# Direct Binding of Ethanol to Bovine Serum Albumin: A Fluorescent and $^{13}C$ NMR Multiplet Relaxation Study†

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ABSTRACT: Molecular mechanisms of ethanol interaction with proteins are not well-understood. In the present study, direct interaction of ethanol with hydrophobic binding sites on fatty acid free bovine serum albumin (BSA) was determined using the fluorescent probe 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS), *cis*-parinaric acid, and $^{13}C$ NMR. The affinity of ethanol for BSA ($K_d$) was $(5.21 \pm 0.31) \times 10^{-2}$ mol. Ethanol (25−200 mmol) competitively inhibited 1,8-ANS binding to BSA in a concentration-dependent manner with a $K_i$ (concentration of ethanol that decreased 1,8-ANS binding by 50%) of 658 mmol. Preincubation of BSA with ethanol significantly decreased *cis*-parinaric acid binding to BSA, indicating interaction of ethanol with hydrophobic fatty acid-binding site(s) on BSA. Furthermore, ethanol was found to act on three of the five fatty acid-binding sites on BSA. These data indicated selectivity in the interaction of ethanol with hydrophobic sites on BSA. $^{13}C$ NMR multiplet relaxation was used to characterize the interaction of ethanol with binding sites on BSA. Detailed analysis of [$^{13}C$]ethanol relaxation data obtained in the presence of increasing BSA concentrations (25−200 mg/mL) led to the conclusion that the ethanol methyl group, as opposed to its hydroxyl group, binds in a hydrophobic pocket(s) on the protein. Ethanol-induced changes in activity of certain proteins may result from direct binding of ethanol to specific hydrophobic binding sites and/or displacement of endogenous ligands from those sites.

Ethanol alters the biological activity of various proteins (Deitrich et al., 1989; Wood et al., 1995; Miller, 1993). The molecular mechanism of how ethanol affects activity of proteins is not well-understood. One hypothesis that has been previously proposed is that ethanol as well as general anesthetics directly binds to certain proteins and such binding alters protein function (Franks & Lieb, 1984). Several studies have examined this hypothesis utilizing the firefly luciferase enzyme as a model protein (Herskovits et al., 1970; Franks & Lieb, 1984, 1985, 1986; Curry et al., 1990; Moss et al., 1991). It was concluded in those studies that certain anesthetics, including ethanol, compete with luciferin for binding to luciferase, which was shown by changes in luminescence intensity. Recently, it was reported that the ATP-dependent activity of protein kinase C (PKC)[1] was significantly inhibited by the presence of 50 mmol of ethanol, with a resulting $IC_{50}$ of 250 mmol (Slater et al., 1993). It was proposed in that study that ethanol binds to a hydrophobic site on the regulatory subunit of PKC, specifically to the diacylglycerol binding site (Slater et al., 1993). Alcohols differing in the number of carbon atoms have also been shown to decrease the current of an ATP ligand-gated ion channel as measured by whole-cell patch clamping of neurons (Li et al., 1994). In order to inhibit ion channel activity, the molecular volume of the alcohol had to be equal to or less than 46.1 mL/mol, and it was concluded that alcohols were acting on a small hydrophobic pocket of the ion channel (Li et al., 1994).

Ethanol and other alcohols may directly bind to a hydrophobic site on proteins, but evidence in support of that hypothesis is based on changes in protein activity and/or an interaction with lipids and proteins. Moreover, a study using proton NMR found that ethanol and other alcohols interacted with a polar amino acid side chain of BSA (Lubas et al., 1979). A conclusion of that study was that the hydroxyl group of the ethanol molecule was acting on the protein, and such a conclusion would argue against ethanol acting exclusively on a hydrophobic pocket. An ethanol molecule contains a polar hydroxyl group and a nonpolar hydrophobic methyl group. Neither the contribution of polar and nonpolar interactions of ethanol with proteins nor the binding geometry and the nature of the purported hydrophobic site(s) on proteins is well-understood.

Two hypotheses were tested in the present study. The first hypothesis was that ethanol directly binds to proteins. This hypothesis was tested using BSA as a model protein and 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) as the

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[1] Abbreviations: 1,8-ANS, 1-anilinonaphthalene-8-sulfonic acid; BSA, bovine serum albumin; DMF, *N*,*N*-dimethylformamide; NMR, nuclear magnetic resonance; rf, radio frequency; PKC, protein kinase C; $F_{mol}$, molar fluorescence.

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fluorescent probe. 1,8-ANS has both hydrophobic and polar domains similar to but larger than the two ethanol domains and has been used to study binding of neurotropic drugs in different membranes (Avdulov et al., 1982, 1986, 1989, 1994). It was further proposed that ethanol binds to a hydrophobic site(s) on BSA. This second hypothesis was tested using fluorescence of *cis*-parinaric acid and $^{13}C$ NMR multiplet relaxation technique. The results of the present study show that ethanol directly binds to BSA. Moreover, ethanol prevents *cis*-parinaric acid binding to three out of five fatty acid-binding sites of BSA, most probably the sites that fatty acids and ANS share. NMR data revealed that the ethanol methyl group and not the ethanol hydroxyl group binds in a hydrophobic pocket(s) on the protein.

## MATERIALS AND METHODS

*Chemicals*. Bovine serum albumin (BSA), fraction V, which is essentially fatty acid free, was purchased from Sigma Chemical Co. (A1887). 1,8-ANS and *cis*-parinaric acid were purchased from Molecular Probes, Inc., (Eugene, OR). Ethanol was obtained from Pharmco Products, Inc., (Bayonne, NJ). Ethanol that had been $^{13}C$-enriched in either the methyl carbon, $^{13}CH_3CH_2OH$, or the methylene carbon, $CH_3{}^{13}CH_2OH$, was purchased from Cambridge Isotope, Inc., Cambridge, MA, and used in a 50:50 mixture to avoid $^{13}C-{}^{13}C$ NMR line splitting.

*Fluorescence Measurements*. An SLM 8100 fluorescence spectrophotometer (SLM-Aminco, Milton-Roy Co., Rochester, NY) and LS 5 Perkin-Elmer fluorimeter (Perkin-Elmer, Oak Brook, IL) were used for the fluorescence measurements. Band-pass slits of 10 and 5 nm were used for fluorescence excitation and emission, respectively. For measurements using 1,8-ANS, the excitation wavelength was set at 380 nm and emission was monitored at 480 nm. For endogenous tryptophan fluorescence, an excitation wavelength of 295 nm was used with emission being detected at 335 nm. *cis*-Parinaric acid was excited at 318 nm, and either emission spectra at 350−450 nm were recorded or emission was detected at 410 nm. BSA was added to a phosphate-buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 6.5 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, 20 mM HEPES, pH adjusted to 7.4 with Tris base) to give a final sample volume of 1 or 2 mL (1.5 $\mu$mol of protein/mL). Disposable 2 mL methacrylate UV-grade fluorimetric cuvettes (Spectrocell, Oreland, PA) or, for enhanced sensitivity, 1 mL quartz cuvettes (Hellma, Germany) were used in all measurements. Ethanol was added to samples approximately 10 min prior to fluorescence excitation. The cuvette temperature was maintained at 36.5 ± 0.1 °C in a thermostated chamber by using a circulating water bath.

*1,8-ANS Binding to BSA*. Two titration procedures were performed to calculate the parameters of 1,8-ANS binding to BSA. In one group of experiments, a fixed concentration of BSA was titrated by the probe: 1,8-ANS (0.2 $\mu$L of a $10^{-2}$ M solution) was added repeatedly to the sample (2 mL of buffer, containing 1.5 $\mu$mol of BSA/mL), the resulting solution was stirred, and measurements were made 15 s after the addition of the probe. In each case, at least 14 probe concentrations were used. In another group of experiments, a fixed concentration of 1,8-ANS was titrated by the protein. BSA was added in 0.375 $\mu$mol increments to a buffer solution, containing 5 $\mu$mol of 1,8-ANS/mL. Association constants ($K_b$) and the number of binding sites ($n$) for the 1,8-ANS−BSA complex were calculated by using linear regression analysis of double-reciprocal plots (Harris, 1971; Vladimirov & Dobretsov, 1980).

In a buffer solution in which both BSA and 1,8-ANS are present, an equilibrium is established between 1,8-ANS molecules in the buffer and 1,8-ANS molecules bound to BSA.

$$\mathrm{ANS} + \mathrm{BSA} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ANS{-}BSA} \tag{1}$$

The relationship between the free 1,8-ANS ($c$) and 1,8-ANS bound to BSA ($r$) and the number of binding sites for 1,8-ANS on BSA ($n$) may be described as

$$K_b = r/[c(n - r)] \tag{2}$$

where $K_b$ is a binding constant.

In order to determine $K_b$ and $n$ for 1,8-ANS a procedure based on the use of the total amount of the probe added to the sample ($a = r + c$) was applied. If $c$ is replaced by ($a - r$) in eq 2, $K_b$ can be expressed as

$$K_b = r/[(a - r)(n - r)] \tag{3}$$

When $n \gg r$, eq 3 can be written as

$$K_b = r/[(a - r)n] \tag{4}$$

which describes a double-reciprocal plot

$$1/r = [1/(K_b a)](1/n) + 1/a \tag{5}$$

When $a \gg r$, eq 3 can be written as

$$1/r = [1/(K_b n)](1/a) + 1/n \tag{6}$$

Though we used 1,8-ANS concentrations in the above equations, experimentally we measure fluorescence intensity ($F$) of 1,8-ANS. For 1,8-ANS, $F$ is related to $r$ as

$$r/a = F/F_{max} \tag{7}$$

where $F_{max}$ is the maximal fluorescence of 1,8-ANS, observed if all of the probe is bound.

$F_{max}$ depends on the number of binding sites for the probe in suspension

$$F_{max} = F_{mol} n \tag{8}$$

where $F_{mol}$ depends on the characteristics of the probe and of the fluorimeter and on the quantum yield of the fluorescence.

$$n = n'(\mathrm{BSA}), \tag{9}$$

where $n'$ is a number of binding sites per mol of BSA and (BSA) is the number of moles of BSA in 1 mL. Using known values of F and (BSA) instead of unknown values of $r$ and $n$, eqs 5 and 6 can be written (a) for the titration of a fixed ANS concentration by BSA

$$1/F = \{1/[K_b a F_{mol} n'(\mathrm{BSA})]\} + 1/(a F_{mol}) \tag{10}$$

and (b) for the titration of a fixed BSA concentration by ANS

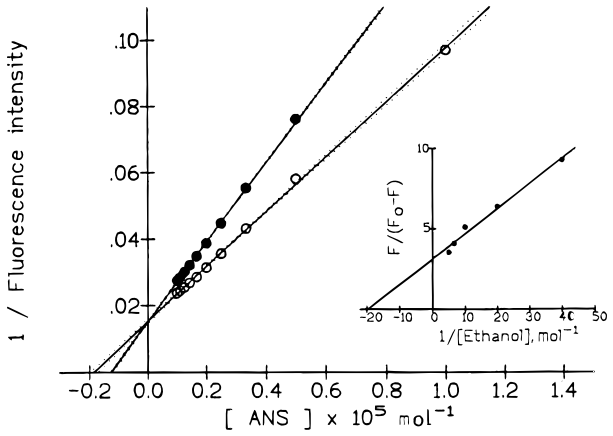


FIGURE 1: Double-reciprocal plot of the effect of ethanol (200 mmol) on 1,8-ANS binding to BSA. Unfilled circles, control; filled circles, 200 mmol of ethanol. Each point is an average of at least eight independent measurements. The dotted lines represent standard deviations of the curves. Inset: Effect of ethanol (25−200 mmol) on 1,8-ANS fluorescence in BSA. The data are presented as a Stern−Volmer plot. The measurements were taken at a fixed 1,8-ANS ($10^{-6}$ mol) and BSA ($1.5 \times 10^{-6}$ mol) concentrations. Ethanol binding constant to BSA was estimated from this plot as an absolute value of the *x*-axis intercept.

$$1/F = \{1/[K_b a F_{mol} n'(\text{BSA})]\} + 1/[n'(\text{BSA}) F_{mol}] \quad (11)$$

$K_b$ was determined as the absolute value of the *x*-intercept of 1/(fluorescence intensity) plotted versus 1/(1,8-ANS concentration) (eq 11, Figure 1). The value $n'$ was calculated from the *y*-intercept of the same plot, taking into account that

$$Y_{intc} = 1/[n'(\text{BSA}) F_{mol}] \quad (12)$$

$F_{mol}$ was obtained from other experiments where 1,8-ANS was titrated with BSA (eq 10). The *y*-intercept of a double-reciprocal plot of fluorescence intensity vs BSA concentration equals

$$Y_{intc} = 1/(F_{mol} a) \quad (13)$$

The total affinity of 1,8-ANS for BSA is represented by $n'/K_d$ (Slavik, 1982), where $K_d$ is the dissociation constant of the 1,8-ANS−BSA complex. Taking into account that $K_d = 1/K_b$, the total affinity of the probe can be characterized by $K_b n'$. Correlation coefficients were greater than 0.996. The Bonferonni-corrected *t*-test was used to determine significant differences in data points.

*cis-Parinaric Acid Assay.* 1 $\mu$mol of *cis*-parinaric acid/mL in DMF solution (0.1 $\mu$L of DMF per 2 mL of buffer) was added to the buffer, which contained 1.5 $\mu$mol of BSA/mL. The solution was stirred for 5 min prior to fluorescence excitation at 318 nm. Fluorescence intensity was recorded in the range 350−500 nm. Ethanol was added to a sample containing BSA 10 min prior to the addition of *cis*-parinaric acid. The fluorescence intensity of *cis*-parinaric acid in the buffer was also recorded after adding 0.1 $\mu$L of DMF solution and stirring for 5 min before recording fluorescence emission.

In another series of experiments 1 $\mu$mol of BSA/mL of buffer was titrated by *cis*-parinaric acid in 1 $\mu$mol/mL increments in the absence and presence of ethanol (100 and 200 mmol). Ethanol was added to the samples 10 min prior to the first addition of *cis*-parinaric acid, and the measurements were made 5 min after each addition of *cis*-parinaric acid.

Table 1: Effects of Ethanol on the 1,8-ANS−BSA Complex Association Constant ($K_b$)[a]

| ethanol, mmol | $K_b$, mol$^{-1}$ |
|---|---|
| 0 | 20576 ± 591 |
| 25 | 17671 ± 379[b] |
| 50 | 16629 ± 267[b] |
| 100 | 15802 ± 254[b] |
| 200 | 13636 ± 165[b] |

[a] Data are presented as (mean ± SEM). Each value is an average of eight independent measurements. [b] $p < 0.001$ as compared with 0 mmol of ethanol as determined by Student's *t*-test, Bonferroni-corrected for multiple comparisons.

*NMR Measurements.* All $^{13}C$ relaxation measurements were performed on a Bruker AM-250 NMR spectrometer at the $^{13}C$ frequency of 62.8 MHz. The temperature was maintained at 37 °C. Spin−lattice relaxation was studied by using the inversion−recovery method. The number of acquisitions was chosen to give a signal to noise ratio greater than 10. At least 12 partially relaxed spectra were acquired for each relaxation measurement. In order to reduce experimental errors arising from radio frequency inhomogeneities, the composite pulse sequence, $90°_x-180°_y-90°_x$ was used. To minimize the error in determining initial relaxation rates, a least-squares method with weighted functions as described in detail by Daragan et al. (1993) was used. The BSA concentration was varied from 0 to 200 mg/mL, and the ethanol concentration was held constant at 200 mmol.

## RESULTS

*1,8-ANS Binding to BSA.* The binding of 1,8-ANS to BSA was saturable. The average association constant for the 1,8-ANS−BSA complex, $K_b$, was $(2.06 \pm 0.06) \times 10^4$ mol$^{-1}$. The number of binding sites ($n'$) was 2.82 ± 0.08 and is in agreement with data reported previously (Naik et al., 1975; Pal & Patra, 1994). The total affinity ($K_b n$) of 1,8-ANS for BSA was $(5.81 \pm 0.21) \times 10^4$ mol$^{-1}$.

*Effects of Ethanol on 1,8-ANS Binding to BSA.* As shown in Figure 1, ethanol inhibited 1,8-ANS binding to BSA and significantly modified the affinity of the probe for the protein (Table 1). The $Y_{intc}$ of 1/(fluorescence intensity) vs 1/(1,8-ANS concentration) plot was not significantly changed in the presence of ethanol (6.55 ± 0.1 in control, 6.54 ± 0.09 in the presence of 100 mmol of ethanol, and 6.65 ± 0.15 in the presence of 200 mmol of ethanol) and was indicative of possible competitive inhibition of 1,8-ANS binding to BSA by ethanol. The possibility of competitive inhibition of 1,8-ANS and ethanol was addressed in the context of a constant number of binding sites, $n'$. On the basis of eqs 12 and 13, ethanol competes with 1,8-ANS for the same binding site(s) if the $F_{mol}$ for bound 1,8-ANS is not affected by addition of ethanol. Experiments showed that for amounts up to 200 mmol of ethanol (highest concentration tested), $F_{mol}$ was not changed significantly.

*Ethanol Binding to BSA.* Ethanol affinity for binding sites on BSA was determined from titration of a standard ANS concentration (1 $\mu$mol) by ethanol (Figure 1, insert). Affinity of ethanol for BSA was $K_b = 19.3 \pm 0.12$ mol$^{-1}$ and was 9-fold lower than the $K_b$ of 1,8-ANS for BSA [(2.06 ± 0.06)

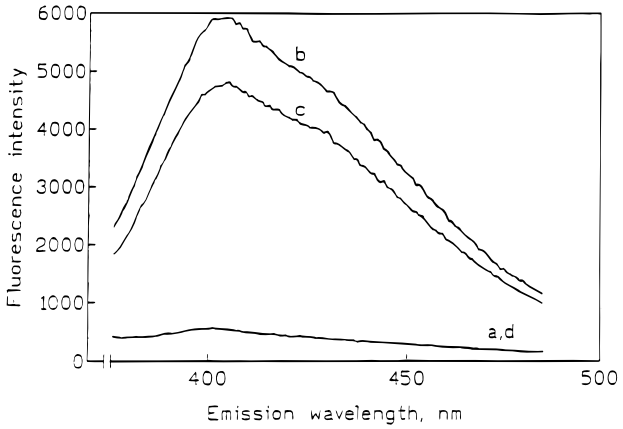


FIGURE 2: Effect of ethanol (200 mmol) on fluorescence of *cis*-parinaric acid ($1 \times 10^{-6}$ mol) in the presence and absence of BSA ($1.5 \times 10^{-6}$ mol). Curve a, fluorescence of *cis*-parinaric acid in the buffer; curve b, fluorescence of *cis*-parinaric acid in the presence of BSA; curve c, fluorescence of *cis*-parinaric acid in the presence of BSA and 200 mmol of ethanol; curve d, fluorescence of *cis*-parinaric acid in buffer in the presence of ethanol.

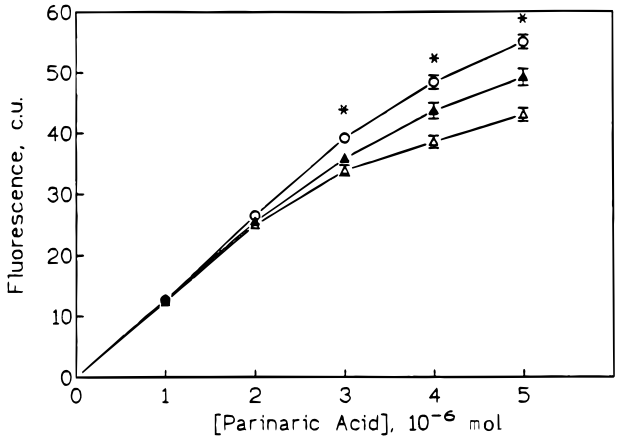


FIGURE 3: Effects of ethanol (100 and 200 mmol) on *cis*-parinaric acid binding to BSA. *cis*-Parinaric acid in $1 \times 10^{-6}$ mol increments was added to $1 \times 10^{-6}$ mol of BSA in the absence (control, unfilled circles) or presence of ethanol (100 mmol, filled triangles; 200 mmol, unfilled triangles). $p \leq 0.001$ as compared with control. Each point is an average of at least four independent measurements.

$\times 10^4$ mol$^{-1}$]. If ethanol and 1,8-ANS share the same binding sites on BSA, the total affinity of ethanol for BSA, $K_b n$, was $(54.4 \pm 0.3) \times 10^1$ mol$^{-1}$. Thus, even though ethanol and 1,8-ANS share the same binding sites on BSA, binding affinities vary considerably. Millimolar concentrations of ethanol are required to inhibit the binding of micromolar concentrations of 1,8-ANS.

It is important to note that at the ethanol concentrations studied, no effect on tryptophan fluorescence in BSA was observed (in conventional units: $9254 \pm 167$ in control, $9267 \pm 154$ in the presence of 100 mmol of ethanol, and $9243 \pm 159$ in the presence of 200 mmol of ethanol). These data indicated that effects of ethanol on 1,8-ANS binding were probably not due to conformational changes in BSA.

*Effect of Ethanol on cis-Parinaric Acid Binding to BSA.* Binding of ethanol to the fatty acid binding sites on BSA was addressed using a fluorescent fatty acid, *cis*-parinaric acid. Fluorescence intensity of *cis*-parinaric acid in buffer (Figure 2a) was markedly increased by the presence of BSA (Figure 2b). The increase in fluorescence intensity resulted from an increase of the quantum yield of the probe in the bound state compared to the free state in solution. Preincubation for 10 min with 200 mmol of ethanol significantly decreased *cis*-parinaric acid fluorescence in the presence of BSA (Figure 2c). The reduction in the fluorescence intensity of *cis*-parinaric acid in the presence of ethanol could be due to decreased binding of *cis*-parinaric acid to BSA or direct quenching of *cis*-parinaric acid fluorescence by ethanol. Figure 2d shows that ethanol had no effect on the fluorescence of *cis*-parinaric acid in the buffer in the absence of BSA. Thus, direct quenching of *cis*-parinaric acid fluorescence by ethanol did not occur. Instead, the data indicate that *cis*-parinaric acid is displaced from the binding sites on BSA and that ethanol interacts with fatty acid binding sites on BSA.

Binding of *cis*-parinaric acid to BSA was saturable (Figure 3) with an average $K_b$ of $(1.01 \pm 0.08) \times 10^5$ mol$^{-1}$ and one molecule of BSA was binding approximately five molecules of *cis*-parinaric acid, which is in agreement with the data of Sklar et al. (1977). Ethanol at 100 and 200 mmol significantly inhibited *cis*-parinaric acid binding to BSA, but did not affect all the binding sites. As seen in Figure 3, ethanol did not influence the binding of the first two molecules of *cis*-parinaric acid to BSA. Significant effects of ethanol were observed only on the binding of 3−5 molecules of *cis*-parinaric acid per molecule of BSA (Figure 3).

*NMR Relaxation.* $^{13}$C NMR multiplet relaxation was used in the present study to examine the dynamic behavior of ethanol in a BSA−water system. The $^{13}$C NMR proton-coupled spectrum of ethanol has two multiplets: a triplet and a quartet arising from the methylene and methyl groups, respectively. Each NMR line of the ethanol $CH_2$ or $CH_3$ multiplet displays different relaxation behavior depending on the rotational mobilities of each group. If the transition rates for outer and inner multiplet lines, $W_o$ and $W_i$, respectively, are averaged, one can write the following equations, which are valid for extreme narrowing conditions (Werbelow & Grant, 1979; Daragan & Mayo, 1993a):

$$\begin{gathered}
\mathrm{CH_2} \\
W_c = (W_o + W_i)/2 \\
W_c = 2\tau_{CH} h^2 \gamma_C{}^2 \gamma_H{}^2/(4\pi^2 r_{CH}{}^6) \\
W_o - W_i = (6/5)\tau_{HCH} h^2 \gamma_C{}^2 \gamma_H{}^2/(4\pi^2 r_{CH}{}^6) \\
\mathrm{CH_3} \\
W_c = (W_o + 3W_i)/4 \\
W_c = 3\tau_{CH} h^2 \gamma_C{}^2 \gamma_H{}^2/(4\pi^2 r_{CH}{}^6) \\
W_o - W_i = (12/5)\tau_{HCH} h^2 \gamma_C{}^2 \gamma_H{}^2/(4\pi^2 r_{CH}{}^6)
\end{gathered} \tag{14}$$

where $h$ is Planck's constant, $\gamma_C$ and $\gamma_H$ are the magnetogyric ratios for carbon and hydrogen nuclei, respectively, and $r_{CH}$ is the internuclear distance between carbon and its bonded hydrogens. $\tau_{CH}$ is the auto-correlation time for rotational motions of the CH bond, and $\tau_{HCH}$ is the cross-correlation time which characterizes the mutual rotational motions of the two CH bond vectors in the

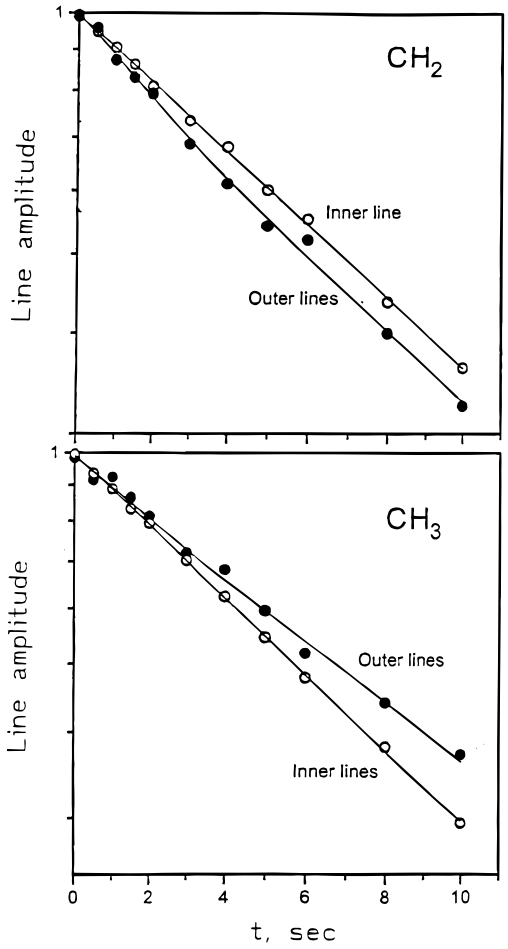


FIGURE 4: Relaxation curves for inner and outer lines (average) of $^{13}C$ NMR multiplet spectra of ethanol in $D_2O$-based buffer, pH = 7.4, containing 125 mg of BSA/mL at 37 °C. Different signs of initial rates (see text), $W_o - W_i$, show different anisotropy of the rotational motion of these groups. Internal rotation of methyl group is rather hindered and correlation time of internal rotation should be comparable with correlation time of overall tumbling. The positive sign of $W_o - W_i$ for the methylene group indicates the relatively high rotational anisotropy of this group.

methyl or methylene groups. The formal definitions of these correlation times can be written as

$$\tau_{CH} = 4\pi\int_0^\infty \langle Y_{20}(\theta_{CH}(t))Y_{20}(\theta_{CH}(0))\rangle \, dt \qquad (15)$$

$$\tau_{HCH} = 4\pi\int_0^\infty \langle Y_{20}(\theta_{CH}(t))Y_{20}(\theta_{CH'}(0))\rangle \, dt$$

where $Y_{20}$ is the second-rank spherical harmonic and $\theta_{CH}$ is the angle that the CH bond makes with some arbitrary axis in the laboratory frame. The definition of the cross-correlation time includes contributions from the molecular geometry, and for this reason, it can be positive when internal motions are very fast with respect to reorientation of the $CH_2$ or $CH_3$ symmetry axes, or it can be negative when internal rotations are relatively slow. Therefore, analysis of cross-correlation times can be very useful in interpreting molecular rotational anisotropy. Figure 4 presents the relaxation curves for the $CH_2$ and $CH_3$ groups of ethanol. One can see that the outer lines of the methylene group relax faster than the inner line and indicates a positive cross-correlation time. For the methyl group, one can observe that relaxation of the inner lines is faster than the relaxation of the outer lines. Cross-correlation times $\tau_{HCH}$ were negative in this case and one can conclude that rotation of the ethanol methyl group was relatively restricted. Four independent correlation times, which were determined from multiplet relaxation experiments, allowed us to develop a model for describing rotational motions of ethanol.

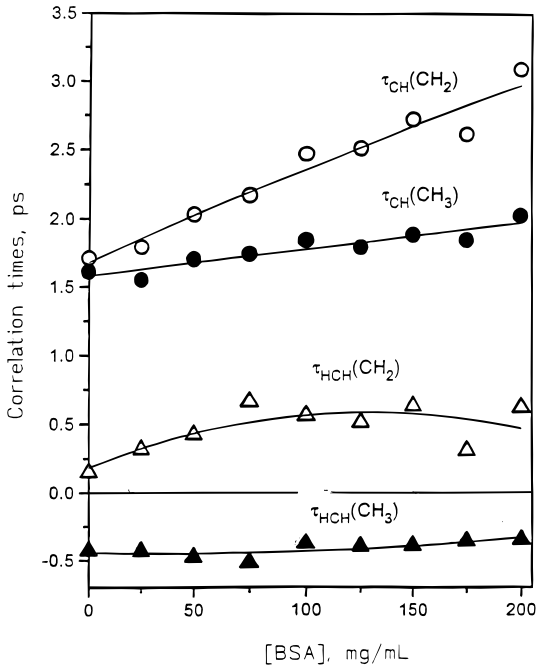


FIGURE 5: BSA concentration dependencies of auto- and cross-correlation times of the rotational motions of the C−H bonds in ethanol.

Figure 5 plots auto- and cross-correlation times for ethanol methylene and methyl groups at different concentrations of BSA. Correlation times shown in this figure were the result of averaging of data taken from at least three independent relaxation measurements. One can see that the concentration dependence of auto-correlation times, $\tau_{CH}$, for the methylene group was more pronounced than that for the methyl group, and is consistent with the result of Lubas et al. (1979). Methylene group cross-correlation times, $\tau_{HCH}$, showed some BSA concentration dependence only at values lower than 75 mg/mL, at which point it appeared to plateau. Moreover, the methylene group cross-correlation term was positive, indicating a high degree of rotational anisotropy. A negative cross-correlation term for the methyl group indicated slow rotational mobility about the C−C bond axis relative to overall motional tumbling of the molecule for the whole range of BSA concentration.

From $\tau_{CH}$ and $\tau_{HCH}$ without using any assumptions about the model of methyl group rotation, the correlation time for reorientation of the ethanol C−C bond, $\tau_{CC}$, can be determined by using the following equation for tetrahedral geometry of methyl group (Daragan & Khazanovich, 1978; Daragan & Mayo, 1993b).

$$\tau_{CC} = 3(\tau_{CH} + 2\tau_{HCH}) \qquad (16)$$

If one considers that internal rotations do not disturb the geometry of the ethanolic fragment $-O-CH_2-C-$, one has three experimental parameters to describe the rotational motion of this fragment: $\tau_{CH}$, $\tau_{HCH}$, and $\tau_{CC}$, all of which can be determined as mentioned above. From these values,

any other three model-dependent motional parameters can be determined.

For the model of anisotropic rotational diffusion of a symmetric top or symmetric ellipsoid, rotations can be characterized by two rotational diffusion coefficients: $D_{\|}$, describing rotation around the symmetry axis, and $D_{\perp}$, describing rotations orthogonal to this axis. As will be shown later, $D_{\|} > D_{\perp}$ for ethanol, and the symmetry axis is the main molecular rotation axis. Since this axis lies in the O−C−C plane, auto- and cross-correlation times can be written as (Werbelow & Grant, 1977)

$$\tau_{ab} = a^{ab}{}_0/D_0 + a^{ab}{}_1/D_1 + a^{ab}{}_2/D_2 \qquad (17)$$

where

$$a^{ab}{}_0 = P_2 \cos(\theta_a)\, P_2 \cos(\theta_b)$$

$$a^{ab}{}_1 = 3 \cos\theta_a \cos\theta_b \sin\theta_a \sin\theta_b \cos(\varphi_a - \varphi_b) \qquad (18)$$

$$a^{ab}{}_1 = (3/4)\sin^2\theta_a \sin^2\theta_b \cos(2\varphi_a - 2\varphi_b)$$

$P_2(x) = 0.5(3x^2 - 1)$ is the second-order Legendre polynomial. $\theta_a$, $\theta_b$, $\varphi_a$, and $\varphi_b$ are the polar angles of the vectors $a$ and $b$ in the coordinate system $X_D$, $Y_D$, $Z_D$, where the $Z_D$ axis is directed along the main rotation axis. When $a = b$ = CH, one has the auto-correlation time $\tau_{CH}$, and when $a$ = CH and $b$ = CH′, one has the cross-correlation time $\tau_{HCH}$. The coefficients $D_m$, $m = 0, 1, 2$, can be written as

$$D_m = 6D_{\perp} + (D_{\|} - D_{\perp})m^2 \qquad (19)$$

Introducing an angle between the main rotation axis and the OC bond, α, and expressing the polar angles $\theta_a$, $\theta_b$, $\varphi_a$, and $\varphi_b$ in terms of α and the geometry of the methylene group, one can determine the parameters $D_{\|}$, $D_{\perp}$, and α from the experimental values of the correlation times $\tau_{CH}(CH_2)$, $\tau_{HCH}(CH_2)$, and $\tau_{CC}$. The results are shown in Figure 6. In general, $D_{\|}$ and $D_{\perp}$ decreased with increasing BSA concentration while α increased. The increasing value of α is particularly meaningful since this means that at higher BSA concentrations, the main axis of molecular rotation becomes more aligned with the ethanol C−C bond. It should be mentioned that values of α in the ethanol−water−BSA mixture are in the range of 18−28°, that is, larger than 5−8°, which was obtained by Zheng et al. (1993) for an ethanol−chloroform mixture. This difference can indicate the sensitivity of parameter α to intermolecular interactions.

In order to study internal rotations of the methyl group relative to the −O−$CH_2$−C− fragment, one must relate rotational correlation times of the methyl group to the components of the rotational diffusion tensor, an angle $\beta$ between the $Z_D$ axis and the C−C bond, and the methyl group geometry. It can be done by making two rotational transformations of the spherical harmonics from the $X_L$, $Y_L$, $Z_L$ to the $X_D$, $Y_D$, $Z_D$ and then to the $X_R$, $Y_R$, $Z_R$ frame. The subscripts L, D, and R indicate laboratory, diffusion, and rotational (connected to the methyl group) frames, respectively. The $Z_R$ axis is directed along the C−C bond. After some calculations, $\tau_{CH}$ and $\tau_{HCH}$ can be written as

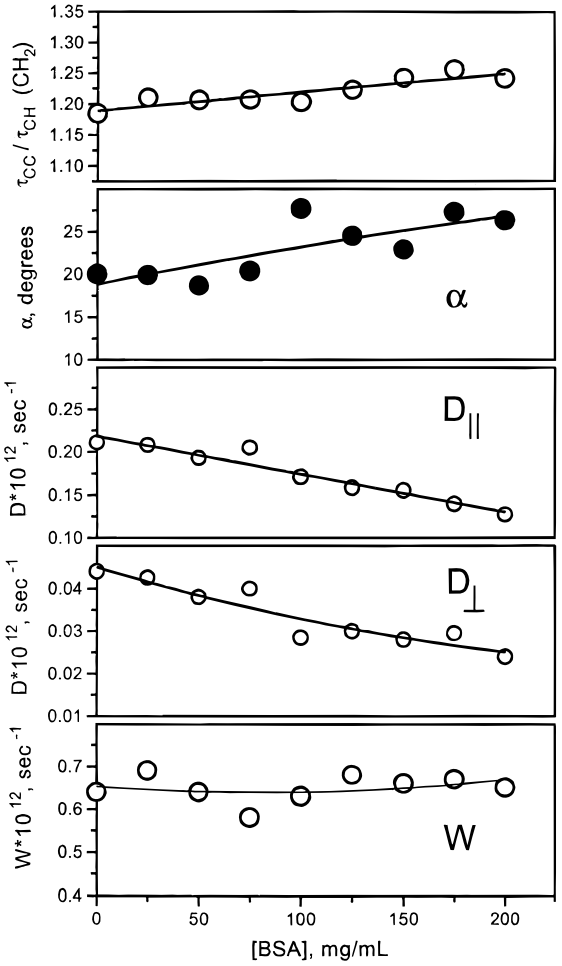


FIGURE 6: BSA concentration dependencies of the parameters for model of anisotropic overall tumbling and three states rotational jumps of the ethanol methyl group.

$$\tau_{CH} = (1/9)\tau_{00} + (8/27)\tau_{11} + (16/27)\tau_{22} - (16\sqrt{2}/27)\tau_{2-1}$$

$$\tau_{HCH} = (1/9)\tau_{00} - (4/27)\tau_{11} - (8/27)\tau_{22} + (8\sqrt{2}/27)\tau_{2-1} \qquad (20)$$

where the correlation times $\tau_{mm}$ for three-state jump model for methyl group rotation (Versmold, 1973) are equal to

$$\tau_{mm'} = \sum_{q=-2}^{2} d^{(2)}{}_{qm}(\beta) d^{(2)}{}_{qm'}(\beta)/[D_q + 3(1 - \delta_{m0})W] \qquad (21)$$

$d^{(2)}{}_{qm}(\beta)$ is the reduced second-rank Wigner rotation matrix, $\delta_{m0}$ is the Kronecker delta. $W$ is the jump rate for the methyl group internal rotation on the C−C bond. Note that $\tau_{00}$ is the correlation time of C−C bond, $\tau_{CC}$, which was defined above. From eq 20 one can easily check the validity of eq 16. By using eqs 20 and 21, one can calculate the jump rate, $W$. As the BSA concentration increased, $W$ increased slightly (Figure 6), indicating increasing internal rotational mobility of the methyl group at higher BSA concentrations.

## DISCUSSION

Effects of ethanol on the activity of different proteins are well documented (Deitrich et al., 1989; Wood et al., 1995; Hunt, 1985). However, the molecular mechanism of these

effects is not established. The purpose of the current study was to determine if ethanol directly binds to a protein and, if it does, to establish the nature of the binding sites.

Previously it was proposed that ethanol directly binds to hydrophobic sites on proteins (Franks & Lieb, 1984). Support for ethanol binding to hydrophobic sites on protein molecules has been based on ethanol-induced changes in protein function, including effects of different chain length alcohols on protein function. For example, effects of ethanol on protein kinase C (PKC) activity is thought to be the result of ethanol direct binding to the regulatory subunit of PKC (Slater et al., 1993). Alcohols, including ethanol, have been reported to inhibit neurotransmitter receptor function by interacting with a small hydrophobic pocket on the protein receptor surface (Li et al., 1994). However, in those studies the hypothesis that ethanol directly binds to hydrophobic sites or pockets on proteins has not been directly examined. Instead, effects of ethanol and other alcohols on protein function have been used as support for this hypothesis. There is also evidence suggesting involvement of the hydrophilic portion of the ethanol molecule in its interaction with proteins. The ethanol hydroxyl group was reported to be involved in hydrogen bond formation with peptide bonds in BSA (Lubas et al., 1979). In the present study, we showed that ethanol directly binds to BSA with low affinity ($K_d$) of $5.34 \times 10^{-2}$ mol, that is, in the millimolar range. This concentration of ethanol is consistent with functional studies showing, that millimolar concentrations of ethanol are required to produce biological effects (Hunt, 1985).

Regarding the nature of the binding site, we conclude that ethanol binds to a hydrophobic binding site(s) on BSA. This conclusion is based on the following lines of evidence. Ethanol decreased fluorescence of the fluorescent fatty acid, *cis*-parinaric acid, in the presence of BSA. This effect indicated interaction of ethanol with a hydrophobic fatty acid binding site on BSA. We also concluded that ethanol was binding to some, but not all fatty acid binding sites on BSA. Approximately five binding sites for *cis*-parinaric acid per molecule of BSA have been reported (Sklar et al., 1977). Our data in Figure 3 suggest that ethanol binds to three out of five fatty acids binding sites on BSA. It was previously reported that up to 2 mol of fatty acids per mole of BSA did not influence ANS binding to BSA (Santos & Spector, 1972). Therefore, the hydrophobic binding sites influenced by ethanol are the ones shared by *cis*-parinaric acid and ANS. An important conclusion derived from the *cis*-parinaric acid experiments was that ethanol has selectivity in the interaction with the hydrophobic sites on BSA.

NMR results indicate that the primary mode of ethanol binding to BSA is through the hydrophobic methyl group. We have arrived at this conclusion by considering four possibilities which could explain the dependence of NMR relaxation derived motional parameters on the BSA concentration (see Figure 7 for illustration). In the first case, ethanol could bind very weakly to BSA and the reduction in molecular mobility could be the result of increased microviscosity of the solution. However, for this to occur, the direction of the main rotation axis would be BSA concentration independent, and the opposite is observed. Secondly, the ethanol hydroxyl group could interact primarily with the protein through hydrogen bonding. In this case, the principle rotation axis would be continually realigning itself in the direction of the O−C bond as the BSA concentration is

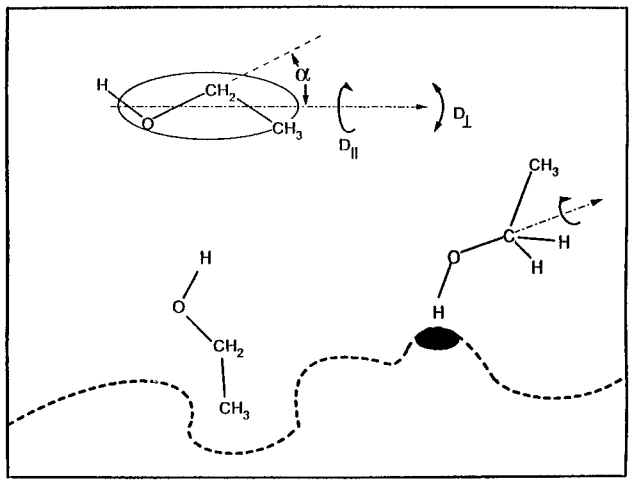


FIGURE 7: Illustration for different ethanol−BSA binding mechanisms.

increased, whereby auto-correlation times for the C−H methylene and C−C bonds would be equal. The opposite tendency is observed. Thirdly, the ethanol methyl group could hydrophobically interact with the protein surface causing reorientation of the main rotation axis to be in the direction of the C−C bond and reducing $CH_3$ mobility (decreasing *W* at higher BSA concentration), which was also not observed. Lastly, ethanol could be binding via methyl group interactions in hydrophobic "pockets" on BSA. Here, realignment of the principle rotation axis would also tend toward coincidence with the C−C bond, whereas $CH_3$ mobilities would not be as significantly affected as in the previous case, since steric restrictions on C−C bond reorientation, as reflected by increases in the methylene group correlation time, would be more pronounced. Furthermore, decreasing the microviscosity near the protein surface can increase the internal mobility of the methyl group. In our opinion the last mechanism is the most probable. In this orientation, the hydroxyl group faces the solvent and protein surface where hydrogen bonding is more favorable and more probable.

The finding that ethanol displaced a fatty acid has important implications with respect to effects of ethanol on liver and brain. It has been reported that chronic ethanol consumption increased the amount of liver−fatty acid binding protein (L−FABP) in liver homogenate of chronic ethanol-treated rats (Pignon et al., 1987). In the same study, it was shown that the binding affinity of palmitate to liver cytosolic proteins was reduced in the chronic ethanol-treated animals and the reduction in binding affinity was attributed to ethanol-induced effects on L−FABP. Acute ethanol administration may directly interfere with binding of fatty acids to proteins. Chronic ethanol consumption may have effects at the level of translation or post-translational modification of L−FABP that would modify fatty acid binding.

It was recently shown that a family of fatty acid binding proteins shared a similar amino acid sequence as the NMDA receptor. The NMDA receptor has been considered to be one of the primary membrane targets of ethanol in brain (Gonzales, 1990). Fatty acids, in particular *cis*-polyunsaturated, *trans*-polyunsaturated, *cis*-monounsaturated, and saturated acids have been reported to be directly involved in the regulation of ion channels (Ordway et al., 1989, 1991).

Regulation of NMDA function was proposed to involve the direct binding of fatty acids to the receptor (Ordway et al., 1989). It is tempting to speculate that effects of ethanol on NMDA function may be due in part to displacement of fatty acids from the receptor and/or direct binding of ethanol to NMDA.

This study demonstrated that ethanol binds to the hydrophobic sites of BSA and that ethanol inhibited binding of a fatty acid and another hydrophobic compound, 1,8-ANS, to BSA. It also was observed that ethanol had a selective effect on the BSA fatty acid binding sites. Some proteins may be more or less affected by ethanol and differences in ethanol effects could depend on the composition of the hydrophobic pocket(s) of proteins.

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