Electrooptical Measurements Demonstrate a Large Permanent Dipole Moment Associated with Acetylcholinesterase

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ABSTRACT Acetylcholinesterase (AChE) from krait (*Bungarus fasciatus*) venom is a soluble, nonamphiphilic monomer of 72 kDa. This snake venom AChE has been analyzed by measurements of the stationary and the transient electric dichroism at different field strengths. The stationary values of the dichroism are consistent with the orientation function for permanent dipoles and are not consistent with the orientation function for induced dipoles. The permanent dipole moment obtained by least-squares fits for a buffer containing 5 mM MES is 1000 D, after correction for the internal directing field, assuming a spherical shape of the protein. The dipole moment decreases with increasing buffer concentration to 880 D at 10 mM MES and 770 D at 20 mM MES. The dichroism decay time constant is 90 ns (±10%), which is clearly larger than the value expected from the size/shape of the protein and indicates contributions from sugar residues attached to the protein. The dichroism rise times observed at low field strengths are larger than the decay times and, thus, support the assignment of a permanent dipole moment, although it has not been possible to approach the limit where the energy of the dipole in the electric field is sufficiently low compared to kT. The experimental value for the permanent dipole moment is similar to that calculated for a model structure of *Bungarus fasciatus* AChE, which has been constructed from its amino acid sequence, in analogy to the crystal structure of AChE from *Torpedo californica*.

INTRODUCTION

The principal biological role of acetylcholinesterase (AChE) is termination of impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (Barnard, 1974). In keeping with this requirement, it is an extremely effective catalyst, especially for a serine hydrolase, acting at rates that are almost diffusion controlled (Quinn, 1987). Unexpectedly, the three-dimensional structure of AChE from *Torpedo californica* revealed that its active site is located at the bottom of a deep and narrow gorge (Sussman et al., 1991). Earlier kinetic studies suggested that the positively charged substrate might be attracted to the active site by a relatively large number of negative charges in its vicinity (Nolte et al., 1980). The crystal structure of AChE did not bear out this prediction, but theoretical calculations indicated that the enzyme possesses a large permanent dipole moment, which may serve to guide the cationic substrate into the gorge leading to the active site (Ripoll et al., 1993; Tan et al., 1993). The contribution of this dipole to the high turnover rate of AChE is the subject of controversy (Shafferman et al., 1994; Antosiewicz et al., 1994, 1995). It seemed therefore that direct experimental establishment of its value would be of importance. The availability of a monomeric species of AChE, purified from the venom of the krait *Bungarus fasciatus* (Cousin et al., manuscript submitted for publication), permitted the use of an electrooptical technique (O'Konski et al., 1959; Porschke, 1987) for direct measurement of its electrical properties. Analysis of the data obtained revealed that the AChE from *B. fasciatus* venom possesses a large permanent dipole moment on the order predicted by the theoretical calculations for a model of the *B. fasciatus* enzyme.

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MATERIALS AND METHODS

Protein isolation and characterization

Dried *B. fasciatus* venom collected from Cambodia was from the stock of the Institut Pasteur (Paris, France). Purification of venom AChE was performed by affinity chromatography as previously described for enzyme from other origins (Dudai and Silman, 1974; Massoulié and Bon, 1976). Briefly, *B. fasciatus* venom was dissolved in distilled water at a concentration of 100 mg/ml, then applied to an ethyldimethylphenyl ammonium Sepharose column (4 ml) previously equilibrated with 0.1 M sodium phosphate buffer (pH 7.4). The gel was washed successively with 30 ml of equilibration buffer and 30 ml of 0.1 M sodium phosphate buffer containing 0.2 M NaCl. AChE adsorbed to the affinity column was eluted with 0.1 M sodium phosphate buffer (pH 7.4) containing 0.4 M NaCl and 0.02 M decamethonium bromide. Active fractions were pooled and dialyzed for 72 h at 4°C against 0.1 M sodium phosphate buffer (pH 7.4), with several bath changes, then against 10 mM morpholinoethanesulfonic acid (MES) (pH 6.5). Homogeneity of purified *B. fasciatus* venom AChE was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

AChE activity was determined by the colorimetric method of Ellman et al. (1961), using 0.75 mM acetylthiocholine iodide as substrate, in the presence of 0.5 mM dithiobisdinitrobenzoic acid and 0.1 M sodium phosphate buffer (pH 7.0). The specific activity of purified AChE from *B. fasciatus* venom was 75,700 Ellman units/mg; 1 Ellman unit is defined as the activity producing an increase in optical density of 1 absorbance unit/min in 1 ml of assay medium (1 cm pathlength) and corresponds to the hydrolysis of 75 nmol substrate/min.

PAGE performed in the presence and the absence of reducing agent, and sucrose gradient centrifugation in the presence and in the absence of detergent showed that krait venom AChE is exclusively constituted of a nonamphiphilic monomer (G_1^{na}) of 72 kDa apparent molecular mass (Cousin et al., manuscript submitted for publication). Fig. 1 shows that the venom enzyme is monomeric, even at the high protein concentration (0.2 mg/ml) used to perform the electric dichroism measurements.

Electric dichroism measurements

Electric dichroism was measured at 20°C by a pulse generator (Grünhagen, 1974) and an optical detection system as described (Porschke, 1980). Samples of purified *B. fasciatus* venom AChE (0.2 to 0.5 mg/ml) were subjected to field pulses in the range of 10–70 kV/cm and 2–5 μs in a cell with 10 mm optical path length and a distance between the Pt electrodes of 6.05 mm. UV radiation damage was avoided by an automatic shutter for short light pulses synchronized to the field pulses. Both UV transmission and electric field strength as a function of time were transiently stored by a Tektronix DSA 602 digitizing signal analyzer. The stationary changes in light intensity and electric field strength were evaluated by using graphic routines on a personal computer. The time constants were determined by an efficient deconvolution routine (Porschke and Jung, 1985). Potential damage to the AChE samples during measurements was tested by taking UV spectra of the sample in the electrooptical cell before and after each experiment; in all cases absorbance did not change by more than 3%. Controls also demonstrated that the enzymatic activity of the samples exposed to the electric field pulses remained unchanged within the limits of experimental accuracy.

RESULTS AND DISCUSSION

Electrooptical measurements

In most experiments, the effect of electric field pulses on solutions of AChE was studied by measuring the absorbance of light polarized parallel to the field vector. Under these conditions electric field pulses induced an increase in the absorbance, $\Delta A_{\parallel}$, of the AChE solutions. Control experiments using light polarized at the magic angle (54.7° with respect to the field vector) showed a small field-induced change in absorbance corresponding to less than 5% of $\Delta A_{\parallel}$, which is mainly due to a temperature jump effect. Because the effect at the magic angle is close to the noise level, it was not used for any further analysis. Experiments using light polarized perpendicular to the field vector showed a decrease in the absorbance, $\Delta A_{\perp}$. The absorbance changes $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ fulfilled the condition $\Delta A_{\parallel} = -2 \times \Delta A_{\perp}$ within the limits of experimental error ($\Delta A_{\parallel}/\Delta A_{\perp} = -2 \pm 10\%$) as expected for a simple field-induced orientation effect.

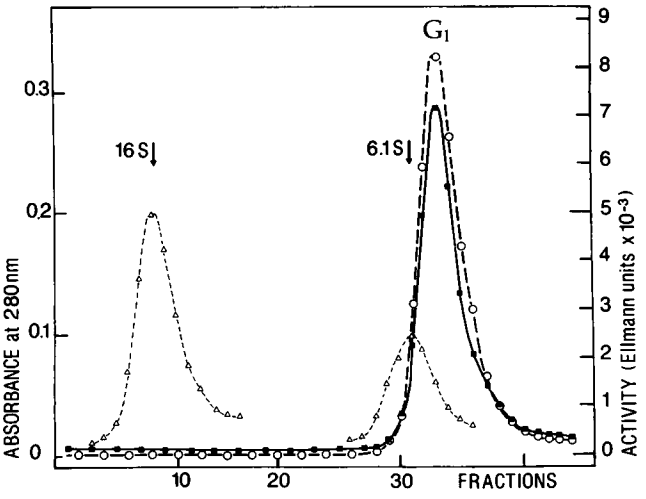

FIGURE 1 Sedimentation analysis of purified AChE from *Bungarus fasciatus* venom. Samples (500 μl) containing 0.34 mg/ml purified krait venom AChE were layered on sucrose gradients (5–20% sucrose, w:v; 10 mM Tris-HCl, pH 6.5; 40 mM $MgCl_2$) and centrifuged for 18 h at 40,000 rpm, at 4°C, in a SW41 rotor (Beckman Instruments). For each fraction absorbance was determined at 280 nm (■) and AChE activity assayed in Ellman reaction medium (○). Sedimentation coefficients were determined by comparison with *Escherichia coli* β-galactosidase (△, 16 S) and alkaline phosphatase from calf intestine (△, 6.1 S), centrifuged in a twin gradient, and included as internal standards in a third gradient layered with diluted enzyme.

Stationary values of the absorbance changes were converted into the stationary linear dichroism according to

$$\frac{\Delta\epsilon}{\bar{\epsilon}} = \frac{\Delta\epsilon_{\parallel} - \Delta\epsilon_{\perp}}{\bar{\epsilon}} = \frac{\Delta A_{\parallel} - \Delta A_{\perp}}{\bar{A}} = \frac{1.5 \cdot \Delta A_{\parallel}}{\bar{A}}, \quad (1)$$

where $\bar{A}$ is the isotropic absorbance, and $\Delta\epsilon_{\parallel}$, $\Delta\epsilon_{\perp}$, and $\bar{\epsilon}$ are the extinction coefficients with indices defined corresponding to those used for the absorbance changes. The stationary values of the linear dichroism were measured as a function of the electric field strength at three different buffer concentrations and fitted to the orientation function for permanent dipole moments (O'Konski et al., 1959)

$$\frac{\Delta\epsilon}{\epsilon} = \left[1 - \frac{3(\coth\beta - 1/\beta)}{\beta}\right] \cdot \left(\frac{\Delta\epsilon}{\epsilon}\right)_{\infty}, \quad (2)$$

where $\beta = \mu_p \cdot E/kT$, μ_p is the permanent dipole moment, E is the electric field strength, kT is the thermal energy, and $(\Delta\epsilon/\epsilon)_{\infty}$ is the electric dichroism at infinite field strength.

For comparison the data have also been fitted according to the orientation function for induced dipoles (Fredericq and Houssier, 1973)

$$\frac{\Delta\epsilon}{\epsilon} = \left[\frac{3}{4}\left\{\left(\frac{e^{\gamma}}{\gamma^{1/2}}\int_0^{\gamma^{1/2}} e^{x^2}\,dx\right) - 1/\gamma\right\} - \frac{1}{2}\right] \cdot \left(\frac{\Delta\epsilon}{\epsilon}\right)_{\infty}, \quad (3)$$

where $\gamma = \alpha E^2/(2kT)$ and α the polarizability.

In all three cases, the permanent dipole function fits the data very well within the limits of experimental accuracy, whereas the orientation function for induced dipoles cannot be used to represent the data, as is shown in Fig. 2 for measurements carried out in 20 mM MES. The dipole moments obtained according to Eq. 2 have to be corrected for the difference between the external, applied electric field strength E_e and the internal directing field strength E_i. Because AChE may be regarded as spherical to a reasonable approximation, the correction factor (Bottcher, 1973) may be calculated according to

$$E_i = [3\epsilon/(2\epsilon + 1)]E_e, \quad (4)$$

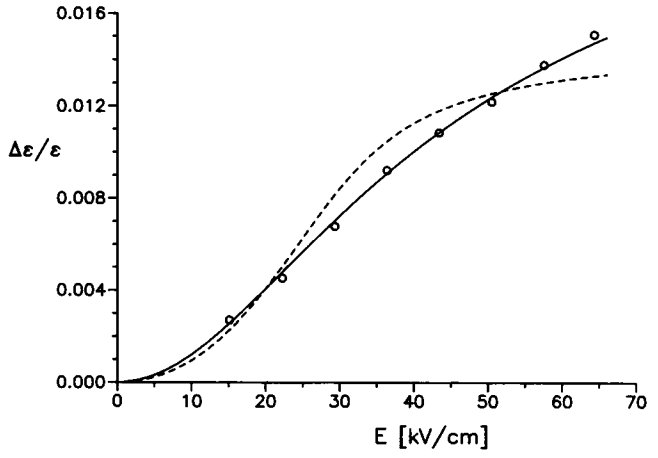

FIGURE 2 Stationary values of the electric dichroism $\Delta\epsilon/\epsilon$ measured for AChE from *B. fasciatus* in 20 mM MES-buffer, pH 7.0, as a function of the electric field strength, *E*. The continuous line represents a least-squares fit of the data by the permanent dipole orientation function with $\mu_p = 3.86 \times 10^{-27}$ Cm and $(\Delta\epsilon/\epsilon)_\infty = 0.026$. The dashed line represents a fit by the induced dipole orientation function with polarizability 3.8×10^{-33} C m^2V^{-1} and $(\Delta\epsilon/\epsilon)_\infty = 0.014$.

where ϵ is the dielectric constant of the solvent. As shown in Table 1, the corrected dipole moments are in the range from 1000 D at 5 mM MES to 770 D at 20 mM MES. Whereas the dipole moments decrease with increasing buffer concentration c_b, the limiting reduced dichroism is virtually independent of c_b. The decrease in the dipole moment with increasing salt concentration presumably reflects some binding of counter-ions to the protein.

It is well known that in some cases the electric field strength dependence of the stationary dichroism suggests the existence of a permanent dipole moment, even when there is no permanent anisotropy of the charge distribution (Diekmann et al., 1982). The standard example is double-helical DNA, where, in a certain range of chain lengths, a permanent dipole is simulated by a saturation of the polarizability. For double-helical DNA it has been possible to distinguish the simulated permanent dipole from a real one by a detailed analysis of the dichroism rise time constants. According to the classical result of Benoit (1951), the electrooptical rise curves of a permanent dipole in the limit of low electric field strengths may be decomposed into two exponentials with time constants $\tau_1^r = \tau^d$ and $\tau_2^r = 3 \times \tau^d$, where τ^d is the time constant of dichroism decay. In the case of double-helical DNA, the rise curves do not show the slow component ($\equiv 3 \times \tau^d$) expected for permanent dipole moments; a detailed analysis of these rise curves has revealed a special polarization mechanism involving ion dissociation (Porschke, 1985). Thus, an analysis of the time constants of AChE under electric field pulses should provide more information about the nature of the dipole moment.

The dichroism decay observed for AChE could be fitted by single exponentials to a satisfactory accuracy (cf. Fig. 3). The average decay time constant was 90 ns (±10%) and was virtually independent of electric field strength. The dichroism rise curves showed a clear dependence on the electric field strength. As shown by the examples in Fig. 3, the rise in dichroism at low field strengths was clearly slower than the decay. This result provides further clear evidence against a standard induced dipole model for *B. fasciatus* venom AChE. It was possible to fit the rise curves by single exponentials (model I), or by a combination of two exponentials with initial zero slope (model II; cf. Porschke, 1985) with about the same accuracy. The fits obtained using model II always provided a first exponential with time constants in the range of 1–3 ns and a second exponential in the range of 70–300 ns, depending on the field strength. The second exponential time constant, τ_2^r, obtained using model II was very close to the single exponential time constant obtained using model I. τ_2^r was a linear function of the reciprocal electric field strength (cf. Fig. 4), which is consistent with the existence of a permanent dipole moment (O'Konski et al., 1959; Porschke, unpublished material). Under the experimental conditions of the present investigation, it has not been possible to approach the limit case of low electric field strength and, thus, to observe a correspondence of τ_1^r with τ^d. However, at the lowest field strength employed, τ_2^r is much larger than τ^d. It should also be noted that, even at the lowest field strength of the present investigation, $\mu_p E$ is approximately of the same magnitude as kT (μ_p, permanent dipole moment; *E*, electric field strength; *kT*, thermal energy), thus confirming that the field strengths were not sufficiently low, because of the rather low optical anisotropy of AChE.

TABLE 1 Electric dipole moments μ_p and limiting values of the reduced linear dichroism $(\Delta\epsilon/\epsilon)_\infty$ for AChE from *Bungarus fasciatus* venom at three different concentrations of MES buffer, c

c (mM)	μ_p (Cm)	$(\Delta\epsilon/\epsilon)_\infty$	μ_p^c (D)
5	$5.03 \cdot 10^{-27}$	0.026	1000
10	$4.43 \cdot 10^{-27}$	0.027	880
20	$3.86 \cdot 10^{-27}$	0.026	770

μ_p and $(\Delta\epsilon/\epsilon)_\infty$ are the values obtained directly from fitting the orientation function to the experimental data. μ_p^c is corrected for the internal directing electric field, assuming a spherical shape for the protein.

Comparison with calculations

Ripoll et al. (1993) calculated a dipole moment of 505 D for AChE from *Torpedo californica* based on its crystal structure, utilizing the finite difference algorithm DELPHI. Antosiewicz et al. (1994) subsequently calculated a dipole moment of 1500 D on the basis of the same structural data. The difference is mainly due to the choice of the center of the coordinate system. AChE has an overall net charge of about $-6e$ per monomer at pH 7 resulting from a large excess of acidic amino acids. In such a case it is important to calculate the dipole moment relative to the center of

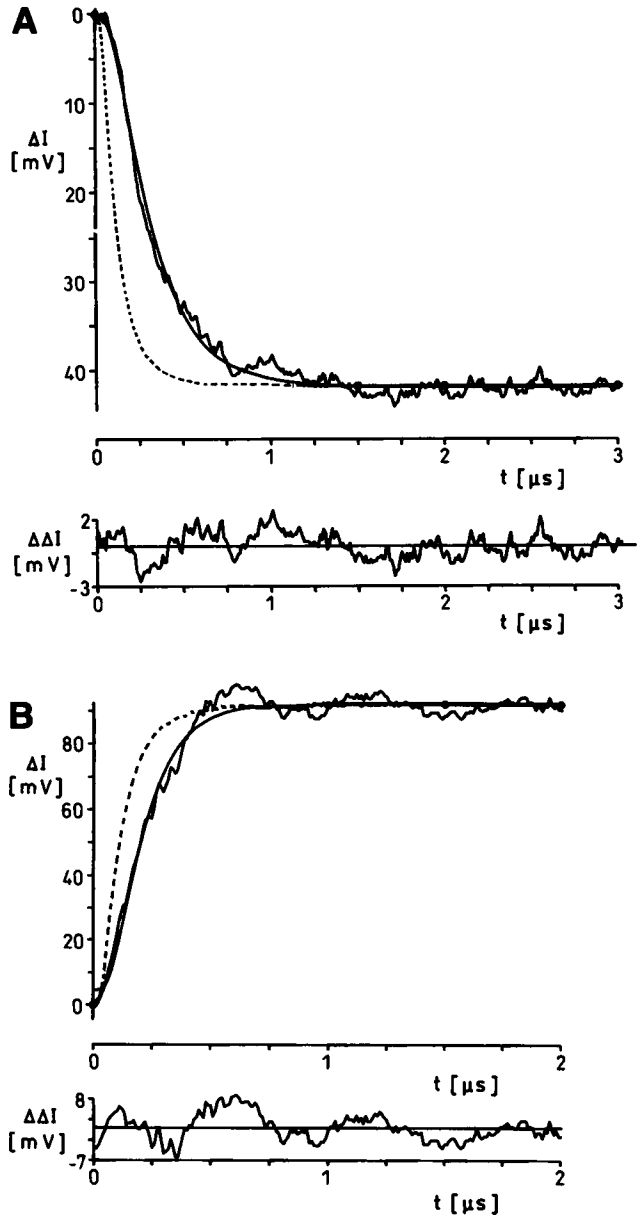

FIGURE 3 Dichroism rise (*a*) and decay (*b*) of krait venom AChE in 5 mM MES at 20°C. The continuous lines with noise represent the experimental data, the dashed lines represent the machine function (cf. Porschke and Jung, 1985) used for deconvolution (birefringence of buffer under the conditions of the measurements), and the full lines without noise represent the least-squares fits: (*a*) by two exponentials according to model II (cf. Porschke and Jung, 1985) τ_1^r = 2 ns and τ_2^r = 203 ns (relaxation induced by a pulse of 24.6 kV/cm); (*b*) by a single exponential with τ^d = 100 ns (relaxation measured after a pulse of 71.9 kV/cm amplitude and 5 μs duration).

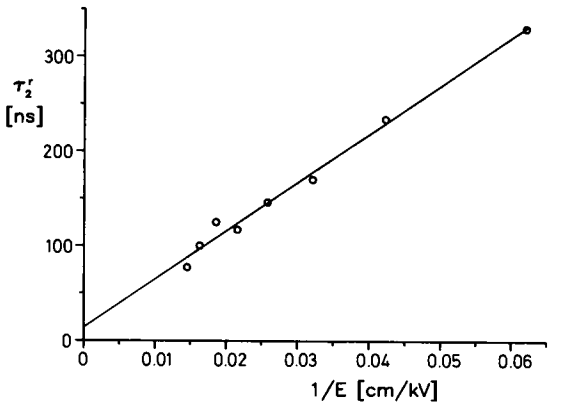

FIGURE 4 Rise time constant τ_2^r obtained according to model II from experimental data obtained in 10 mM MES-buffer at 20°C, pH 7.0, as a function of the inverse of electric field strength, 1/*E*. The straight line represents a linear regression with intercept 13.4 ns and slope 5.1 × 10^3 ns · kV cm^{-1}.

diffusion, if it is to have any physical meaning (Mysels, 1953; Antosiewicz and Porschke, 1989a). The dipole moment determined by Antosiewicz et al. (1994) was indeed so calculated.

For comparison with the present experimental data we have used a model structure of *B. fasciatus* AChE (Cousin et al., manuscript submitted for publication). This model was constructed using the first approach mode of the Swiss-Model automatic modeling server from Expasy. The construction was based on the amino acid sequence of *B. fasciatus* AChE and the x-ray structure of *Torpedo* AChE. The dipole moment, the limiting value of the electric dichroism, and the dichroism decay time constant were calculated from this model by a procedure that had been proved to be valid for the case of chymotrypsin (Antosiewicz and Porschke, 1989b). The dipole moment calculated for pH 7 is 1830 D (simple addition of the contributions from charged residues, with respect to the center of diffusion, ion atmosphere effects not considered), the limiting value of the electric dichroism at 280 nm is 0.053, and the dichroism decay time constant is 46 ns. We have also calculated these parameters for *Torpedo* AChE according to the same procedure using the data stored in the Brookhaven Protein Data Bank (1ACE.PDB): the dipole moment calculated for pH 7 is 1600 D, the limiting value of the electric dichroism is −0.24 (280 nm), and the dichroism decay time constant is 40 ns. An illustration of the structures, the dipole vector, and isopotential surfaces is given in Fig. 5.

A comparison of the calculated (46 ns) and experimental (90 ns) decay time constants indicates that there is a substantial difference between the model structure and the structure in solution. This difference cannot be explained by a dimerization of the venom AChE at the high protein concentration used for electrooptical measurements, because Fig. 1 unambiguously indicates that the enzyme from *B. fasciatus* venom remains monomeric at the concentrations used for these experiments. The venom AChE contains about 7% carbohydrates (Cousin et al., manuscript submitted for publication), which have not been included in the model structure used for the calculations. These carbohydrates clearly lead to an increase of the effective hydrodynamic dimensions and thus explain the difference between calculated and experimental decay time constants.

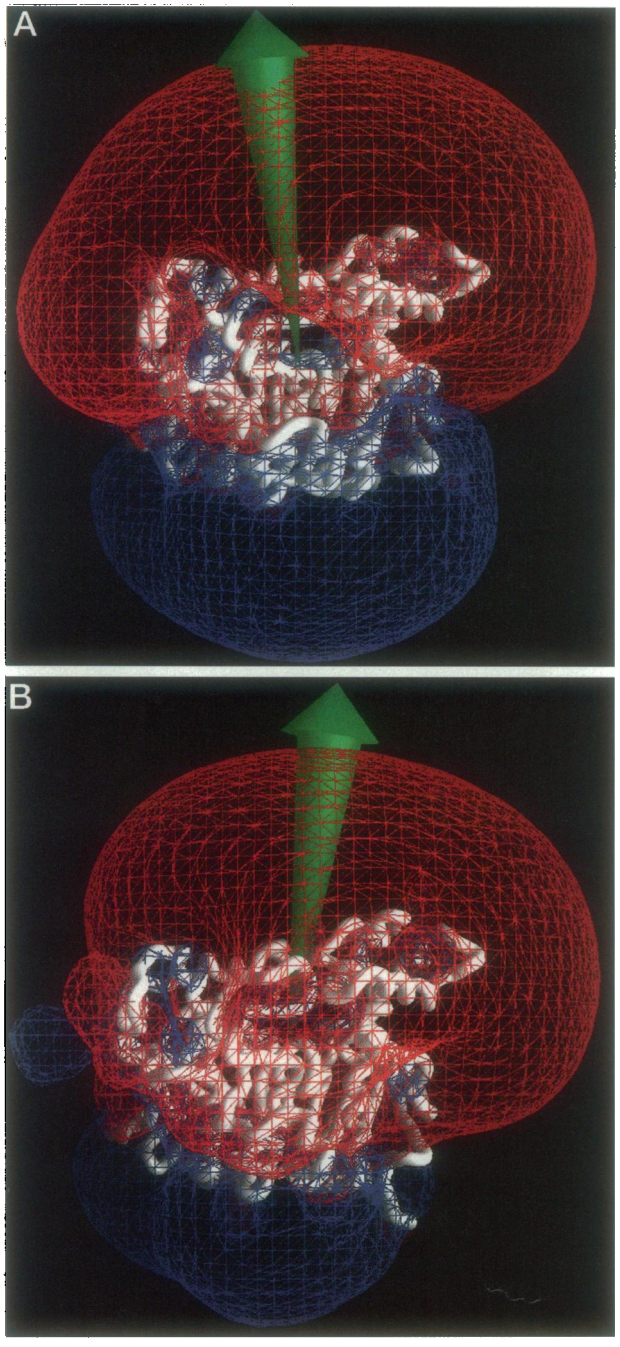

FIGURE 5 (*a*) Electrostatic diagram of *Torpedo* AChE as prepared by application of GRASP, using the coordinates of PDB entry 1ace, all acid and basic groups ionized at pH 7, full atom charge set (e.g., a +1 or −1 charge assigned to each charged group), and in the same view as Ripoll et al. (1993). The molecular backbone is represented by a backbone worm, the +1 and −1 isopotential surfaces are shown as cross-hatched blue and red surfaces, respectively, and the green arrow represents the dipole moment due to the fixed charges (not drawn to scale). (*b*) Electrostatic diagram of a homology model for *B. fasciatus* AChE, in the style used in *a*.

Because these carbohydrates also bear negative charges, it is very likely that these residues are also the main reason for the difference between calculated and experimental dipole moments. Thus, the comparison between calculated and experimental data can only be qualitative at present.

CONCLUSIONS

The electrooptical measurements show that AChE from snake venom bears a permanent electric dipole moment in the range of about 1000 D. The dipole moment calculated for a model of *B. fasciatus* AChE is on the same order of magnitude but is clearly higher than the experimental value. The difference between calculated and experimental values appears to be mainly due to the attachment of carbohydrate residues, which have not been included in the calculations.

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