Original Paper

Pharmacology

Pharmacology 2002;64:152–159

Received: February 28, 2001
Accepted after revision: April 5, 2001

Binding of the Active Metabolite of Chloral Hydrate, 2,2,2-Trichloroethanol, to Serum Albumin Demonstrated Using Tryptophan Fluorescence Quenching

Ken Solt[a] Jonas S. Johansson[a, b]

[a]Department of Anesthesia and [b]Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa., USA

Key Words

Anesthetic-protein interaction · Trichloroethanol · Fluorescence · Anesthetic mechanisms · Albumin · Chloral hydrate

Abstract

Chloral hydrate, a sedative/hypnotic agent widely used in the pediatric population, is converted to the active metabolite 2,2,2-trichloroethanol (TCE) in the liver. Tryptophan fluorescence quenching has been used previously to show that halothane and chloroform bind saturably to serum albumin, and a similar approach is used here to demonstrate that TCE also binds to albumin. TCE quenches the steady-state tryptophan fluorescence of bovine serum albumin (BSA) in a concentration-dependent, saturable manner with a K_D = 3.3 ± 0.3 mmol/l. Unlike halothane and chloroform, however, TCE also elicits a concentration-dependent blue-shift in the fluorescence emission spectrum of BSA and human serum albumin. This indicates that TCE induces a conformational change in the protein, causing the tryptophan to experience a change in its chemical environment, thus shifting the peak of the emission spectrum. Circular dichroism spectroscopy revealed a decrease in the α-helical content of BSA from 65.8 ± 0.4 to 62.9 ± 0.6% when TCE was present at a concentration of 30 mmol/l, providing further evidence for a conformational change. There is evidence that TCE potentiates the action of ligand-gated ion channels such as the $GABA_A$ and 5-HT_3 receptors, and the present results suggest that anesthetic alcohols may act by binding to these proteins *and* inducing structural changes that may in turn alter protein function.

Introduction

Chloral hydrate is an orally administered sedative/hypnotic agent that is widely used in the pediatric population [1]. It is rapidly converted in the liver by alcohol dehydrogenase to 2,2,2-trichloroethanol (TCE), which is thought to be the active compound responsible for its pharmacological effects [2]. The mechanism of action of TCE is unknown, although it has been shown that TCE potentiates γ-aminobutyric acid (GABA) function in mouse hippocampal neurons [3], and enhances 5-hydroxytryptamine$_3$ (5-HT_3) receptor function in the rat vagus nerve [4].

This hydrophobic molecule is likely bound to the carrier protein serum albumin when present in the bloodstream, and this binding interaction provides a model system for studying the potential effects of TCE on its functional target in vivo. Quenching of native tryptophan flu-

KARGER

Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0031–7012/02/0643–0152$18.50/0

Accessible online at:
www.karger.com/journals/pha

Jonas S. Johansson
778 Dulles, Hospital of the University of Pennsylvania
3400 Spruce St, Philadelphia, PA 19104 (USA)
Tel. +1 215 349 5472, Fax +1 215 349 5078
E-Mail johansso@mail.med.upenn.edu

orescence has been utilized previously do demonstrate the saturable binding of the general anesthetics halothane and chloroform to serum albumin [5, 6]. TCE is known to be an efficient quencher of protein fluorescence, and has been shown to cause 'exalted quenching' of tryptophan residues in bovine and human serum albumin, attributed to hydrophobic interactions between TCE and protein [7]. Here we use fluorescence quenching to demonstrate that TCE not only binds to albumin, but unlike halothane and chloroform, elicits a conformational change in the protein. Changes in protein structure following the binding of anesthetic agents are likely to be responsible for changes in protein function. Studies with model proteins that interact with anesthetic molecules therefore provide guidelines for plausible structural changes expected when these important clinical compounds perturb the function of ligand-gated ion channels.

Materials and Methods

Materials

TCE (99+%) was purchased from Aldrich Chemical Company (Milwaukee, Wisc.). Human serum albumin (HSA), bovine serum albumin (BSA) and N-acetyl tryptophanamide (NATA) were purchased from Sigma Chemical Company (St. Louis, Mo.). All other chemicals used were reagent grade.

Experiments were conducted with 1 μmol/l protein, in an aqueous buffer solution of 130 mmol/l NaCl and 20 mmol/l sodium phosphate, at pH 7.0. Protein concentration was determined with a Shimadzu (Columbia, Md.) model UV-160U UV-visible recording spectrophotometer, using extinction coefficients of 45,000 $(mol/l)^{-1} \cdot cm^{-1}$ for BSA and 36,000 $(mol/l)^{-1} \cdot cm^{-1}$ for HSA, both at 279 nm [8]. All spectral measurements were made using a 10-mm pathlength quartz cuvette.

Protein solutions were drawn up in 10-cm^3 gas-tight Hamilton (Reno, Nev.) syringes, and TCE was added to a final concentration of 30 mmol/l. The mixture was stirred with a small magnetic stir bar for 60–90 min, allowing for complete equilibration of TCE with the protein in buffer solution. TCE may react chemically with tryptophan residues under the influence of ultraviolet light [9], so care was taken to wrap the syringes with aluminum foil to minimize light exposure. For each experiment, the solution of protein equilibrated with TCE was diluted by a stock solution of protein alone, to achieve the final concentrations of TCE noted in the figures.

Fluorescence Spectroscopy

Fluorescence spectra were recorded with a Shimadzu (Columbia, Md.) Spectrofluorometer RF-5301PC connected to a PC running Windows. Personal Fluorescence Software for RF-5301PC (Shimadzu, version 1.2) was used to analyze the data. Emission spectra were taken using an excitation wavelength of 295 nm, to minimize excitation by radiationless transfer from tyrosine residues [8]. Excitation and emission slit widths were set at 10 and 1.5 nm, respectively. Scan speed was set to 'fast' (8.3 nm/s) in order to minimize the possibility of a photochemical reaction between TCE and tryptophan residues. To ensure that the amount of UV light exposure required to take a fluorescence spectrum would not cause a significant reaction, a solution of BSA with 15 mmol/l TCE was subjected to two scans taken in succession, and no detectable change was observed in the emission spectrum. Inner filter effects [10] were found to be negligible (absorbance by TCE was undetectable at both emission and excitation wavelengths). An absorption spectrum taken from 200 to 400 nm revealed a single peak for TCE at 209 nm (spectrum not shown), allowing calculation of the extinction coefficient for TCE [44 $(mol/l)^{-1} \cdot cm^{-1}$] at this wavelength.

A baseline fluorescence spectrum of buffer solution alone (without protein or TCE) was subtracted from all spectra. The 'peak pick' and 'point pick' programs were used to obtain the fluorescence intensity and the wavelength of each emission peak, respectively.

Circular Dichroism Spectroscopy

Circular dichroism spectra were recorded with a DS spectropolarimeter (Aviv Model 62, Lakewood, N.J.). BSA (1 μmol/l) in a 10 mmol/l potassium phosphate buffer at pH 7.0 was equilibrated with 30 mmol/l TCE in a gas-tight Hamilton syringe. Spectra were taken using a 2-mm pathlength quartz cell. The temperature of the cell holder was kept at 25.0 ± 0.1 °C. The bandwidth was 1.0 nm, with a scan step of 0.5 nm and an average scan time of 3.0 s per sampling point.

Curve Fitting and Statistics

Best-fit curves were generated using the Prism™ (Graphpad Inc., version 2.01, 1996) program for Windows. Data are expressed as mean ± standard deviation.

Results

Representative fluorescence spectra obtained during one experiment with BSA are shown in figure 1. TCE causes a concentration-dependent decrease in the native tryptophan fluorescence of BSA. Taking F_0 as the native fluorescence in the absence of quencher, it follows that

$$Q = (F_0 - F)/F_0 \quad (1)$$

where F = the fluorescence in the presence of quencher, and Q = the quenched fluorescence. Quenching is a function of the maximum fluorescence that can be quenched (Q_{max}) and the affinity of TCE for the binding sites in BSA close to the two tryptophan residues (the average dissociation constant, K_D). It follows from mass law considerations that

$$Q = (Q_{max} \cdot [TCE])/(K_D + [TCE]) \quad (2)$$

Figure 2 (curve A) is a plot of Q versus [TCE] for BSA at pH 7.0. Using a best-fit curve derived from equation 2, the Q_{max} = 0.93 ± 0.02 and the K_D = 3.3 ± 0.3 mmol/l indicate that both of the tryptophan residues in BSA are effectively quenched by TCE. Fluorescence lifetime studies have been used to show that TCE is a static quencher

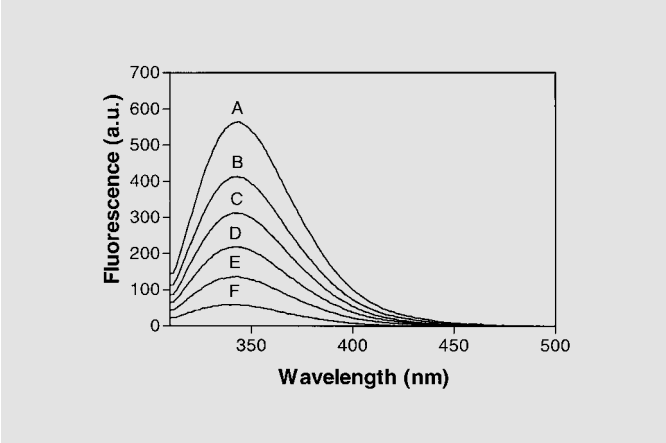

Fig. 1. Fluorescence spectra of 1 μmol/l bovine serum albumin in pH 7.0 buffer, equilibrated with TCE at the following concentrations: 0 mmol/l (curve A), 1 mmol/l (curve B), 3 mmol/l (curve C), 7 mmol/l (curve D), 15 mmol/l (curve E) and 30 mmol/l (curve F).

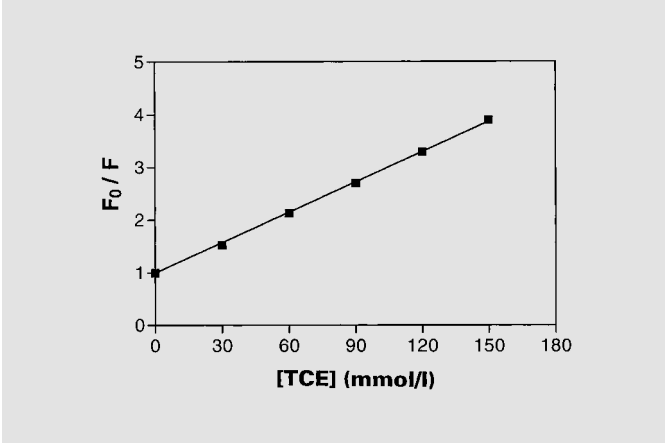

Fig. 3. Stern-Volmer plot showing TCE quenching of NATA in methanol. Data points are the average of three experiments, with error bars representing the standard deviation. The data points were fit to the classic Stern-Volmer equation, $F_0/F = 1 + K_{sv} \cdot [TCE]$, where F_0 and F are, respectively, the fluorescence intensity in the absence and presence of a given concentration of TCE, and K_{sv} is the collisional quenching constant. Error bars are contained within the data points.

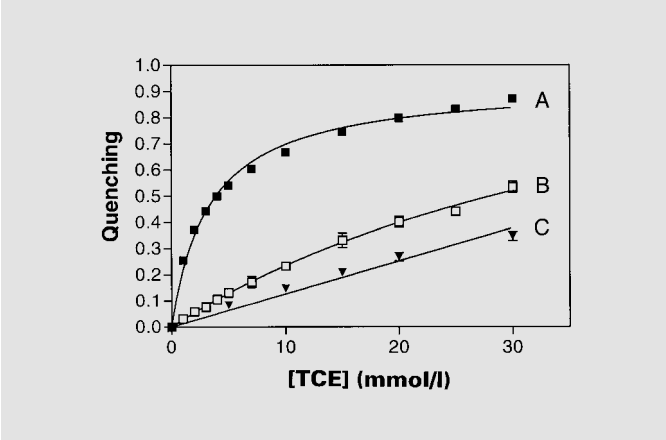

Fig. 2. Quenching of 1 μmol/l BSA by TCE at pH 7.0 (curve A) and pH 3.0 (curve B). Curve C represents quenching of 2 μmol/l NATA by TCE at pH 7.0. Data points are the average of three experiments, with error bars representing the standard deviation. Curves A and B were generated using equation 2. Curve C is a least-squares linear regression fit. Where error bars are not apparent they are contained within the data points.

of the *Streptomyces* subtilisin inhibitor [11], and static quenching due to binding of TCE is the likely mechanism with BSA as well, because the quenching is much more effective compared to what is seen with NATA (fig. 2, curve C).

In order to determine the importance of intact native tertiary structure for TCE binding, experiments were carried out in pH 3.0 buffer (fig. 2, curve B). At this pH, albumin changes shape from the 'N' (normal) form to the 'E' (expanded) conformation [8]. In the 'E' form, the albumin molecule is an elongated 'series of balls and strings' [12], and the change in the environment of the tryptophan residues causes a blue-shift of the fluorescence emission peak (consistent with a less polar environment), along with a concomitant decrease in quantum yield. As shown in figure 2 (curve B), this change in tertiary structure results in a marked loss of binding. The concentration of TCE required to achieve half-maximal quenching is approximately 10-fold higher at pH 3.0 (about 30 mmol/l compared to 3.3 mmol/l at pH 7.0).

As a control, experiments were carried out with NATA in pH 7.0 buffer to study the collisional (dynamic) quenching of free tryptophan by TCE. The results (fig. 2, curve C) show that only about 30% of the total fluorescence is quenched at a TCE concentration of 30 mmol/l. The published dynamic quenching constant for indole in water is 21 $(mol/l)^{-1}$ [7], which corresponds to a TCE concentration of 48 mmol/l to achieve half-maximal quenching, in agreement with the present results. To confirm that indole and NATA behave similarly, a Stern-Volmer plot of NATA quenching in methanol was obtained (fig. 3), yielding a quenching constant, K_{sv}, of 19.1 ± 0.1 $(mol/l)^{-1}$, in agreement with the published dynamic quenching constant of 21 $(mol/l)^{-1}$. These results suggest that

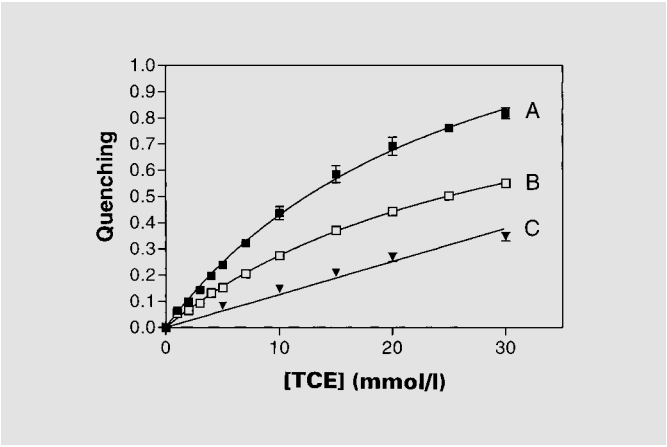

Fig. 4. Quenching of 1 µmol/l human serum albumin by TCE at pH 7.0 (curve A) and pH 3.0 (curve B). Quenching of 2 µmol/l NATA by TCE at pH 7.0 (curve C). Data points are the average of three experiments, with error bars representing the standard deviation. Curves A and B were generated using equation 2. Curve C is a least-squares linear regression fit. Where error bars are not apparent they are contained within the data points.

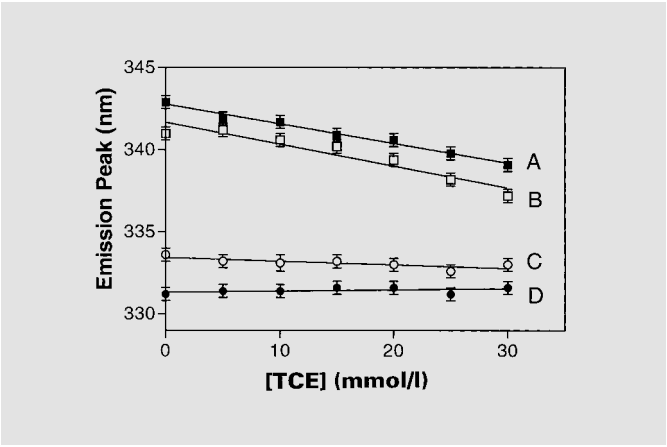

Fig. 5. Plots of fluorescence emission peak wavelength vs. concentration of TCE for BSA at pH 7.0 (curve A) , HSA at pH 7.0 (curve B), HSA at pH 3.0 (curve C) and BSA at pH 3.0 (curve D). Data points are the average of three experiments, with error bars representing the standard deviation. Because the sensitivity of the 'point pick' program used to find emission peaks was ±0.4 nm, this number was used to produce error bars when the calculated SD was less than 0.4 nm.

quenching of NATA by TCE is also due to random collisions. The quenching observed with NATA at pH 7.0 is similar in magnitude to the quenching observed with BSA at pH 3.0 (fig. 2, curve C and B, respectively), indicating that when the native tertiary structure of BSA is lost, most quenching can be accounted for by random collisions of TCE with tryptophan residues exposed to solvent, and not from binding interactions.

Figure 4 shows quenching data for HSA. The data for NATA at pH 7.0 (curve C) is the same as in figure 2 and is provided for comparison. Unlike halothane and chloroform [5, 6], which have more comparable dissociation constants for HSA and BSA during the fluorescence quenching method (3.9 vs. 1.8 mmol/l, respectively, for halothane, and 5.6 vs. 2.7 mmol/l, respectively, for chloroform), TCE exhibits much weaker binding to HSA than BSA. Indeed, because the binding interaction is so weak, it is difficult to generate a precise binding curve over the concentration range studied. The use of TCE concentrations higher than 30 mmol/l was avoided, however, because high concentrations of TCE may cause solvent changes that influence protein conformation and stability, and are unrelated to binding effects. Furthermore, experimental error increases as fluorescence signals decrease at high levels of quenching, due to signal-to-noise constraints.

Assuming a Q_{max} of 1.0, however, we can use figure 4 (curve A) to estimate the point of half-maximal quenching as the K_D for HSA at pH 7.0 (approximately 12 mmol/l). At pH 3.0 (figure 4, curve B), tertiary structure is lost as albumin assumes the 'E' conformation, and HSA behaves similarly to BSA (fig. 2, curve B) under these conditions.

BSA has two tryptophan residues, at positions 134 and 212. HSA has only one at position 214, which is considered to be analogous to tryptophan 212 in BSA [8]. Figure 2 shows that TCE is able to quench the fluorescence of both tryptophan residues in BSA, indicating the presence of at least two binding sites. The K_D found for BSA using a best-fit curve (3.3 mmol/l) is therefore the average K_D for the two binding sites. To a first approximation, the analogous tryptophans (212 in BSA and 214 in HSA) can be considered to be in similar chemical and structural environment with equal binding affinities for TCE. Assuming this, and applying the K_D for HSA (12 mmol/l) to the site in BSA close to the conserved tryptophan 212, it is possible to calculate the approximate affinity of TCE for the second binding site close to tryptophan 134 in BSA, by fitting the data in figure 1 (curve A) to an equation of the form [5]

$$Q = (0.37 \cdot [TCE])/(12 + [TCE]) + (0.63 \cdot [TCE])/(K_d^{134} + [TCE]) \quad (3)$$

where TCE concentrations are expressed in millimoles per liter; 0.63 and 0.37 are the experimentally determined

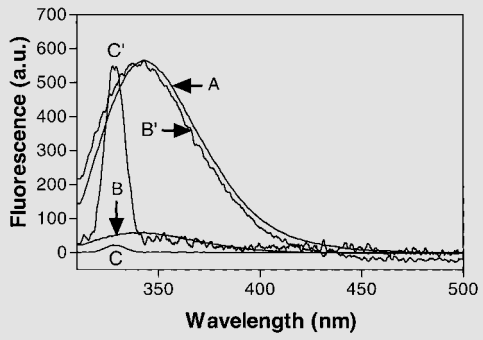

Fig. 6. Fluorescence emission spectra for BSA (curve A), BSA with 30 mmol/l TCE (curve B) and BSA with 30 mmol/l TCE multiplied by a factor of 9.5 (curve B'). Curve C shows the water Raman band at 330 nm, observed with 295 nm excitation. Curve C' is curve C multiplied by a factor of 25.

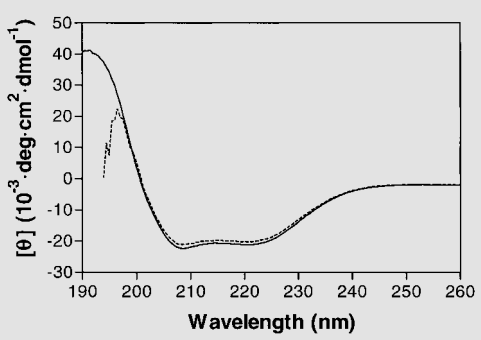

Fig. 7. Circular dichroism spectra for 1 µmol/l BSA (——) and 1 µmol/l BSA with 30 mmol/l TCE (– – –). For each condition, four individual spectra were obtained and the data averaged to produce the curves shown.

contributions of each tryptophan residue to the total fluorescence at 344 nm [8], and K_d^{134} = the dissociation constant of the tryptophan 134 site. This equation allows a value of 2.1 ± 0.1 mmol/l to be calculated for the K_d^{134} of this more exposed site. The correlation coefficient improves slightly when the data is fitted to this two-affinity model (0.997 vs. 0.992 for the one-affinity model).

As shown in figure 5, TCE causes a concentration-dependent shift in the tryptophan fluorescence emission peak for both BSA and HSA at pH 7.0 (curves A and B, respectively), but not at pH 3.0 (curves C and D for HSA and BSA, respectively). There was also no peak shift observed for NATA at pH 7.0 (data not shown). Figure 6 compares the fluorescence spectrum of BSA at pH 7.0 (curve A, solid line), and the spectrum of BSA with 30 mmol/l TCE multiplied by a factor of 9.5 (dashed line), to further demonstrate the presence of the peak shift. For BSA, the fluorescence emission peak shifted from 342.9 ± 0.4 to 339.1 ± 0.4 nm, and for HSA, the peak moved from 341.0 ± 0.4 to 337.2 ± 0.4 nm, at TCE concentrations of 0 and 30 mmol/l, respectively. (The sensitivity of the 'point pick' program used to identify emission peaks was 0.4 nm, and where the SD of three experiments was less than 0.4 nm, this number was used instead of the calculated SD to express the experimental error.)

These results show that upon binding to albumin at pH 7.0, TCE is causing a change in the chemical environment of the tryptophan residue(s). The blue-shift in the spectrum suggests that as the TCE concentration is increased, the tryptophan is experiencing a more hydrophobic environment. This effect cannot be attributed merely to the presence of TCE in the proximity of tryptophan, since no peak shift is observed with uncoiled BSA at pH 3.0, nor with NATA. The results indicate that TCE binding causes a conformational change in serum albumin close to physiological pH, an effect which was not observed previously with halothane or chloroform [5, 6]. Furthermore, the similar results obtained with BSA and HSA (fig. 5) suggest that the effect is mediated by an analogous binding site on the two proteins, perhaps the one close to the conserved tryptophan (212 for BSA, 214 for HSA), or alternatively a third binding site without a nearby tryptophan (and hence undetectable by our methods).

In order to further test whether TCE is indeed causing a conformational change in the protein at pH 7.0, circular dichroism spectra were obtained for BSA with and without 30 mmol/l TCE (figure 7). At 208 and 222 nm, the two minima observed in the negative absorption bands, BSA without TCE exhibited negative ellipticities of 22,250 ± 170 and 21,040 ± 140, respectively, whereas BSA with 30 mmol/l TCE exhibited negative ellipticities of 21,000 ± 380 and 20,120 ± 210 at the same wavelengths (data are expressed as mean ± SD of four experiments). Using $-[\theta]_{222}$ = 32,000 for a 100% α-helical content [13], our value of $[\theta]_{222}$ for BSA at pH 7.0 is consistent with 65.8 ± 0.4% α-helical content, in agreement with published values ranging from 65 to 67% [12]. With 30 mmol/l TCE, however, the observed ellipticity at

222 nm is consistent with an α-helical content of only 62.9 ± 0.6%. These subtle but important differences between the CD spectra confirm that TCE is causing a change in the conformation of albumin.

Discussion

Various studies have been conducted to assess the effect of TCE on ligand-gated ion channels, which are thought to be important sites of general anesthetic action [14, 15]. TCE increases the magnitude of depolarization observed with 5-HT_3 receptors in the rat vagus nerve, with a reported EC_{50} of 3.6 ± 0.9 mmol/l at a 5-HT concentration of 0.3 μmol/l [4]. In mouse hippocampal neurons, TCE potentiates the GABA-activated chloride current in a concentration-dependent manner, with an observed EC_{50} of 3.0 ± 1.4 mmol/l [3]. TCE has also been shown to inhibit N-methyl-*D*-aspartate receptor function in rat mesencephalic and cortical neurons, with IC_{50} values of 2.76 ± 0.04 and 1.12 ± 0.38 mmol/l, respectively [16]. These data all correspond well with the observed K_D for BSA at the site close to tryptophan 134 (2.1 mmol/l), suggesting that BSA may serve as a good model for how TCE interacts with these large ligand-gated ion channels. BSA is architecturally better understood than these membrane proteins, and is therefore amenable to biophysical studies of higher structural resolution, which may provide information on how bound anesthetic molecules might alter protein function. Further, when mice were given a dose of TCE equivalent to the ED_{50} value (200 mg/kg), the level of TCE in blood was found to be 4–5 mg/ml [17], corresponding to a concentration range of 27–34 mmol/l. The sites on BSA therefore have an average K_D that is approximately 10 times smaller than the whole animal EC_{50} value. The lack of correspondence between the EC_{50} values for TCE action on the 5-HT_3 and $GABA_A$ receptors, and the K_D for binding to BSA, compared to the mouse ED_{50} value, represent further examples of how it is difficult to predict a priori what the K_D of an in vivo target will be based on whole animal potency data [18].

In BSA, tryptophan residue 134 is more exposed to the aqueous solvent than the conserved tryptophan 212 [8], and greater accessibility of TCE to this protein region may account for the lower K_D observed for BSA. Previous studies with BSA using fluorescence quenching showed that halothane also favors this site (K_D = 1.3 mmol/l) over the more buried site (K_D = 3.9 mmol/l), although the difference in affinities is not as large as that observed for TCE (K_D values of 2.1 and 12 mmol/l, respectively). Photoaffinity labeling studies have identified at least four distinct binding sites for halothane in BSA [19], an x-ray crystallography has revealed four fatty acid binding sites in HSA [20]. TCE may also bind to more sites than the two identified in the current study. The approach using fluorescence quenching does not provide information about binding sites without a nearby tryptophan.

Another important difference between the results with TCE and the earlier studies with halothane and chloroform is the presence of a shift in the fluorescence emission peak. Both BSA and HSA exhibit an incremental blue-shift in the emission peak as the concentration of TCE increases. Fluorescence is blue-shifted if the local electric field is oriented in a direction counter to the tryptophan dipole change during excitation. This will occur if a positive charge is near the pyrrole ring, or if a negative charge is near the benzene ring of the indole group [21]. For BSA at pH 3.0 and NATA at pH 7.0 no shift in emission peak is observed, indicating that the shift is probably not due to any direct interaction between the tryptophan and the TCE. Rather, the binding of TCE is likely to elicit a conformational change in the protein, causing the tryptophan (presumably the conserved residue at position 212, since similar changes were determined for HSA) to experience a slightly different electric field induced by charge redistribution in the protein matrix [22]. Emission peak shifts have been reported previously with TCE for human adenosine deaminase, but the shift was accounted for by a heterogeneous emission spectrum produced by several tryptophan residues in distinct environments interacting differently with TCE, and not by a change in protein conformation [23]. The results obtained with the single-tryptophan protein HSA, however, strongly suggest that TCE is indeed inducing a conformational change.

This conclusion is also supported by the results obtained with CD spectroscopy, which suggest that TCE binding to BSA is associated with a 4.4–5.6% decrease in α-helical content. Some of the decrease in the CD signal may however be due to changes in the tryptophan environments as noted above, since aromatic residues also contribute to the far-UV spectra [24]. BSA has 582 residues, and assuming 66% helicity, there would normally be on the order of 384 backbone amide groups involved in α-helix formation. The CD results indicate that 17–21 of these amide hydrogen bonds are being disrupted upon addition of TCE, suggesting that there are more than two TCE molecules bound to each molecule of BSA. Disruption of hydrogen-bonded networks in biological macromolecules as a mechanism of anesthetic action has been proposed previously [25, 26], and may apply in the case of

the alcohols. This does not represent a nonspecific cosolvent effect because under such conditions alcohols will increase protein α-helical content [27]. Earlier studies with the inhaled anesthetics halothane and chloroform failed to detect any secondary structural changes in BSA and also in a small four-α-helix bundle protein [5, 6, 28]. The current results therefore indicate that alcohols and halogenated alkanes interact quite differently with similar sites on a protein, presumably related to the greater hydrogen-bonding potential of the alcohol group.

Eight amino acid side chains (lysine, tyrosine, glutamine, asparagine, glutamic acid, aspartic acid, cysteine and histidine) and the peptide bond are known to quench tryptophan fluorescence [29, 30]. Therefore, a change in protein conformation alone may alter the degree of quenching observed, if the spatial relationships among quenching amino acid residues and the indole ring are altered. This may explain why the observed Q_{max} for BSA at pH 7.0 is only 0.93 ± 0.02 for TCE, while the Q_{max} is 0.99 ± 0.04 for halothane; and 1.01 ± 0.02 for chloroform [5, 6]. However, the excellent correlation coefficients obtained from our best-fit curves for BSA at pH 7.0 (0.992 and 0.997 for one- and two- affinity models, respectively), along with the observed Q_{max} close to unity (0.93), suggest that changes in fluorescence quenching due to conformational changes elicited by TCE are likely to be small.

The mechanism whereby TCE quenches typtophan fluorescence in proteins probably involves internal conversion due to electron exchange, and possibly a contribution from heavy atom enhanced intersystem crossing secondary to spin orbital coupling [31, 32]. Both mechanisms require close contact (electron overlap) between TCE and the indole ring, although there is some evidence that quenching can occur at distances of 3–5 Å [32, 33]. The ability of TCE to quench tryptophan fluorescence in BSA and other macromolecules therefore reports directly on the binding location of TCE in the protein matrix. The results with BSA indicate that TCE is binding in the vicinity of tryptophan 134 and tryptophan 212, as previously reported for halothane and chloroform [5, 6]. We are not able to determine the total number of binding sites for TCE per BSA molecule using the fluorescence quenching approach. The finding that TCE quenches the fluorescence of both tryptophan residues in BSA suggests that TCE is binding to at least two sites. Binding of TCE to other sites in BSA cannot be detected using this approach.

The in vivo targets responsible for the pharmacological effects of TCE may include ligand-gated ion channels such as the $GABA_A$ and 5-HT_3 receptors. These large membrane proteins are not amenable to the kinds of structural analyses reported here for albumin. The results of the current study lend support to the possibility of a protein target which undergoes a conformational change upon binding TCE. The results also suggest that anesthetic alcohols may bind tightly enough to receptor proteins to induce significant structural changes, which may subsequently alter protein function. Anesthetic-induced structural changes in their potential targets have been difficult to detect experimentally to date [15], but are ultimately responsible for altering protein activity.

Acknowledgement

This work was supported by NIH GM55876.

References

1 Oaks DL, Robertson AF, Broderson R: The effect of chloral hydrate and its metabolites, trichloroethanol and trichloroacetic acid, on bilirubin-albumin binding. Pharmacol Toxicol 1992;71:196–197.

2 Hobbs WR, Rall TW, Verdoorn TA: Hypnotics and sedatives; ethanol; in Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Gilman AG (eds): Goodman and Gilman's: The Pharmacological Basis of Therapeutics, ed 9. New York, McGraw-Hill, 1996, p 381.

3 Peoples RW, Weight FF: Trichloroethanol potentiation of gamma-aminobutyric acid-activated chloride current in mouse hippocampal neurones. Br J Pharmacol 1994;113:555–563.

4 Bentley KR, Barnes NM: 5-Hydroxytryptamine$_3$ (5-HT_3) receptor-mediated depolarisation of the rat isolated vagus nerve: Modulation by trichloroethanol and related alcohols. Eur J Pharmacol 1998;354:25–31.

5 Johansson JS, Eckenhoff RG, Dutton PL: Binding of halothane to serum albumin demonstrated using tryptophan fluorescence. Anesthesiology 1995;83:316–324.

6 Johansson JS: Binding of the volatile anesthetic chloroform to albumin demonstrated using tryptophan fluorescence quenching. J Biol Chem 1997;272:17961–17965.

7 Eftink MR, Zajicek JL, Ghiron CA: A hydrophobic quencher of protein fluorescence: 2,2,2-Trichloroethanol. Biochim Biophys Acta 1977; 491:473–481.

8 Peters T: Serum albumin. Adv Protein Chem 1985;37:161–245.

9 Casas-Finet JR, Wilson SH, Karpel RL: Selective photochemical modification by trichloroethanol of tryptophan residues in proteins with a high tyrosine-to-tryptophan ratio. Anal Biochem 1992;205:27–35.

10 Lakowicz JR: Principles of Fluorescence Spectroscopy. New York, Plenum, 1983.

11 Komiyama T, Miwa M: Fluorescence quenching as an indicator for the exposure of tryptophyl residues in *Streptomyces* subtilisin inhibitor. J Biochem 1980;87:1029–1036.

12 Carter DC, Ho JX: Structure of serum albumin. Adv Protein Chem 1994;45:153–203.

13 Lau SYM, Taneja AK, Hodges RS: Synthesis of a model protein of defined secondary and quaternary structure. J Biol Chem 1984;259: 13253–13261.
14 Franks NP, Lieb WR: Molecular and cellular mechanisms of general anaesthesia. Nature 1994;367:607–614.
15 Eckenhoff RG, Johansson JS: Molecular interactions between inhaled anesthetics and proteins. Pharmacol Rev 1997;49:343–367.
16 Scheibler P, Kronfeld A, Illes P, Allgaier C: Trichloroethanol impairs NMDA receptor function in rat mesencephalic and cortical neurones. Eur J Pharmacol 1999;366:R1–R2.
17 Owen BE, Taberner PV: Studies on the hypnotic effects of chloral hydrate and ethanol and their metabolism in vivo and in vitro. Biochem Pharmacol 1980;29:3011–3016.
18 Eckenhoff RG, Johansson JS: On the relevance of 'clinically relevant concentrations' of inhaled anesthetics in in vitro experiments. Anesthesiology 1999;91:856–860.
19 Eckenhoff RG, Shuman H: Halothane binding to soluble proteins determined by photoaffinity labeling. Anesthesiology 1993;79:96–106.
20 Curry S Mandelkow H Brick P, Franks N: Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites. Nat Struct Biol 1998;5:827–835.
21 Callis PR: ^1L_a and ^1L_b transitions of tryptophan: Applications of theory and experimental observations to fluorescence of proteins. Methods Enzymol 1997;278:113–150.
22 Laberge M: Intrinsic protein electric fields: Basic non-covalent interactions and relationship to protein-induced Stark effects. Biochim Biophys Acta 1998;1386:305–330.
23 Philips AV, Robbins DJ, Coleman MS: Immunoaffinity purification and fluorescence studies of human adenosine deaminase. Biochemistry 1987;26:2893–2903.
24 Woody RW: Circular dichroism. Methods Enzymol 1995;246:34–71.
25 Shibata A, Yamamoto M, Yamashita T, Chiou J-S, Kamaya H, Ueda I: Biphasic effects of alcohols on the phase transition of poly(*L*-lysine) between α-helix and β-sheet conformations. Biochemistry 1992;31:5728–5733.
26 Brockerhoff H, Brockerhoff S, Box LL: Mechanism of anesthesia: The potency of four derivatives of octane corresponds to their hydrogen bonding capacity. Lipids 1986;21:405–408.
27 Hirota N, Mizuno K, Goto Y: Group additive contributions to the alcohol-induced α-helix formation of melittin: Implication for the mechanism of alcohol effects on proteins. J Mol Biol 1998;275:365–378.
28 Johansson JS, Rabanal F, Dutton PL: Binding of the volatile anesthetic halothane to the hydrophobic core of a tetra-α-helix bundle protein. J Pharmacol Exp Ther 1996;279:56–61.
29 Chen Y, Liu B, Yu H-T, Barkley MD: The peptide bond quenches indole fluorescence. J Am Chem Soc 1996;118:9271–9278.
30 Chen Y, Barkley MD: Toward understanding tryptophan fluorescence in proteins. Biochemistry 1998;37:9976–9982.
31 Cowan DO, Drisko RLE: The photodimerization of acenaphthylene. Heavy-atom solvent effects. J Am Chem Soc 1970;92:6281–6285.
32 Eftink MR, Ghiron CA: Fluorescence quenching studies with proteins. Anal Biochem 1981; 114:199–227.
33 Basu G, Anglos D, Kuki A: Fluorescence quenching in a strongly helical peptide series: The role of noncovalent pathways in modulating electronic interactions. Biochemistry 1993; 32:3067–3076.