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Effects of drug-binding on the thermal denaturation of human serum albumin

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Abstract: Asymmetric thermograms of defatted albumin, alone and in the presence of two model drugs, have been obtained in phosphate buffers at three pH values. The albumin is less thermally stable in the N form, but is protected by both drugs. The nonsteroidal antiinflammatory benoxaprofen offers more protection than warfarin against thermal denaturation.

Keywords: *HSA; DSC; thermogram; warfarin; benoxaprofen; stabilization; conformation; unfolding.*

Introduction

Albumin is the most abundant protein circulating in blood, being an important carrier for drugs and endogenous substances like fatty acids, bilirubin and some hormones [1]. One of the physico-chemical properties of human serum albumin (HSA) is the conformational change occurring near neutral pH. This conformational change is referred as a neutral to base transition or N–B transition. The involvement of this N–B transition in drug–HSA interactions has been reported by several research groups [2–4]. The high affinity binding sites of most drugs can be divided into at least two binding sites, the so-called warfarin site, or site I and the diazepam site or site II. The binding of benoxaprofen to HSA has also been shown by circular dichroism and dialysis to be dependent on the N–B conformational change of the protein, however, benoxaprofen goes to the second major binding site (i.e. the non-warfarin site) on HSA [5]. Furthermore, benoxaprofen has been shown by microcalorimetric titration to have a very high affinity for HSA [6]. pH and fatty acid-induced conformation changes in HSA have also been studied by spectroscopic measurements [1].

One way to examine the stability of the folded conformation of globular proteins in solution is to measure the thermodynamics of their denaturation behaviour [7], which provides quantitative estimates of their conformational stability. The thermal stability of the protein can be examined precisely and directly over a much broader concentration range by calorimetry than by spectroscopy. Subtle changes in protein folding, not detectable by spectroscopic methods, are often measurable by high resolution differential scanning calorimetry (DSC) [8]. Moreover, the presence of domains in the intact protein can be demonstrated by analysing the process of the melting of the native structure [8–10]. Studies on the thermal stability of HSA has been the subject of an increasing number of investigations [11–15]. Recent crystallographic studies on HSA [16–18] and serum albumin from horse [19] have shown that HSA consists of three homologous domains, each of which has two subdomains with common structural motifs. The hydrophobic cavity in domain IIA accommodates warfarin and the one in IIIA diazepam [18] and, therefore, benoxaprofen. This is in agreement with the reported primary structure of the protein [20, 21].

Shrake and Ross investigated the thermal stability of HSA by DSC, and observed a major difference in thermograms displayed by unfatted and defatted HSA [14]. These re-

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searchers also studied the thermal stability of HSA as a function of added caprylate and/or N-acetyl tryptophan [12, 13], two ligands, used as thermal stabilizers in the USA [22]. In a recent communication, Markovich and Schraiber [23] reported a calorimetric investigation of the phenoxymethyl penicillin HSA interaction.

In this study, DSC has been utilized to investigate the effects of drug binding at different pH on the thermal denaturation of HSA. The drugs warfarin and benoxaprofen, have been chosen to represent the two different classes which bind to site I and site II of the protein, respectively. Spectroscopic studies [3, 5] of the binding characteristics of these two drugs with HSA have shown that both have very high affinity for albumin. The current investigations enable the relative effects of these drug–protein interactions at the two major binding sites to be investigated in terms of the thermal stability of the HSA.

Materials and Methods

Chemicals and reagents

Human serum albumin, fatty acid free, was obtained from the Sigma Chemical Co. (St Louis, MO, USA). Monobasic and dibasic sodium phosphate, analytical reagent grade, were obtained from Mallinckrodt (St Louis, MO, USA). Sodium benoxaprofen and sodium warfarin were obtained from Eli Lilly (Indianapolis, IN, USA) and DuPont Pharmaceuticals (Wilmington, DE, USA), respectively. All chemicals were used without further purification.

Human serum albumin solutions were prepared by dissolving the dry powder in 100 mM Na–phosphate buffer stock solutions of pH 5.3, 7.0 and 8.4, respectively. Before the calorimetric experiments the protein solutions were dialysed over night at 4°C and centrifuged in an airfuge to remove aggregates. The dialysate was used to fill the reference cell and to obtain the instrumental baseline. The protein concentration (about 1.6 mg ml^{-1}) was determined spectroscopically. Stock solutions of the drugs were prepared by weighing the appropriate amount in an aliquot of the dialysate. An aliquot of these stock solutions was added to the protein solutions to yield molar ratios of drug to protein of 3:1, in an attempt to saturate the primary binding site [5, 6, 24]. Finally the protein concentration was corrected for the dilution effect.

Calorimetry

DSC experiments were performed on a Microcal MC-2D high-precision scanning calorimeter (Microcal Inc., Amherst, MA, USA). All scans were performed at an actual scan rate of 1.25°C min^{-1} under an excess pressure of N_2 (1.5 atm) to prevent the appearance of bubbles on heating and loss of solvent by evaporation. The calorimeter was interfaced to an IBM-Personal Computer AT using a 12 bit analogue/digital conversion board (Data Translation DT-2801) for automatic data collection. Data were analysed using the software package provided by the manufacturer of the instrument. After subtraction of the stored buffer–buffer baseline the thermograms were normalized to standard heat capacity units taking into account the amount of the protein present in the volume of the calorimeter cell, 1.204 ml and then digitally filtered to remove short-term noise without distortion of the peak shape. The calorimetric enthalpy (ΔH_{cal}) of the transitions was calculated from the excess heat capacity function, which was obtained from the normalized thermograms (Figs 1–3) after baseline adjustment, and equals the area under the curve. These excess heat capacity curves were then subjected to deconvolution analysis [25–27].

Results and Discussion

Figure 4 presents the thermal denaturation profile, the heat capacity function (Cp vs T) of defatted HSA in 100 mM phosphate buffer, pH 7.0. This thermogram is characterized by an endotherm having a melting temperature, T_m, of 62.5°C, where T_m is defined as the temperature at maximum excess heat capacity (ACp_{max}). Furthermore, the thermogram has an asymmetric shape with a shoulder on the high temperature side.

Figure 4 also shows the reproducibility of the DSC profile of HSA at pH 7.0, scanned immediately after preparing the stock solution (full line), and after storage for 3 weeks at 4°C (dotted line), confirming negligible deterioration in the sample under these conditions. The shape and the thermodynamic characteristics of the curve agree well with the ones obtained by Tiktopulo *et al.* [11] under comparable experimental conditions. However, in

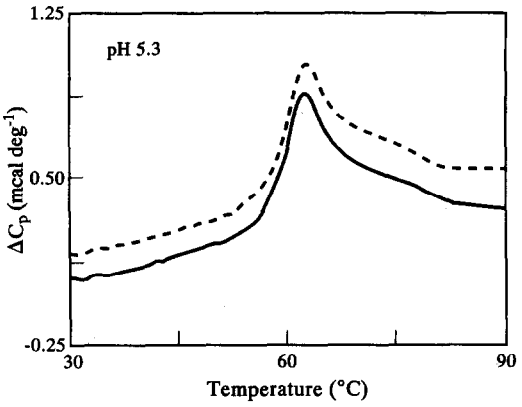

Figure 1
Calorimetric raw data (mcal deg^{-1}) of defatted HSA (1.6 mg ml^{-1}) in 0.1 M phosphate buffer pH 7.0 at a scan rate of 1.25°C min^{-1}; (———) immediately after preparation; (-----) after storage for 3 weeks at 4°C.

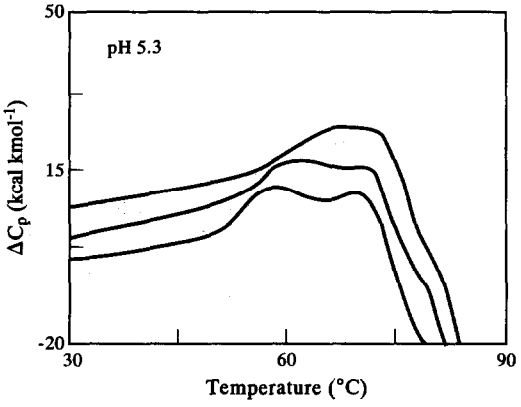

Figure 2
Thermograms of defatted HSA at pH 5.3 and 0.1 M phosphate. Lower curve is HSA alone; middle curve is of HSA–warfarin complexes; upper curve is of HSA–benoxaprofen complexes.

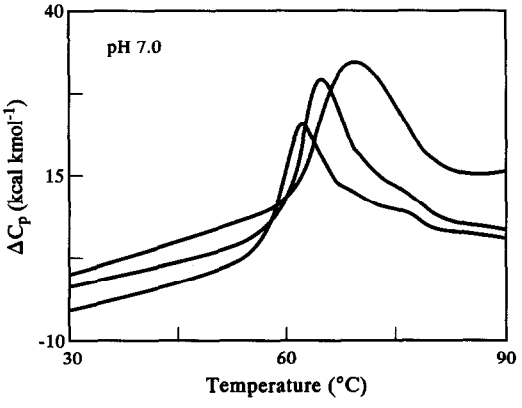

Figure 3
As Fig. 2 but at pH 7.0.

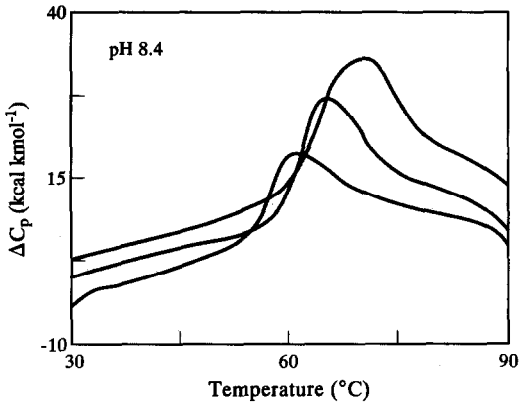

Figure 4
As Fig. 2 but at pH 8.4.

the present study the protein sample was used without any modification, whereas Tiktopulo *et al.* protected the free-SH group with *l*-cysteine. Tiktopulo *et al.* reported that the repeated treatment of the protein with activated carbon did not result in further changes in the shape of the curve, from which it followed that the complexity of the melting curve of the defatted protein was not a consequence of an incomplete purification of the protein molecules. However, this is in contrast to the thermograms obtained by Ross and Shrake [15] for monomeric HSA at pH 7.0. They report [14, 15], the denaturation of defatted human albumin monomer, as monitored by DSC to be monophasic by the presence of a single endotherm, but in the case of undefatted monomer, the thermal profile exhibited two endothermic peaks in the absence of any stabilizers. According to their interpretation, the two transitions did not represent the accepted domain structure [5, 6, 18] of the protein but resulted from an uneven distribution of long chain fatty acids, due to the preexistence of the heterogeneity in the protein or the heterogeneity that developed during the DSC experiments. These differences in the denaturation patern obtained by the two research groups can also be ascribed to the differences in the experimental conditions, namely ionic strength, protein concentration or bound fatty acids, etc.

Analysis of excess heat capacity curves requires thermodynamic equilibrium in the protein throughout the whole unfolding process, however, this equilibrium criterion is not fulfilled for HSA as judged by the results obtained from rescans of once heated samples,

which show no endothermic event. Nevertheless, Sturtevant and coworkers [28–30] have recently shown that thermograms can be interpreted using equilibrium thermodynamics in spite of the calorimetric irreversibility, if the process leading to the irreversible alteration is slow compared to the unfolding of the protein as observed with aggregation of HSA samples [11, 15]. The complex shape of the endotherms as well as the ratio of calorimetric to van't Hoff enthalpy, which is larger than one for all investigated samples, indicate that the unfolding process involves intermediates. The van't Hoff enthalpy was calculated from the parameters of Table 1 by the standard formula

$$\Delta H_{vH} = RT_m^2 \Delta C_{p,max}/\Delta H_{cal}$$

where T_m is the peak temperature at maximum excess heat capacity, $\Delta C_{p,max}$, and R is the gas constant. The above conclusion is supported by fragmental analysis which has shown that HSA consists of three compact globular domains [20], in agreement with the model of Brown *et al.* [21] and recent crystallographic studies [16–19]. Furthermore, deconvolution of the thermograms at pH levels 7.0 and 8.4, (Figs 2 and 3) gave three independent compact globular domains with enthalpies of 108, 70 and 68 kcal mol^{-1}, characterized by the van't Hoff and calorimetric enthalpies being equal [8, 9]. This result is in good agreement with Tiktopulo *et al.* [11]. It should be noted that because of the large transition width of about 40°C and difficulties in the baseline adjustment, the uncertainty in the calculated calorimetry enthalpy (ΔH_{cal}) is approximately 5%. Thermograms recorded at pH 5.3 (Fig. 1) cannot be deconvoluted due to the overlapping of unfolding and aggregation of HSA, indicated by the appearance of the exothermic event and the turbidity of the samples after withdrawal from the DSC cells. However, the biphasic nature of the thermogram at pH 5.3 clearly supports a multidomain structure of the protein.

The results (Figs 1–3 and Table 1) suggest that the HSA is more heat stable near the physiological pH and that biphasic behaviour is more apparent when HSA is in the N form at pH 5.3 when peak temperatures of 58 and 68°C are observed. At pH 5.3 aggregation of the protein, even in the presence of drugs is observed before the unfolding is complete. The biphasic character of the asymmetric thermogram is less obvious at pH 8.4; at pH 7.0 the thermogram shows an endotherm around 62°C with a shoulder at higher temperatures. Correlation of this pH dependency with the N → B transformation in albumin, which occurs over the pH range 5–8 is difficult with the limited amount of data reported here but albumin appears more vulnerable to heat in the N conformation.

The data, summarized in Table 1 clearly indicate that both warfarin and benoxaprofen stabilize HSA against heat at the pHs investigated without significantly altering the enthalpy of the unfolding. At pH 5.3 HSA is predominantly in the N form whereas at pH 8.4 the B form is favoured. Although drugs gave protection to HSA in the N form as shown by the increase of the peak temperature, they offered little protection from the aggregation seen at the higher temperatures, aggregation and denaturation overlap when HSA is in the N form. This is in contrast to the observations made at pH 8.4 when HSA is mainly in the B form (Fig. 3).

Comparison of the thermograms obtained at pHs 7.0 and 8.4 (Figs 2 and 3) shows also a significant increase in denaturation tempera-

Table 1
Calorimetric data derived from the data for HSA–drug complexes

HSA	pH	T_{max} (°C)	$\Delta C_{p,max}$ (kcal $kmol^{-1}$)	ΔH_{cal} (kcal mol^{-1})
Without drugs		62.5	20.8	238.3
+ Warfarin	7.0	65.1	24.2	254.9
+ Benoxaprofen		69.0	20.2	244.6
Without drugs		60.5	14.0	209.0
+ Warfarin	8.4	65.4	19.3	249.6
+ Benoxaprofen		69.5	18.6	246.0
Without drugs		~58/68	~8.7/6.8	(166)
+ Warfarin	5.3	~62/69	~8.5/7.0	(146)
+ Benoxaprofen		~66/70	~8.3/7.8	(147)

T_m is the peak temperature at maximum heat capacity difference (ΔCp_{max}). ΔH_{cal} is the calorimetric enthalpy of the unfolding of the HSA.

ture of HSA in the presence of both drugs, the effect being more pronounced for the non-steroidal antiinflammatory benoxaprofen. Qualitatively, similar effects were observed at pH 5.3 (Fig. 1). The presence of benoxaprofen significantly alters the shape of the thermogram at all pHs, giving a more symmetrical form. A possible explanation of this observation is increased interaction of the three domains causing a more uniform melting. Furthermore, a closer inspection reveals that the thermograms, obtained for the HSA–benoxaprofen complexes at pH levels of 7.0 and 8.4 with half denaturation around 69°C, are identical within the experimental limits. Also in the presence of warfarin very similar heat capacity functions are observed at these pHs, exhibiting a peak temperature of about 65°C.

These features indicate that the binding of benoxaprofen and warfarin induces distinct changes of the thermal stability of the HSA domains and markedly affects the N–B transition. However, only 2-dimensional DSC (27), i.e. recording a number of thermograms in the pH range of interest to experimentally define a heat capacity surface in terms of temperature and pH, will allow detailed analysis in order to make any conclusions about the stability of the individual domains and to correlate the binding of these drugs to eventual conformational changes, as reported for spectroscopic studies [3, 5, 6, 24]. Nevertheless, these results clearly show that the thermal stability of HSA is increased upon the binding of warfarin and benoxaprofen. It has already been suggested that similar interaction of anti-flammatory agents like benoxaprofen with membrane proteins may be the basis of their multiple effects on cells, and preliminary screening of antiflammatory drugs includes a test involving their ability to protect HSA from heat coagulation [31].

If one assumes, that increasing the thermal stability of proteins is a general property of anti-inflammatory agents, then DSC investigations of albumin interactions with known nonsteroidal antiinflammatory drugs will show if DSC is a useful probe. It should also prove to be a useful tool in understanding the N → B conformational change, and showing different behaviours at the two major drug binding sites on albumin.

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