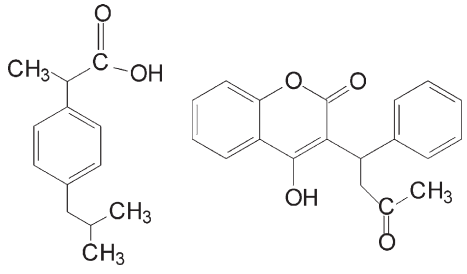


Fig. 1. Ribbon representation of the HSA structure with interacting ligands rendered as ball-and-stick models. Sub-domains are colored as follows: IA, blue; IB, cyan; IIA, dark green; IIB, light green; IIIA, orange; IIIB, red. Heme (red) occupies its primary binding site. Ibuprofen (magenta) occupies both primary (Sudlow's site II) and secondary binding sites. Warfarin (purple) occupies its primary binding site (Sudlow's site I). Other fatty acid sites are occupied by myristate ions (yellow). N- and C-termini of the protein are labelled. Atomic coordinates are taken from PDB entries 1O9X, 2BXD, and 2BXG [8,17].

acts as a nitric oxide depot and carrier, leading to covalent modification(s) of (macro)molecules [1,3,12–18].

The interaction of ligands with HSA occurs mainly in two regions. According to the Sudlow's nomenclature, bulky heterocyclic anions bind to site I (located in sub-domain IIA), whereas site II (located in sub-domain IIIA) is preferred by aromatic carboxylates with an extended conformation. Remarkably, ibuprofen, a non-steroidal anti-inflammatory agent, and warfarin, an anti-coagulant drug, are considered as stereotypical ligands for Sudlow's site II and Sudlow's site I, respectively (Chart 1) [4,6–8,13].



Warfarin binds to Sudlow's site I with $K_d = 3.0 \times 10^{-6}$ M, in a pocket formed by the packing of all six helices of sub-domain IIA (Fig. 1) [19,20]. The binding site has two sub-chambers that accommodate different portions of the warfarin molecule. The interaction between warfarin and HSA appears to be dominated by hydrophobic contacts, although specific electrostatic interaction are observed [19].

Ibuprofen binds primarily to Sudlow's site II with $K_d = 3.7 \times 10^{-7}$ M [20]. Site II is composed of all six helices of sub-domain IIIA and it is topologically similar to site I, with the exception that it may accommodate two fatty acid anions. It is stereoselective and shows different affinity for stereoisomers. A secondary ibuprofen site has been located at the interface between sub-domains IIA and IIB (Fig. 1) [8].

Heme binds with $K_d = 1.0 \times 10^{-7}$ M to a site located in sub-domain IB, with the tetrapyrrole ring arranged in a D-shaped cavity limited by Tyr-138 and Tyr-161 that provide π–π stacking interaction with the porphyrin and supply a donor oxygen (from Tyr-161) coordinating the heme iron. Heme propionates point towards the interface between domains I and III and are stabilized by salt bridges with His-146 and Lys-190 [16,17]. It has been recently proposed that HSA evolved to specifically bind heme, thus suggesting a possible role for HSA in heme transport and homeostasis [10]. Heme and warfarin binding sites are spectroscopically- and functionally-coupled, thus indicating that Sudlow's site I ligands act as allosteric effectors for heme binding and vice versa [20,21].

HSA is subject to reversible conformational transitions by allosteric effectors and pH changes. Between pH 2.7 and 4.3, HSA assumes the fast-migrating (F) form, characterized by an increase in viscosity, much lower solubility and a significant loss in helical content. Between pH 4.3 and 8.0, HSA displays the neutral (N) form that is characterized by heart-shaped structure. At pH greater than 8.0, HSA changes conformation to the basic form (B) characterized by a loss of α-helix and an increased affinity for some ligands [3,20–25].

HSA has served as a model multidomain protein to investigate how interdomain interactions affect the global folding/unfolding process [26–32]. By using different experimental techniques to follow thermal or chaotropic denaturation of HSA, it has been observed that the HSA N state unfolds sequentially, with domain I (i.e., the heme binding domain) always unfolding after domain II [30–32]. It has been suggested that unfolding of the HSA N form occurs through the formation of a molten globule [32], and a similar mechanism has been proposed for the urea-induced unfolding of HSA in the F state [31].

Heme binding to HSA endows the protein with peculiar optical absorbance and magnetic spectroscopic properties that

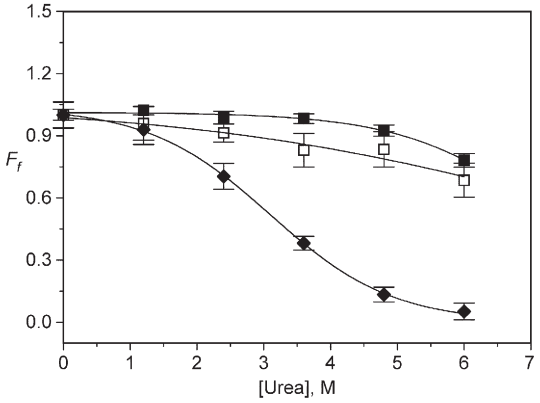


Fig. 2. Effect of pH on urea-induced unfolding of heme-HSA at 298 K obtained by measuring the protein folded fraction (F_f) according to Eq. (2). Solid diamonds: F conformation, pH 4.0. Solid squares: N conformation, pH 7.0. Open squares: B conformation, pH 9.0. Error bars indicate standard errors from four repeats.

Table 1
ΔG^0_U and m values for the urea-induced (0 to 6 M) unfolding of 1.0×10^{-4} M heme-HSA in the F state (i.e., at pH=4.0), in the N state (i.e., at pH=7.0), and in the B state (i.e., at pH=9.0), at 298 K, according to Eq. (2)

HSA state	ΔG^0_U (kJ mol^{-1})	m (kJ L mol^{-2})
F-state (pH=4.0)	17.0±0.4	5.2±0.4
N-state (pH=7.0)	16.8±0.4	2.3±0.7
B-state (pH=9.0)	8.7±0.8	1.0±0.1

can be used to follow ligand- and pH-dependent conformational transition(s) [20,23–25,33,34]. Electronic absorption and NMR relaxometric data indicate the occurrence of a high-spin Fe(III) center with a strong paramagnetic contribution due to a cluster of water molecules buried nearby [20,23]. This contribution may be employed to follow a number of events involving the conformational state of the heme-protein. Here, it has been utilized to follow the effect of drug(s) on the chaotropic reversible unfolding of three different conformational states (F, N, and B) of heme-HSA, allowing the determination of the thermodynamic parameters of protein stability in the absence and presence of stereotypical ligands of Sudlow's site I and II.

2. Experimental

All reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) of highest purity available and were used without further purification. Heme-HSA was prepared by adding the appropriate volume of 1.2×10^{-2} M heme, dissolved in 1.0×10^{-1} M NaOH, to a 1.0×10^{-3} M HSA solution in 0.1 M phosphate buffer pH 7.0, 298 K. In the final solution heme-HSA concentration was 1.0×10^{-4} M. The ten-fold excess of HSA ensured that heme was only bound to HSA primary binding site. The actual concentration of the HSA stock solution was determined by using the Bradford method [35]. The actual concentration of the heme stock solution was checked as bis-imidazolate complex in sodium dodecylsulfate micelles with the extinction coefficient value of 14.5 cm^{-1} mM^{-1} at 535 nm [36]. Under all the experimental conditions, no free heme was present in the reaction mixtures.

Urea was added to heme-HSA solution to obtain final urea concentration of 6 M. Different volumes of solution with and without chaotropic agent were mixed to obtain different concentrations of urea. Heme-HSA solution in the presence of 5 M GnCl was prepared by dissolving GnCl in the heme-HSA solution. Different volumes of solution with and without chaotropic agent were mixed to obtain different GnCl concentrations. Sample pH was adjusted by adding a few μl of HCl or NaOH concentrated solutions.

Ibuprofen stock solution (4.8×10^{-2} M) was prepared by dissolving the drug in pure water. Warfarin stock solution (2.4×10^{-2} M) was prepared by dissolving the drug in pure water at pH 10.0 until it dissolved, then adjusting to pH 7.0 with HCl. Drug solutions were then mixed with heme-HSA solution to reach final ibuprofen and warfarin concentrations 1.6×10^{-2} M and 1.0×10^{-2} M, respectively.

Water proton T_1 measurements at 0.04 MHz were obtained on a Stelar Spinmaster-FFC fast-field-cycling relaxometer (Stelar, Mede, PV, Italy) with 16 experiments in 4 scans. Briefly, macroscopic magnetization is created by inserting the sample in a magnetic field of 2.35×10^{-1} T (corresponding to 10 MHz proton Larmor frequency), then the magnetic field is switched to 9.4×10^{-3} T (corresponding to 0.04 MHz proton Larmor frequency) for a variable time. During this evolution interval the macroscopic magnetization changes with a time constant corresponding to the T_1 value at 0.04 MHz. At the end, the magnetic field is switched to 2.21×10^{-1} T (corresponding to 9.4 MHz proton Larmor frequency) and the magnetization is converted into an observable NMR signal by a radiofrequency pulse at 9.4 MHz. The intensity of the signal is proportional to the magnetization at the end of the evolution interval. The reproducibility in T_1 measurements was ±0.5%. The temperature was controlled by a Stelar VTC-91 airflow heater and checked in the sample cavity with a mercury thermometer.

The molar fraction of the folded protein (F_f) resulted from the change of the observed relaxation rate ($R_{1,obs}$) as a consequence of chaotropic unfolding:

$$F_f = \frac{R_{1,obs} - R_{1,blank}}{R^0_1 - R_{1,blank}} \quad (1)$$

$R_{1,blank}$ is the observed relaxation rate in the presence of excess urea or GnCl (regardless of the nature of the chaotropic

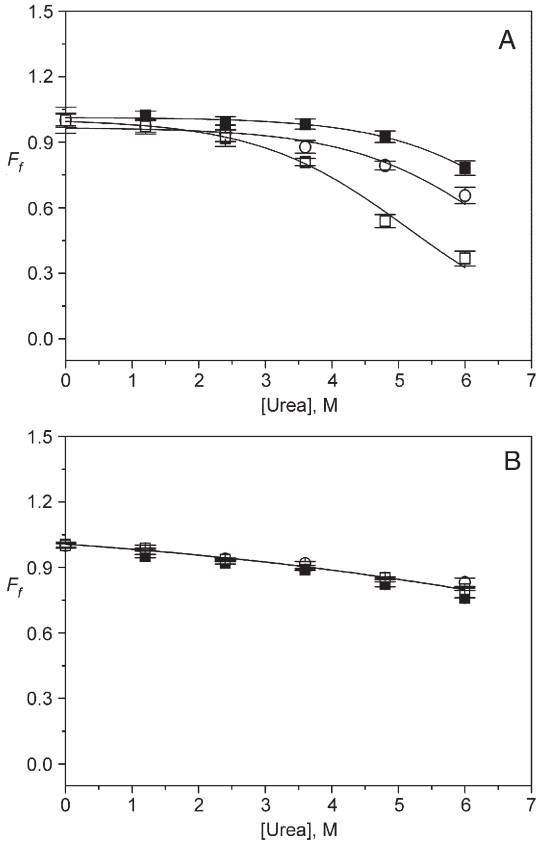


Fig. 3. Effect of temperature on urea-induced unfolding of heme-HSA in the N conformation (pH 7.0) obtained by measuring the protein folded fraction (F_f) according to Eq. (2) at 298 K (full squares), 311 K (open circles), and 321 K (open squares). Curves were measured in the absence of drugs (panel A) and in the presence of ibuprofen (panel B). Error bars indicate standard errors from four repeats.

Table 2
ΔG^0_U values calculated by Eq. (2) for heme-HSA 1.0×10^{-4} M in the F state (i.e., at pH=4.0), in the N state (i.e., at pH 7.0), and in the B state (i.e., at pH=9.0), in the presence of different urea (0 to 6 M) or GnCl (0 to 5 M) concentrations, at three different temperatures

T (K)	F-state (pH=4.0)	N-state (pH=7.0)		B-state (pH=9.0)
	Urea	Urea	GnCl	Urea
298	17.0±0.4	16.8±0.4	13.1±0.3	8.7±0.8
311	15.1±0.9	15.0±0.4	11.4±0.3	6.0±1.6
321	11.1±1.1	11.7±0.3	9.1±0.4	5.4±0.22

agent), with no significant differences for the three different HSA conformational states considered and without being affected by the presence of interacting drug(s). R^0_1 is the observed relaxation rate of heme-HSA in the absence of chaotropic agent. Reversibility of the unfolding process was investigated by dilution of the protein solution containing the highest concentration of the chaotropic agent with a protein solution without denaturant. Reversible urea- or GnCl-induced unfolding data were analysed according to Eq. (2) [37]. The coefficient m is proportional to the increase in the protein exposed surface area on unfolding.

$$F_f = \frac{1}{1+\exp[-(\Delta G^0_U - m\times[\text{denaturant}])/RT]} \quad (2)$$

Standard Gibbs free energy values ΔG^0_U for the global unfolding process allowed to determine melting enthalpy ΔH_m, melting temperature T_m, and specific heat ΔC_p, according to Eq. (3) [38].

$$\Delta G^0_U = \Delta H_m \times \left(1-\frac{T}{T_m}\right) - \Delta C_p \times [(T_m - T) + T \times \ln(T - T_m)] \quad (3)$$

The three-dimensional structures of HSA complexed with heme, myristate, ibuprofen, and warfarin were downloaded from the Protein Data Bank (PDB entries: 1O9X, 2BXD, and 2BXG) [8,17]. Ribbon representation of HSA with ball-and-stick representation of ligands was drawn with the Swiss-PdbViewer [39].

3. Results

Fig. 2 reports the urea-induced unfolding of heme-HSA at three different pH values, 298 K. At pH 9.0, heme-HSA is in the basic form (B) and shows a structural stability comparable to that of the N form (pH 7.0). On the other hand, at pH 4.0 the protein (in the F form) is easily unfolded by urea at 298 K. According to Eq. (2), ΔG^0_U (kJ mol^{-1}) and m (kJ L mol^{-2}) values can be estimated for the F form (pH 4.0), the N form (pH 7.0), and the B form (pH 9.0), (Table 1). Under all the experimental conditions urea-induced heme-HSA unfolding was completely reversible.

By increasing the temperature, urea-induced unfolding is facilitated. Fig. 3, Panel A shows urea-induced unfolding isotherms at three different temperatures. By considering that m value is not expected to change upon temperature in the temperature range considered, data have been fitted using Eq. (2) by taking constant the m value to an average value of 2.3±0.5 kJ L mol^{-2}. Values of ΔG^0_U at 298 K, 311 K, and 321 K, respectively, are reported in Table 2. These data are consistent with T_m=339±1 K and ΔH_m=(2.7±0.1)×10^2 kJ mol^{-1}, according to the Gibbs–Helmholtz equation (Eq. (3)). When the anti-inflammatory drug ibuprofen is added, unfolding isotherms become mostly unaffected by temperature (Fig. 3, Panel B).

Chaotropic unfolding of heme-HSA has been obtained by means of GnCl, as well. This denaturant has a remarkably stronger effect compared to urea, therefore global unfolding of the protein is complete in the whole concentration range explored. Fig. 4, Panel A shows the unfolding isotherms measured at three different temperatures, with a complete unfolding occurring at denaturant concentration higher than 4 M. In this case, an m value of 6.5±1.0 kJ L mol^{-2} is averaged, and values of ΔG^0_U are obtained at 298 K, 311 K, and 321 K, respectively (Table 2). According to the Gibbs–Helmholtz equation (Eq. (3)), these data are consistent with a melting temperature of 339±1 K, as obtained with urea, and ΔH_m=(2.1±0.1)×10^2 kJ mol^{-1}. Also in this case the stabilizing effect of ibuprofen is remarkable, as it may be appreciated from Fig. 4, Panel B. The unfolding isotherms are almost independent on temperature, thus making the quantitative determination of the melting temperature unaffordable. Under all the experimental conditions GnCl-induced heme-HSA unfolding was completely reversible.

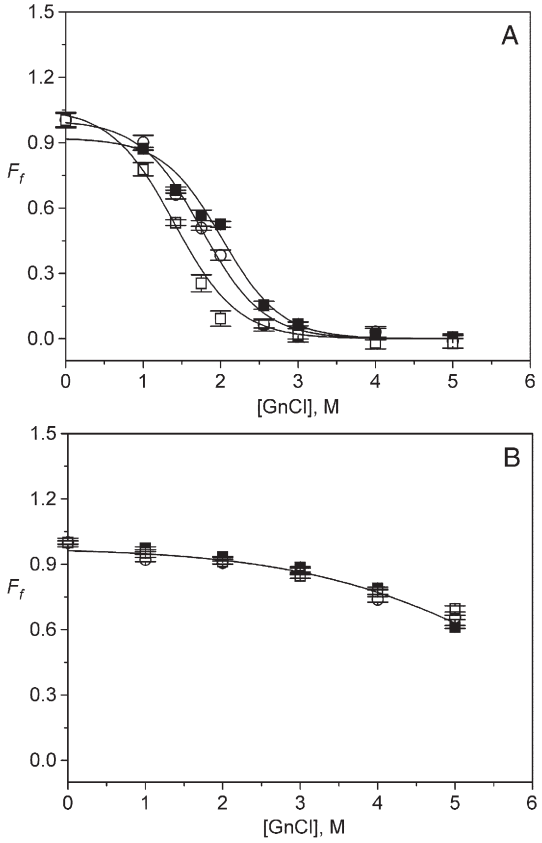


Fig. 4. Effect of temperature on GnCl-induced unfolding of heme-HSA in the N conformation (pH 7.0) obtained by measuring the protein folded fraction (F_f) according to Eq. (2) at 298 K (full squares), 311 K (open circles), and 321 K (open squares). Curves were measured in the absence of drugs (panel A) and in the presence of ibuprofen (panel B). Error bars indicate standard errors from four repeats.

Fig. 5 reports urea-induced unfolding of HSA at pH 9.0 (i.e., in the B state) in the absence of drugs (Panel A) and in the presence of either ibuprofen or warfarin (Panels B and C, respectively). Panel A shows that the stability of the B form is decreased and it becomes more sensitive to the temperature than the N form. An m value of 1.0 ± 0.1 kJ L mol^{-2} is averaged for the analysis of the curves using Eq. (2), and values of ΔG^0_U are obtained at 298 K, 311 K, and 321 K, respectively (Table 2). According to the Gibbs–Helmholtz equation (Eq. (3)), these data are consistent with $T_m=331\pm2$ K, which is sensibly lower than the corresponding value measured at pH 7.0 (i.e., in the N state), and $\Delta H_m=(2.1\pm0.1)\times10^2$ kJ mol^{-1}. In presence of drugs, heme-HSA in the B form increases its stability and urea-induced unfolding isotherms become mostly unaffected by temperature (Panels B and C).

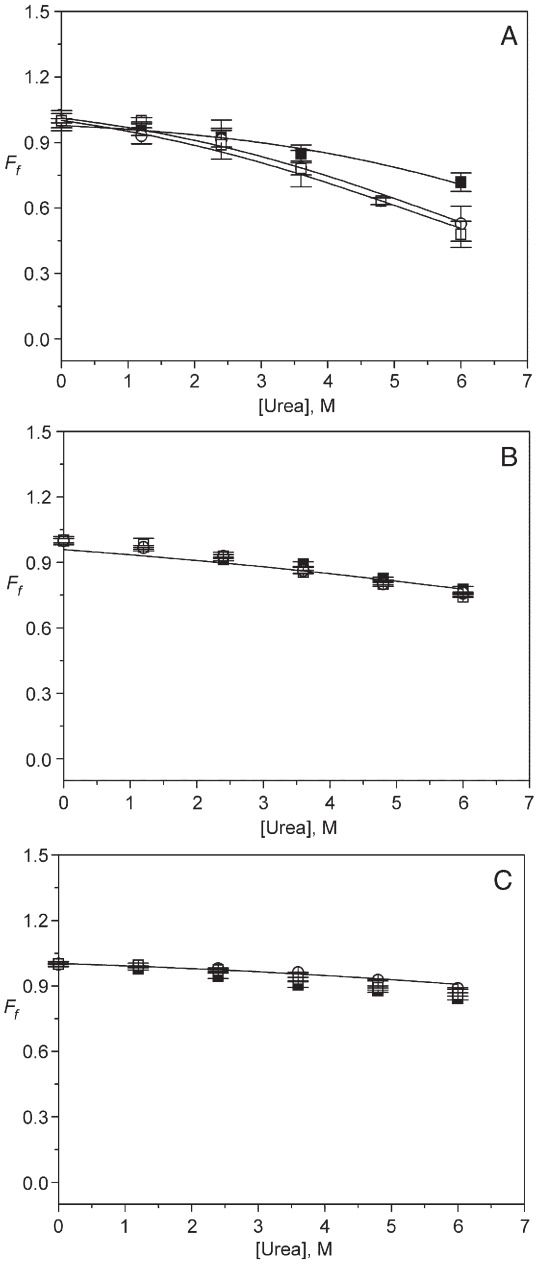


Fig. 5. Effect of temperature on urea-induced unfolding of heme-HSA in the B conformation (pH 9.0) obtained by measuring the protein folded fraction (F_f) according to Eq. (2) at 298 K (full squares), 311 K (open circles), and 321 K (open squares). Curves were measured in the absence of drugs (panel A) and in the presence of ibuprofen (panel B) and warfarin (panel C). Error bars indicate standard errors from four repeats.

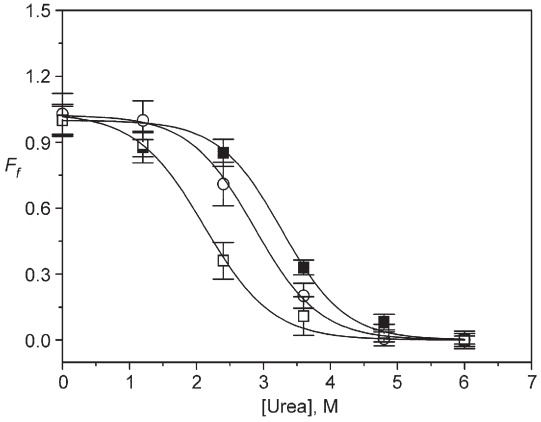


Fig. 6. Effect of temperature on urea-induced unfolding of heme-HSA in the F conformation (pH 4.0) obtained by measuring the protein folded fraction (F_f) according to Eq. (2), at 298 K (full squares), 311 K (open circles), and 321 K (open squares). Curves were measured in the absence of drugs. Error bars indicate standard errors from four repeats.

Fig. 6 reports urea-induced unfolding curves for heme-HSA at pH 4.0 (i.e., in the F conformation) at three different temperatures. Compared to Fig. 3, Panel A, urea is sufficient to cause complete unfolding of heme-HSA in the F state, thus indicating that the tertiary structure is loosened at pH 4.0. An average m value of 5.2 ± 0.2 kJ L mol^{-2} is used for the analysis of the curves using Eq. (2), and values of ΔG^0_U are obtained at 298 K, 311 K, and 321 K, respectively (Table 2). According to the Gibbs–Helmholtz equation (Eq. (3)), these data are consistent with $T_m=337\pm2$ K, not too different from the values reported above, and $\Delta H_m=(2.6\pm0.3)\times10^2$ kJ mol^{-1}, similar to the corresponding value measured at pH 7.0 (i.e., in the N state).

4. Discussion

Fast-field-cycling NMR relaxometry allows to follow chaotropic unfolding of paramagnetic proteins by measuring relaxivity changes at the proton Larmor frequency where they are most prominent. Water molecules located in the proximity of paramagnetic heme in the three-dimensional structure are used as high sensitivity reporters of changes in the folding state. According to this principle, thermodynamic parameters for the chemical unfolding of heme-HSA have been obtained. In particular, the ΔG^0_U value obtained from urea-induced denaturation of heme-HSA in the N state (i.e., at pH 7.0) is more than two-fold larger than those measured for the B and the F species (Table 1), thus accounting for an increased stability of the HSA structure. On the other hand, m values reported in Table 1 indicate that the conformational entropy of HSA in the B state is sensibly different with respect to that of the N state; as a consequence, the conformational B state appears to be stabilized by an entropic contribution. These results are consistent with thermodynamic parameters obtained for heme-free HSA using different spectroscopic and calorimetric techniques [28,40].

Also the melting temperature (T_m) values obtained from chemical denaturation of heme-HSA in the N state (i.e., at pH 7.0) according to the Gibbs–Helmholtz equation (Eq. (3)) are in agreement with previous reports [41] and are the same in urea- and GnCl-induced unfolding experiments. As expected [42], GnCl appears to exert a greater denaturant effect, thus lowering the melting enthalpy value significantly with respect to urea.

In both urea- and GnCl-induced unfolding processes the anti-inflammatory drug ibuprofen is able to dramatically stabilize the HSA N state. It has been proposed that unfolding of domain I always follows unfolding of domain II [30,32]. Although ibuprofen is a stereotypical Sudlow's site II ligand, it also binds to a cleft close to the warfarin site at the interface between sub-domains IIA and IIB (Fig. 1) [8]. It might therefore be suggested that stabilization of domain II also prevents the full unfolding of heme-HSA. Low solubility of warfarin at neutral pH does not allow to verify the hypothesis by directly occupying Sudlow's site I.

The effect of ibuprofen and warfarin on the thermodynamic parameters of heme-HSA unfolding may be followed at pH 9.0, with the protein in its conformational B state. As described above, the B conformation is enthalpically less stable with respect to the N conformation, but overall the structure is stabilized by a strong entropic contribution as witnessed by the strong reduction of the *m* value. This becomes evident in terms of a 10 degree reduction of the melting temperature, whereas the melting enthalpy does not change significantly. Both drugs display a stabilizing and almost superimposable effect, thus confirming that stabilization of domain II is crucial for the overall stability of the HSA fold [30–32].

At pH 4.0 HSA assumes the F conformation, supposed to differ from the N form for a partial unfolding of domain III and a consequent loosening of contacts between domain III and domain II [31,43]. Thermodynamic parameters obtained in the present work are in keeping with this hypothesis; actually, the change in the unfolding free energy is almost superimposable to that of the N state, but the *m* value increases significantly; consequently, the reduced stability of the F form is mainly due to the weakening of hydrophobic contacts. The low pH value prevents the dissolution of ibuprofen and warfarin, therefore any potential stabilization effect cannot be evaluated.

5. Conclusions

Chaotropic reversible unfolding of heme-HSA in the F, N, and B conformational states (i.e., at pH 4.0, 7.0, and 9.0) is governed by different thermodynamic regimes, with the B form showing an entropic stabilization of the structure that compensates an enthalpic destabilization, and the F form easily unfolding under entropic control. Occupancy of drug binding sites located in domain II by ibuprofen and warfarin dramatically hinders global unfolding of heme-HSA in both N and B states.

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