CMLS, Cell. Mol. Life Sci. 57 (2000) 265–275
1420-682X/00/020265-11 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Chemical properties of alcohols and their protein binding sites

D. S. Dwyer* and R. J. Bradley

LSU Medical Center-Shreveport, Departments of Psychiatry and Pharmacology, 1501 Kings Highway, Shreveport (Louisiana 71130, USA), Fax + 1 318 675 6148, e-mail: ddwyer@lsumc.edu

Received 10 June 1999; received after revision 30 August 1999; accepted 26 October 1999

Abstract. Alcohols affect a wide array of biological processes including protein folding, neurotransmission and immune responses. It is becoming clear that many of these effects are mediated by direct binding to proteins such as neurotransmitter receptors and signaling molecules. This review summarizes the unique chemical properties of alcohols which contribute to their biological effects. It is concluded that alcohols act mainly as hydrogen bond donors whose binding to the polypeptide chain is stabilized by hydrophobic interactions. The electronegativity of the O atom may also play a role in stabilizing contacts with the protein. Properties of alcohol binding sites have been derived from X-ray crystal structures of alcohol-protein complexes and from mutagenesis studies of ion channels and enzymes that bind alcohols. Common amino acid sequences and structural features are shared among the protein segments that are involved in alcohol binding. The alcohol binding site is thought to consist of a hydrogen bond acceptor in a turn or loop region that is often situated at the N-terminal end of an α-helix. The methylene chain of the alcohol molecule appears to be accommodated by a hydrophobic groove formed by two or more structural elements, frequently a turn and an α-helix. Binding at these sites may alter the local protein structure or displace bound solvent molecules and perturb the function of key proteins.

Key words. Alcohol; chemistry; hydrogen bonds; ethanol; protein structure.

Introduction

Alcohols can dramatically affect both the structure and function of a wide variety of cellular proteins and, ultimately, complex biological activities such as neurotransmission and immune responses. The neurobiological effects of ethanol have been thoroughly summarized in numerous review articles (e.g. see [1–4]). This review is focused primarily on the chemistry of alcohols and the nature of their binding sites on various protein molecules. Although ethanol, and especially the longer-chain aliphatic alcohols, can partition into membranes and cause nonspecific changes in neural activity (at high concentrations), we are interested in specific interactions with proteins which occur at physiologically relevant concentrations.

It has been known for some time that at high concentrations (typically > 0.5 M) alcohols produce structural changes in peptides and proteins [5–11]. The formation of secondary structure (α-helices) is promoted by alcohols [5–8], whereas tertiary structure is often disrupted and can lead to denaturation of the protein molecule [9–11]. At lower concentrations, the effects of alcohols are more subtle, although significant. For instance, ethanol has been shown to inhibit the function of various ion channel proteins [12–15], neurotransmitter receptors [16–24], enzymes [25, 26] and adhesion molecules [27] in the range of 1–50 mM. Behavioral

* Corresponding author.

effects of ethanol are produced at these same concentrations; the longer-chain alcohols (e.g. butanol) are even more potent [28]. This latter phenomenon led to the formulation of the Meyer-Overton rule, which states that the anesthetic potency of a compound is related to its oil:water partition coefficient (as discussed in [29]). As a general principle, the longer the methylene chain of an alcohol the greater its activity, when measured as either a behavioral effect or as a change in protein folding.

It is not yet known exactly how the alcohol molecule produces such significant changes in biological activity. Alcohols can directly alter protein folding, and the physical binding of alcohols to proteins in solution has been conclusively demonstrated [30–34]. Therefore, a reasonable assumption would appear to be that many of the biological effects of alcohols are due to pharmacological interactions with important proteins, such as ion channels and signal transduction molecules. Recent mutational analyses have lent support to this notion by localizing discrete binding sites for alcohols on ion channel proteins [35–37]. In view of these observations, we have reviewed what is known about alcohol binding to proteins with the goal of identifying the molecular basis for these interactions.

Clues about the nature of alcohol-protein interactions have been drawn from several different areas. (i) The chemistry of alcohols has been briefly discussed from the standpoint that the protein binding site should be complementary to the alcohol molecule (in terms of its molecular features). We have sought to provide a comprehensive summary of the chemical properties of alcohols in the context of their biological effects. (ii) The three-dimensional (3-D) structures of proteins with alcohol molecules bound in their crystal forms have been inspected to characterize the mode of binding. We have detected structural and sequence similarities among the alcohol binding sites that have not previously been appreciated. (iii) Data have been reviewed from recent studies aimed at mapping the binding of alcohols to various proteins using mutagenesis approaches. A general model of an alcohol binding site has been proposed here that may help to guide future mutagenesis studies. Taken together, the findings provide an emerging picture of the biochemical features of alcohol binding sites on proteins. As the picture becomes clearer, we may eventually gain insight into how alcohols produce their important biological effects.

Chemistry of alcohols

A list of selected alcohols and their pertinent physicochemical features in relation to water is shown in table 1. The hydroxyl moiety endows the alcohols with several important properties. First, alcohols are weak acids on the order of water [38, 39] except for the fluoroalcohols [such as trifluoroethanol (TFE) and hexafluoroisopropanol (HFIP)], which are stronger acids than water [40, 41]. It is important to note that the pK_a's for the alcohols shown here were determined in aqueous solutions [38, 39]. The ordering of the relative acidities for the nonhalogenated alcohols is actually reversed in the vapor phase (butanol > ethanol > methanol) in keeping with the expected contributions of the alkyl groups [42]. The behavior in aqueous solutions is affected by the bulky aliphatic chains which produce steric interference with stabilizing ion-dipole interactions. The phase-dependent reversal in the properties of alcohols is an

Table 1. Chemical properties of various alcohols in comparison to water.

	Dielectric constant*	Dipole moment*	Refractive index*	pK_a§	Δv_{AH} (cm^{-1})‖ (pyridine)	Δv_{AH} (cm^{-1})‖ (dioxane)	Boiling point, °C*
Water	78.5	1.84	1.333	15.7	–	–	100
Methanol	32.6	1.70	1.329	15.5	287	126	65
Ethanol	24.3	1.69	1.361	15.9	276	123	78
Propanol	20.1	1.58	1.385	16.1	286	–	97
Isopropanol	18.3	1.58	1.377	~25	269	–	82
Butanol	17.1	1.66	1.399	~16	274	–	117
TFE	26.6	2.52‡	1.291	12.4¶	428	214	74
HFIP	~10†	–	1.275	9.3¶	–	342	59
Ethanediol	37.7	2.28	1.432	15.1	–	–	198

* These values were obtained from the *CRC Handbook of Chemistry and Physics* [87] unless otherwise noted.
† This value for HFIP (hexafluoroisopropanol) was estimated in [52].
‡ This value for TFE (trifluoroethanol) was reported in [49].
§ Except where noted, the pK_a values were taken from [38] and [39].
¶ These values were determined by Dyatkin et al. [41].
‖ These data represent the shifts in the O-H stretching frequency of the various alcohols as measured by IR spectroscopy in the presence of the hydrogen bond acceptors pyridine and *p*-dioxane. The values were derived from tables in [44].
The dash (–) indicates that comparable data were not available.

important point because butanol has been found [43] to be an even more potent anesthetic in the gas phase (as compared with the aqueous phase), which coincides with its maximum acidity. Second, alcohols are good hydrogen bond donors. For the most part, their strength in hydrogen bonding follows the pK_a's as judged by infrared spectroscopy (IR) analysis of bond stretching in the presence of suitable hydrogen bond acceptors (table 1) [44]. Branching of the aliphatic chain of an alcohol decreases both its ability to donate hydrogen bonds and its anesthetic potency. For instance, propanol and isopropanol display frequency shifts of 286 cm^{-1} vs. 269 cm^{-1} and have effective anesthetic doses of 13.3 mmol/kg and 18.4 mmol/kg, respectively [28]. Third, alcohols can also act as hydrogen bond acceptors with varying effectiveness. Consequently, alcohols self-associate, which is evident from the elevated boiling points compared with the corresponding alkanes (e.g. ethane and propane boil at -88.5 °C and -42 °C, respectively). Nevertheless, methanol, ethanol and propanol all have boiling points below that of water, suggesting that water is a better hydrogen bond acceptor than the alcohols [44]. This interpretation is complicated by the fact that water, as opposed to alcohol, can donate two hydrogens for bonding rather than one, although the second bond is much weaker than the first [45].

Llinas and Klein [46] have observed that water behaves more like a proton acceptor (base) than a proton donor in a model system that mimics interactions with the polypeptide chain. In contrast, TFE behaves mainly as a proton donor at peptide carbonyl groups and reacts poorly with the amide groups which can serve as hydrogen bond donors [46]. The differences between water and alcohols in this system may be due, in part, to the different geometries of the hydrogen bonds that can be formed. The H-O-H angle has been measured experimentally to be 104.52° [44], whereas the C-O-H angle in methanol was found to be 110.15° [38] and is around 109° in the other alcohols as observed in molecular dynamics simulations [D. Dwyer, unpublished observations] consistent with the sp^3 hybridization. The volume and rotational freedom of the aliphatic chains will also tend to create steric hindrance at the O atom of the alcohols, thereby reducing hydrogen bond acceptance at this site. It has been suggested that the effects of alcohols on protein structure may be due to desolvation of the peptide amide groups (relative to the situation in water which is a better hydrogen bond acceptor) and subsequent destabilization of planar peptide bonds [47, 48]. If this is so, there should be some correlation between the ability of an alcohol to accept hydrogen bonds and its ability to induce structural changes in proteins which appears to be the case [49].

A comparison between TFE and ethanol may be informative. TFE is a poor hydrogen bond acceptor compared with ethanol but a much better hydrogen bond donor (table 1, frequency shift data). It also has more potent biological effects than ethanol at receptors activated by acetylcholine [50] and adenosine triphosphate (ATP) [51] and in protein folding paradigms [52–54]. However, TFE is more polar than ethanol (dielectric constant, -26.6 vs. 24.3), which contradicts the Meyer-Overton rule. Therefore, it appears that for closely related alcohol molecules (of the same chain length), the ability to disrupt protein function may largely be determined by hydrogen bond donor strength. These data lend support to the ideas of Sandorfy [55] that anesthetic potency is related to the capacity of a compound to donate hydrogen bonds to an acceptor site. Moreover, the data are consistent with the findings of Abraham et al. [43] that the potency of an anesthetic molecule decreases as its strength as a hydrogen bond acceptor increases. However, hydrogen bond donation is not the complete story. The overall hydrophobicity of alcohol molecules clearly contributes to their relative effectiveness because methanol is less potent than the longer chain aliphatic *n*-alcohols, although it is more acidic in aqueous solutions than these molecules (e.g. propanol and butanol). Taken together, the data suggest that alcohols behave mainly as hydrogen bond donors whose binding is stabilized by hydrophobic interactions. Alcohols may displace water molecules from specific sites (including catalytic sites or transition states of the protein) because the aliphatic chain forms more favorable contacts with nonpolar groups (typically, amino acid side chains) in the vicinity. A recent review has discussed in greater detail the notion that there is effective competition between alcohol and water molecules at key sites on proteins and glycolipids [56].

For a given alcohol, there is generally a close correspondence between its behavioral effects (intoxication) and its effects on protein structure [6, 9, 11, 28]. Thus, butanol is more potent than propanol or ethanol at inducing conformational changes, and halogenated alcohols, such as TFE and HFIP (which, incidentally, is a major metabolite of the experimental anesthetic, sevoflurane), produce the greatest effects on protein structure [52–54]. Therefore, a better understanding of the anesthetic properties of alcohols may be achieved by defining more precisely how alcohols alter protein structure. Moreover, it follows that the chemical properties of the alcohols will determine the nature of their binding site on a protein. The most salient features of the alcohol molecule that are likely to contribute to stable binding are (i) the hydroxyl group involved in hydrogen bond donation, (ii) the electronegative O atom and (iii) the aliphatic chain. One could already propose a model

for the protein binding site on the basis of complementary features that would include (i) a hydrogen bond acceptor site, (ii) net positive charge in the vicinity and (iii) a hydrophobic groove or cleft. Eyring et al. [57] have argued persuasively that anesthetics act at hydrophobic sites on proteins, and Abraham et al. [43] have suggested there should be a hydrogen bond acceptor group near the actual binding site.

Alcohol binding sites: crystallographic studies

What is actually known about the sites where alcohols interact with proteins? The importance of hydrogen bonding and hydrophobic interactions have already been discussed. A variety of studies suggests that there may be a size limitation to the binding site—the so-called cut-off phenomenon—because alcohols beyond a certain chain length (typically about 8–12 methylene groups, depending upon the system) have reduced anesthetic potency [16, 17, 28, 58–62]. Generally, the data suggest that the binding site is a limited groove or pocket lined by nonpolar amino acid side chains. Thus, long chain alcohols that exceed a critical length may experience steric hindrance at the alcohol binding site, although other plausible explanations have also been offered [34]. The findings of Strassmair et al. [31] indicate that the alcohol moiety prefers to hydrogen-bond at peptide carbonyl groups. These groups could conceivably be located anywhere along the peptide chain and may already participate in hydrogen bonds with other donors (e.g. in an α-helix) [63]. An attractive site for alcohol binding might be turns that include serine or threonine residues whose side-chain hydroxyls can interact with the peptide main chain. Alternatively, alcohols could form hydrogen bonds with side chain hydroxyl groups of serine, threonine and even tyrosine rather than with main chain carbonyl or amide groups.

One way to identify common motifs involved in alcohol binding would be to examine the structure of alcohol-protein complexes that have been solved by X-ray crystallography. Several such complexes have been resolved at sufficiently high resolution. For most of these structures, the protein crystals were formed in solutions that contained high concentrations of alcohol ($\sim$ 7.5–60%) [64–66]. Therefore, the conditions were not physiological. Nevertheless, the data imply that alcohols (in aqueous mixtures) can bind to discrete sites on proteins in a highly reproducible manner. This view is supported by the fact that ethanol molecules were bound at the same locations in two independently derived crystal structures of porcine pepsin [65, 67].

For this review, the structures of four separate alcohol-protein complexes were inspected using a Silicon Graphics Indigo^2 workstation and molecular modeling software from Molecular Simulations (San Diego, CA, USA). Atomic coordinates were obtained from the Protein Data Bank (Brookhaven National Laboratories) [68] for alcohol dehydrogenase (from horse liver; 1hld) [69], pepsin (3pep), crambin (1cbn) and calmodulin (1cll). Alcohol molecules were located in the crystal structures and atoms within 6 Å were noted and further investigated for possible contacts. Several points emerged from this analysis.

1) The bound alcohol was typically sandwiched between two structural elements—one of these was often an α-helix (see fig. 1). The segments involved in binding have been represented as ribbon structures in the diagram. In many cases, the alcohol molecule was located near the N-terminal end of the helix (a later section will discuss this point in greater detail).

2) The atomic interactions between the alcohol and the protein were varied. There were examples of hydrogen bond donation to backbone carbonyl groups, for instance at S36 and I128 of pepsin. In other cases, the binding was mediated by hydrogen bonding to side-chain groups, e.g. S48 and H67 of alcohol dehydrogenase, S219 of pepsin and N46 of crambin. In calmodulin, it appeared that alcohol binding was mainly stabilized by hydrophobic (van der Waals) interactions between the methyl group of ethanol and the side chain methylene groups at L32, F68 and M71. Hydrophobic interactions appeared to stabilize alcohol binding in all of the complexes analyzed. In addition, a zinc ion (bound at residues C46, H67 and C174) stabilized the interaction between benzyl alcohol and alcohol dehydrogenase. Calmodulin and lignin peroxidase (described below) are also metalloproteins; however, in these molecules, the metal complexes are quite distant from the alcohol binding site.

3) Despite the varied nature of the contacts in the complexes, there were common themes at the level of the amino acid sequences. As shown in figure 2A, the segments involved in binding typically comprised a central polar region flanked by hydrophobic bookends (one to two nonpolar residues). The central region included a number of amino acids with a high propensity for turn/coil conformations, such as glycine, proline and serine. This is consistent with the actual 3-D structure of these segments, which have been depicted in figure 1. The amino acid sequences were aligned here mainly on the basis of the start sites of the helical regions (indicated by the closed circles)—when they were present. Otherwise, existing regions of homology (such as the sequences GTI or PSI) were used to guide the alignment. This method revealed additional regions of similarity among the segments, including ones from other proteins that bind alcohols (as discussed below). In general, the data from crystal structures with bound alcohol molecules suggested that turn segments were

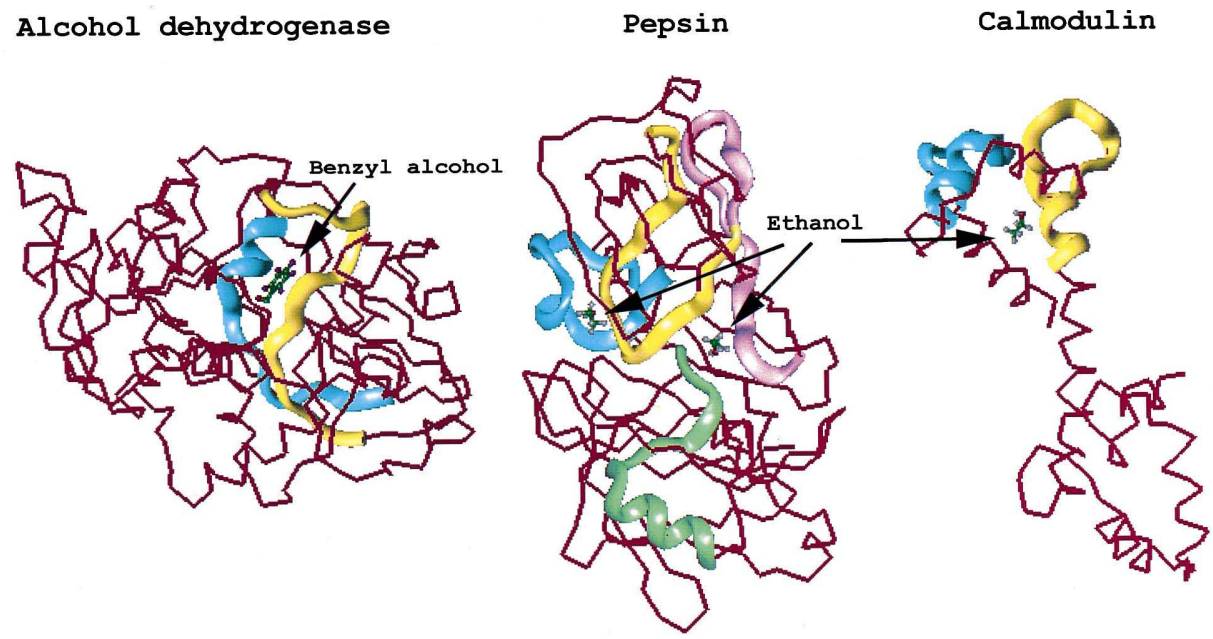

Figure 1. High resolution 3-D structures of proteins that include bound alcohol molecules. The structures are based on the atomic coordinates from the Protein Data Bank. Segments that are involved in binding have been rendered as ribbon structures and are color-coded as follows: alcohol dehydrogenase 37–52 (blue), 57–73 (yellow); pepsin 10–27 (magenta), 28–44 (yellow), 120–137 (blue), 217–234 (green); and calmodulin 19–36 (blue), 55–72 (yellow). Arrows highlight the positions of the bound alcohol molecules.

preferred binding sites, particularly when the turn was located just upstream from an α-helix.

Although general themes for an alcohol binding site have emerged from this analysis, the situation is clearly more complex than our preliminary observations suggest. Alcohol molecules could bind to proteins at additional sites that bear little or no relationship to the general motif presented here. Furthermore, protein sites that resemble this motif may not in every case bind alcohol with a detectable affinity. It is problematic to estimate how frequently these types of situations will arise, in part due to the difficulty in distinguishing between alcohol binding and its effects in many of the systems that have been studied. For the most part, data on alcohol binding have been derived from functional studies that reveal little about the possibility of additional binding sites that are functionally silent.

Alcohol binding sites: mutagenesis approaches

Recently, mutagenesis studies of several proteins have provided additional insights into the nature of alcohol binding sites [35–37, 70]. For these studies, the proteins were mutated at specific residues, and the effects on alcohol binding or the response to alcohol were then measured. Using this approach, a potential alcohol/anesthetic binding site has been mapped to a region near the transmembrane segments, TM2/TM3, of the glycine receptor (Gly-R) [35], TM2 of the α- and β-subunits of the acetylcholine receptor (AChR) [37] and TM4/TM5 of the voltage-gated K^+ channel, *Shaw* variant (K-*Shaw*) [36]. Point mutations that altered the response to ethanol were located near the putative aqueous channels of these proteins. Mutation of S267 in the glycine receptor attenuated the response to ethanol. In contrast, a similar mutation in the α-subunit of the AChR (replacing S252 with a hydrophobic amino acid) enhanced the blocking effects of long-chain alcohols [37]. The same general strategy has been applied to a soluble enzyme (lignin peroxidase) to map the binding site for veratryl alcohol [70]. Mutation of a glutamic acid at position 146 disrupted alcohol binding to the protein. The findings from these various studies support two earlier conclusions. (i) Alcohol molecules can interact directly with proteins to produce their effects. (ii) There are discrete sites on proteins that are functionally affected by alcohols. These sites might either coincide with the actual binding site (as in the case of lignin peroxidase), or they may be sites that allosterically affect alcohol binding, i.e. mutations in one region of the protein may indirectly alter binding at another site.

The mapping information from these studies has provided additional clues about the alcohol binding site. At the level of the amino acid sequences, there are certain

similarities between the ion channels (in the regions where mutations affected the response to ethanol) and other members of the panel of alcohol binding proteins (fig. 2B). The central polar region is present as well as the hydrophobic bookends. Alignment of the various binding segments revealed additional homology, such as the SIXA sequence (found in pepsin, the β-subunit of the AChR and the gamma aminobutyric acid (GABA) receptor) and the sequence KELG (found in calmodulin and the potassium channels). The sequence of lignin peroxidase also conforms to the general motif and includes the sequence LVPEP, which is similar to that found in a segment of pepsin (LVTPLP) that interacts with ethanol. There are also structural similarities among the proteins that are worth noting. According to secondary structure predictions (both Chou-Fasman [71] and GOR II [72]), the regions of Gly-R, K-*Shaw* depicted here should adopt a turn-helix conformation in the native protein (the start of each putative helix has been indicated by an open circle). Mutations that affect the response to ethanol map to these turn regions. The same general pattern is observed for the alcohol binding site in lignin peroxidase, whose crystal structure is known [73]. Finally, a similar analysis was performed on adenylate kinase because this protein binds halothane at a specific site defined by X-ray crystallography [74]. Halothane is not an alcohol, but like alcohol it is a general anesthetic. By including the sequence of adenylate kinase in the analysis, it may be possible to identify certain themes that are common to anesthetic binding sites. The helical segments involved in halothane binding have been shown (fig. 2B), and they bear some resemblance to those defined earlier from crystallographic and mutagenesis studies.

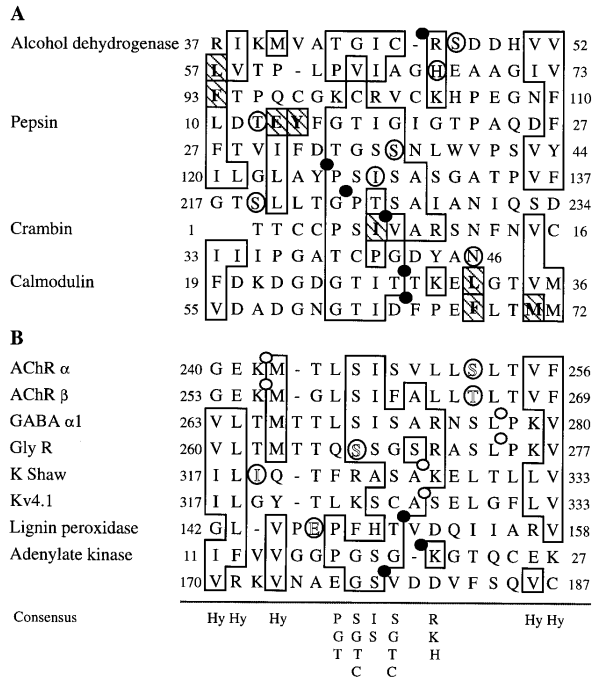

Figure 2. Amino acid sequences of protein segments involved in alcohol binding as defined by either high-resolution 3-D structures (*A*) or mutagenesis studies (*B*). The single-letter code has been used to represent the sequences, and the numbering indicates the position of each sequence within the protein. Homologous regions have been indicated by enclosure in boxes. X-ray crystallography has defined residues that make contact with alcohols via hydrogen bonding (circled amino acids) or van der Waals interactions (boxed with hash marks). Pepsin, crambin and calmodulin had ethanol bound in the crystal structures, whereas alcohol dehydrogenase was solved with 2,3,4,5,6-penta-fluorobenzyl alcohol in the binding site. In (*B*), mutations that affected the response to alcohol have been circled, and the letters have been outlined to indicate that these amino acids have not been confirmed as contact residues. The small, filled circles indicate the start of an α-helix, whereas the open circles mark the start of a putative helical segment as determined by secondary structure predictions. A consensus sequence is shown at the bottom of the figure (Hy stands for hydrophobic residues). Some of the segments shown here have also been highlighted in figure 1. The sequence information concerning adenylate kinase was derived from [74] and [88].

Alcohol binding sites: theoretical considerations

Data from high-resolution crystal structures and mutation analyses suggest that alcohol molecules can bind to discrete sites on proteins that share certain general features, including regions of amino acid sequence homology. The binding site typically includes a turn or loop segment that is adjacent to an α-helix. Hydrogen bonding via the alcohol moiety provides a significant contribution to the binding energy; however, hydrophobic interactions also play an important role. Older studies suggest a possible specific location for the hydrogen bond site with respect to the folded protein structure. Twenty years ago, Hol et al. [75] analyzed the phosphate/nucleotide binding sites on various proteins and proposed a biophysical basis for the interaction. They noted that phosphate groups (including nicotinamide adenine dinucleotide (NAD) cofactors) were preferentially bound at the N-terminal region of an α-helix and suggested that this site was favored because the net positive charge of the helix dipole stabilized contacts with negatively charged species. A considerable body of evidence now supports this concept, which appears to apply not only to phosphate moieties but to other small molecules as well (e.g. staurosporine [76] and forskolin [77, 78]). Therefore, it seems entirely possible that there may be a distinct binding site for alcohols (and general anesthetics) formed by an equivalent motif that is similar among different proteins [2, 79]. Although it is likely that other binding motifs may exist, this analysis may provide a useful framework or starting point for future studies.

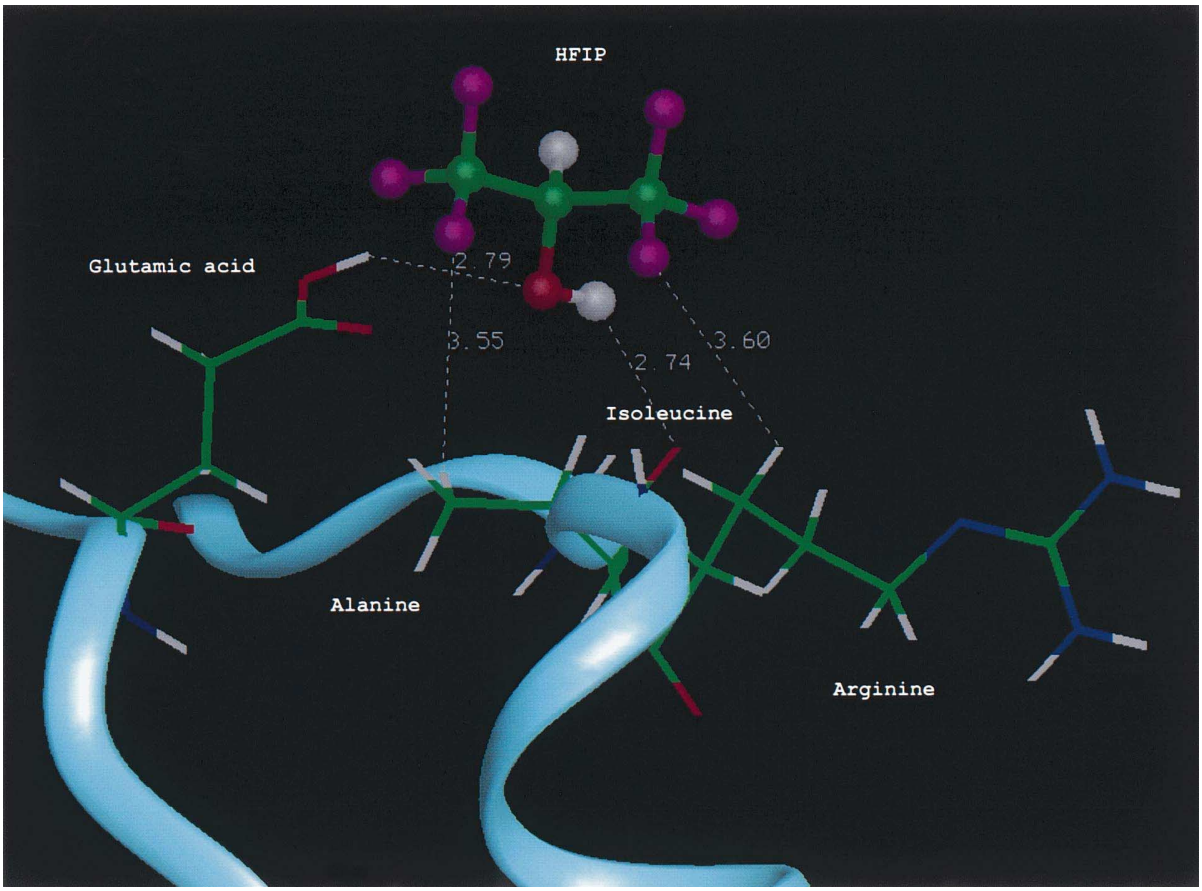

Figure 3. Molecular model of an alcohol binding site. The structure of the alcohol binding region of horse liver alcohol dehydrogenase (1hld) [69] was adapted to construct a plausible model site. HFIP (hexafluoroisopropanol) was docked into this site to illustrate the type of molecular interactions that have been observed in the various crystal structures. There is hydrogen bonding (bond lengths indicated in Å) between the hydroxyl group of HFIP and the carbonyl group of isoleucine (labeled) as well as the hydroxyl group of glutamic acid. Binding is stabilized in this model by hydrophobic interactions with methylene groups of arginine and alanine. The HFIP molecule is located at the N-terminal end of an α-helix. Theoretically, ethanol would make the same contacts as HFIP except for the interaction with arginine because there is no methyl group at an equivalent position in ethyl alcohol. Atoms in the molecules have been colored according to the following scheme: green, carbon; red, oxygen; white, hydrogen; blue, nitrogen; and purple, fluorine.

One possibility that is consistent with the available data would be that the alcohol binding site consists of a pocket formed by a turn or loop (at the N-terminal end of an α-helix) and an adjacent α-helix. A close-up view of a molecular model of a generic alcohol binding site is depicted in figure 3. HFIP was selected to illustrate binding to this site because it has potent effects on a variety of proteins, yet it has a relatively simple structure. The model, which is based on horse liver alcohol dehydrogenase [69], is intended to show the types of molecular contacts (e.g. hydrogen bonding and van der Waals interactions) that might contribute to alcohol binding. Regions of proteins that resemble the model structure would constitute special sites for several reasons. (i) Turns are rich in peptide carbonyl groups that normally form hydrogen bonds with the solvent [63]. Alcohols could readily displace water molecules from these sites. (ii) The net positive charge due to the dipole moment of the helix would tend to promote interactions with electronegative groups [75], including the alcohol moiety. (iii) Packing of α-helices could provide a hydrophobic pocket or groove for the methylene chain of the alcohol [57, 79]. The proposed binding site would be expected to be a common feature of ion channel proteins, which comprise multiple membrane-spanning helices. Moreover, according to this model, alcohol binding sites may partially overlap those for phosphates/nucleotides. This might explain, in part, why ethanol affects the activity of certain kinases, such as protein kinase C [80, 81]. Other studies from our labo-

ratory support the possibility of overlap between binding sites for alcohols and phosphates/nucleotides. Thus, the alcohol-binding segment from crambin (fig. 2) has previously been identified as a duplication unit—a protein structural motif that appears to mediate protein-protein and protein-nucleotide interactions [82].

Functional consequences of alcohol binding

Although specific biochemical features of a putative alcohol binding site have been outlined above, several important questions remain. (i) Is there a single, functional binding site for alcohols on proteins that are affected by these compounds or do multiple binding sites exist? (ii) Are all binding sites available to alcohol molecules at all times, or are new sites created during transition states of the protein (e.g. after opening an ion conductance channel)? (iii) What are the possible effects of alcohol binding at the functional site(s)? In response to the first question, it is clear that more than a single binding site for alcohols may exist on a given protein. This conclusion is supported by the crystal structure of pepsin (which includes two bound ethanol molecules) and by functional data obtained in electrophysiology studies of the acetylcholine receptor (AChR).

Studies of the AChR from muscle [16, 17, 83, 84] and from Torpedo [85, 86] have established that there is a site for small chain alcohols (< hexanol) that is associated with facilitation of channel opening. This effect of the alcohols is measured as a reduction in the apparent dissociation constant for the agonist, ACh [16, 17]. Rapid perfusion techniques (in conjunction with patch clamping) have shown that the ethanol-induced facilitatory effect is due to an increased probability of channel opening and a decreased probability of channel closing [84]. The facilitating ability of *n*-alcohols increases with chain length (methanol < ethanol < propanol < butanol), but there is a cutoff at hexanol and above where little or no facilitatory effect is observed [17, 85]. Alcohols also block AChR function by binding to a second site on the receptor that may be exposed in the open channel conformation [17, 85, 86]. Binding at this site blocks conductance only after the channel opens, and the potency increases from ethanol to octanol in this case [17]. This point is germane to the second question and suggests that new binding sites can indeed be created during conformational changes in proteins. It is interesting to note that the facilitatory site and the channel blocking site show different cutoffs. The differences cannot be attributed to either the receptor or the preparations being examined—these are the same in both cases. Rather, the difference in cutoff length could reflect the steric limitations of two distinct binding sites.

There are several reasons for believing that the facilitatory and blocking effects of alcohols are mediated by two separate sites on the AChR. (i) As mentioned, the cutoff for aliphatic chain length is different for facilitation (hexanol) and inhibition (octanol) by alcohols. (ii) The facilitatory effect occurs prior to channel activation, whereas the blocking effect occurs only after the channel has been opened. (iii) The separate site hypothesis is also supported by studies of small congeners of ethanol with similar structure but increasing molecular volume [50]. These are TFE, trichloroethanol (TCE) and tribromoethanol (TBE). TFE is more potent than ethanol at inducing the facilitatory effect on the AChR, whereas TCE and TBE never facilitate channel opening but only show a blocking effect [50]. It is significant that ethanol and TFE are both small enough in diameter to pass through an open channel, whereas TCE and TBE are too bulky to traverse the channel and may therefore block it. Thus, blockade of ion conductance is pharmacologically distinct from facilitation of channel opening, but requires an open state of the channel for its expression. As with the ion channels, other proteins may reveal binding sites for alcohols during transition states that involve conformational changes. These binding sites would have been effectively occupied in the resting state of the protein by water molecules or side-chain groups (e.g. threonine) that are mimicked by the alcohol moiety.

In response to the third question, alcohols may bind to proteins and affect function in one of several ways. First, alcohols may compete for binding with critical water molecules. The result might be perturbation of local structure or interference with protein activity (e.g. conductance) that requires bound water. Second, the binding of alcohols might influence the activity of cofactors or other associated molecules (e.g. lipids or carbohydrates) that normally regulate the function of the protein. Third, alcohol molecules, acting as cosolvents, may alter the local conformation of the protein by increasing the flexibility of the polypeptide backbone. As we have discussed elsewhere [48], this effect may involve hydrogen bonding to peptide carbonyl groups or dehydration at peptide amide groups. The alcohols are most effective at altering peptide structure when they are positioned for optimum hydrogen bonding to the backbone. Although alcohols may affect protein function by additional mechanisms, the main point is that they act by binding to discrete regions of proteins via a limited range of chemical interactions. By understanding the chemistry of alcohols and their binding sites, we may learn how ethanol affects higher order function such as behavior and immunity.

Conclusion

Alcohols can generally be considered effective hydrogen bond donors with weak acidic properties and aliphatic chains which appear to stabilize important hydrophobic interactions. They would be expected to bind at a mirror image site—hydrogen bond acceptor (base) with a hydrophobic cleft nearby—of limited dimensions, perhaps $14 \times 4 \times 3$ Å based on cutoff effects. Alcohol binding sites defined by X-ray crystallographic analysis and mutagenesis studies suggest certain shared features at the sequence and structural levels. Alcohol binding to these sites may displace bound water molecules or in other ways distort the local peptide structure of an ion channel (or other) protein and interfere with its normal function. An attractive possibility might be that alcohol binding leads to local changes in electron density (delocalization) along the peptide backbone and altered flexibility of a discrete peptide segment. The affected peptide segment might be located in the vicinity of a conductance channel or an active site and might be involved in the modulation of protein function. In some cases, the alcohol binding site may be exposed during transition states of the protein, such as channel opening. Ultimately, the chemistry of the alcohols determines how and where these molecules produce their important biological effects.

Acknowledegements. This research was supported by funding from the Department of Psychiatry, LSUMC-Shreveport.

1 Deitrich R. A., Dunwiddie T. V. and Harris R. A. (1989) Mechanism of action of ethanol: initial central nervous system actions. Pharmacol. Rev. **41:** 489–537
2 Peoples R. W., Li C. and Weight F. F. (1996) Lipid vs protein theories of alcohol action in the nervous system. Annu. Rev. Pharmacol. Toxicol. **36:** 185–201
3 Crews F. T., Morrow A. L., Criswell H. and Breese G. (1996) Effects of ethanol on ion channels. Int. Rev. Neurobiol. **39:** 283–367
4 Diamond I. and Gordon A. S. (1997) Cellular and molecular neuroscience of alcoholism. Physiol. Rev. **77:** 1–20
5 Goodman M., Listowsky I., Masuda Y. and Boardman F. (1963) Conformational aspects of polypeptides. VIII. Helical assignments via far ultraviolet absorption spectra and optical activity. Biopolymers **1:** 33–42
6 Conio G., Patrone E. and Brighetti S. (1970) The effect of aliphatic alcohols on the helix-coil transition of poly-L-ornithine and poly-L-glutamic acid. J. Biol. Chem. **245:** 3335–3340
7 Lehrman S. R., Tuls J. L. and Lund M. (1990) Peptide α-helicity in aqueous trifluoroethanol: correlations with predicted α-helicity and the secondary structure of the corresponding regions of bovine growth hormone. Biochemistry **29:** 5590–5596
8 Nelson J. W. and Kallenbach N. R. (1986) Stabilization of the ribonuclease S-peptide alpha-helix by trifluoroethanol. Proteins **1:** 211–217
9 Schrier E. E. and Scheraga H. A. (1962) The effect of aqueous alcohol solutions on the thermal transition of ribonuclease. Biochim. Biophys. Acta **64:** 408–408
10 Brandts J. F. and Hunt L. (1967) The thermodynamics of protein denaturation. III. The denaturation of ribonuclease in water and aqueous urea and aqueous ethanol mixtures. J. Am. Chem. Soc. **89:** 4826–4838
11 Herskovits T. T., Gadegbeku B. and Jaillet H. (1970) On the structural stability and solvent denaturation of proteins. I. Denaturation by the alcohols and glycols. J. Biol. Chem. **245:** 2588–2598
12 Moore S. D., Madamba S. G. and Siggins G. R. (1990) Ethanol diminishes a voltage-dependent K^+ current, the M-current, in CA1 hippocampal pyramidal neurons in vitro. Brain Res. **516:** 222–228
13 Covarrubias M. and Rubin E. (1993) Ethanol selectively blocks a noninactivating K^+ current expressed in *Xenopus* oocytes. Proc. Natl. Acad. Sci. USA **90:** 6957–6960
14 Messing R. O., Carpenter C. L., Diamond I. and Greenberg D. A. (1986) Ethanol regulates calcium channels in clonal neural cells. Proc. Natl. Acad. Sci. USA **83:** 6213–6215
15 Treistman S. N., Bayley H., Lemos J. R., Wang X., Nordmann J. J. and Grant A. J. (1991) Effects of ethanol on calcium channels, potassium channels and vasopressin release. Ann. N. Y. Acad. Sci. **625:** 249–263
16 Bradley R. J., Peper K. and Sterz R. (1980) The effects of ethanol at the frog endplate. Nature **284:** 60–62
17 Bradley R. J., Sterz R. and Peper K. (1984) The effects of alcohols and diols at the nicotinic acetylcholine receptor of the neuromuscular junction. Brain Res. **295:** 101–112
18 Suzdak P. D., Schwartz R. D. and Paul S. M. (1987) Alcohols stimulate GABA receptor-mediated chloride uptake in brain vesicles: Correlation with intoxication potency. Brain Res. **444:** 340–344
19 Allan A. M. and Harris R. A. (1987) Acute and chronic ethanol treatments alter GABA receptor-operated chloride channels. Pharmacol. Biochem. Behav. **27:** 665–670
20 Lima-Landman M. T. and Albuquerque E. X. (1989) Ethanol potentiates and blocks NMDA-activated single-channel currents in rat hippocampal pyramidal cells. FEBS Lett. **247:** 61–67
21 Dildy-Mayfield J. E. and Leslie S. W. (1989) Ethanol inhibits NMDA-induced increases in free intracellular Ca^{2+} in dissociated brain cells. Brain Res. **499:** 383–387
22 Hoffman P. L., Rabe C. S., Moses F. and Tabakoff B. (1989) N-methyl-D-aspartate receptors and ethanol: inhibition of calcium flux and cyclic GMP production. J. Neurochem. **52:** 1937–1940
23 Engblom A. C. and Akerman K. E. (1991) Effect of ethanol on GABA and glycine receptor-coupled Cl^- fluxes in rat brain synaptoneurosomes. J. Neurochem. **57:** 384–390
24 Aguayo L. G. and Pancetti F. C. (1994) Ethanol modulation of the gamma-aminobutyric acid A- and glycine-activated Cl^- current in cultured mouse neurons. J. Pharmacol. Exp. Ther. **270:** 61–69
25 Ahmed S. A. and Miles E. W. (1994) Aliphatic alcohols stabilize an alternative conformation of the tryptophan synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium*. J. Biol. Chem. **269:** 16486–16492
26 Chen Q. and LaBella F. S. (1997) Inhibition of nitric oxide synthase by straight chain and cyclic alcohols. Eur. J. Pharmacol. **321:** 355–360
27 Charness M. E., Safran R. M. and Perides G. (1994) Ethanol inhibits neural cell-cell adhesion. J. Biol. Chem. **269:** 9304–9309
28 McCreery M. J. and Hunt W. A. (1978) Physico-chemical correlates of alcohol intoxication. Neuropharmacol. **17:** 451–461
29 Seeman P. (1972) The membrane actions of anesthetics and tranquilizers. Pharmacol. Rev. **24:** 583–655
30 Russell A. E. and Cooper D. R. (1969) Structural and functional factors in the hydrogen bonding of polar organic solvents to acid-soluble collagen. Effect on renaturation kinetics and thermal stability. Biochemistry **8:** 3980–3990

31 Strassmair H., Engel J. and Zundel G. (1969) Binding of alcohols to the peptide CO-group of poly-L-proline in the I and II conformation. I. Demonstration of the binding by infrared spectroscopy and optical rotatory dispersion. Biopolymers **8:** 237–246
32 Pitner T. P. and Urry D. W. (1972) Proton magnetic resonance studies in trifluoroethanol, solvent mixtures as a means of delineating peptide protons. J. Am. Chem. Soc. **94:** 1399–1400
33 Bull H. B. and Breese K. (1978) Interaction of alcohols with proteins. Biopolymers **17:** 2121–2131
34 Eckenhoff R. G., Tanner J. W. and Johansson J. S. (1999) Steric hindrance is not required for n-alkanol cutoff in soluble proteins. Mol. Pharmacol. **56:** 414–418
35 Mihic S. J., Ye Q., Wick M. J., Koltchine V. V., Kraskowski M. D., Finn S. E. et al. (1997) Sites of alcohol and volatile anaesthetic action on $GABA_A$ and glycine receptors. Nature **389:** 385–389
36 Covarrubias M., Vyas T. B., Escobar L. and Wei A. (1995) Alcohols inhibit a cloned potassium channel at a discrete saturable site. Insights into the molecular basis of general anesthesia. J. Biol. Chem. **270:** 19408–19416
37 Forman S. (1997) Homologous mutations on different subunits cause unequal but additive effects on n-alcohol block in the nicotinic receptor pore. Biophys. J. **72:** 2170–2179
38 Hine J. and Hine M. (1952) The relative acidity of water, methanol and other weak acids in isopropyl alcohol solution. J. Am. Chem. Soc. **74:** 5266–5271
39 Ballinger P. and Long F. A. (1960) Acid ionization constants of alcohols. II. Acidities of some substituted methanols and related compounds. J. Am. Chem. Soc. **82:** 795–798
40 Ballinger P. and Long F. A. (1959) Acid ionization constants of alcohols. I. Trifluoroethanol in the solvents H_2O and D_2O. J. Am. Chem. Soc. **81:** 1050–1053
41 Dyatkin B. L., Mochalina E. P. and Knunyants I. L. (1965) The acidic properties of fluorine-containing alcohols, hydroxylamines and oximes. Tetrahedron **21:** 291–295
42 Morrison R. T. and Boyd R. N. (1973) Organic Chemistry, Allyn and Bacon, Boston
43 Abraham M. H., Lieb W. R. and Franks N. P. (1991) Role of hydrogen bonding in general anesthesia. J. Pharmaceut. Sci. **80:** 719–724
44 Joesten M. D. and Schaad L. J. (1974) Hydrogen Bonding, Marcel Dekker, New York
45 Huyskens P. L. (1977) Factors governing the influence of a first hydrogen bond on the formation of a second one by the same molecule or ion. J. Am. Chem. Soc. **99:** 2578–2582
46 Llinas M. and Klein M. P. (1975) Charge relay at the peptide bond. A proton magnetic resonance study of solvation effects on the amide electron density distribution. J. Am. Chem. Soc. **97:** 4731–4737
47 Cammers-Goodwin A., Allen T. J., Oslick S. L., McClure K. F., Lee J. H. and Kemp D. S. (1996) Mechanism of stabilization of helical conformations of polypeptides by water containing trifluoroethanol. J. Am. Chem. Soc. **118:** 3082–3090
48 Dwyer D. S. (1999) Molecular simulation of the effects of alcohols on peptide structure. Biopolymers **49:** 635–645
49 Rajan R. and Balaram P. (1996) A model for the interaction of trifluoroethanol with peptides and proteins. Int. J. Peptide Protein Res. **48:** 328–336
50 Sterz R., Hermes M., Peper K. and Bradley R. J. (1981) Effects of halogenated derivatives of ethanol on the nicotine acetylcholine receptor. Brain Res. **230:** 434–438
51 Li C., Peoples R. W. and Weight F. F. (1994) Alcohol action on a neuronal membrane receptor: evidence for a direct interaction with the receptor protein. Proc. Natl. Acad. Sci. USA **91:** 8200–8204
52 Beaudette N. V., Okabayashi H. and Fasman G. D. (1982) Conformational effects of organic solvents on histone complexes. Biochemistry **21:** 1765–1772
53 Jackson M. and Mantsch H. H. (1992) Halogenated alcohols as solvents for proteins: FTIR spectroscopic studies. Biochim. Biophys. Acta **1118:** 139–143
54 Hirota N., Mizuno K. and Goto Y. (1997) Comparative α-helix formation in β-lactoglobulin and melittin induced by hexafluoroisopropanol. Protein Sci. **6:** 416–421
55 Sandorfy C. (1978) Intermolecular interactions and anesthesia. Anesthesiol. **48:** 357–359
56 Klemm W. R. (1998) Biological water and its role in the effects of alcohol. Alcohol **15:** 249–267
57 Eyring H., Woodbury J. W. and D'Arrigo J. S. (1973) A molecular mechanism of general anesthesia. Anesthesiol. **38:** 415–424
58 Franks N. P. and Lieb W. R. (1985) Mapping of general anaesthetic target sites provides a molecular basis for cutoff effects. Nature **316:** 349–351
59 Alifimoff J. K., Firestone L. L. and Miller K. W. (1989) Anaesthetic potencies of primary alkanols: implications for the molecular dimensions of the anaesthetic site. Br. J. Pharmacol. **96:** 9–16
60 Chu B. and Treistman S. N. (1997) Modulation of two cloned potassium channels by 1-alkanols demonstrates different cutoffs. Alcohol. Clin. Exp. Res. **21:** 1103–1107
61 Peoples R. W. and Weight F. F. (1995) Cutoff in potency implicates alcohol inhibition of N-methyl-D-aspartate receptors in alcohol intoxication. Proc. Natl. Acad. Sci. USA **92:** 2825–2829
62 Dildy-Mayfield J. E., Mihic S. J., Liu Y., Deitrich R. A. and Harris R. A. (1996) Actions of long chain alcohols on $GABA_A$ but not glutamate receptors correlate with their in vivo effects. Br. J. Pharmacol. **118:** 378–384
63 Baker E. N. and Hubbard R. E. (1984) Hydrogen bonding in globular proteins. Prog. Biophys. Mol. Biol. **44:** 97–179
64 Teeter M. M., Roe S. M. and Heo N. H. (1993) Atomic resolution (0.83 Å) crystal structure of the hydrophobic protein crambin at 130 K. J. Mol. Biol. **230:** 292–311
65 Abad-Zapatero C., Rydel T. J. and Erickson J. (1990) Revised 2.3 Å structure of porcine pepsin: evidence for a flexible subdomain. Proteins **8:** 62–81
66 Chattopadhyaya R., Meador W. E., Means A. R. and Quiocho F. A. (1992) Calmodulin structure refined at 1.7 Å resolution. J. Mol. Biol. **228:** 1177–1192
67 Andreeva N. S., Zdanov A. S., Gustchina A. E. and Federov A. A. (1984) Structure of ethanol-inhibited porcine pepsin at 2-Å resolution and binding of the methyl ester of phenylalanyl-diiodotyrosine to the enzyme. J. Biol. Chem. **259:** 11353–11365
68 Bernstein F. C., Koetzle T. F., Willaims J. B., Meyer E. F. Jr, Brice M. D., Rodgers J. R. et al. (1977) The Protein Data Bank: a computer-based archival file for macromolecular structures. J. Mol. Biol. **112:** 535–542
69 Ramaswamy S., Eklund H. and Plapp B. V. (1994) Structures of horse liver alcohol dehydrogenase complexed with NAD and substituted benzyl alcohols. Biochemistry **33:** 5230–5237
70 Ambert-Balay K., Fuchs S. M. and Tien M. (1999) Identification of the veratryl alcohol binding site in lignin peroxidase by site-directed mutagenesis. FASEB J. **13:** A1566
71 Chou P. Y. and Fasman G. D. (1978) Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. **47:** 45–148
72 Garnier J. and Robson B. (1989) The GOR method for predicting secondary structures in proteins. In Prediction of Protein Structure and the Principles of Protein Conformation, p. 417, Fasman G. D. (ed): Plenum Press, New York
73 Poulos T. L., Edwards S. L., Wariishi H. and Gold M. H. (1993) Crystallographic refinement of lignin peroxidase at 2 Å. J. Biol. Chem. **268:** 4429–4440
74 Sachsenheimer W., Pai E. F., Schulz G. E. and Schirmer R. H. (1977) Halothane binds in the adenine-specific niche of crystalline adenylate kinase. FEBS Lett. **79:** 310–312
75 Hol W. G. J., van Duijnen P. T. and Berendsen H. J. C. (1978) The α-helix dipole and the properties of proteins. Nature **273:** 443–446

76 Lawrie A. M., Noble E. M., Tunnah P., Brown N. R., Johnson L. N. and Endicott J. A. (1997) Protein kinase inhibition by staurosporine revealed in details of the molecular interaction with CDK2. Nature Struct. Biol. **4:** 796–801
77 Tesmer J. J. G., Sunahara R. K., Gilman A. G. and Sprang S. R. (1997) Crystal structure of the catalytic domains of adenylyl cyclase in a complex with $G_{s\alpha}$-GTPγS. Science **278:** 1907–1916
78 Skiba N. P. and Hamm H. E. (1998) How Gsα activates adenylyl cyclase. Nature Struct. Biol. **5:** 88–92
79 Franks N. P. and Lieb W. R. (1994) Molecular and cellular mechanisms of general anaesthesia. Nature **367:** 607–614
80 Skwish S. and Shain W. (1990) Ethanol and diolein stimulate PKC translocation in astroglial cells. Life Sci. **47:** 1037–1042
81 Slater S. J., Cox K. J., Lombardi J. V., Ho C., Kelly M. B., Rubin E. et al. (1993) Inhibition of protein kinase C by alcohols and anaesthetics. Nature **364:** 82–84
82 Dwyer D. S. (1999) Assembly of exons from unitary transposable genetic elements: implications for the evolution of protein-protein interactions. J. Theoret. Biol. **194:** 11–27
83 Sterz R., Peper K., Simon J., Edge M., Pagala M. and Bradley R. J. (1986) Agonist and blocking effects of choline at the neuromuscular junction. Brain Res. **385:** 99–114
84 Dilger J. P., Liu Y., Roper J. F. and Bradley R. J. (1994) Effects of ethanol on ACh receptor channels. Biophys. J. **66:** A9
85 Wood S. C., Forman S. A. and Miller K. W. (1991) Short chain and long chain alkanols have different sites of action on nicotinic acetylcholine receptor channels from *Torpedo*. Mol. Pharmacol. **39:** 332–338
86 Tonner P. H. and Miller K. W. (1995) Molecular sites of general anaesthetic action on acetylcholine receptors. Eur. J. Anaesthesiol. **12:** 21–30
87 Weast R. C., Lide D. R., Astle M. J. and Beyer W. H. (1989) CRC Handbook of Chemistry and Physics, CRC Press, Boca Raton
88 Heil A., Muller G., Noda L., Pinder T., Schirmer H., Schirmer I. et al. (1974) The amino acid sequence of porcine adenylate kinase from skeletal muscle. Eur. J. Biochem. **43:** 131–144