Truncated human serum albumin retains general anaesthetic binding activity

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Multiple binding sites for anaesthetics in HSA (human serum albumin) make solution studies difficult to interpret. In the present study, we expressed the wild-type HSA domain 3 (wtHSA_{d3}), a peptide with two known anaesthetic binding sites in a yeast expression system. We also expressed a site-directed mutant of domain 3 (Y411Wd3). The stability and secondary structure of the constructed fragments were determined by HX (hydrogen–tritium exchange) and CD spectroscopy. The binding of two general anaesthetics, 2-bromo-2-chloro-1,1,1-trifluoroethane and propofol, to wtHSA_{d3} and Y411Wd3 was determined using isothermal titration calorimetry, HX and intrinsic tryptophan fluorescence quenching. Although the expressed fragments are less stable than intact wtHSA as indicated by both CD and HX, they retain the sec-

ondary structure and anaesthetic-binding characteristics of an intact HSA molecule, but with fewer binding sites. Y411Wd3 had decreased affinity for propofol but not for 2-bromo-2-chloro-1,1,1-trifluoroethane, consistent with steric hindrance. Retention of structural features and anaesthetic binding properties with fewer binding sites in this truncated protein provide feasibility for using scaled-down models of otherwise intractable systems to gain an understanding of anaesthetic binding requirements and binding–stability relationships.

Key words: anaesthesia, anaesthetic, halothane, human serum albumin, isothermal titration calorimetry, protein stability.

INTRODUCTION

It is now widely appreciated that many potential targets of inhaled anaesthetics have multiple binding sites for these small, relatively featureless drugs [1–5]. For example, various methods have revealed at least seven binding sites for halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) on HSA (human serum albumin) [6,7], whereas the nicotinic acetylcholine receptor, a ligand-gated ion channel thought to be relevant for anaesthetic action, has far more [8,8a]. In addition to making the relationship between binding and function complex, this also makes the energetics for each site and the effects of mutations ambiguous in the biologically relevant solution phase [6]. A strategy to solve this problem has been to truncate and study the isolated domains [9]. However, it is not clear if domain–domain interactions are required to stabilize allosterically these low-affinity drug-binding sites.

HSA has three homologous domains, each approx. 22 kDa in size. Solution and crystallographic studies with wild-type HSA have indicated that both halothane, a volatile general anaesthetic, and propofol, an injectable general anaesthetic, bind to a common site in domain 3 [6,7] (Figure 1). A previous study indicates that another volatile general anaesthetic, isoflurane, binds this same site [10], raising the likelihood that this site resembles those in the central nervous system responsible for the action shared by the three compounds, anaesthesia. If the truncated domain 3 can retain its structural feature and anaesthetic binding character, the smaller peptide should facilitate structural and dynamics studies using NMR or molecular dynamic simulations. Also the fewer binding sites should allow for a better correlation of binding energetics with the structural detail, which will be especially useful with site-directed mutants. Thus we expressed wild-type HSA

domain 3 (wtHSA_{d3}) in a yeast expression system to test whether the anaesthetic binding character is maintained; we also expressed a site-directed mutant (Y411Wd3) to test whether weak π -cation interactions contribute to general anaesthetic binding in the common site.

EXPERIMENTAL

Materials

HSA (essentially fatty acid free) was purchased from Sigma (St. Louis, MO, U.S.A.) and used without further purification. Halothane was obtained from Halocarbon Laboratories (Hackensack, NJ, U.S.A.). The thymol preservative and water in halothane were removed before use with an aluminium oxide column. ³HOH (³H-labelled water) was obtained from ICN (Costa Mesa, CA, U.S.A.) at 100 mCi/ml. All other chemicals were of reagent grade or better, obtained from Sigma.

Expression and purification of domain 3 and its mutant Y411W (Tyr⁴¹¹ → Trp) of HSA

For the synthesis and purification of HSA fragments (amino acid positions 381–585), we used a modified method based on a previously published technique [11]. Using pHIL-D2 HSA as template, DNA sequence for domain 3 was amplified by PCR. The amplified fragment was digested with the DNA restriction enzymes XhoI and EcoRI, and was ligated into pPIC9 (Invitrogen, San Diego, CA, U.S.A.) in frame with the alpha mating factor signal sequence, which was shown to be cleaved at the expected position, liberating the translation product of the cloned cDNA fragment for the domain. QuickChange[®] XL site-directed

Abbreviations used: halothane, 2-bromo-2-chloro-1,1,1-trifluoroethane; HSA, human serum albumin; ³HOH, ³H-labelled water; HX, hydrogen–tritium exchange; ITC, isothermal titration calorimetry; PFR, protection factor ratio; wtHSA_{d3}, wild-type HSA domain 3.

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Figure 1 Shared anaesthetic binding site in domain 3

The images are processed for an optimal view of the ligand and some lining residues, and are derived from the intact HSA–ligand complexes; 1E7A for propofol (PFL) and 1E7B for halothane (HLT).

mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) was used for Y411Wd3 construction. The methods used to verify the DNA sequence of a yeast clone expressing the domain were identical with that used to analyse clones expressing full-length HSA. Since HSA domains bind to Cibacron Blue Sepharose 6B resin (Sigma), the domain 3 and its Y411W mutant were purified and defatted using the same methods as used for full-length wild-type HSA described previously [12,13].

CD spectroscopy

Spectra were recorded on a model 62 DS spectropolarimeter (Aviv, Lakewood, NJ, U.S.A.) using 2 mm pathlength quartz cells, a bandwidth of 1.0 nm, a scan step of 0.5 nm and a time constant of 3.0 s. The buffer was 10 mM sodium phosphate (pH 7.0), and the cell-holder was maintained at $25.0 \pm 0.1^\circ\text{C}$. For CD determination, $5.5 \mu\text{M}$ of wtHSA_{d3} or Y411Wd3 and $2.5 \mu\text{M}$ of intact HSA were used. The obtained CD data were analysed using a web-based program (<http://www.embl-heidelberg.de/~andrade/k2d.html>) to allow secondary structure assignment [14]. Stability of the HSA fragments was determined by monitoring the mean residue ellipticity at 222 nm ($[\theta]_{222}$) in the presence of increasing guanidine concentration in 10 mM sodium phosphate buffer (pH 7.0). The measured $[\theta]_{222}$ were plotted as a function of the concentration of guanidine. The changes in the far-UV CD signal showed that the guanidine hydrochloride-induced unfolding of HSA followed a two-state process [15]. Thus we fitted CD denaturation data to an equation describing the denaturation of the two-state-monomeric protein as follows:

$$Y = [\exp(\Delta G - mx)]/[1 + \exp(\Delta G - mx)]$$

where ΔG is the conformational stability of the protein and m is the slope of the unfolding transition. The melting point is calculated as $\Delta G/m$.

Intrinsic fluorescence quenching

Quenching of tryptophan and tyrosine fluorescence were used to study halothane binding as described previously [16]. Briefly, a stock solution of halothane was prepared in 130 mM NaCl/20 mM NaHPO₄ (pH 7) buffer at a concentration of 10 mM and loaded into a gas-tight Hamilton syringe (Hamilton, Reno, NV, U.S.A.). Different concentrations of halothane were equilibrated with 1 mM wtHSA_{d3} (or mutants) in a 10 mm path-length quartz cuvette (1.5 ml) with a polytetrafluorethylene stopper during fluorescence measurements. The point of 50% inhibition (IC_{50}) of the fluorescence maxima in halothane-free species was calculated with Hill plots. Fluorescence measurements were performed with a Spectrofluorimeter RF-5301PC (Shimadzu, Columbia, MD, U.S.A.). All fluorescence measurements were carried out at room temperature ($25 \pm 1^\circ\text{C}$). Excitation and emission slit widths were 3 and 5 nm respectively. The background fluorescence was subtracted from each emission spectrum. The single tryptophan residue in Y411Wd3 was excited at a wavelength of 295 nm and tyrosine residues were excited at a wavelength of 275 nm. Emission spectra were recorded from 310 to 450 nm.

ITC (isothermal-titration calorimetry) measurements

The thermodynamic parameters for the anaesthetic–domain 3-peptide interactions at 20°C were determined using an isothermal titration calorimeter (model VP-ITC; MicroCal, Northampton, MA, U.S.A.). The ITC instrument consists of a matched pair of sample and reference vessels (1.43 ml) enclosed in an adiabatic enclosure and a rotating stirrer-syringe for titrating aliquots of the ligand solution into the sample vessel. The sample vessel contained the peptide solution with concentration around 0.04 mM. The peptide concentrations were determined before titration. The reference vessel contained water. Stock ligand solutions (0.5 mM for propofol and 10 mM for halothane) were loaded into the injector and titrated after 5 min into the HSA sample solution.

Origin 5.0 (Microcal Software) was used to analyse the titration heat profiles. The model used to fit the ITC data is based on the equation, $Q = M_t V_0 (n_1 \Theta_1 \Delta H_1 + n_2 \Theta_2 \Delta H_2)$, where Q is the heat content after any injection; M_t is the total concentration of the macromolecule in the active cell; V_0 is the volume, n_1 and n_2 are the numbers of binding sites for set 1 and set 2, Θ_1 and Θ_2 are the fraction of sites occupied by the ligand for set 1 and set 2 and ΔH_1 and ΔH_2 are the molar heats of ligand-binding for set 1 and set 2 respectively. Θ is related to its respective K and the free concentration of ligand $[X]$ by the equations $K = \Theta / \{(1 - \Theta)[X]\}$, where $[X] = X_t - M_t(n_1 \Theta_1 + n_2 \Theta_2)$ and X_t is the total concentration of ligand. Note that M_t , X_t and V_0 are known. The parameters n , ΔH and K for each set are adjusted to minimize the sum of squares of differences between the measured heats and those predicted from this model using the Marquardt method. Once K and ΔH are determined, ΔS can be calculated using the standard formulas $\Delta G = \Delta H - T \Delta S = -RT \ln K$.

HX (hydrogen–tritium exchange)

HX is a sensitive method of measuring changes in protein stability due to ligand-binding [17,18]. HSA and domain solutions (1 mg/ml) were incubated with 5–10 mCi of ^3HOH in 1 M GdnCl and 0.1 M NaH_2PO_4 buffer (pH 8.5), for at least 18 h at room temperature for exchange-in. Free ^3HOH was removed and the buffer was changed with a PD-10 gel-filtration column (Sigma), thereby initiating exchange-out with 0.5 M GdnCl and 0.1 M NaH_2PO_4 buffer (pH 7.4). After recovery from the column, the protein solution was immediately transferred into prefilled Hamilton gas-tight syringes containing 7.0 mM halothane or 0.5 mM propofol in exchange-out buffer and equipped with repeaters. Aliquots were precipitated with 2 ml of ice-cold 20% (v/v) trichloroacetic acid at timed intervals for at least 6 h. The precipitated protein was rapidly vacuum-filtered through Whatman GF/B filters (Whatman International, Maidstone, U.K.) and washed with 8 ml of ice-cold 2% trichloroacetic acid. ^3H retained by the protein was determined by liquid-scintillation counting. Exchange-out buffer conditions were adjusted to focus on the most protected final 5–10% of hydrogens over a 6 h period, to ensure that global unfolding events were being monitored. PFR (protection factor ratio) for the given hydrogen atoms were determined from the exchange-out curves as described previously [17,19,20]. The changes in free energy change ($\Delta\Delta G$) were determined ($\Delta\Delta G = -RT \ln(\text{PFR})$). Negative values reflect stabilization (slower exchange-out). Given $\text{PFR} = (1 + [\text{H}]/K_D)^n$, the change in free energy is related to ligand concentration $[\text{H}]$, affinity K_D and stoichiometry n .

Analysis of the binding-site geometry

Atomic coordinates of intact HSA (1AO6 [21], 1E7A and 1E7B [6]) were obtained from the Protein Databank (<http://www.rcsb.org/pdb>). Cavity volume (the volume enclosed by the molecular surface of wall atoms) of the anaesthetic binding site in domain 3 were determined from intact HSA by CASTp, a web-based program for cavity and pocket determination (<http://cast.engr.uic.edu/cast/>) [22].

RESULTS

Secondary structure and stability

Far-UV CD spectroscopy demonstrated that the secondary structure of the wtHSAAd3 and the Y411W mutant are predominantly

Figure 2 Far UV-CD of wtHSAAd3 and Y411Wd3

Proteins were dissolved in 10 mM sodium phosphate buffer (pH 7.0), at a concentration of 2.5 μM HSA or 5.5 μM (domain). The CD signals of intact wtHSA and the domains are overlapped.

Figure 3 HX of HSA and the two fragments

(a) The change in exchange-out rate reflects the change in stability of the domain peptides. The wtHSAAd3 peptide is 1.4 kcal/mol less stable than intact wtHSA. (b) The Y411W mutation is 0.3 kcal/mol more stable than wtHSAAd3.

α -helical (Figure 2), not distinguishable from that of wtHSA, which is approx. 60% α -helical as determined by us and others [23,23a]. Using HX, we found that wtHSAAd3 is less stable ($\Delta\Delta G$ is 1.4 kcal/mol; 1 cal = 4.184 J) than intact wtHSA (Figure 3a). Relative to the wtHSAAd3, the Y411W mutation stabilizes the peptide slightly ($\Delta\Delta G = -0.3$ kcal/mol, Figure 3b). This same rank order of stability is also indicated by sensitivity to guanidine denaturation monitored by CD. The denaturation midpoint was estimated to be 2.0, 2.6 and 2.8 M guanidine for wtHSAAd3, Y411Wd3 and intact HSA respectively. The denaturation midpoint for HSA is the same as reported previously [15].

Halothane binding

Fluorescence

The wavelength maxima for tyrosine fluorescence in wtHSAAd3 was observed at 307 nm. A maximum of 44–47% quenching of

Figure 4 Intrinsic fluorescence quenching

Tyrosine was excited at 280 nm and tryptophan was excited at 295 nm. ●, wtHSAd3; and ■, Y411Wd3. Since there are four tyrosine residues in domain 3, lack of full quenching indicates not all are accessible to halothane, in agreement with the crystal structures.

the fluorescence emission from tyrosine residues could be reached by 10 mM halothane in wtHSAd3 with an IC_{50} of 1.8 ± 0.3 mM (Figure 4). The wavelength maximum for tryptophan fluorescence

in Y411Wd3 was observed at 330 nm and a maximum of 86–92 % quenching was reached by 10 mM halothane with an IC_{50} of 1.9 ± 0.4 mM (Figure 4).

ITC

The interaction between halothane and the two domain peptides is exothermic. Hyperbolic rather than the ideal sigmoid isothermal titration curves were observed even when sequential injections were performed (Figures 5a and 5b). Both the curve for wtHSAd3 and Y411Wd3 can be best fitted to the sequential binding (co-operative binding) model with $n = 2$. No significant difference in association constants for halothane was detected between the wild-type peptide and the mutant. However, a larger enthalpy change was observed in wtHSAd3 compared with the mutant. The thermodynamic parameters are presented in Table 1.

Hydrogen exchange

Halothane binding stabilized both wtHSAd3 and Y411Wd3 significantly, but produced larger $\Delta\Delta G$ values in wtHSAd3 (-1.3 kcal/mol, Figure 6, upper panel) than in Y411Wd3 (-0.9 kcal/mol, Figure 6, lower panel).

Figure 5 Halothane ITC

(a) Halothane titration into wtHSAd3 and (b) halothane titration into Y411Wd3. In both cases, an exothermic process was noted. The derived parameters are presented in Table 1. Each downward peak represents heat change of each titration and sequential titrations were performed.

Table 1 Thermodynamic parameters for anaesthetic interaction

The isothermal curve was best-fitted to the sequential binding model with $n = 2$ for halothane interaction with domains and propofol with the wild-type (wtHSAd3). The isothermal curve was best-fitted to the one-site model with $n = 1.2$ for propofol with the mutant (Y411Wd3). Data are presented as mean $\pm$ S.D. χ^2 for halothane was 3.3 in wtHSAd3 and 1.5 in Y411Wd3. χ^2 for propofol was 282 in wtHSAd3 and 563 in Y411Wd3. Halo, halothane; PFL, propofol.

	Set 1			Set 2		
	$K_1 (1 \times 10^3 \cdot M^{-1})$	ΔH_1 (kcal/mol)	ΔS_1 (cal $\cdot$ mol $^{-1} \cdot$ K $^{-1}$)	$K_2 (1 \times 10^3 M^{-1})$	ΔH_2 (kcal/mol)	ΔS_2 (cal $\cdot$ mol $^{-1} \cdot$ K $^{-1}$)
wtHSAd3-Halo	0.7 ± 0.2	-3.8 ± 1.0	-0.1	0.2 ± 0.1	-7.9 ± 1.4	-16.1
Y411Wd3-Halo	0.8 ± 0.1	-2.5 ± 0.4	4.6	0.2 ± 0.01	-5.7 ± 5.3	-8.9
wtHSAd3-PFL	32 ± 4	-3.2 ± 0.2	9.5	2 ± 1	-2.1 ± 0.5	7.9
Y411Wd3-PFL	8 ± 2	-2.3 ± 0.7	10.0

Figure 6 Halothane effects on stability by HX

Upper panel, exchange-curves for wtHSA, in the presence and absence of 7 mM halothane; and lower panel, analogous set of curves for Y411Wd3. Halothane enhanced the stability of both peptides.

Propofol binding

ITC

The interaction between propofol and the two domain peptides is also exothermic with hyperbolic titration curves (Figures 7a and 7b). Titration of 0.5 mM of propofol into wtHSA, produced relatively larger heat release compared with Y411Wd3. The isothermal curve for wtHSA was best fitted with a sequential binding (co-operative binding) model for $n = 2$. However, the isothermal curve for Y411Wd3 was best fitted with the one-site

Figure 8 Propofol and halothane binding competition

Halothane was titrated into 0.04 mM wtHSA after pre-equilibration with propofol. No binding signal was observed, indicating occupancy of halothane binding sites by propofol.

model for $n = 1.2$. The thermodynamic parameters are presented in Table 1. To determine the similarity of the binding site between halothane and propofol, a propofol-saturated wtHSA sample (0.04 mM) was further titrated with halothane, and no significant heat change was observed (Figure 8).

Hydrogen exchange

Propofol binding stabilized both wtHSA and Y411Wd3 significantly, with a larger $\Delta\Delta G$ in wtHSA (-1.3 kcal/mol, Figure 9, upper panel) than in Y411Wd3 (-0.3 kcal/mol, Figure 9, lower panel).

Figure 7 Propofol ITC

Propofol titration (a) into wtHSA and (b) into Y411Wd3. Less heat release was observed for the Y411Wd3 peptide. The derived parameters are presented in Table 1.

Figure 9 Propofol effects on stability by HX

The upper panel shows the exchange-out curves for wtHSA in the presence or absence of 0.5 mM of propofol (PFL) and the lower panel shows the analogous set of curves for Y411Wd3. Note the greater stabilization of wtHSA as compared with Y411Wd3 and for propofol as compared with halothane.

Binding cavity information

Since the high-resolution structure of isolated HSA domains were not available, we used the structure of intact HSA for cavity calculation. As determined by CASTp using a $1.4 \text{ \AA}$ ($1 \text{ \AA} = 10^{-10} \text{ m}$) radius probe, the calculated internal cavity surface volume of the Tyr⁴¹¹ binding site for both halothane and propofol in domain 3 is around $340 \text{ \AA}^3$ in unliganded molecule (1AO6) and around $500 \text{ \AA}^3$ in a liganded complex (1E7A and 1E7B). The second propofol pocket has a calculated volume of $810 \text{ \AA}^3$.

DISCUSSION

The major finding of this study is that, relative to intact HSA, the structure and anaesthetic binding character of the constructed domain 3 of HSA is well maintained. At the level of a secondary structure, no significant changes from intact HSA were detected, predominantly α -helical (60%), and anaesthetic binding affinity as reflected by fluorescence and ITC is altered by less than 2-fold. This provides confidence in the general strategy of truncation to study ligand-binding–structure relationships in otherwise intractable systems.

Propofol binding

Two propofol binding sites were reported in HSA both in crystallographic [6] and solution studies [7]. Both of these sites reside in domain 3, and one is of somewhat higher affinity than the other [6,24]. Consistent with this, our current ITC results indicated the existence of two propofol binding sites of differing affinity in the truncated peptide, and the expected stabilization was observed. The Y411W mutation appeared to eliminate a site or at least to lower significantly the binding to a site, reflected by both ITC and HX results. The most reasonable explanation is that the larger tryptophan residue ($\sim 35 \text{ \AA}^3$ difference) sterically hindered access of propofol to the Tyr⁴¹¹ site. Because the larger tryptophan residue might have improved van der Waals interactions within

this cavity, this estimated volume difference might underestimate the actual change. For example, a Tyr to Trp mutation in the active site of cyclodextrin glycosyltransferase decreased the volume of the binding site from $930 \text{ \AA}^3$ in wild-type (1CDG) [25] to $740 \text{ \AA}^3$ in Y195W (1CGY) [26] as determined by CASTp. Thus, in the case of propofol, it was not possible to estimate the contribution of π -cation interactions due to steric hindrance, but in the unaltered peptide, propofol binding is well maintained.

Halothane binding

Previous ITC studies of intact HSA found approx. eight binding sites for halothane, highly consistent with the crystal structure of the halothane–HSA complex [6,12,24]. For the isolated domain 3, two binding sites with different affinity were suggested. The K_A for the intact HSA was previously reported [10,12] as approx. 600 M^{-1} , whereas for domain 3 ‘high’ affinity site, it is essentially unchanged at approx. 700 M^{-1} . The crystal complex reported only a single halothane site in domain 3. However, the predicted second site is of lower affinity (200 M^{-1}) compared with the first, and thus would have been very poorly occupied under the reported crystal conditions. Both the crystal structure predicted from previous studies and our results show that the Tyr⁴¹¹ site can accommodate either halothane or propofol, so it seems reasonable that the other, lower affinity, propofol site may be the lower affinity halothane site. This was confirmed by the competition experiments. The lower affinity probably caused by this cavity being too large for the smaller halothane ($\sim 93 \text{ \AA}^3$). As determined by CASTp, the size of the cavity is more than double the size of the Tyr⁴¹¹ cavity ($> 800 \text{ \AA}^3$ versus $340\text{--}500 \text{ \AA}^3$).

Unlike propofol, the mutation did not significantly alter halothane binding parameters. This is most probably due to the smaller size of halothane relative to propofol. Also, the higher density π -electron cloud of the indole group must not contribute significantly to the energetics of halothane binding over that offered by the wild-type tyrosine residue, unless there are other offsetting unfavourable interactions. This is consistent with our previous conclusions with respect to π -cation interactions in HSA [10,12], but is in contrast with conclusions from a designed 4-helix bundle system [27].

Altered features and limitations

We noted differences in stability as reflected by both HX and CD results. The isolated domain was 1.4 kcal/mol less stable than the parent molecule, indicating that domain–domain interactions serve to stabilize the individual domain folded structure. This may have important implications for extrapolating binding and structural relationships to any functional effects of the protein. The decrease in stability suggests lower energetic barriers for accessing conformations underlying functional changes, and since the present study suggests that anaesthetic binding carries roughly the same binding free energy, the relationship between binding and functional change could be skewed. Thus it would appear that caution is required for extrapolating functional effects from truncated systems. The Y411W mutation did not alter the secondary structure, but the domain stability was increased somewhat. The most probable explanation is that the larger tryptophan residue recovered a portion of the cavity destabilizing effect by partial refilling.

Summary

Truncation of the wtHSA molecule to the isolated domain 3 does not significantly alter the secondary structure or anaesthetic binding properties, indicating feasibility as a general strategy for examining binding–structure relationships in otherwise

intractable systems. Domain stability is significantly decreased after truncation, however, suggesting that the relationship between binding and functional change in other protein systems could be altered by this strategy.

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