Continuum Solvent Model Studies of the Interactions of an Anticonvulsant Drug with a Lipid Bilayer

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ABSTRACT Valproic acid (VPA) is a short, branched fatty acid with broad-spectrum anticonvulsant activity. It has been suggested that VPA acts directly on the plasma membrane. We calculated the free energy of interaction of VPA with a model lipid bilayer using simulated annealing and the continuum solvent model. Our calculations indicate that VPA is likely to partition into the bilayer both in its neutral and charged forms, as expected from such an amphipathic molecule. The calculations also show that VPA may migrate (flip-flop) across the membrane; according to our (theoretical) study, the most likely flip-flop path at neutral pH involves protonation of VPA pending its insertion into the lipid bilayer and deprotonation upon departure from the other side of the bilayer. Recently, the flip-flop of long fatty acids across lipid bilayers was studied using fluorescence and NMR spectroscopies. However, the measured value of the flip-flop rate appears to depend on the method used in these studies. Our calculated value of the flip-flop rate constant, 20/s, agrees with some of these studies. The limitations of the model and the implications of the study for VPA and other fatty acids are discussed.

INTRODUCTION

Valproic acid (VPA) is a short-chain, branched fatty acid with broad-spectrum anticonvulsant activity and has been used for the last 20 years for the treatment of generalized epilepsy (Fariello et al., 1995). Recently, VPA has also been used for the treatment of other diseases, such as bipolar disorders and migraines (Loscher, 1999).

The exact mechanism of VPA's anticonvulsant action is still unknown. Because of its anticonvulsant activity against a broad spectrum of seizure types, it has repeatedly been suggested that VPA acts through a combination of several mechanisms (Loscher, 1999). Several studies suggest that the anti-seizure action of VPA results from its effect on gamma-aminobutyric acid (GABA)-mediated neurotransmission in the brain. VPA acts against seizures induced by GABA antagonists (Frey and Loscher, 1976; Worms and Lloyed, 1981) and by inhibitors of GABA synthesis (Dren et al., 1979), suggesting that its mechanism of action may involve the increase of brain GABA levels. This suggestion is supported by studies that demonstrate the ability of VPA to increase GABA levels in vivo (Godin et al., 1969), to inhibit GABA-degrading enzymes in vitro (Godin et al., 1969; Harvey et al., 1975), and to increase the activity of a GABA-producing enzyme (Loscher, 1980, 1981a,b; Phillips and Fowler, 1982; Teberner et al., 1980). Other studies suggest that the anticonvulsant action of VPA is a result of its effect on sodium (McLean and Macdonald, 1986) and potassium (Franceschetti et al., 1986) channels, but the data are inconsistent (Loscher, 1999).

VPA has no known specific binding site, which suggests that it may act directly on the plasma membrane, possibly as a membrane-perturbing agent. This suggestion is supported by several studies. Keane et al. (1983) have demonstrated that the anticonvulsant potency of VPA analogs increases with their chain length and water/octanol partitioning, suggesting that the activity of VPA depends on its lipophilicity. Goldstein and co-workers have found that VPA and analogous compounds are membrane-disordering agents (Lyon and Goldstein, 1980) and that their membrane-disordering potency in vitro correlates with their anticonvulsant activity (Perlman and Goldstein, 1984).

The transverse diffusion (flip-flop) of fatty acids across lipid bilayers has recently been studied by several research groups. The characterization of this process is important for the understanding of the general behavior of membrane lipids. It may also be important in understanding relevant physiological processes. In the case of VPA, for example, the flip-flop of the drug across the plasma membrane of neurons has been implicated in the late anticonvulsant effect of the drug (Loscher, 1999). Despite extensive research, the flip-flop process is far from being clearly understood. For example, different flip-flop rates have been measured by studies that employed different methods and/or protocols.

In this work, we used simulated annealing and continuum solvent models to study the energetics of VPA-membrane interactions, with a focus on the flip-flop of VPA across lipid bilayers. We calculated the free energy of VPA association with the lipid bilayer in different configurations and used these free energy values to determine the most likely path for the flip-flop of VPA across the bilayer.

MATERIALS AND METHODS

The total free energy difference between VPA in the membrane and in the aqueous phase (ΔG_{tot}) can be decomposed into a sum of differences of the

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following terms: the electrostatic (ΔG_{elc}) and nonpolar (ΔG_{np}) contributions to the solvation free energy, lipid perturbation effects (ΔG_{lip}), molecule immobilization effects (ΔG_{imm}), and effects due to changes in the pK_a of titratable groups (ΔG_{pKa}) (Ben-Tal et al., 1996a; Engelman and Steitz, 1981; Fattal and Ben-Shaul, 1993; Honig and Hubbell, 1984; Jacobs and White, 1989; Jahnig, 1983; Milik and Skolnick, 1993; reviewed by White and Wimley, 1999; Kessel and Ben-Tal, 2001):

$$\Delta G_{tot} = \Delta G_{elc} + \Delta G_{np} + \Delta G_{lip} + \Delta G_{imm} + \Delta G_{pKa} \quad (1)$$

The studies mentioned above (Lyon and Goldstein, 1980; Perlman and Goldstein, 1984) indicate that the main contribution to the transfer free energy comes from the solvation free energy, ΔG_{sol}, defined as:

$$\Delta G_{sol} = \Delta G_{elc} + \Delta G_{np} \quad (2)$$

ΔG_{sol} is the free energy of transfer of VPA from water to a bulk hydrocarbon phase. It accounts for electrostatic contributions resulting from changes in the solvent dielectric constant as well as for van der Waals and solvent structure effects, which are grouped in the nonpolar term and together define the classic hydrophobic effect. We calculated ΔG_{sol} using the continuum solvent model. This method has been described in detail in our earlier studies of the membrane association of polyalanine α-helices (Ben-Tal et al., 1996a), alamethicin (Kessel et al., 2000a,b), and monensin-cation complexes (Ben-Tal et al., 2000a). Here we present a brief outline, with emphasis on the minor changes we made to adapt the method to VPA.

Electrostatic contributions

Calculations were based on a continuum solvent model in which electrostatic contributions are obtained from finite difference solutions to the Poisson-Boltzmann equation (the FDPB method) (Honig and Nicholls, 1995; Honig et al., 1993). Three-dimensional model structures of VPA were generated using the Builder and Discover modules of Insight II (MSI, San Diego, CA), as described below. VPA was represented in atomic detail, with atomic radii and partial charges defined at the coordinates of each nucleus. The charges and radii were taken from PARSE, a parameter set that was derived to reproduce gas phase-to-water (Sitkoff et al., 1994) and liquid alkane-to-water (Sitkoff et al., 1996) solvation free energies of small organic molecules.

In the FDPB calculations reported here, the boundary between VPA and the solvents (water or membrane) was set at the contact surface between the van der Waals surface of the complex and a solvent probe (defined here as having a 1.4 Å radius; see Sharp et al., 1991). VPA and the lipid bilayer were assigned a dielectric constant of 2, and water a dielectric constant of 80. The system was mapped onto a lattice of 65^3 grid points, with a resolution of 3 points per Å, and the Poisson equation was numerically solved for the electrostatic potential. The electrostatic free energy was calculated by integration over the potential multiplied by the charge distribution in space.

Nonpolar contributions

The nonpolar contributions to the solvation free energy, G_{np}, were assumed to be proportional to the water-accessible surface area (A) of VPA through the expression:

$$G_{np} = \gamma A + b \quad (3)$$

We used the parameters, $\gamma = 0.0278$ kcal/(mol Å^2) and $b = -1.71$ kcal/mol, that had previously been derived from the partitioning of alkanes between liquid alkane and water (Sitkoff et al., 1996), and had successfully been used in our previous studies (Ben-Tal et al., 1996a,b, 1997, 2000a; Kessel et al., 2000a,b). The total area of VPA that is accessible to lipids in a particular configuration was calculated with a modified Shrake-Rupley (Shrake and Rupley, 1973) algorithm (Sridharan et al., 1992).

Estimates of ΔG_{lip}

ΔG_{lip} is the free energy penalty resulting from the interference of the solute with the conformational freedom of the lipid bilayer chains. $\Delta G_{lip} = 2.3$ kcal/mol has been previously calculated for the insertion of polyalanine α-helices into the lipid bilayer (Ben-Shaul et al., 1996; Ben-Tal et al., 1996a). However, VPA is much smaller and significantly less rigid than a peptide, and its effects on the conformational freedom of the lipid bilayer chains are likely to be negligible. Thus, we used $\Delta G_{lip} = 0$ kcal/mol in our calculations.

Estimates of ΔG_{imm}

ΔG_{imm} is the free energy penalty resulting from the confinement of the external translational and rotational motion of VPA inside the membrane. We have estimated an upper bound value of 3.7 kcal/mol for the insertion of a polyalanine α-helix into lipid bilayers (Ben-Shaul et al., 1996; Ben-Tal et al., 1996a) and a value of 1.3 kcal/mol for the adsorption of the basic peptide pentalysine on membranes containing acidic lipids (Ben-Tal et al., 2000b). We used the latter estimate here because our calculations show that VPA is likely to be at the water-bilayer interface, its association thus resembling an adsorption rather than an insertion process.

Estimates of ΔG_{pKa}

The transmembrane insertion of the negatively charged form of VPA, valproate, may involve the unfavorable exposure of the charged COO^- group to the hydrophobic region of the lipid bilayer. The high free energy penalty involved in the process may be lowered if the carboxyl group is neutralized by protonation (e.g., Hamilton, 1998; Honig and Hubbell, 1984). This involves a free energy penalty, ΔG_{pKa}, given by:

$$\Delta G_{pKa} = -2.3 k_B T(pK_a - pH) \quad (4)$$

where k_B is the Boltzmann constant, T is the absolute temperature and K_a is the ionization equilibrium constant of valproate. pK_a and pH were assigned the values of 4.6 (Loscher, 1999) and 7, respectively. Substituting these values into Eq. 4, $\Delta G_{pKa} = 3.3$ kcal/mol.

Calculation of the flip-flop rate of VPA across bilayers

The translocation of VPA across the membrane involves a free energy barrier that results from the insertion of the polar carboxyl group into the hydrophobic core of the lipid bilayer. The translocation rate is given by Schulten et al. (1981) and Wilson and Pohorille (1996)

$$k = \{((F_1F_2)^{0.5}D)/(2\pi k_B T)\}e^{-\Delta\Delta G/k_B T}, \quad (5)$$

where D is the diffusion coefficient of VPA in a uniform medium. For lack of experimental data on D for VPA, we relied on measurements (Pfeiffer et al., 1989) and calculations (Essmann and Berkowitz, 1999) of the lateral motion of phosphatidylcholine in dipalmitoylphosphatidylcholine (DPPC) bilayers. Based on these studies, $D \approx 10^9$ Å^2/s. F_1 and F_2 in Eq. 5 are the force constants, i.e., the second derivatives of the free energy of the system with respect to the distance between VPA and the bilayer, in the orientations separated by the free energy barrier.

$\Delta\Delta G$ is the free energy difference between the VPA-membrane system above (ΔG_2) and below (ΔG_1) the barrier, calculated using Eq. 1:

$$\Delta\Delta G = \Delta G_2 - \Delta G_1, \quad (6)$$

where ΔG_1 and ΔG_2 are the free energies of transfer of VPA from the aqueous phase to configurations 1 and 2 (below and above the barrier, respectively)in the lipid bilayer.

Models of VPA

Initial structures of the uncharged (Valproic acid) and charged (Valproate) forms of VPA (Fig. 1) and valproate were generated by Insight/Builder (MSI). These initial structures were used in simulated annealing molecular dynamics simulations to generate different conformations, some of which were used in the continuum solvent model calculations.

Molecular dynamics simulations

VPA is amphipathic and is, therefore, likely to be at the water-bilayer interface. Because the value of the dielectric constant in this region is not known, we used two extreme values in the simulated annealing procedure: 1 and 80. The charges of the VPA atoms were taken from the CVFF forcefield. The calculations (Insight II/Discover3; MSI) were based on the standard protocol given by Rapaport (1995) (http://molvis.chem.indiana.edu/app guide/InsightII/sim ann/sim ann inp.html) and include the following.

Initiation

VPA structure was minimized using the steepest-descent, conjugate-gradient, and Newton algorithms to a final convergence of 0.001 kcal/(mol Å). The molecule was then heated to 1000 K during a 2000-fs molecular dynamics simulation using the RATTLE procedure and 3-fs time step.

Simulated annealing

The simulated annealing procedure included two repetitive steps: 1) molecular dynamics simulation, in which the system was heated to 1000° K over a period of 2000 fs using a 1 fs time step, and 2) 1000 fs molecular dynamics simulations using a time step of 1 fs, during which the system was cooled down to 300° K. The resulting conformation was saved. The simulated annealing procedure was repeated 100 times, and 100 conformations of VPA were obtained for each form of VPA, i.e., 100 conformations for VPA and 100 conformations for valproate.

Final minimization

Each of the 100 conformers that were generated in the simulated annealing was minimized using the steepest-descent, conjugate-gradient, and Newton algorithms to a final convergence of 0.001 kcal/(mol Å).

Analysis and grouping of conformers

The VPA conformers were grouped according to the distances and dihedral angles between the carbon atoms of VPA. Two structural descriptors were found to be the most informative in defining the conformational difference between the conformers: the distance (d) between carbons C5 and C8 and the dihedral angle (α) between carbon atoms C5-C4-C7-C8 (Fig. 1). This process yielded 30 different groups of conformations for each form of VPA (i.e., charged and uncharged).

Selection of conformers

Of each of the 30 conformation groups generated in the previous step, 12 structures were selected, according to their internal energy. Of these structures, six final conformations for VPA and six conformations for valproate were selected as follows: two of the lowest internal energy, two of medium, and two of the highest internal energy.

Choosing the initial VPA-membrane configuration

VPA is amphipathic (Fig. 1). Therefore, it is likely to associate with lipid bilayers in surface orientations, with its nonpolar tail buried inside the

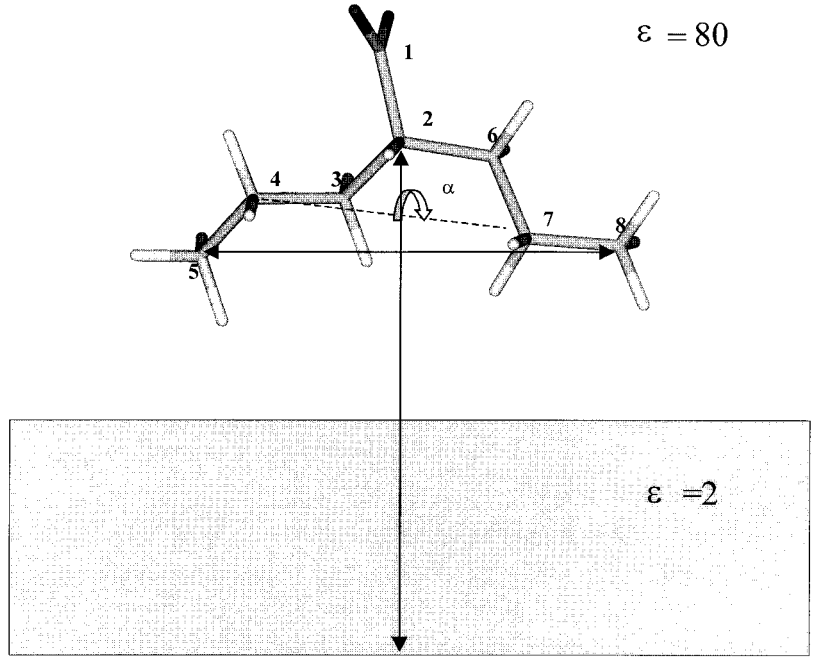

FIGURE 1 The initial structure of VPA and a hypothetical process of VPA translocation across a lipid bilayer. The sticks model of VPA is displayed with INSIGHT (MSI). Carbon atoms are gray, hydrogen atoms are white, and oxygen atoms are black. The carbon atoms are numbered, and the distance d and angle α used to classify conformers are indicated. The hydrophobic core of the membrane is depicted by shaded gray; only one leaflet is shown. The distance h is measured between the geometrical center of VPA and the mid-plane of the lipid bilayer.

hydrocarbon region of the bilayer and its polar carboxyl group protruding into the aqueous solution. Thus, for the continuum-solvent model calculations, VPA was oriented with the axis that separates the two oxygen atoms of the carboxyl group and the aliphatic tails of the molecule parallel to the normal of the lipid bilayer (Fig. 1). In the calculations, numerous orientations of VPA were sampled around the initial VPA-membrane configuration. The orientations were sampled by translating the molecule along the *h* axis (the normal of the lipid bilayer) and rotating it around the *x* and *y* axes (which lie parallel to the bilayer plane). We sampled ~20 configurations involving *h* translations and ~20 configurations involving rotations around each of the *x* and *y* axes. Overall, ~60 configurations were sampled.

TABLE 1 Calculated free energy values for the association of valproic acid/valproate with the lipid bilayer in the most favorable conformation and orientation

Molecule*	ΔG_{solv}[†] (kcal/mol)	ΔG_{imm}[‡] (kcal/mol)	ΔG_{lip}[§] (kcal/mol)	ΔG_{tot}[¶] (kcal/mol)
VPA	−6.4	1.3	0.0	−5.1
Valproate	−5.3	1.3	0.0	−4.0

*The protonation state of VPA.
[†]The solvation free energy (Eq. 2).
[‡]The molecule immobilization free energy.
[§]The lipid perturbation free energy.
[¶]The total free energy (Eq. 1).

RESULTS

The most probable orientation of VPA in the membrane

VPA and valproate were each placed in different configurations with respect to the lipid bilayer, and the free energy of each molecule-membrane configuration was calculated as described in Materials and Methods. We sampled ~60 VPA-membrane configurations around this orientation to find the one with the most negative free energy, which represents the most probable membrane-associated orientation (Fig. 2). In this orientation, the molecule was inserted into the hydrocarbon region of the bilayer, with its hydrophobic region dissolved in the bilayer and its polar COOH/COO^- group at the bilayer-water interface.

The free energies of transfer of valproate and VPA from the aqueous phase into these membrane-associated orientations were −5.3 kcal/mol and −6.4 kcal/mol, respectively (Table 1). The orientations of the ionic and non-ionic forms of VPA in the membrane that were associated with these free energy values were nearly identical, and the reason for the ~1.1 kcal/mol difference in ΔG_{sol} is probably the differences in image-charge repulsion of a full charge versus a set of dipoles.

The total free energies of transfer of valproate and VPA from the aqueous phase into these membrane-associated orientations were −4.0 kcal/mol and −5.1 kcal/mol (Table 1). This indicates that both forms of the molecule are likely to partition into membranes, in accordance with measurements of other fatty acids (Anel et al., 1993; Doody et al., 1980; Hamilton, 1998; Hamilton and Kamp, 1999; Kamp et al., 1995; Kleinfeld et al., 1997; Miyazaki et al., 1992; Peitzsch and McLaughlin, 1993; Ptak et al., 1980; Rooney et al., 1983; Zhang et al., 1996).

The free energy of membrane association of different VPA conformers

VPA may assume different conformations in solution and in the lipid bilayer. To determine the effect of the conformational freedom of the molecule on its free energy of association with the membrane, we repeated the calculations of Table 1 for different conformations, generated by simulated annealing. To provide the selected conformations with an initial orientation inside the bilayer, each conformation was superimposed on the conformation previously found with the most negative transfer free energy. For each conformation, different VPA-membrane configurations were sampled around the initial orientation to find the one of most negative transfer free energy, representing the most probable membrane-associated orientation. The solvation, immobilization, lipid perturbation, and total free energies of transfer of the different VPA and valproate conformations from the aqueous solution into this membrane-associated orientation are listed in Table 2. The results demonstrate only slight differences in $\Delta G_{sol,}$ and hence in ΔG_{tot}, between these conformations, indicating that the conformational freedom of VPA should not, in essence, be affected by the transfer from a polar to a nonpolar phase.

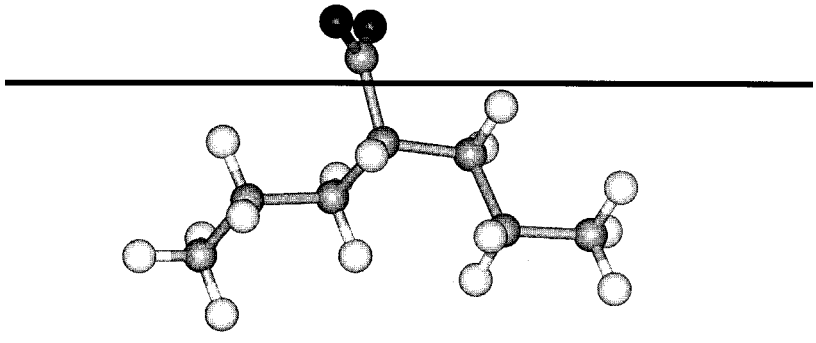

FIGURE 2 Schematic representation of the most probable orientation of VPA in the lipid bilayer. The balls-and-sticks model of VPA is displayed with INSIGHT (MSI). Carbon atoms are gray, hydrogen atoms are white, and oxygen atoms are black. The white line represents the boundary between the aqueous phase at the top and the lipid bilayer at the bottom.

TABLE 2 Calculated free energy values for the association of different VPA and valproate conformations with the lipid bilayer

Molecule*	Conformation†	ΔG_{solv}‡ (kcal/mol)	ΔG_{imm}§ (kcal/mol)	ΔG_{lip}¶ (kcal/mol)	ΔG_{tot}‖ (kcal/mol)
VPA	1	−6.4	1.3	0.0	−5.1
	2	−6.4	1.3	0.0	−5.1
	3	−6.2	1.3	0.0	−4.9
	4	−6.2	1.3	0.0	−4.9
	5	−5.9	1.3	0.0	−4.6
	6	−5.5	1.3	0.0	−4.2
Valproate	1	−5.3	1.3	0.0	−4.0
	2	−5.3	1.3	0.0	−4.0
	3	−5.3	1.3	0.0	−4.0
	4	−5.2	1.3	0.0	−3.9
	5	−5.2	1.3	0.0	−3.9
	6	−4.9	1.3	0.0	−3.6

*The protonation state of VPA.
†An index marking the conformation of VPA. Conformations 1, 3, and 5 of VPA and 1, 2, and 3 of valproate were produced by calculations in a medium of dielectric constant of 1, whereas conformations 2, 4, and 6 of VPA and 4, 5, and 6 of valproate were produced by calculations in a medium of dielectric constant of 80. The conformations in each group are sorted according to their free energies.
‡The solvation free energy (Eq. 2).
§The molecule immobilization free energy.
¶The lipid perturbation free energy.
‖The total free energy (Eq. 1).

The free energy of translocation of VPA and valproate across the lipid bilayer

In some seizure models, VPA has both immediate and late anticonvulsant effects. The late effect of the drug may be explained by free diffusion of VPA across the plasma membrane of the neuron (Loscher, 1999). Thus, we calculated the free energy of translocation of VPA across the lipid bilayer (flip-flop motion) to better understand the kinetics of this process. We considered three different translocation processes. In the first, VPA would translocate as VPA. In the second, it would translocate as valproate (i.e., charged). In the third, which we consider the most probable path, VPA would exist as valproate in the aqueous solution and become protonated pending its insertion into the bilayer. These flip-flop paths are described in detail in the following subsections. For the calculations, we used the most probable conformation and orientation of VPA from previous calculations (Fig. 2; Table 1).

Translocation of VPA across the lipid bilayer

At low pH, the carboxylic group of VPA is protonated, i.e., neutrally charged. Fig. 3 *A* presents the electrostatic, nonpolar, and solvation free energy terms for the translocation of VPA across a lipid bilayer as a function of the distance h between the geometrical center of the molecule and the membrane mid-plane. Our putative translocation started at $h = 18$ Å, where the hydrophobic tail of the molecule is just in contact with the bilayer, and ended at $h = -18$ Å, where the molecule is at the other end of the bilayer with its hydrophilic COOH group just in contact with the bilayer. This process would involve a large free energy penalty, which corresponds to a configuration of VPA where the COOH group of the molecule is inserted inside the bilayer and the hydrophobic tail protrudes into the aqueous phase. Alternatively, the free energy penalty could be avoided by changing the direction of VPA, when it is fully immersed inside the bilayer, so that the molecule is able to leave the membrane using the same path of insertion. The free energy curve obtained for this flip-flop path is depicted in Fig. 3 *A* by the solid line. This path was characterized by a single energy barrier of $\Delta\Delta G \approx 6$ kcal/mol, resulting from the insertion of the COOH group into the hydrophobic core of the lipid bilayer. The curvatures (or force constants) F_1 and F_2 of the orientations separated by the free energy barrier were 1.4 kcal/(mol Å^2) and 0.7 kcal/(mol Å^2), respectively. Substituting these values in Eq. 5 gives a flip-flop rate of ~2000/s; that is, on average, VPA crosses pure lipid membranes every ~0.5 ms.

Translocation of valproate across the lipid bilayer

At physiological pH, VPA is deprotonated and has a net charge of −1. Fig. 3 *B* presents the electrostatic, nonpolar, and solvation free energy curves for the translocation of valproate across a lipid bilayer, as a function of the distance h between the geometrical center of the molecule and the membrane mid-plane. Again, the translocation of valproate started at $h = 18$ Å, where the hydrophobic tail of the molecule is just in contact with the bilayer and ended at $h = -18$ Å, where the molecule is at the other end of the bilayer with its hydrophilic COO^- group just in contact with the bilayer. Again, the very large free energy penalty could be somewhat reduced by

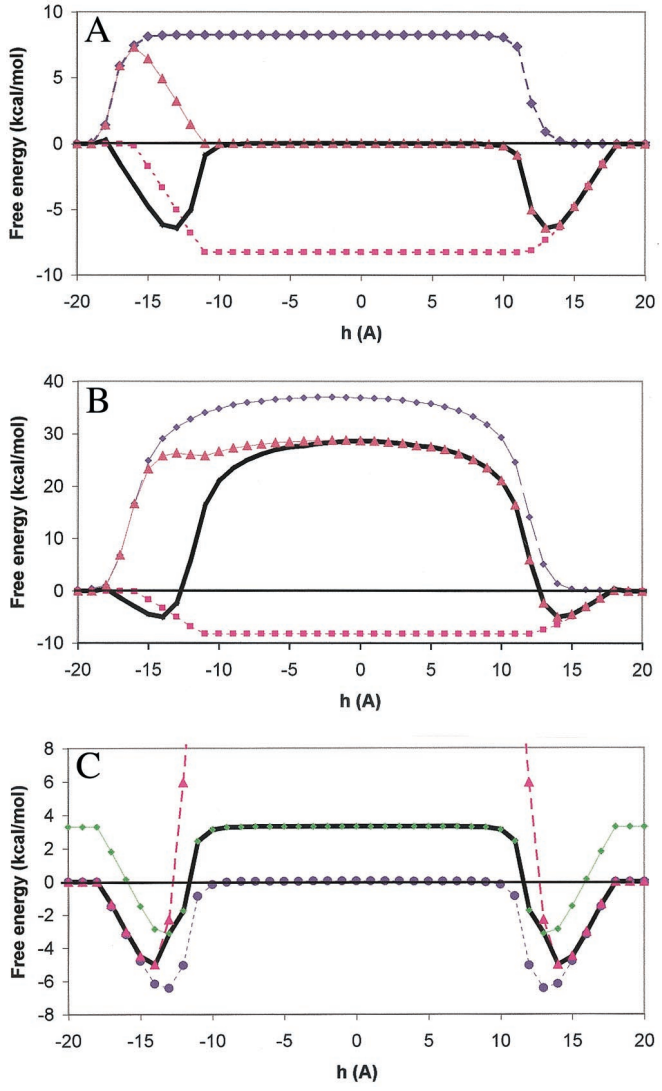

FIGURE 3 (*A* and *B*)The translocation of valproic acid (*A*) and valproate (*B*) across the lipid bilayer, in the orientation of Fig. 1. The electrostatic (♦), nonpolar (■), and solvation (▲) free energies of the VPA-membrane system are presented as a function of the distance *h* between the geometrical center of the molecule and the membrane mid-plane. The solid line in *A* and *B* presents an alternative flip-flop path, in which VPA reverses its orientation in the membrane by a 180° rotation after the complete insertion of the carboxyl group inside the bilayer. (*C*) The minimal free energy path for the flip-flop of valproate across the membrane. ▲, ΔG_{sol} for the flip-flop of valproate in its charged form, taken from *B*; ●, ΔG_{sol} for the flip-flop of valproic acid (neutral form), taken from *A*; ♦ sum of ΔG_{pKa} (the free energy of discharging valproate in the aqueous phase) and ΔG_{sol} for its flip-flop in the neutral form. The solid line denotes the minimal free energy path for the flip-flop of (negatively charged) valproate across the membrane. Note the curve crossing at $h = -13.5$ Å and $h = 13.5$ Å, where the valproate becomes protonated to reduce the free energy of translocation across the membrane. The zero of the free energy was chosen at $h = \infty$. The membrane width was 30 Å. The calculations were carried out on a lattice of 65^3 points with a resolution of 3 grid points per Å as described in Materials and Methods.

rotating the molecule inside the bilayer. This leads to the free energy curve depicted by the solid line in Fig. 3 *B*. The free energy barrier for this flip-flop process was still very large and would lead to a translocation rate of $\sim 5 \times 10^{-15}$/s.

The minimal free energy path for VPA translocation across the lipid bilayer

As mentioned above, water-soluble VPA is deprotonated at physiological pH. The insertion of the charged carboxylic group of valproate into the lipid bilayer results in a very large electrostatic penalty. This indicates that the carboxylic group should probably become protonated pending its insertion into the bilayer. The COO^- group has a p*K*a of 4.6 (Loscher, 1999), and its protonation in the aqueous solution (pH = 7) involves a free energy penalty of ~3.3 kcal/mol (Eq. 4). Fig. 3 *C* suggests the minimal free energy path for the flip-flop of valproate across the lipid bilayer. It shows the ΔG_{sol} curve obtained for the flip-flop of VPA (Fig. 3 *A*) superimposed on the ΔG_{sol} curve obtained for the flip-flop of valproate (Fig. 3 *B*). It is evident from Fig. 3 *C* that although ΔG_{sol} of these two processes is very different in the bilayer interior, it is very similar in the aqueous phase and in the membrane-water interface. This is due to the polarity of water, which is reflected in the model by the high dielectric constant of water.

Fig. 3 *C* also presents a third hypothetical process for the flip-flop of valproate across the bilayer. In this process valproate would become protonated before it is inserted into the bilayer. The free energy values of the protonated molecule are the sum of ΔG_{sol} of valproate and $\Delta G_{pKa} = 3.3$ kcal/mol. The minimal free energy path for the flip-flop of valproate across the bilayer is depicted by the solid line in Fig. 3 *C*. The protonation of the COO^- upon entering the bilayer ($h = -16.5$ Å) and the deprotonation of the COOH group upon exiting the bilayer ($h = 13.5$ Å) appear as a curve crossing. The free energy barrier for the translocation of valproate was 8.8 kcal/mol, and F_1 and F_2 were 0.5 kcal/(mol Å^2) and 0.7 kcal/(mol Å^2), respectively. These correspond to a translocation rate of ~20/s.

DISCUSSION

The main hypothesis in this study was that VPA interacts with lipid bilayers (Keane et al., 1983; Lyon and Goldstein, 1980; Perlman and Goldstein, 1984; Loscher, 1999), and so we used a continuum solvent model and a slab representation of the lipid bilayer to study these interactions. The model is well documented (reviewed by Kessel and Ben-Tal, 2001) and has been quite successful in studies of hydrophobic peptides, such as alamethicin, that interact mainly with the hydrocarbon region of the bilayer. However, its implementation for small molecules, such as VPA, which may interact with the polar headgroup region of the bilayer, needs to be considered. Thus, we begin by discussing a number of approximations used in the study.

The model structure of VPA may seem uncertain, considering the different conformations VPA can assume as reflected by our simulated annealing calculations. However, our continuum-solvent model calculations indicate that, despite the significant differences in the internal energies of these conformations, the values obtained for their free energy of transfer from the aqueous phase into the lipid bilayer are very similar, provided that the polar carbonyl group is kept outside the hydrocarbon core of the bilayer.

The description of the lipid bilayer as a slab of low dielectric constant obscures all atomic detail of VPA-bilayer interactions. However, this is the standard representation of the hydrocarbon region of lipid bilayers (e.g., Ben-Tal et al., 1996a; Bernèche et al., 1998; Biggin et at., 1997; Kessel et al., 2000a,b) and is likely to provide a reasonable model of bilayer effects on electrostatic interactions. The calculated solvation free energy values depend strongly on the value assigned to the inner dielectric constant and on the choice of the set of atomic partial charges and radii. PARSE yields accurate transfer free energies between water and liquid alkane for small organic molecules, among them acetic acid and propionic acid, which chemically resemble VPA (Sitkoff et al., 1996). Therefore, PARSE is likely to provide a very good approximation of the water-hydrocarbon region solvation properties of VPA. Moreover, the nonpolar surface tension coefficient used in PARSE, which is deduced from the partitioning of nonpolar molecules between water and liquid alkane, is nearly identical to that recently reported for the transfer of nonpolar molecules into lipid bilayers (Buser et al., 1994; Thorgeirsson et al., 1996). We have recently used the same methodology to successfully study amide hydrogen-bond formation (Ben-Tal et al., 1997), polyalanine α-helix insertion into lipid bilayers (Ben-Tal et al., 1996a), alamethicin-membrane interactions (Kessel et al., 2000a,b), helix-helix interactions in lipid bilayers (Ben-Tal and Honig, 1996), and membrane permeability of monensin-cation complexes (Ben-Tal et al., 2000a).

The main uncertainty in our model of the lipid bilayer results from its complete neglect of the polar headgroup region of the bilayer, which is presumably where VPA is adsorbed. Because the dielectric constant in this region is estimated to be between 25 and 40 (Ashcroft et al., 1981), the polar headgroup region might most appropriately be regarded as part of the aqueous phase defined in this study. Our calculations suggest that the carboxyl group of VPA is in the vicinity of the polar headgroups and may therefore interact with them.

Measurements of the partitioning of fatty acids of different chain lengths (N_{carbon} = 10, 12, 14, 16) between the aqueous phase and phospholipid vesicles suggest that the first four carbons following the COO^- group of VPA would reside in the polar headgroup region of the bilayer (Peitzsch and McLaughlin, 1993). If this is the case, then VPA is most likely to be located at the water-bilayer interface, and the horizontal line in Fig. 2 should best be regarded as the boundary between the aqueous phase and the polar headgroup region, rather than that between the polar headgroups and the hydrocarbon region.

If one can indeed deduce the behavior of VPA in lipid bilayers from the behavior of long-chain fatty acids, the free energy of VPA association with the membrane may be significantly different from the calculated value. The results of Peitzsch and McLaughlin show a linear dependency of the association free energy on N_{carbon}. According to their measurements, a free energy value of $\Delta G_{tot} = -4$, calculated here for VPA, whose chains are composed of 7 carbon atoms, should correspond to the membrane partitioning of a lipid chain of 12 carbon atoms. However, such extrapolation is inaccurate, as Peitzsch and McLaughlin's measurements were carried out on straight-chain fatty acids, whereas VPA is a branched fatty acid.

Keane et al. (1983) studied the water/octanol partition of VPA and analogs. Octanol, being amphiphilic, resembles the structure of phospholipids, and the partition of solutes to this medium may be considered as an approximation of water/lipid bilayer partition. The measured water/octanol partition coefficient, 562.3, corresponds to a free energy value of -3.75 kcal/mol. This value is very close to our calculated value, but given all the approximations used in our model, the nearly perfect agreement is probably fortuitous.

Experimental data from many labs indicate that there is a difference of ~100–1000-fold in the association constants of the ionic and non-ionic forms of fatty acids with lipid membranes (Miyazaki et al., 1992; Peitzsch and McLaughlin, 1993; Rooney et al., 1983). This difference translates into a 3–4-kcal/mol difference in the corresponding association free energies, compared with only 1.1 kcal/mol in our model. The remaining 2–3-kcal/mol difference may be attributed to the p*K*a shift due to interactions of the fatty acid with the polar headgroups (Miyazaki et al., 1992; Ptak et al., 1980), which are missing in our model.

Our calculations demonstrate that VPA is likely to partition into lipid bilayers, as would be expected from its molecular structure; this is in qualitative agreement with measurements using fatty acids with longer chains (Anel et al., 1993; Doody et al., 1980; Hamilton, 1998; Hamilton and Kamp, 1999; Kamp et al., 1995; Kleinfeld et al., 1997; Miyazaki et al., 1992; Peitzsch and McLaughlin, 1993; Ptak et al., 1980; Rooney et al., 1983; Zheng and Gierasch, 1996). As already discussed above, the location of VPA in the lipid bilayer is probably different from the one produced by our calculations. However, the accompanying difference in the free energy of association should be very small. The reasons for this are that the free energy of association of VPA with the lipid bilayer is very similar to the measured free energy of transfer of VPA from water to octanol, and that experimental data show that there is very good correlation between the free energies of transfer of, e.g., the

amino acids from water to octanol and from water to the bilayer-water interface (Wimley and White, 1996; Thorgeirsson et al., 1996).

The flip-flop rate of VPA across the bilayer is determined by a barrier, corresponding to the free energy difference between VPA in the configuration of Fig. 2 and VPA when it is fully immersed in the hydrocarbon region of the membrane. Thus, for the calculations of VPA flip-flop, the differences in ΔG_{tot} (i.e., $\Delta\Delta G$ in Eq. 5), rather than its absolute value, are important, and these are likely to be more accurate. It should be noticed, however, that the possibility that the flip-flop is facilitated by membrane defects (Wilson and Pohorille, 1996) was not considered here. Thus, the calculated average flip-flop time should best be regarded as a lower bound to the real value.

We characterized the flip-flop process by a single free energy barrier, which results from the insertion of the carboxylic group of VPA into the hydrocarbon region of the bilayer. Experimental data demonstrate that the association of VPA with the lipid bilayer is likely to involve the polar headgroup region of the bilayer (Peitzsch and McLaughlin, 1993). This suggests that the flip-flop process should include two energy barriers. The first barrier would result from the transfer of VPA from the aqueous solution into the polar headgroup region of the bilayer, and the second would result from the transfer of VPA from the polar headgroup region into the hydrocarbon region of the bilayer. Both free energy barriers result from the electrostatic penalty of transferring the carboxyl group of VPA between regions of different polarity, i.e., media of different dielectric constants. The dielectric constant of the headgroup region is between 25 and 40, whereas the dielectric constant of the hydrocarbon region is much lower (~2). Thus, the free energy barrier associated with the transition from water to the headgroup region should be insignificant compared with the free energy barrier, due to the transition between the headgroups and the hydrocarbon regions of the bilayer. Thus, the model on which our flip-flop calculations are based should reliably describe the flip-flop kinetics.

We have calculated the free energy curves for the flip-flop of VPA (Fig. 3 *A*) and valproate (Fig. 3 *B*) across the lipid bilayer. The calculations indicate that, although water-soluble VPA is deprotonated at physiological pH, it is likely to become protonated pending its insertion into the bilayer due to the high electrostatic free energy penalty of the insertion of the charged carboxylic group into the lipid bilayer. Thus, we suggest a minimal free energy path (Fig. 3 *C*, black solid line) for the flip-flop of VPA across the lipid bilayer. This process includes the protonation-coupled insertion of valproate into the bilayer via its hydrophobic tails, diffusion of the molecule across the hydrophobic core of the bilayer, and protonation-coupled exit into the aqueous solution, carboxyl group first. The kinetic rate coefficient obtained for this path, ~20/s, is many orders of magnitude larger than that obtained for the charged form.

We are not aware of any experimental measurement of the flip-flop rate of VPA across lipid bilayers. However, the flip-flop rate of other fatty acids in different lipid bilayer systems has been measured using different techniques. Measurements using NMR spectroscopy (reviewed by Hamilton, 1998) indicate that the p*K*a of fatty acids increases to ~7.5 upon membrane association, presumably due to their interactions with the polar headgroups of the lipid bilayer. The p*K*a shift results in ~50% of the fatty acid molecules being protonated at physiological pH. This allows the flip-flop process to occur very rapidly (within milliseconds), as confirmed by fluorescent studies carried out by this group. The fast flip-flop rates obtained in these measurements are indeed very close to our calculated value for VPA in its non-ionic form (2000/s).

In contrast, fluorescent studies carried out by Kleinfeld and co-workers (e.g., Kleinfeld et al., 1997) indicate that the flip-flop rate of fatty acids across lipid bilayers is much slower, with rate constants in the range of 0.1–100/s, depending on the type of fatty acid and the lipid bilayer. According to these measurements, the exact value of the flip-flop rate constant depends on the length of the fatty acid chain, on its level of saturation, on the properties of the lipid membrane, and on the temperature. Our theoretical model is not detailed enough to take these parameters into account, and in any event, VPA was not used in these studies, which prevents us from directly comparing our calculated rate constant with the measured value. However, our calculated value of ~20/s is in accord with the measurements. The uncertainties resulting from neglect of the polar headgroup region of the lipid bilayer by our model have been discussed above. It should be mentioned, in this respect, that the inclusion of the headgroup region would allow us to observe the p*K*a shift of the carboxyl group of VPA. As a result, the calculated flip-flop rate would be expected to be considerably higher and might be closer to the value reported by Hamilton and co-workers.

Regardless of the exact value of the flip-flop rate constant, measurements indicate that the non-ionic fatty acids flip-flop is ~100 times faster than the one of the corresponding ionic fatty acids (Doody et al., 1980). This measured ratio is in very good agreement with our calculated ratio of 2000/s vs. 20/s, i.e., a ratio of 100.

In the brain, the rapid uptake of VPA across the membranes of the blood-brain barrier is mediated by a monocarboxylic acid carrier (Adkison and Shen, 1996). The immediate anticonvulsant effect of VPA in some seizure models can be explained by its effect on neuronal extracellular sites. However, a late anticonvulsant effect of the drug has also been observed, and this effect can be explained by the slow diffusion of VPA across the plasma membrane of the neuron and its action on neuronal intracellular sites (Loscher, 1999). Thus, the characterization of the transmembrane diffusion of VPA may help us to better understand relevant physiological processes. It may also help us to characterize

the transmembrane diffusion of other amphipathic molecules present in biological membranes, such as phospholipids.

The results described above, together with the observation that the anticonvulsant activity of VPA and its analogs correlates with their water/octanol partitioning (Keane et al., 1983) and bilayer-perturbing ability (Perlman and Goldstein, 1984), further support the hypothesis that the mechanism of action of VPA involves a direct membrane effect. Many studies implicate complex biochemical systems, such as the GABA system, in VPA action. One may wonder how the nonspecific effect of VPA on the lipid bilayer can influence the operation of these complex systems. One possibility is that VPA actually acts directly on specific membrane proteins, which are an integral part of these systems, with the membrane binding of VPA serving to increase its local concentration in the vicinity of these target proteins. The activity of membrane-bound proteins is influenced by their interactions with membrane lipids. This has already been demonstrated for several enzymes, such as Na^+/K^+ ATPase (Chong et al., 1985), β-hydroxybutyrate dehydrogenase (Cortese et al., 1989), and protein kinase C (Newton, 1993). In the case of protein kinase C, the enzymatic activity has been shown to be affected both by specific and nonspecific interactions with membrane lipids and by the physical properties of the lipid bilayer (reviewed by Mosior and McLaughlin, 1992). Thus, an alternative explanation for VPA action is that its direct membrane effect may indirectly change the activity of the target membrane proteins by influencing their interactions with the lipid bilayer.

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