Saturable Ethanol Binding in Rat Liver Microsomes*

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The binding of ethanol to rat liver microsomes is shown to be saturable at clinically relevant ethanol concentrations, whereas this effect is not observed in extracted microsomal phospholipids. Brief exposure of the microsomes to heat abolishes saturable ethanol binding. Equilibrium binding data analysis, although only approximate in this context, suggests the presence of at least two groups of specific sites: high capacity sites with affinities near the pharmacological range and low capacity sites at lesser levels. The results indicate that the specificity of ethanol for tissue is considerably greater than previously recognized.

**EXPERIMENTAL PROCEDURES**

Membrane Preparation—Liver microsomes were isolated from chow-fed Sprague-Dawley rats (Zivic Miller Laboratories, Allisson Park, PA) weighing 300–350 g, as described previously (6, 7), except that the microsomal fraction was washed three times. The pellet was resuspended at a concentration of 10–15 mg/ml and stored under argon at –20 °C. Protein was determined by Peterson's modified Lowry method (8). Cytosol P-450 assays (9) indicated 0.818 ± 0.034 nmol/mg protein, in agreement with others (10). Heat-treated microsomes were placed in boiling water for 5 min prior to being subjected to the protocols below.

Microsomal phospholipids were extracted by the Bligh-Dyer procedure (11) and separated from neutral lipids by silicic acid chromatography (99%) (12). Solvents, which contained 0.01% butylated hydroxytoluene to prevent oxidation, were flushed with N2. Lipids were stored under argon in CHCl3 at –20 °C. Multilamellar vesicles were prepared by vigorous vortexing in buffer (10 mM Hepes, 100 mM KCl, pH 7.4) at a concentration of 10 mg/ml.

Ethanol Binding Assay—Ethanol binding was determined using a modification of the dual radiolabel centrifugal technique originally described by Katz and Diamond (13). Binding to liposomes was performed as described by J anes et al. (14), except that [3H]labeled sucrose was used as a bulk water marker. The binding is expressed as the molar partition coefficients of the hydrated membrane. Binding to microsomes is performed as described below. Since the binding assay is a ratio method, it is insensitive to uncertainties in the specific activity of the sample, to evaporation, or to the adsorption of radiolabels to the sample tubes.

Radioisotopes were obtained from DuPont NEN ([3H]ethanol, [3H]water, [3H]glycine) or Amersham Corp. ([3H]sucrose). Radioisotopes were deemed pure if partitioning between octanol/water or butanol/water was independent of the bulk solvent ratio at 1:1 and 1:10 (15).

Centrifugal Separations—Typically, 0.7 ml of the microsomal suspension was transferred into a sealed 10-ml Oak Ridge polycarbonate centrifuge tube (Nalgene Co., Rochester, NY), along with 40 μl of [3H]water (9.0 μCi), 12 μl of aqueous [14C]ethanol (1.35 μCi), and 4.3 ml of buffer (10 mM Hepes, 100 mM KCl, pH 7.4). The microsomal dispersion was equilibrated at 37 °C in a water bath for 1 h and then centrifuged for 1 h at 37 °C at 130,000 × g in a centrifuge and rotor that were previously equilibrated at 37 °C. Centrifuged samples were placed immediately in a water bath at 37 °C. The uncertainty in the temperature throughout the procedure was typically ±1 °C. Four 100-μl aliquots of the supernatant and the pellet were transferred rapidly into tared glass scintillation vials that were immediately tightly sealed with Polyseal cone caps (Fisher Scientific, Pittsburgh, PA) and again weighed (±0.1 mg). Additional supernatant was occasionally reserved for protein determinations to assess the centrifugal separation (±99%). Typical weights were 100 mg for the supernatants and 45–65 mg for the pellets.

The four supernatant samples were divided into two pairs. To one pair, a nonradioisotopic microsomal pellet (∼50 mg) was added. Subsequently, 2 ml of tissue solubilizer (TS-2, Research Products International, Mount Prospect, IL) was added to all five vials. The vials were transferred to a 45 °C heating bath for at least 20 min for solubilization and were stored in the dark overnight. To the solubilized sample was added 9 ml of Bioase II scintillation fluid (Research Products International, Mount Prospect, IL) and 1.5 ml of water. After pretreatment the samples were stored for 4 days in the dark to achieve a stable quench.

Liquid Scintillation Analysis—Two sets of quench standards were prepared, counted several times, averaged, fit to a polynomial, and stored. Mock samples of known activity were used to ensure discrimination of radionuclides. One set of quench standards was prepared with microsomes as a color quenching agent in the presence of excess water and was used for the microsomal pellets and the supernatants which contained added cold microsomal pellet. The other set was prepared without microsomes and used with the supernatants that lacked tissue. Equality of the supernatant activities under the two quench curves provided a verification of the counting fidelity.

The radioisotopes were counted in a Packard Tri-Carb model 1900CA liquid scintillation analyzer (Packard Instrument Co., Downers Grove, IL) equipped with a barium-133 external γ-ray source, using a dual window analysis (0–9.1 keV; 9.1–156 keV). The instrument was calibrated weekly. Typical counting times were 5 min.

Calculation of Binding Constants—Ethanol binding was determined according to Katz and Diamond (13, 16, 17). The determinations required the discrimination of bulk trapped water from the bound water of hydration in the microsomal pellet (16). The total pellet water was determined using [H2O] as a marker. The trapped bulk water was determined as that water accessible to labeled glycine. Binding con-
The concentration dependence of ethanol binding to the rat liver microsomal fraction at 37 °C is shown (8). The line represents a fit to a model, which contains two specific and one nonspecific components. The binding behavior at low ethanol concentrations is inset.

The concentration dependence of ethanol binding to the rat liver microsomal fraction is shown in Fig. 1. At the lowest ethanol concentration examined (5 μM) binding was greatest (0.73 molal unit). Ethanol binding progressively declined with increasing ethanol concentrations before stabilizing at ethanol levels of approximately 200–400 mM. For comparison, the clinical effects of ethanol occur at 5–100 mM. At the low ethanol concentrations shown in the inset (5 μM–1 mM), a sharper concentration dependence was evident.

Given the compositional complexity of the microsomal fraction, the scatter in the data, and the possibility that the higher ethanol concentrations used may be perturbing (see the lipid solubility data shown below), quantitative analysis of the ethanol binding curves must be viewed with considerable caution. A parsimonious first approximation, represented by the fit in Fig. 1, suggests the presence of (at least) two specific binding components and one nonspecific binding component. The first specific component exhibits a high affinity and a low capacity (Kd = 120 ± 70 μM; Bmax = 40 ± 20 μmol/kg membrane). The second specific component exhibits a lower affinity and a higher capacity (Kd = 110 ± 60 mM; Bmax = 50 ± 30 mmol/kg membrane). A third component is nonspecific and represents the residual binding at saturation (0.00 ± 0.17 molal unit). The actual situation may be considerably more complicated than the three-component binding model employed. Nonetheless, the saturable binding behavior observed is consistent with specific sites being filled at clinically relevant ethanol concentrations with site densities in the range of 20–80 mmol/kg of membrane.

The absolute level of ethanol binding is dependent on the hydration of the microsomal fraction. Hydration, measured as water which is inaccessible to glycine, is 0.35 ± 0.10 g/g, dry weight, and is independent of ethanol concentration. This value is in good agreement with the literature value of 0.34 (19). Such considerations shift the binding curve by a constant amount but do not alter the shape of the ethanol binding curve nor any of the fitted parameters for the specific binding components (16). The level of hydration does affect the nonspecific component of binding, which will be higher if some of the bulk water is inaccessible to glycine.

The linearized form (Scatchard plot) of the binding curve is shown in Fig. 2 together with the nonlinear fit described above. Site densities of 20–80 mmol/kg of membrane are corroborated in this format.

Estimates of site densities from Scatchard plots are subject to error if the data are restricted to low site occupancies (20). The Klotz format of the binding curve is shown in Fig. 3 along with the nonlinear fit described above. The fit includes the inflection point and the initial stages of the plateau, although higher specific site densities can be envisioned from the data. Further definition of the plateau was precluded because of perturbations at higher ethanol levels and the increasingly large error bars in the specific binding. The Klotz fit further reinforces the ethanol dependence of ethanol binding and the substantial specific site densities.

To address the role of bulk membrane lipids in saturable ethanol binding, the binding of ethanol to multilamellar liposomes composed of extracted microsomal phospholipids was examined, as shown in Fig. 4. No negative concentration dependence that would be indicative of saturable binding was observed in the liposomes. By contrast, a small concentration-dependent increase in ethanol’s lipid solubility is observed.

To address the role of macromolecular conformation in saturable ethanol binding, microsomes were subjected briefly to heat (5 min in boiling water), and the binding assay was repeated at 37 °C with 5 μM and 400 mM ethanol, as shown in Fig. 5. Thermal treatment appeared to expose additional nonspecific (or innumerable specific) sites without altering microsomal hydration. Saturable ethanol binding at pharmacologically relevant ethanol concentrations was abolished.
DISCUSSION

Despite much effort, the elucidation of specific binding modalties of alcohols and anesthetics in tissue has proven elusive. Typically, high lipid solubilities tend to dwarf any specific effects (but see Ref. 21). Ethanol, by contrast, exhibits such a low affinity for lipids that specific effects predominate and can be observed directly. However, because the binding is so weak with respect to the aqueous ethanol concentrations, its determination requires exacting protocols that are able to distinguish low levels of tissue binding from the comparatively high levels of aqueous ethanol that are contained between and inside the microsomal vesicles.

Evidence of ethanol-dependent ethanol binding at clinically relevant concentrations is shown above. Quantification of the binding behavior through traditional ligand binding analyses offers risks of overinterpretation in this context but provides a useful first approximation of the binding behavior. A group of high affinity site(s) of very low capacity (20–60 μmol/kg) saturates at concentrations below the onset of ethanol’s behavioral effects. A second group of site(s) appears to be of much higher capacity (20–80 mmol/kg) and exhibits an affinity within the pharmacological range (50–170 mM). The specific site densities obtained substantially exceed the density of known sources of ethanol binding in microsomes that derive from the cytochrome P450 of the microsomal ethanol-oxidizing system (0.8 nmol/mg protein).

Extracted microsomal phospholipids exhibit a weak concentration dependence, which would be expected for a partitioning mechanism without site specificity (5). Thermal denaturation abolishes saturable ethanol binding and is consistent with the presence of defined structural features within proteins or their interfaces as important determinants of site specificity.

The number of specific sites of weak affinity in the microsomes appears to be considerably greater than has been previously recognized. Assuming that on average the protein concentration of the microsomal fraction is 12 mmol/kg (22), the specific site density ranges from approximately two to seven sites per protein. No individual protein is likely to account for such widespread binding, which is more likely to arise from common structural motifs in proteins or at their interfaces. An abundance of specific sites with weak, yet clinically relevant, affinities is consistent with evidence for the numerous processes that are sensitive to ethanol. The results raise the possibility that there exist commonly found protein microstructures that are inherently ethanol-sensitive.

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