Mapping the Polarity Profiles of General Anesthetic Target Sites Using *n*-Alkane-(α,ω)-diols†

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ABSTRACT: The effects of the homologous series of *n*-alkane-(α,ω)-diols have been studied on the inhibition of the purified firefly luciferase enzyme from *Photinus pyralis*, the inhibition of the purified bacterial luciferase enzyme from *Vibrio harveyi*, and the induction of general anesthesia in *Xenopus laevis* tadpoles. All but one of the diols tested were found to be reversible general anesthetics. The diols inhibited firefly luciferase by competing with its normal substrate firefly luciferin, and they inhibited bacterial luciferase by competing with the substrate *n*-decanal. For all but the smallest agent (1,4-butanediol), only a single diol molecule was found to be involved in the inhibition of the enzymes. Inhibition constants K_i were determined for the enzymes, and general anesthetic EC_{50} concentrations were determined for tadpoles. These data were then used in conjunction with previously determined *n*-alkane and *n*-alcohol data to calculate, as a function of chain length, the incremental standard Gibbs free energies $\Delta(\Delta G^0)$ for adding apolar $-CH_2-$ groups and for converting apolar terminal $-CH_3$ groups to polar $-CH_2OH$ groups. The resulting plots of $\Delta(\Delta G^0)$ versus chain length gave a consistent mapping of the polarity profiles of the anesthetic-binding pockets. They clearly reveal the existence of two substantial and distinct polar regions in the anesthetic-binding pocket of firefly luciferase but only one such region for bacterial luciferase and for the unknown target sites underlying general anesthesia. The polarities and geometric properties of these different binding sites for straight-chain anesthetics are discussed in terms of simple models.

The unknown target sites in the central nervous system to which general anesthetics bind to produce reversible loss of consciousness have long been known to possess substantial hydrophobic character. This has been deduced from the good correlations of general anesthetic potency with solubility in fat-like solvents such as olive oil (Meyer, 1899; Overton, 1901; Miller et al., 1967). More recently, however, it has become apparent that these sites also have significant polar characteristics. This became apparent when it was noticed that very poor correlations of anesthetic potency are obtained with partition coefficients between water and purely hydrophobic solvents such as *n*-hexadecane, in contrast to the excellent correlations found with amphiphilic solvents such as *n*-octanol [Franks & Lieb, 1978; see also Katz and Simon (1977)]. In addition, some of the characteristics of these polar regions are beginning to be unraveled. For example, it has recently been shown (Abraham et al., 1991) that these target sites are able to accept (but not to donate) hydrogen bonds from anesthetics almost as well as can be done by water.

In the present study, we have investigated the manner in which polar and apolar regions are distributed in a number of different anesthetic-binding sites. The basic idea behind the method we have used is to compare the potencies of compounds which have comparable sizes but differ in their possession of simple polar groups (terminal hydroxyl –OH groups) or a simple apolar group (the methylene $-CH_2-$ group). For this purpose, we have used the homologous series of *n*-alkanes, *n*-alcohols, and *n*-alkane-(α,ω)-diols. For each chain length, it is possible to calculate from experimental potency data the incremental standard Gibbs free energies for adding these polar or apolar groups. By comparing these free energies at each chain length, it is then possible to map out the polarity profiles of the anesthetic-binding sites to which these compounds bind and to check for consistency in the resulting maps.

The anesthetic-binding sites we have looked at are those on the firefly luciferase enzyme, on the bacterial luciferase enzyme, and on the primary target sites underlying the induction of general anesthesia. We have chosen the luciferase enzymes because extensive data for their inhibition by *n*-alkanes and *n*-alcohols are available in the literature and because their anesthetic-binding pockets have contrasting properties (Franks & Lieb, 1985; Curry et al., 1990). Of equal importance, the anesthetic-binding pocket of firefly luciferase is currently the best available protein model of the unknown target sites underlying general anesthesia (Miller, 1985; Franks & Lieb, 1987), being able to account for the potencies of a diverse range of general anesthetics over a potency range of about 100 000-fold (Franks & Lieb, 1984).

Experimental Procedures

Chemicals. The *n*-alkane-(α,ω)-diols were purchased from Aldrich, with the exception of 1,7-heptanediol, which was obtained from Lancaster Synthesis. Their purities were C4, 99+%; C5, 99%; C6, 99+%; C7, 98%; C8, 96%; C9, 97%; C10, 99%; C12, 96%; C14, 99%; and C16, 98%. The *n*-alkane-(α,ω)-diols with 11, 13, and 15 carbons were not available commercially. Other reagents were as described previously (Franks & Lieb, 1984; Curry et al., 1990).

Purification of Luciferase Enzymes. Pure crystals of the enzyme firefly luciferase were obtained from desiccated lanterns of the North American firefly *Photinus pyralis* using an affinity chromatography procedure (Branchini et al., 1980; Franks & Lieb, 1984). Bacterial luciferase was extracted and purified from *Vibrio harveyi* bacteria (strain MB20) as de-

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scribed in detail elsewhere (Curry et al., 1990). The highly purified enzymes were stored as stock solutions in 0.4 M ammonium sulfate/1 mM EDTA, pH 7.8 at 4 °C (firefly enzyme) or in 50 mM potassium phosphate/0.1 mM dithiothreitol, pH 7.0 at −20 °C (bacterial enzyme).

Assays of Luciferase Activities. The activities of the enzymes were assayed at 25 ± 1 °C as described previously for firefly luciferase (Franks & Lieb, 1984) and for bacterial luciferase (Curry et al., 1990), under conditions where the ATP-binding site of the firefly enzyme and the flavin-binding site of the bacterial enzyme were effectively saturated (final ATP and $FMNH_2$ concentrations were 2 mM and 100 μM, respectively). Luciferase activity was taken as the peak light intensity (the maximum rate of photon emission). Some of the inhibitors induced significant changes in the rate of luminescent decay which, in the case of the bacterial enzyme, have been shown (Curry et al., 1990) to be due to the inhibitors stabilizing an intermediate form of the enzyme. This has the effect of somewhat underestimating the derived values of K_i, as discussed in detail elsewhere (Curry et al., 1990); no corrections were made for this effect. The *n*-alkane-(α,ω)-diols from C4 to C9 inclusive were added as concentrated aqueous solutions directly to the assay vials. Those from C10 to C16 were added as concentrated ethanolic solutions, such that the final ethanol concentrations never exceeded 23 mM [<3% of the ethanol K_i = 820 mM for inhibiting firefly luciferase (Franks & Lieb, 1985) and of the ethanol EC_{50} = 1560 mM for inhibiting bacterial luciferase (Curry et al., 1990)]; the controls nonetheless always contained the same concentrations of ethanol.

Analysis of Enzyme Inhibition Data. For both enzymes, inhibition by the *n*-alkane-(α,ω)-diols was competitive in nature, with the diols competing for the binding of luciferin (firefly luciferase) or *n*-decanal (bacterial luciferase). Inhibition constants K_i and their standard errors and the number N of diol molecules involved in the inhibition were determined from plots of $f(A)$ or $\sqrt{f(A)}$ versus anesthetic concentration [A], where the function $f(A)$ is the factor by which the apparent Michaelis constant for the competing substrate (firefly luciferin or *n*-decanal) changes with anesthetic concentration [A]. It can be shown [see, for example, Franks and Lieb (1984)] that if N anesthetic molecules can bind independently to the enzyme with the same inhibition constant K_i but the presence of only one is necessary to cause inhibition then $f(A) = (1 + [A]/K_i)^N$. Mean inhibition constants K_i and stoichiometries N were determined by plotting $f(A)$ and $\sqrt{f(A)}$ versus [A]. In all cases either the $f(A)$ or the $\sqrt{f(A)}$ plot was linear (showing that N = 1 or 2, respectively), and the inhibition constants K_i were determined from the slopes of the linear plots using weighted least-squares regression analysis as described in detail by Curry et al. (1990).

Determination of General Anesthetic Potencies. General anesthetic EC_{50} concentrations were determined for 17–24-day-old *Xenopus laevis* tadpoles (length, 1.2 ± 0.2 cm) at 20 ± 0.5 °C. Six tadpoles were placed in each of three to six beakers containing 300 mL of tap water, with or without dissolved anesthetic. The anesthetic end point was defined as the lack of a swimming response following a gentle prod with the rounded end of a smooth glass rod. The number of anesthetized tadpoles was recorded as a function of time up to about 1.5–3 h. When a steady state was reached, the tadpoles were returned to tap water without anesthetic and tested for recovery; almost all tadpoles recovered, but the few which did not were not included in the following analysis. EC_{50} concentrations and their estimated errors were determined from the final dose–response curves either by interpolation or by the method of Waud (1972).

Calculation of Incremental Standard Gibbs Free Energies for Methylene and Hydroxyl Groups. For the luciferase enzymes, these were calculated from inhibition constants for the homologous series of *n*-alkanes (Franks & Lieb, 1985; Curry et al., 1990), *n*-alcohols (Franks & Lieb, 1985; Curry et al., 1990), and *n*-alkane-(α,ω)-diols (this paper), using the following formulas:

$$\Delta(\Delta G^0_{CH_2}) = RT \ln \left[\frac{K_{i,n}^{alkane}}{K_{i,n-1}^{alkane}} \right] \quad (1a)$$

or

$$\Delta(\Delta G^0_{CH_2}) = RT \ln \left[\frac{K_{i,n}^{alcohol}}{K_{i,n-1}^{alcohol}} \right] \quad (1b)$$

$$\Delta(\Delta G^0_{1st\text{-}OH}) = RT \ln \left[\frac{K_{i,n}^{alcohol}}{2K_{i,n}^{alkane}} \right] \quad (2)$$

$$\Delta(\Delta G^0_{2nd\text{-}OH}) = RT \ln \left[\frac{2K_{i,n}^{diol}}{K_{i,n}^{alcohol}} \right] \quad (3)$$

where R is the gas constant, T is the absolute temperature, the K_i terms are inhibition constants [superscript = type of compound and subscript (n or $n - 1$) = number of carbon atoms], and the incremental free energies are for n carbon atoms on the final anesthetic molecule and refer to adding a methylene ($-CH_2-$) group (eqs 1a and 1b), substituting a hydroxyl (−OH) group for a hydrogen atom on one of two terminal methyl groups (eq 2), and substituting a hydroxyl group for a hydrogen atom on the remaining terminal methyl group (eq 3). The factor 2 which appears in eqs 2–3 is a statistical factor due to the different symmetries of the symmetric *n*-alkane and *n*-alkane-(α,ω)-diol molecules compared to the asymmetric *n*-alcohol molecules. The exact value of this factor is of course model-dependent, and we have considered the simplest case, where a given asymmetric *n*-alcohol molecule binds to a given site predominantly in one orientation. A symmetric molecule (an alkane or diol), as opposed to an asymmetric alcohol molecule, can then bind to the site in two indistinguishable orientations. This results in an effective doubling of the intrinsic "on" rate constant and hence a halving of the observed inhibition (dissociation) constant K_i for the symmetric molecules compared to the asymmetric molecules. In order to compare symmetric with asymmetric ligands, a factor of 2 is therefore required to multiply the K_i for the symmetric molecules if the required values of $\Delta(\Delta G^0)$ are to be placed on the same free energy scale.

For the unknown primary target sites underlying general anesthesia, tadpole EC_{50} values for *n*-alcohols (Alifimoff et al., 1989) and *n*-alkane-(α,ω)-diols (this paper) were used to calculate the corresponding *apparent* incremental standard Gibbs free energies $\Delta(\Delta G^0)$ for the addition of a methylene group and for substituting a hydroxyl group for a hydrogen atom on the remaining terminal methylene group using

$$\Delta(\Delta G^0_{CH_2}) = RT \ln \left[\frac{EC_{50,n}^{alcohol}}{EC_{50,n-1}^{alcohol}} \right] \quad (4)$$

$$\Delta(\Delta G^0_{2nd\text{-}OH}) = RT \ln \left[\frac{2EC_{50,n}^{diol}}{EC_{50,n}^{alcohol}} \right] \quad (5)$$

Table I: *n*-Alkane-(α,ω)-diol Inhibition Constants K_i for Luciferase Enzymes and EC_{50} Concentrations for General Anesthesia in Tadpoles[a]

(α,ω)-diol	firefly luciferase		bacterial luciferase		anesthesia
	K_i	N	K_i	N	EC_{50}
C4	240 ± 5 mM	2	759 ± 59 mM	2	toxic
C5	5.43 ± 0.45 mM	1	8.39 ± 0.97 mM	1	19 ± 2 mM
C6	3.08 ± 0.75 mM	1	6.80 ± 0.47 mM	1	25 ± 5 mM
C7	345 ± 27 μM	1	386 ± 41 μM	1	3.3 ± 0.3 mM
C8	71.6 ± 4.5 μM	1	90.3 ± 8.2 μM	1	960 ± 90 μM
C9	208 ± 30 μM	1	94.8 ± 6.4 μM	1	640 ± 110 μM
C10	36 ± 1 μM	1	38.8 ± 3.4 μM	1	250 ± 50 μM
C12	920 ± 40 nM	1	2.74 ± 0.22 μM	1	45 ± 15 μM
C14	340 ± 14 nM	1	2.11 ± 0.29 μM	1	not tested
C16	350 ± 30 nM	1	1.49 ± 0.15 μM	1	not tested

[a] The errors are standard errors in the mean, and N is the number of diol molecules involved in the inhibition of the luciferase enzymes. EC_{50} concentrations for the enzymes can be calculated (Franks & Lieb, 1984; Curry et al., 1990) from their K_i values using $EC_{50} = 2K_i$ for $N = 1$ and $EC_{50} = 0.732K_i$ for $N = 2$.

where the subscripts and superscripts on the EC_{50} terms have the same meaning as those on the K_i terms in eqs 1–3.

Standard errors were calculated for all of the above incremental free energies using the standard errors for the diols listed in Table I together with standard errors for the *n*-alkanes and *n*-alcohols either published (Curry et al., 1990; Alifimoff et al., 1989) or, in the case of firefly luciferase, determined from an analysis of our earlier data (Franks & Lieb, 1984).

Results and Discussion

Inhibition of Firefly Luciferase. Inhibition by the series of *n*-alkane-(α,ω)-diols of the highly purified firefly luciferase enzyme from *P. pyralis* was studied under conditions where the ATP-binding site was effectively saturated. In this state, the enzyme is highly sensitive to a diverse range of anesthetic agents (Franks & Lieb, 1984), in contrast to the low-ATP state which is relatively insensitive to anesthetic inhibition (Moss et al., 1991). The *n*-alkane-(α,ω)-diols inhibit the enzyme by competing with the natural substrate firefly luciferin (see Figure 1a), as previously demonstrated for a wide variety of other anesthetic agents (Franks & Lieb, 1984). The number of diol molecules involved in the inhibition was found to be two for 1,4-butanediol but only one for all of the other *n*-alkane-(α,ω)-diols. These stoichiometries N and the inhibition constants K_i for all of the diols investigated are listed in Table I.

Inhibition of Bacterial Luciferase. The corresponding results for inhibition of the highly purified bacterial luciferase enzyme from *V. harveyi* are also given in Table I. In order to minimize possible effects (Middleton & Smith, 1976) of the *n*-alkane-(α,ω)-diols on the binding of flavin ($FMNH_2$), we studied this enzyme under conditions where the flavin-binding site was effectively saturated. The diols inhibit the bacterial enzyme by competing with the long-chain substrate *n*-decanal (Figure 1b), as found previously (Curry et al., 1990) for a wide range of general anesthetics. As with the firefly enzyme, two diol molecules were found to be involved in the inhibition by 1,4-butanediol (Figure 2a) but only one diol molecule was found (see Figure 2b for a typical example) for all of the other *n*-alkane-(α,ω)-diols investigated.

Induction of General Anesthesia. We tested each of the available *n*-alkane-(α,ω)-diols from C4 upward for which the corresponding *n*-alcohol is a general anesthetic (the C13 and higher *n*-alcohols have no anesthetic potency). We found that all of these diols acted as general anesthetics, with the exception of 1,4-butanediol. (0.5 M 1,4-butanediol did not anesthetize tadpoles, but their tails became curled, which is indicative of a toxic effect. We therefore did not test higher levels of this agent.) The *n*-alkane-(α,ω)-diols reversibly suppressed the reflex swimming response in 50% of a population of *X. laevis* tadpoles at the EC_{50} concentrations listed in Table I.

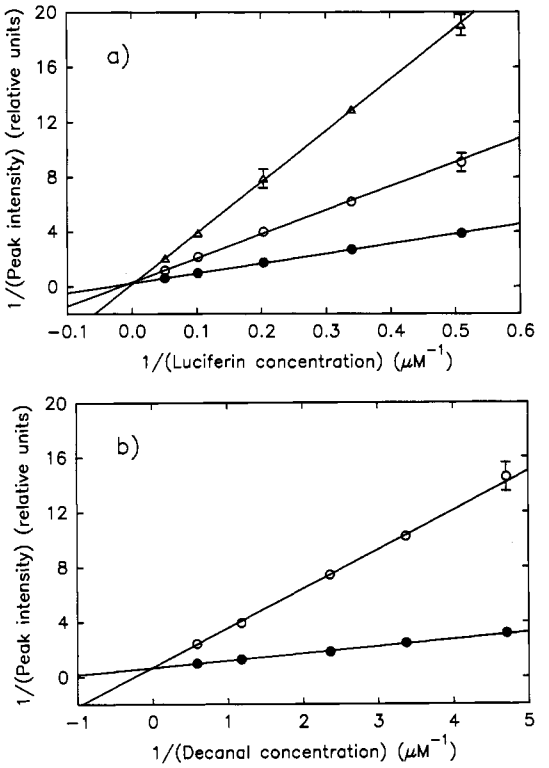

FIGURE 1: Competitive nature of the inhibition of the luciferase enzymes by *n*-alkane-(α,ω)-diols. (a) Firefly luciferase. Double-reciprocal plots of enzyme activity versus luciferin concentration are shown at different concentrations of 1,4-butanediol: (●) control; (○) 170 mM; (△) 340 mM. Error bars (±SE) are shown where larger than the symbols. The straight lines were fitted by the method of weighted least squares, using weighting factors proportional to the squares of the enzyme activities (peak light intensities). (b) Bacterial luciferase. Double-reciprocal plots of enzyme activity versus *n*-decanal concentration are shown under control conditions (●) and in the presence of 1,7-heptanediol (○) at 1.67 mM. Error bars and straight lines are as in (a).

Strategy for Mapping of Anesthetic-Binding Sites. In order to map out the polarities and geometries of the different anesthetic-binding sites, we took advantage of the fact that the effects of the homologous series of *n*-alcohols are quantitatively known for all three systems and those of the series of *n*-alkanes are known for both of the luciferase enzymes. The basic strategy was to use the known inhibition constants K_i for the enzymes and the known EC_{50} concentrations for general anesthesia for these homologous series to calculate, as a function of chain length, the incremental standard Gibbs free energies $\Delta(\Delta G^0)$ for adding an apolar methylene group ($-CH_2-$) and

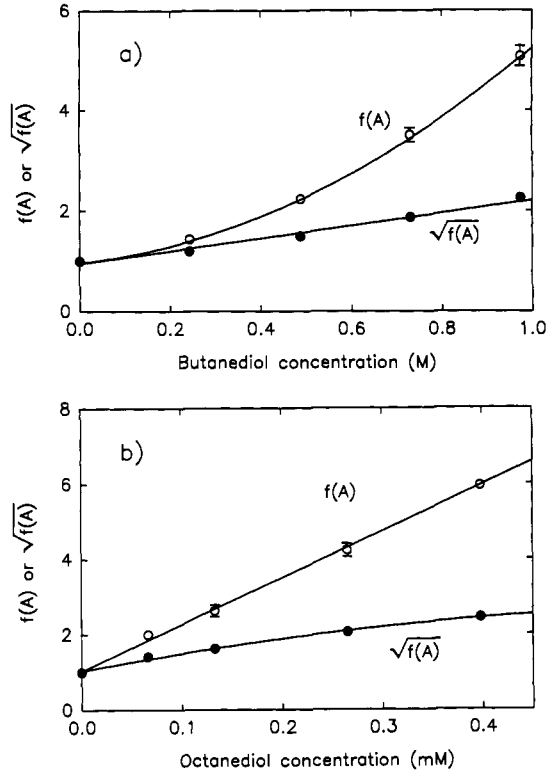

FIGURE 2: The number of *n*-alkane-(α,ω)-diol molecules involved in the inhibition of the bacterial luciferase enzyme is two for 1,4-butanediol but only one for the longer chain diols. The effects of the diols (C4 and C8) are plotted as the functions $f(A)$ (○) and $\sqrt{f(A)}$ (●), where $f(A)$ is the factor by which the apparent Michaelis constant for *n*-decanal increases with anesthetic concentration [A] (see Experimental Procedures). (a) 1,4-butanediol. Error bars (±SE) are shown where larger than the symbols. Values of $f(A)$ and the weighted least-squares regression straight lines were calculated as described by Curry et al. (1990). (b) 1,8-octanediol. Error bars and straight lines are as in (a). Notice that in (a) the $f(A)$ plot (○) is parabolic while the $\sqrt{f(A)}$ plot (●) is linear (consistent with two molecules of butanediol being involved in the inhibition), while in (b) the $f(A)$ plot (○) but not the $\sqrt{f(A)}$ plot (●) is linear (consistent with only one molecule of octanediol being involved in the inhibition). See Experimental Procedures for a discussion of the interpretation of $f(A)$ and $\sqrt{f(A)}$ plots.

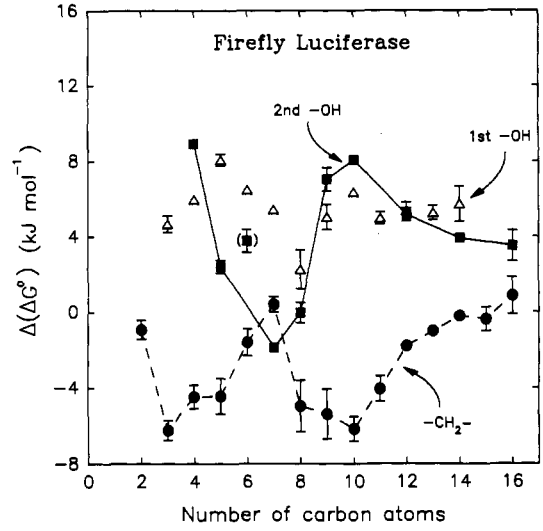

FIGURE 3: Polarity profile of the anesthetic-binding pocket on firefly luciferase. Values (±SE) of incremental standard Gibbs free energies $\Delta(\Delta G^0)$ were calculated using eqs 1–3 as described under Experimental Procedures. (■) $\Delta(\Delta G^0_{2nd\text{-}OH})$, for substituting a hydroxyl group for a hydrogen atom on an *n*-alcohol terminal methyl group, to obtain the corresponding *n*-alkane-(α,ω)-diol. (△) $\Delta(\Delta G^0_{1st\text{-}OH})$, for substituting a hydroxyl group for a hydrogen atom on an *n*-alkane terminal methyl group, to obtain the corresponding *n*-alcohol. (●) $\Delta(\Delta G^0_{CH_2})$, mean of values for adding a methylene group to an *n*-alkane and *n*-alcohol where both values could be calculated (C4–C14) or else value for only an *n*-alcohol (C2, C3, C15, C16). The abscissa is the chain length, expressed as the number of carbon atoms in the final anesthetic molecule. Error bars (±SE) are shown where larger than the symbols.

for converting the apolar terminal methyl groups ($-CH_3$) to polar $-CH_2OH$ groups. If adding an apolar group or converting a polar group to an apolar group makes the ligand bind more tightly (i.e., decreases the free energy), this will indicate the presence of a hydrophobic region in the site. (It should be stressed that this analysis assumes that our observed K_i values for the enzymes and EC_{50} concentrations for anesthesia reflect true binding affinities. Direct binding measurements, however, have not yet been performed on either the luciferase enzymes or, of course, on the unknown targets underlying general anesthesia). Moreover, if the analysis is valid, one would expect the pattern obtained by adding an apolar $-CH_2-$ group to be the opposite of that obtained by converting an apolar $-CH_3$ to a polar $-CH_2OH$ group. We shall see below that this was indeed realized.

Finally, by including other information on the target sites, we shall attempt to formulate simple pictures of the various anesthetic-binding sites which rationalize the observed binding patterns. It should be stressed that at this stage our descriptions of these binding site pictures cannot be, and are not intended to be, exact representations; this will require the use of structural techniques such as X-ray crystallography. However, it is hoped that they will clarify the thermodynamic results (by showing how they *can* be explained in a consistent manner) and suggest future experimental approaches to mapping the polarity profiles of anesthetic-binding sites.

The data for the *n*-alkane-(α,ω)-diols obtained in the present study are listed in Table I. Notice that the enzyme data are expressed as inhibition constants K_i, whereas the general anesthesia data are expressed as EC_{50} concentrations. To compare enzyme with general anesthesia data, the former can be converted to EC_{50} concentrations using the formulas given in the table. Also, notice that all the values for 1,6-hexanediol (C6) in Table I are higher than expected from the overall trends of their neighboring values. This was true for this agent acting on both of the luciferase enzymes and on animals. It is unlikely that this was due to an impurity, since not only was 1,6-hexanediol extremely pure (99+%), but its potencies were *less* than expected. We have no explanation for this anomalous behavior. In the figures which follow (Figures 3–5), we have plotted all data relating to C6 but placed the data symbols in brackets and ignored them in our consideration of overall trends.

The Firefly Luciferase Anesthetic-Binding Pocket. This pocket normally binds the physiological substrate firefly luciferin (Moss et al., 1991), which is a large heterocyclic molecule that is predominantly apolar but has a polar phenol hydroxyl group at one end and a polar carboxylic acid group at its other end. This is the pocket to which the *n*-alkane-(α,ω)-diols bind, and one would predict from the structure of firefly luciferin that it is a predominantly hydrophobic pocket or cleft, with polar patches at each end. If so, this should be reflected in the pattern of binding of the homologous series of *n*-alkane-(α,ω)-diols, *n*-alcohols, and *n*-alkanes.

In order to map out the polarity profile of this pocket, we used the inhibition constants K_i for the *n*-alkane-(α,ω)-diols (Table I), the *n*-alcohols (Franks & Lieb, 1985), and the *n*-alkanes (Franks & Lieb, 1985) to calculate (eqs 1–3), as a function of chain length, the following incremental standard

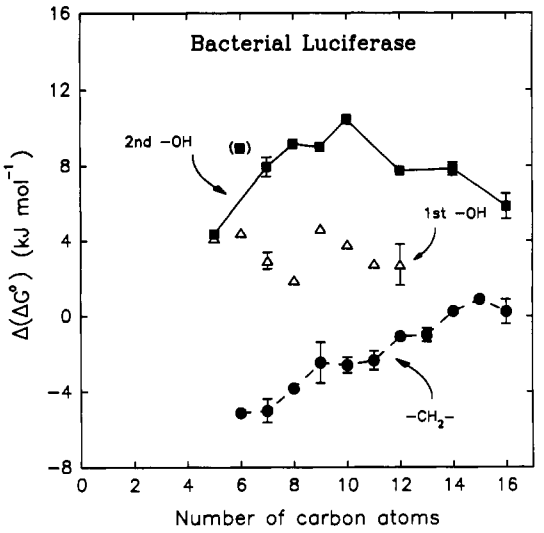

FIGURE 4: Polarity profile of the anesthetic-binding pocket on bacterial luciferase. Values (±SE) of $\Delta(\Delta G^0)$ were calculated using eqs 1–3 as described under Experimental Procedures. (■) $\Delta(\Delta G^0_{\text{2nd-OH}})$, for substituting a hydroxyl group for a hydrogen atom on an *n*-alcohol terminal methyl group, to obtain the corresponding *n*-alkane-(α,ω)-diol. (▲) $\Delta(\Delta G^0_{\text{1st-OH}})$, for substituting a hydroxyl group for a hydrogen atom on an *n*-alkane terminal methyl group, to obtain the corresponding *n*-alcohol. (●) $\Delta(\Delta G^0_{\text{CH}_2})$, mean of values for adding a methylene group to an *n*-alkane and *n*-alcohol where both values could be calculated (C6–C12) or else value for only an *n*-alcohol (C13–C16). The abscissa is the chain length, expressed as the number of carbon atoms in the final anesthetic molecule. Error bars (±SE) are shown where larger than the symbols.

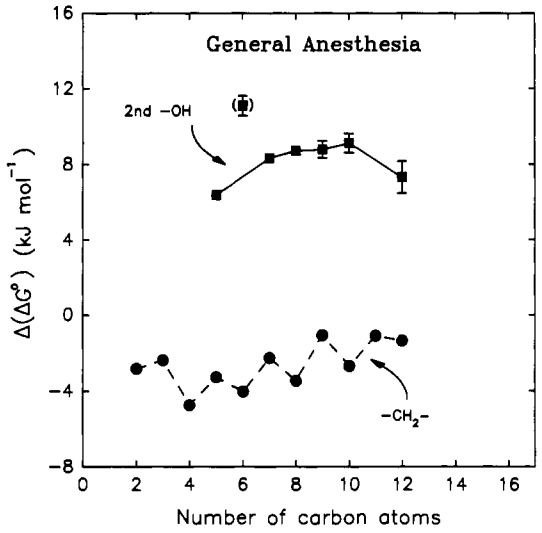

FIGURE 5: Polarity profile of the binding sites for straight-chain general anesthetics on the unknown primary targets underlying general anesthesia. Values (±SE) of the apparent $\Delta(\Delta G^0)$ were calculated using eqs 4–5 as described under Experimental Procedures. (■) $\Delta(\Delta G^0_{\text{2nd-OH}})$, for substituting a hydroxyl group for a hydrogen atom on an *n*-alcohol terminal methyl group, to obtain the corresponding *n*-alkane-(α,ω)-diol. (●) $\Delta(\Delta G^0_{\text{CH}_2})$, for adding a methylene group to an *n*-alcohol [data of Alifimoff et al. (1989)]. The abscissa is the chain length, expressed as the number of carbon atoms in the final anesthetic molecule. Error bars (±SE) are shown where larger than the symbols.

Gibbs free energies: $\Delta(\Delta G^0_{\text{CH}_2})$, $\Delta(\Delta G^0_{\text{1st-OH}})$, and $\Delta(\Delta G^0_{\text{2nd-OH}})$. These are respectively the changes in binding free energies when progressing from a given *n*-alkane or *n*-alcohol to one larger in size by one methylene group (eq 1), from an *n*-alkane to an *n*-alcohol with the same number of carbon atoms (eq 2), and from an *n*-alcohol to an *n*-alkane-(α,ω)-diol with the same number of carbon atoms (eq 3). [For estimates of $\Delta(\Delta G^0_{\text{CH}_2})$, we have pooled where possible the *n*-alkane and *n*-alcohol data and plotted the weighted means (±SE) of the values calculated from eqs 1a and 1b. Estimates obtained from considering *n*-alkane or *n*-alcohol data separately did not give significantly different patterns, so pooled estimates were considered to be more reliable (even though this procedure resulted in larger standard errors).] A negative value for $\Delta(\Delta G^0)$ implies that the converted (final) ligand binds tighter to the enzyme than the original ligand. The resulting values are plotted in Figure 3 as a function of the number of carbon atoms in the final ligand molecule.

It can be seen from this figure that the free energy profiles of $\Delta(\Delta G^0_{\text{CH}_2})$ and of $\Delta(\Delta G^0_{\text{2nd-OH}})$ are essentially mirror images of each other. Thus, it appears that $\Delta(\Delta G^0_{\text{CH}_2})$ and $\Delta(\Delta G^0_{\text{2nd-OH}})$ are mapping out the same polar features on the anesthetic-binding pocket: a polar "patch" centered at C7 and a more extended polar region from about C12 onward. In view of the structure of firefly luciferin, the C7 polar "patch" is most likely at one end of the pocket and the extended polar region is at the other end.

Since both $\Delta(\Delta G^0_{\text{CH}_2})$ and $\Delta(\Delta G^0_{\text{2nd-OH}})$ tend toward zero at long chain lengths, the extended polar region probably includes the bulk aqueous phase; this is supported by previous studies of the cutoff phenomena with the series of *n*-alkanes and *n*-alcohols inhibiting this enzyme (Franks & Lieb, 1985). The relative constancy of $\Delta(\Delta G^0_{\text{1st-OH}})$, on the other hand, suggests that the position of the hydroxyl group is relatively fixed in the series of *n*-alcohols and "anchored" at one or other of the polar regions; the positive values of $\Delta(\Delta G^0_{\text{1st-OH}})$ show that this hydroxyl group is not in as favorable a polar evironment as it would be in water. [The fluctuations in $\Delta(\Delta G^0_{\text{1st-OH}})$ may be partly due to the need to satisfy the hydrophobic interactions of the hydrocarbon chains on the *n*-alcohols, which would tend to "pull" the *n*-alcohol hydroxyl group into an unfavorable apolar environment.]

It may be useful to have a picture in mind to rationalize the above ideas. A very simple (though certainly not unique) model of the anesthetic-binding pocket on firefly luciferase, which is consistent with the above and previous (Franks & Lieb, 1984, 1985) data, as well as with the structure of firefly luciferin, might be pictured as follows. The pocket extends from external aqueous solution into a wide cavity with hydrophobic walls and a polar patch at its "bottom". The depth of the pocket is sufficient to accommodate about seven methylene groups, and its width is sufficient to hold two methylene chains side by side. When a short *n*-alkane-(α,ω)-diol molecule (such as C4) binds, one of its hydroxyl terminals is anchored to one or other of the polar regions and its methylene chain runs along the apolar wall, forcing the second hydroxyl terminal into an unfavorable apolar environment. When the chain length approaches C7, however, both terminal hydroxyls can be favorably accommodated at one or other of the two polar regions, resulting in the minimum in the curve of $\Delta(\Delta G^0_{\text{2nd-OH}})$ seen in Figure 3. As the chain gets still longer, however, it must bend back, thus forcing the second hydroxyl once again along the apolar wall of the pocket, so that at about C9 the incremental free energy becomes unfavorable again. Finally, as the chain length becomes very large (C12 and longer), both terminal hydroxyl groups can position themselves in the extended polar region (facing the aqueous solution) at the top of the pocket, and the incremental free energy of the second hydroxyl group tends toward zero.

The results of Figure 3 show that the inhibition constants K_i for the series of *n*-alkane-(α,ω)-diols, combined with those for the corresponding *n*-alcohols, allows one to calculate values for $\Delta(\Delta G^0_{\text{2nd-OH}})$ which map out the polarity profile of a hy-

drophobic binding pocket in a self-consistent manner. We shall use this method below to map out the polarity profiles of anesthetic-binding pockets in a bacterial luciferase enzyme and in the primary target sites underlying general anesthesia.

The Bacterial Luciferase Anesthetic-Binding Pocket. We have shown previously (Curry et al., 1990) that a wide variety of anesthetics inhibit the bacterial luciferase enzyme from *V. harveyi* by competing for the binding of a long-chain *n*-aldehyde substrate, and we have now found that the *n*-alkane-(α,ω)-diols inhibit in the same manner. The simplest assumption is that anesthetics and diols bind to the same site that normally binds the long-chain *n*-aldehyde substrate (*n*-decanal in our experiments). Consistent with this interpretation is the fact that long-chain *n*-alcohols bind well but bulky anesthetics bind poorly to bacterial luciferase (Curry et al., 1990, 1991), as might be expected if the anesthetic-binding site is a long and narrow pocket, evolved to accommodate the long and narrow *n*-aldehyde substrate.

Since the long-chain *n*-aldehyde substrate is a predominantly apolar molecule, possessing only a single polar group, one would not expect a priori the binding pocket to have the two substantial polar regions found for firefly luciferase. This expectation is borne out by the $\Delta(\Delta G^0_{\text{2nd-OH}})$ values plotted in Figure 4. In contrast to the values for the anesthetic-binding pocket on firefly luciferase (Figure 3), the $\Delta(\Delta G^0_{\text{2nd-OH}})$ values for bacterial luciferase are always positive and relatively large (between 4 and 11 kJ mol^{-1}). This, in addition to the fact that the value of $\Delta(\Delta G^0_{\text{2nd-OH}})$ is usually much greater than that of $\Delta(\Delta G^0_{\text{1st-OH}})$, suggests that the second terminal hydroxyl group on the *n*-alkane-(α,ω)-diols is forced to bind in a relatively apolar region of the bacterial luciferase pocket. In other words, in contrast to the situation for firefly luciferase, there do not appear to be two substantial polar regions in the anesthetic-binding pocket on bacterial luciferase. However, on the basis of the leveling off of the *n*-alkane-(α,ω)-diol (Table I) and *n*-alcohol (Curry et al., 1990) K_i values at long chain lengths, there does appear to be an extended polar region at the "top" of the pocket which extends out into the aqueous solution. This can also be seen from the $\Delta(\Delta G^0_{\text{CH}_2})$ values plotted in Figure 4, which approach zero as the carbon chain becomes very long.

In analogy to the model proposed above for the anesthetic-binding pocket of firefly luciferase, it is instructive to construct a comparable model for bacterial luciferase that again is not unique but is consistent with the experimental data and the structure of the long-chain *n*-aldehyde substrate. The model anesthetic-binding site for bacterial luciferase is a long, narrow, and predominantly hydrophobic pocket or cleft with a polar mouth, extending from the external aqueous solution to a depth sufficient to accommodate about 10–12 methylene groups. In contrast to the firefly pocket, the bacterial pocket is so narrow that it can accommodate only one methylene chain width, and chain bending is not possible. When an *n*-alkane-(α,ω)-diol molecule binds, one of its hydroxyl terminals is anchored to the polar mouth but the second hydroxyl terminal is forced into the unfavorable apolar environment of the apolar pocket wall. When the chain length becomes longer than about C10 and the pocket is filled up, because of the restriction on chain bending both terminal hydroxyl groups cannot (as they can for the firefly enzyme) extend out of the pocket into the aqueous solution, and hence $\Delta(\Delta G^0_{\text{2nd-OH}})$, although decreasing somewhat, does not approach zero but remains high, between about 6 and 8 kJ mol^{-1}.

The Primary Target Sites Underlying General Anesthesia. In contrast to the purified firefly and bacterial luciferase proteins discussed above, the primary target sites in the central nervous system involved in the induction of general anesthesia are presently unknown and are likely to be heterogeneous. Nonetheless, it is possible to get some idea of the polarity profiles of the target sites which are most sensitive to the type of straight-chain anesthetic agents used in mapping the luciferase pockets by employing the EC_{50} concentrations of the *n*-alkane-(α,ω)-diols (Table I) and the *n*-alcohols (Alifimoff et al., 1989) determined for tadpoles of *X. laevis* and *Rana pipiens*, respectively. (There are, unfortunately, no reliable data for tadpole general anesthesia produced by the *n*-alkanes.) Assuming that differences between these two species of tadpole are unimportant, as is suggested by the results of Curry et al. (1991) for *n*-hexanol, it is possible to calculate apparent binding free energies and to get some idea of the properties of these target sites.

We have calculated the *apparent* incremental standard Gibbs free energies $\Delta(\Delta G^0_{\text{CH}_2})$ and $\Delta(\Delta G^0_{\text{2nd-OH}})$ using eqs 4–5 and plotted the results in Figure 5. Comparing the free energy plots of Figure 5 with those of Figures 3 and 4, it is apparent at once that the results for general anesthesia are broadly similar to those for bacterial luciferase but quite distinct from those for firefly luciferase. Thus, it appears that the polarity profile of the target sites underlying general anesthesia has more in common with the bacterial luciferase than with the firefly luciferase profile. In particular, there is little indication in the $\Delta(\Delta G^0_{\text{2nd-OH}})$ general anesthesia data (Figure 5) of the existence of the polar patch at about C7 which is a strong feature of the firefly luciferase data (Figure 3) and, as shown by Franks and Lieb (1985), also a feature of earlier tadpole *n*-alcohol data (Vernon, 1913; Meyer & Hemmi, 1935; Brink & Posternak, 1948). There is also no indication of such a polar patch in the $\Delta(\Delta G^0_{\text{CH}_2})$ general anesthesia data (Figure 5), on the basis of (see eq 4) the recent and probably more reliable tadpole *n*-alcohol measurements of Alifimoff et al. (1989) [see also Elliott and McElwee (1988)]. On the other hand, there is evidence to support the existence of at least one polar region in the target sites underlying general anesthesia (see the introduction). It seems likely, on the basis of the approach of $\Delta(\Delta G^0_{\text{CH}_2})$ toward zero at long chain lengths (Figure 5), that there is a polar region that binds methylene groups only as the sites become full. The simplest interpretation is that this polar region faces onto the external aqueous solution.

Where $\Delta(\Delta G^0_{\text{CH}_2})$ values can be compared (from C6 to C12 inclusive), those for general anesthesia (Figure 5) are on average about 1 kJ mol^{-1} more positive than those for bacterial luciferase (Figure 4). In addition, it is clear from the data in Table I that the diol EC_{50} concentrations for general anesthesia are comparable to those for bacterial luciferase at short chain lengths but considerably larger (by a factor of 3–8) at higher chain lengths. These observations are consistent with the idea that the target sites underlying general anesthesia, while similar to those on bacterial luciferase, are somewhat less hydrophobic.

A simple model for the straight-chain anesthetic-binding sites on the primary protein targets underlying general anesthesia can be proposed that is similar to that for the anesthetic-binding pocket on bacterial luciferase. It would be a long, narrow hydrophobic pocket extending inward from the external surface of the protein target, where it faces water. That part of the pocket facing water would have polar characteristics, while the remainder of the pocket, extending into the protein, would be predominantly hydrophobic (though less hydrophobic than the corresponding region of the bacterial luciferase pocket). When an *n*-alkane-(α,ω)-diol molecule

binds, one of its hydroxyl terminals is anchored to the polar mouth but, since chain bending is not possible (due to the narrowness of the pocket), the second hydroxyl terminal is forced into the unfavorable environment of the apolar pocket wall.

Previous studies (Curry et al., 1990, 1991) have shown that bulky anesthetics (such as cycloalcohols and the widely used inhalational agents halothane and isoflurane) can be good general anesthetics and inhibitors of firefly luciferase, but they are relatively poor inhibitors of bacterial luciferase. Such bulky agents must bind to general anesthesia target sites having different geometries from those revealed here using straight-chain anesthetics. Thus, our results strongly suggest that different classes of the diverse range of known anesthetic agents are able to produce general anesthesia by binding to distinctly different target sites in the central nervous system.

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