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Research Article

Assessment of the Dissociation Energetics of Some Selected Ligand Drugs Bound on Human Serum Albumin by Differential Scanning Calorimetry

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Abstract. Drug-protein binding may play a role in the thermal energetics of protein denaturation and could lead to the determination of its equilibrium dissociation parameter. The aim of this study was to assess the energetics of a drug that was bound to human serum albumin (HSA) during thermal denaturation. Drugs that were bound at a single high-affinity primary binding site on HSA, including diazepam and ibuprofen, were employed. Commercial HSA was treated with charcoal to remove stabilizers and adjusted to 20% *w/v* in a pH 7.4 buffered solution. Serial concentrations of individual drugs up to 0.16 mmole/g-protein were added to the cleaned HSA solutions whereas diazepam was added to a commercial HSA solution. Samples were subjected to differential scanning calorimetry (DSC) set to run from 37 to 90°C at 3.0°C/min. Binding of the drug slightly increased the denaturing temperature of the cleaned HSA due to a shift in the equilibrium toward the native protein bound with the drug. Diazepam depressed the denaturing temperature of the commercial HSA by competing with the stabilizers already bound to the primary site of the HSA. This yielded not only the HSA-stabilizer but also the HSA-diazepam type complexes that exhibited a different denaturation process. A rational approximation of the Lumry-Eyring protein denaturation model was used to treat the DSC endotherms. The approximated scheme: $N \rightarrow^{k_3 K} P$ was successfully fitted to the data. It was used to determine the dissociation parameters for diazepam and ibuprofen bound to the HSA. These results were comparable to those obtained from other methods.

KEY WORDS: human serum albumin; Lumry-Eyring model; differential scanning calorimetry; dissociation energetics.

INTRODUCTION

Plasma proteins especially serum albumin, α_1-acid glycoprotein, and lipoproteins bind to a majority of drugs. This binding exerts significant effects on a drug's pharmacokinetics and pharmacodynamics. Binding can control the systemic distribution of active drug moieties and can also provide a reservoir for a long duration of action which in turn will have an impact on the efficacy and safety of the drug (1). Among these proteins, human serum albumin (HSA) has been considered to be the major binding component. HSA is by far the most abundant protein in blood plasma, and most drugs will undergo binding to HSA to various degrees (2). HSA consists of 585 amino acid residues with three homologous α-helical domains, and two drug binding sites (3,4) that are primarily responsible for acidic and neutral drugs (1). The ligand-binding characteristics of HSA have been extensively reviewed (5,6) while many techniques have been utilized to study the protein binding interactions. To name some of them: X-ray diffraction (7), fluorescence spectroscopy (8), circular dichroism spectroscopy (9), high-performance affinity chromatography (10), and equilibrium dialysis (11).

Most of the aforementioned techniques are conducted isothermally whereas most binding interactions are temperature dependent (12). In addition to these techniques, calorimetry may very well be suitable to detect the binding phenomena (13). Many workers have applied the theory of molecular unfolding to the calorimetric studies of ligand-protein interactions (9,13,14). With a combination of isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC) they were successful in estimating the binding parameter. Although DSC has been a most useful method to resolve the energetics of transition, perhaps its most useful applicability is to study the interaction of macromolecule which is not common (14). Recently, Faroongsarng and Kongprasertkit (15) used a simple but popular model of protein denaturation to scrutinize how sodium caprylate protected HSA from heat treatment using DSC. This was the so-called Lumry-Eyring model, a model of a two-step process that described the conformational changes that occurred in proteins during heat denaturation. The model was able to describe the energetics of the HSA complex and may also

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be useful for investigating drug-protein interactions. The aim of this study was to assess the energetics of the ligand binding interaction of some selected drugs to HSA by means of the Lumry-Eyring model during protein denaturation, induced by DSC, under thermal agitation.

Lumry-Eyring Model on the Denaturation of HSA Linked with a Drug Ligand

DSC measures the heat flow through a sample as a function of the temperature with a controlled temperature program. By this means, the thermal energetics associated with the transition in a system can be determined. For a protein denaturation experiment, the DSC data fit very well with the simple two-step Lumry-Eyring model described in Scheme I (16):

$$N \overset{K0}{\rightleftarrows} U \overset{k3}{\rightarrow} P$$

Scheme I

where, N, U, and P are native, unfolding, and final states of the protein. K_0 and k_3 are the equilibrium and rate parameters that correspond to the reversible transition between N and U, *i.e.*, $K_0 = \frac{[U]}{[N]}$ and the irreversible alteration from U to P, respectively.

It is well known that HSA possesses an extraordinary capacity for drug binding. Therefore, we considered the simplest case where a drug ligand, L, binds to a single high-affinity primary site (n=1) on the native HSA stoichiometry forming a 1:1 molar ratio complex. It is assumed that the complexes with other molar ratios where the drug can bind, *e.g.*, secondary binding sites are negligible. When the binding equilibrium is established, it follows that:

$$NL \overset{k}{\rightleftarrows} N + L \tag{1}$$

where, κ is a dissociation equilibrium parameter; $\kappa = \frac{[N][L]}{[NL]}$. Furthermore upon thermal induction, the ligand bound native form of the protein may also undergo a two-step denaturation which is in the same fashion as in Scheme I:

$$NL \underset{L}{\curvearrowright} \overset{K}{\rightleftarrows} U \overset{k3}{\rightarrow} P$$

Thus, by analogy to Eq. 1, the equilibrium for the unfolding coupled to the ligand dissociation based on Scheme I(a) can be written as (17):

$$NL \overset{K}{\rightleftarrows} U + L \tag{2}$$

where U undergoes further change to be P with a rate k_3. Then, its equilibrium parameter turns to $K = \frac{[U][L]}{[NL]}$. Thus, the relationship between the equilibrium parameters for the protein unfolding and binding to the ligand is now formed:

$$K = \kappa \cdot K_0 \tag{3}$$

Therefore, the standard Gibb's free energy changes for the unfolding and binding equilibria are related through:

$$\Delta G_K^0 = \Delta G_\kappa^0 + \Delta G_{K_0}^0 \tag{4}$$

The Rational Approximation of the Lumry-Eyring Protein Denaturation Model

An approximation to the Lumry-Eyring model was made by treating the two-step process as one simple step: for HSA, the rate at which the irreversible conversion occurred; k_3 in Scheme I as well as I(a) was sufficiently slow and was assumed to be a rate determining step (18). With K_0 less than 1, Scheme I can therefore be acceptably approximated as:

$$N \overset{k_3 K_0}{\rightarrow} P$$

Scheme II

This can also be done for Scheme I(a):

$$NL \overset{k_3 K}{\rightarrow} P + L$$

Scheme II(a)

Notice that both the equilibrium and the rate parameters are temperature dependent which can be described by an equation of the Arrhenius type, *e.g.*,

$$k_3 = \exp\left\{-\frac{E_{k_3}}{R}\left[\frac{1}{T} - \frac{1}{T_r}\right]\right\} \tag{5}$$

where E_{k_3} and T_r are the activation energy for the rate parameter, k_3, and a reference absolute temperature that corresponded to an Arrhenius-type constant, respectively.

The mole fraction of the native protein bound with the ligand drug (x_{NL}) is written as: (16)

$$x_{NL} = \exp\left\{-\frac{1}{\upsilon}\int_{T_0}^{T} k_{\text{app}} \cdot dT\right\} \tag{6}$$

The notation v is the rate of the temperature increment. k_{app} is an apparent parameter of the model. It is equal to either the product k_3K_0 or k_3K depending upon the approximated scheme. x_{NL} that corresponded to T was obtained from the DSC thermogram, *i.e.*, x_{NL}=1−f_T where f_T is the fraction of the DSC tracing at temperature T (15). The equation of the Arrhenius type that describes k_{app} is then plugged into Eq. 4:

$$x_{NL} = \exp\left\{-\frac{1}{\upsilon}\int_{T_0}^{T} \exp\left[-\frac{E_{\text{app}}}{R}\left(\frac{1}{T} - \frac{1}{T_k}\right)\right] \cdot dT\right\} \tag{7}$$

where E_{app} and T_k are the apparent activation energy corresponding to k_{app} and the absolute temperature at which k is equal to 1 min^{-1} that corresponds to the Arrhenius constant (16), respectively. R is the gas constant. The exponent of Eq. 5 as well as 6 is the integral of a temperature-dependent parameter from a low temperature T_0 where x_{NL} was negligible to a

temperature T. It has no analytical solution but can be approximated with a reasonably acceptable error (19) which turns Eq. 5 to:

$$x_{NL} = \exp\left\{ -\frac{1}{\upsilon}\exp\left(E_{\text{app}} \Big/ RT_k\right) \cdot \frac{T^2 \cdot \exp\left(-\frac{E_{\text{app}}}{RT}\right)}{\frac{E_{\text{app}}}{R}} \right\} \quad (8)$$

The value of E_{app} is assigned as the energetics of the denaturation of the protein complex. It is expected that the energetics of Schemes II and II(a) will be related to $(E_{k_3} + \Delta G^0_{K_0})$ and $(E_{k_3} + \Delta G^0_K)$, respectively. If the kinetics of the model does not considerably change when it comes to the drug ligand binding, it is then further assumed that E_{k_3} in Schemes II and II(a) may essentially be identical. Thus, the difference of the E_{app}s between Schemes II(a) and II may correspond to the energetics of the dissociation of the drug ligand binding, E_κ:

$$E_\kappa = E^{\text{II(a)}}_{\text{app}} - E^{\text{II}}_{\text{app}} \quad (9)$$

where $E_{\text{app}}{}^{\text{II}}$ and $E_{\text{app}}{}^{\text{II (a)}}$ are E_{app}s for Schemes II and II(a), respectively.

MATERIAL AND METHOD

Diazepam and ibuprofen that both exhibit a single primary binding site with a high affinity (n=1) on HSA (11,20,21) were chosen as model drugs. Because of the restricted use and distribution by the Royal Thai Narcotics Act, diazepam BP 2007 (99.0–101.0% of $C_{16}H_{13}ClN_2O$, calculated on the dried substance) was obtained as a gift from the Government Pharmaceutical Organization, Thailand. The ibuprofen (Pharmaceutical secondary standard traceable to USP, PhEur, and BP, Fluka Analytical) was from Sigma-Aldrich, Saint Louis, MO. Pharmaceutical-grade commercial human serum albumin (HSA) USP was obtained as a 20% solution form (Albutein®, Grifols International, Barcelona, Spain). Other chemicals were AR grade or equivalent. The substances were used without further purification.

Removal of the Stabilizers from Commercial HSA by Charcoal Treatment

The method for removal of the stabilizers from commercial HSA was taken from Chen (22) and modified by Faroongsarng and Kongprasertkit (15). Briefly, the HSA product was mixed with an equal volume (10 mL each) of distilled water. The mixture was adjusted to pH 3.0 with 0.2 N HCl. The stabilizers were removed from the mixture by mixing it thoroughly with 1 g of activated charcoal for 1 h at ~2–4°C. The activated charcoal was then removed by centrifugation at 20,200×g for 20 min under the same cool conditions. The supernatant was taken and adjusted to pH 7.0 by the addition of 0.2 N NaOH. In addition, detection of residual stabilizers was checked using an HPLC method modified from Dengler *et al.* (23) to ensure the obtained HSA was clean. No traces of stabilizers were found in the samples. Finally, the cleaned HSA was lyophilized, yielded a solid powder, and was used for further studies.

Sample Preparation

Prior to use, the cleaned HSA in its lyophilized powder form was accurately weighed and dissolved in the pH 7.4 phosphate buffer solution to yield a 20% *w*/*v* HSA solution. A stock solution of diazepam was prepared by dissolving an accurately weighed amount of drug into methanol at a 1 mg/mL concentration whereas that of the ibuprofen was 10 mg/mL in a pH 7.4 phosphate buffer solution. The individual stock solutions were serially diluted with pH 7.4 phosphate buffer solution. The individual samples were prepared by mixing equal volumes of the drug ligand solutions with the abovementioned HSA solutions to obtain concentration levels of 0.00125, 0.0025, 0.005, 0.01, 0.04, 0.08, and 0.16 mmole/g-protein for diazepam and those of 0.0047, 0.0082, 0.0093, 0.01, 0.04, 0.08, and 0.16 mmole/g-protein for ibuprofen.

Commercial HSA was doped with diazepam in the pH 7.4 phosphate buffer at various concentrations. The accurately weighed HSA product and individual solutions of diazepam in appropriate serial dilution were mixed to yield the mixtures having diazepam concentrations of 0.000125, 0.00125, 0.0025, 0.005, 0.010, 0.02, 0.04, 0.08, and 0.16 mmole/g-protein. The individual samples were subjected to further DSC temperature scanning experiments.

The DSC Temperature Scanning Experiment

The Perkin-Elmer differential scanning calorimeter (DSC 8000, Perkin-Elmer Crop., Norwalk, CT, USA) equipped with an intercooler II accessory was employed. Prior to operation, the machine was calibrated with standard zinc and indium to ensure the accuracy and precision of the obtained heat of transitions and for the corresponding temperatures. Approximately, 15 μL of liquid sample was accurately weighed and placed in a tightly sealed aluminum pan. While being purged by nitrogen gas, the individual sample pan was subjected to a scan against an empty pan as a reference. With a loading temperature of 30°C, the running program included (1) pre-heating from 30°C to 37°C, (2) equilibrating at 37°C for 15 min, and (3) heating from 37°C to 90°C at a rate of 3.0°C/min. Three replicates were done for each of the samples. All the DSC thermograms were analyzed using Pyris® software (version 11.0.0.0449, Perkin-Elmer Crop., Norwalk, CT, USA). In addition, in-house software was used to analyze the energetics of each of the DSC thermograms for the protein denaturation in accordance with Eq. 6. The thermograms for the commercial and cleaned HSA, commercial HSA doped with serial concentrations of diazepam, and cleaned HSA doped with diazepam and with ibuprofen in various concentrations were used in the analysis. The x_{NL} that corresponded to T was obtained from a DSC thermogram using the technique described in the reference (15). It was found that nonlinear regressions were successful with an r^2 of between 0.9850 and 0.9946.

RESULTS AND DISCUSSION

Thermal Denaturation of Cleaned HSA Bound with Diazepam or Ibuprofen Monitored by DSC

Cleaned HSA with a drug ligand, either diazepam or ibuprofen, mixed at various ligand concentration levels were

thermally scanned by DSC. Some of the thermograms of their denaturation scans are shown in Fig. 1. HSA denaturation is an endothermic transition in which its thermogram is referred to as the DSC endotherm. While HSA was reported to be denatured at 60–65°C (14,15), its denaturation with diazepam and ibuprofen peaked at somewhat higher temperatures. For example, as shown in Fig. 1 (I) and (III), the protein equilibrated with diazepam and ibuprofen in the respective serial concentration ranges of 0.00125–0.005 (up to 0.33 molar ratio) and 0.0047–0.01 mmole/g-HSA (up to 0.67 molar ratio) denatured at ~67–70°C and ~68–72°C, respectively. And for each of the model drugs, it was also observed that the endotherm shifts slightly upwards with an increase of the drug ligand concentration. The larger the drug concentration, the greater was the increase in the peak temperature observed. In thermodynamic theory, it has been stated that a protein ligand interaction always denatured at an increased temperature regardless of the structural consequences (17). In addition, the ligand did not bind to the unfolded form of the protein, or in other words, the native protein eliminated the binding ligand instantaneously before unfolding (24). Whereas in the meantime, an equilibrium was established. Any increase in the drug ligand concentration would shift the equilibrium toward the native protein bound with ligand (*NL* species in Eq. 2). Thus, a higher temperature would be necessary to unfold the protein which in turn produced a slight increase in the denaturing temperature.

Nevertheless, it was observed that the HSA equilibrated with diazepam in excess, *i.e.*, more than 1:1 molar ratio, had a denaturing temperature that was only slightly higher, *i.e.*, ~70–71°C (Fig. 1 (II)). Similar results were obtained for the case of a molar excess of ibuprofen (data not shown). It seems that an increase in the denaturing temperature *via* the equilibrium might not be sufficient to protect HSA from heat sterilization. However, there may be the other mechanism that could stabilize and protect HSA from heat stress. Arakawa and Kita (25) suggested that an added stabilizer might slow down the irreversible step, *i.e.*, k_3 in Scheme I(a). But, this suggestion seems to be unlikely since the ligand does not interact with the unfolded protein.

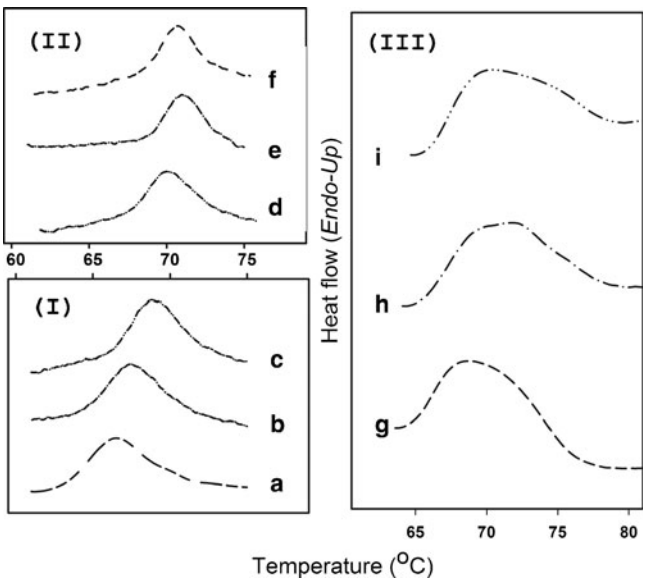

Fig. 1. DSC thermograms of heat-induced denaturation of stabilizers removed HSA incubated with diazepam (**I**) diazepam in excess (**II**), and ibuprofen (**III**) at concentration levels of *a* 0.00125, *b* 0.0025, and *c* 0.005, *d* 0.01, *e* 0.04, and *f* 0.08 mmole/g-protein for diazepam and *g* 0.0047, *h* 0.0082, and *i* 0.0093 mmole/g-protein for ibuprofen, respectively

Effect of Diazepam on the Denaturation of Commercial HSA

The commercial HSA was doped with diazepam at various concentration levels. Their DSC endotherms of denaturation are illustrated in Fig. 2. The HSA product had been stabilized with sodium caprylate and sodium acetyltryptophanate (Albutein®, Grifols International, Barcelona, Spain). The spectroscopic analysis revealed that one of the targets of oxidative stress on HSA was a tryptophanyl residue (26). Thus, added acetyltryptophanate may function as an antioxidant rather than as a heat protector (27). While tryptophanate exhibited only one binding site (28), caprylate bound to seven out of the nine fatty acid sites on the protein (29). It was found that the stabilizers competed with each other since they shared a common binding site with the association constants (K_a) of 3×10^5–2×10^6 M^{-1} and 4–5×10^4 M^{-1} for caprylate and tryptophanate ions, respectively (11). This was greater by almost two orders of magnitude in affinity. The caprylate ligand might replace tryptophanate on the protein binding which allowed the free tryptophanate to provide the activity against oxidative stress. As seen in Fig. 2a, the thermal denaturation of the commercial HSA peaks was at about 77–80°C. It was reported that sodium caprylate plays a role in heat resistance (27). Furthermore, with the Lumry-Eyring protein denaturation model, it was found that the heat protection by the caprylate ion may occur *via* binding to the

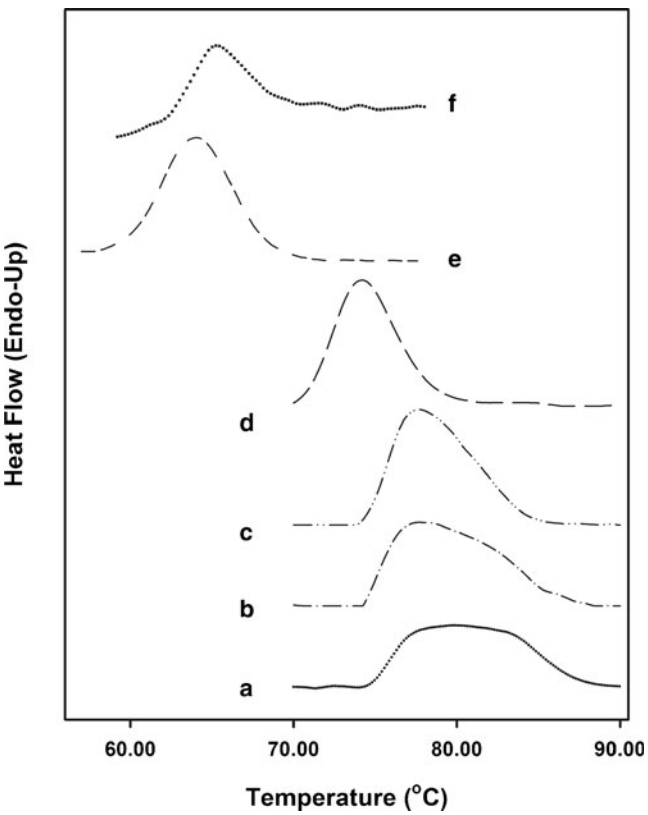

Fig. 2. DSC thermograms of commercial HSA doped with diazepam in various concentrations of: 0 (*a*), 0.0025 (*b*), 0.01 (*c*), 0.02 (*d*), and 0.08 mmole/g-HSA (*e*) compared with that of cleaned HSA (*f*)

native form of HSA (15). The binding interaction may modify the kinetics of HSA denaturation that decreases the protein unfolding rate turning the process into an unfolding dominant. As a consequence, a marked increase in the denaturing temperature was observed.

Surprisingly, as seen in Fig. 2, the addition of diazepam depressed the denaturing temperature of the commercial HSA. It can be lowered down to the same temperature level as that of the cleaned HSA (Fig. 2e *cf.* f). On the HSA, diazepam has two classes of binding sites, a primary high-affinity single site (n=1) and secondary moderate to low-affinity double sites (n=2) (20,28). The primary K_a of diazepam was found in the range of between 3.5 and $4.\times10^5$ M^{-1}; that is comparable with the primary K_a of caprylate, and the mutual reductions in HSA binding were found in mixtures of the protein with caprylate ion plus diazepam (11). It was therefore deduced that the binding of caprylate and diazepam to HSA takes place according to the competitive scheme. Adding diazepam to commercial HSA may produce two types of complexes classified according to their primary binding characteristics including HSA-caprylate and HSA-diazepam types. Interaction with diazepam can increase the denaturing temperature only up to ~71°C. On the other hand, the HSA-caprylate type complex formed in the commercial form of HSA denatured at a temperature that was as high as 77°C (Fig. 2a). Owing to the binding competition, the mole fraction of the HSA-caprylate type complex became less with the increase in diazepam concentration. It was therefore reasonable to assume that in the DSC experiment for the mixture of substances under study that the heat of transition was directly proportional to the mole fractions of the components. The apparent thermal transition was then the summation of its components. Thus, the greater the amount of the HSA-diazepam type complex present, the more the denaturing temperature became depressed.

Thermal Energetics of HSA-Drug Complexes

As the denaturing temperature was always below 74°C (Fig. 1), the denaturation of HSA-diazepam type complex may favor Scheme II(a) (18). To examine whether or not a drug ligand binding interaction can cause a protein complex to denature that fits Scheme II(a), the energetics of the transition of the HSA-drug complex was assessed. A rational approximation of the Lumry-Eyring model was carried out on each of the DSC endotherms from the commercial HSA doped with diazepam at various concentration levels. The E_{app} obtained from the DSC data fitting was plotted against the concentration of the added diazepam in millimole per g-protein and is illustrated in Fig. 3. Prior to adding diazepam, the E_{app} value for the commercial HSA denaturation was ~50 Kcal/mol and designated as the energetic state of the protein unfolding by the dominant scheme (15). As seen in Fig. 3, adding diazepam caused the E_{app} value to markedly increase and level off at ~140 Kcal/mol. It seemed that the energetics of denaturation transfered from one state to the other. Moreover, the E_{app} approached the activation energy of the denaturation of the cleaned HSA (~150 Kcal/mol: dash line in Fig. 3) when diazepam was added at approximately 0.01 mmole/g-protein or more. As previously discussed, adding diazepam to commercial HSA yielded both the HSA-caprylate type and the HSA-diazepam type complexes. These two types of complexes exhibited different states of E_{app} during denaturation, *i.e.*, E_{app} values of ~50 and ~150 Kcal/mol for HSA-caprylate type complex and the HSA-diazepam one corresponded to the protein unfolding dominant scheme and Scheme II(a), respectively. The apparent E_{app} was dependent on the proportion of the type of the individual complex population. When more diazepam was added, the greater was the proportion of the HSA-diazepam type complex, and hence, the energetic state of Scheme II(a) denaturation dominated and yielded an apparent change in the energetic state. The observed E_{app} was able to approach that of the cleaned HSA when excess diazepam was added. Nevertheless, there might be a minor disagreement between the E_{app} values of the clean HSA and the plateau region of the curve in Fig. 3. This may be because both the caprypate and diazepam ligands bind to multiple sites on the protein. The secondary bindings may cause allosteric interactions (30) to cause deviations in the E_{app} values. In addition, the remaining HSA-caprylate type complex present was due to the fact that diazepam cannot fully replace the caprylate ligand. This type of complex still denatures with a low level of E_{app} value, and this possibly reduces the apparent energetics.

Energetics of HSA-Drug Dissociation

Nonlinear regressions were also carried out in accord with Eq. 6 on the DSC denaturing endotherms of the HSA-diazepam and HSA-ibuprofen complexes. Their E_{app}s were plotted against the drug concentrations (Fig. 4). Notice that the drug concentrations recruited in the plots did not exceed the 1:1 molar ratio to ensure that the drug ligand was available only to the primary high-affinity site of the protein. The secondary binding sites would in turn be involved at a higher drug to HSA ratio (31). All the E_{app} values in Fig. 4 indicate that the protein denaturation might be approximated as Scheme II(a) rather than by the protein unfolding dominant scheme. This is because the E_{app} values were greater than that of the protein unfolding dominant scheme but did approach that of Scheme II(a). As shown in Eq. 7, the energetics of the binding dissociation may be estimated as the difference in energetics of denaturation with and without the ligand binding. The E_{app}^{II} value was estimated from the E_{app} of the cleaned HSA denaturation (151.3 Kcal/mol) whereas the $E_{app}^{II\ (a)}$ was from that of a HSA-drug complex. In order to avoid the deviations in the case of the HSA-drug complex where the drug ligand might disturb the binding equilibrium, it was recommended that E_{app} at an indefinitely diluted drug ligand solution should be the estimator for $E_{app}^{II\ (a)}$. With the limit data coordinates, a linear regression was done on each of the plots of Fig. 4 and the regression lines were extrapolated to the ordinate axis so as to obtain $E_{app}^{II\ (a)}$s. Correlation coefficients of 0.8970 and 0.7739 for the diazepam- and ibuprofen-protein complexes, respectively, were found. This is due to the fact that the energetic variables used were from the rational approximation that was based on limited assumptions. The fluctuations are obvious as observed (Fig. 4). In addition, a few recruited coordinates might also have an effect on the statistical results.

The estimates of $E_{app}^{II\ (a)}$, E_κ, and κ for the drug protein binding under study are given in Table I as well as the dissociation equilibrium parameters for the HSA-diazepam and HSA-ibuprofen interactions previously reported in the literature. Notice that κ of the current study was calculated based on an Arrhenius type equation:

$$\kappa_T = \exp\left\{-\frac{E_\kappa}{R}\left(\frac{1}{T}-\frac{1}{T_0}\right)\right\} \tag{10}$$

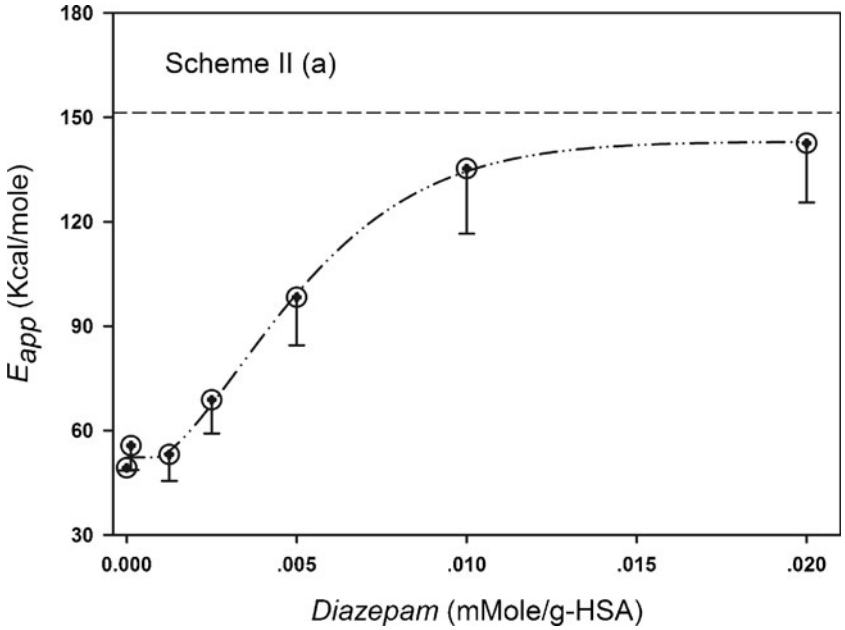

Fig. 3. The plot of the apparent activation energy (E_{app}); the energetics were obtained from a nonlinear fitting according to Eq. 6 against the added diazepam for the pharmaceutical-grade HSA (commercial HSA: *circle legends*). For the guide for the eye that was used to demonstrate the change of energy level in the case of the commercial HSA in which caprylate-HSA and HSA-diazepam complexes coexist, a *trend line* was drawn by the empirical best fit

where, T_0 is the denaturing temperature of the protein complex with a indefinitely diluted ligand concentration. T_0 might be estimated by the temperature for the cleaned HSA denaturation that was found to be 61.48°C. To match with the literature, the T of the experimental conditions including 293.15K (20°C) and 298.15K (25°C) were chosen for the HSA-diazepam and HSA-ibuprofen complexes, respectively. It was found that the obtained κs were comparable to those from the literature. Thermodynamically, the Gibb's free energy; $\Delta G_\kappa^{\ 0}$ is related to κ as: $\Delta G_\kappa^{\ 0} = -RT\ln\kappa_T$. By substituting these values in Eq. 8, $\Delta G_\kappa^{\ 0}$ can be described as a function of E_κ:

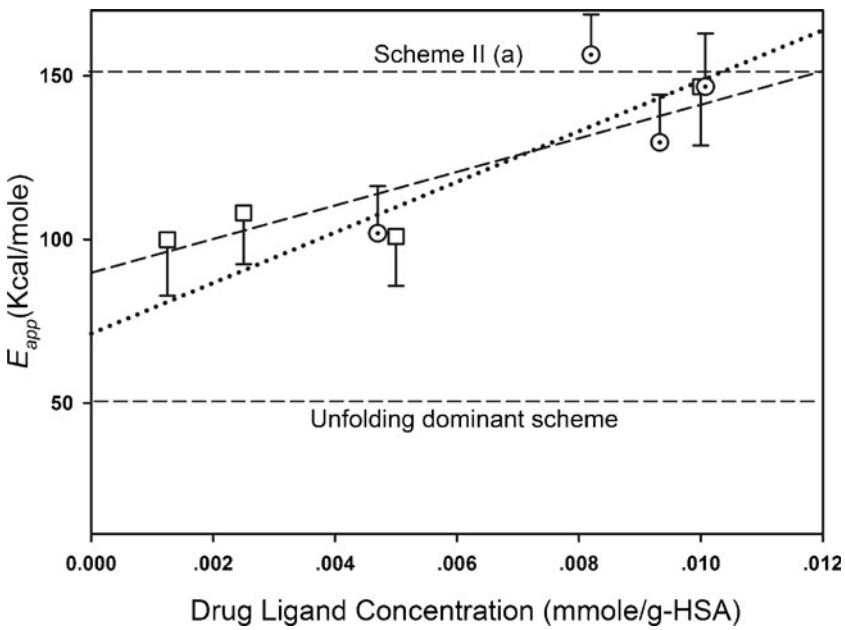

Fig. 4. The plot of the apparent activation energy (E_{app}); the energetics were obtained from a nonlinear fitting according to Eq. 6 against the added diazepam (*square legends*) and ibuprofen (*circle legends*). *Trend lines* including *dashed* (- -) and *dotted* (· ·) *lines* for protein complexes of diazepam and ibuprofen, respectively, are drawn using linear regressions. The *upper gray dashed line* represents the energetic level of denaturation of the cleaned HSA whereas the lower one does the same for the commercial HSA

Table I. Energetic Information Including the Estimates of Apparent Activation Energy ($E_{app}^{II\ (a)}$) and Energetics of the Binding Dissociation (E_κ) as well as Its Dissociation Parameter (κ for the Drug Protein Binding Under Study Obtained from the Rational Approximation of the Lumry-Eyring Model)

Complex (pH 7.4)	$E_{app}^{II\ (a)}$ ±SE (Kcal/mol)[a]	E_κ ±SE (Kcal/mol)[b]	κ; μM This study (range)[c]	κ; μM (reference: conditions)[d]
HSA-diazepam	89.8±9.6	61.5±10.6	2.0 (0.07; 8.8)	2.1 (28: 20°C; pH 7.0) 2.6 (11: 20°C; pH 7.0)
HSA-ibuprofen	71.2±12.7	80.1±13.5	0.39 (0.03; 4.8)	0.28 (20: 25°C; pH 7.4) 1.7 (32: 37°C; pH 7.4)

HSA human serum albumin

[a] The apparent energy of Scheme II(a); $E_{app}^{II\ (a)}$ was estimated from the intercept of the trend lines in Fig. 4. The standard error (SE) of the estimate for each of the complex series is also shown

[b] E_κ; the energetics of the binding dissociation: $E_\kappa = E_{app}^{II(a)} - E_{app}^{II}$ where E_{app}^{II} was obtained from the nonlinear fitting of the DSC data of the cleaned HSA denaturation according to Eq. 6

[c] Dissociation equilibrium parameters in μM ($\times 10^{-6}$ M) at the temperature of the experimental condition. To match with other references, T was chosen at 20°C and 25°C for HSA-diazepam and HSA-ibuprofen complexes, respectively. The range of κ was calculated from a margin of estimation for E_κ, *i.e.*, E_κ −SE and E_κ +SE

[d] Dissociation of the equilibrium parameters obtained from the literature with a variety of experimental conditions, *i.e.*, temperatures of 20–37°C with a pH of 7.0–7.4

$$\Delta G_\kappa^0 = E_\kappa\left(\frac{T_0 - T}{T_0}\right) \tag{11}$$

It was of interest that the $\Delta G_\kappa{}^0$ for the ibuprofen binding interaction was calculated using Eq. 9 to be 8.7±1.5 Kcal/mol whereas that previously measured by microcalorimetry was 7.1 Kcal/mol (−29.7 KJ/mol: Cheruvallath *et al.* (31) reported the value as the free energy of association that possessed a negative sign). Ibuprofen consists of an enantiomer and its HSA binding seems to be stereospecific toward the S-ibuprofen as it showed a somewhat higher Gibb's free energy change (7.7 Kcal/mol (31)). It was found that the $\Delta G_\kappa{}^0$ calculated in the current study may be essentially in agreement with those microcalorimetrically determined. Although the dissociation equilibrium parameter for each ligand bound to HSA was dependent on the methods of determination as well as the drug's enantiomer, the previously reported assays for the parameter were around 10^{-7}–10^{-6} M for diazepam and ibuprofen ligands (11,20,21,28,32). This was also the case in the current study. Taking into account the cost-effectiveness and labor-saving process, DSC temperature scanning could serve as an alternative means to investigate drug-protein interactions. However, the data needs to be interpreted with caution. This is due to the fact that the method is not only based on rational approximations according to the limited model but the nature of the Arrhenius-type treatment often introduces noticeable deviations to the results. It is noted that the estimated range of κ in this method was as wide as 10^{-8}–10^{-6} M for both drugs (Table I) which is one order of magnitude wider than the other previously reported tests.

CONCLUSION

This differential scanning calorimetric experiment has demonstrated that the drug ligand binding to HSA did contribute to the thermal energetics of protein denaturation. Using the approximated scheme of Lumry-Eyring model, the dissociation equilibrium parameters were determined. According to the scheme derivation, only the drugs bound with a single high-affinity primary binding site on HSA were tested. It was found that the scheme could determine the dissociation parameters of diazepam and ibuprofen bound with HSA in which the obtained results were comparable to those of other methods. Nevertheless, caution should be exercised for the interpretations as the estimated range of the parameters was 10^{-8}–10^{-6} M whereas other methods were in the range of 10^{-7}–10^{-6} M for both drugs.

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