Interaction of partially unfolded forms of *Torpedo* acetylcholinesterase with liposomes

IRINA SHIN,¹ ISRAEL SILMAN,¹ AND LEV M. WEINER²

¹ Department of Neurobiology, The Weizmann Institute of Science, Rehovot 76100, Israel

² Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot, 76100, Israel

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Abstract

A water-soluble dimeric form of acetylcholinesterase from electric organ tissue of *Torpedo californica* was obtained by solubilization with phosphatidylinositol-specific phospholipase C of the glycoposphatidylinositol-anchored species, followed by purification by affinity chromatography. The water-soluble species, in its catalytically active native conformation, did not interact with unilamellar vesicles of dimyristoylphosphatidylcholine. We previously showed that either chemical modification or exposure to low concentrations of guanidine hydrochloride converted the native enzyme to compact, partially unfolded species with the physicochemical characteristics of the molten globule state. In the present study, it was shown that such molten globule species, whether produced by mild denaturation or by chemical modification, interacted efficiently with small unilamellar vesicles. Binding was not accompanied by significant vesicle fusion, but transient leakage occurred at the time of binding. The bound acetylcholinesterase reduced the transition temperature of the vesicles slightly, and NMR data suggested that it interacted primarily with the head-group region of the bilayer. The effects of tryptic digestion of the bound acetylcholinesterase were monitored by gel electrophoresis under denaturing conditions. It was found that a single polypeptide, of mass ~5 kDa, remained associated with the vesicles. Sequencing revealed that this is a tryptic peptide corresponding to the sequence Glu 268–Lys 315. This polypeptide contains the longest hydrophobic sequence in the protein, Leu 274–Met 308, as identified on the basis of hydropathy plots. Inspection of the three-dimensional structure of acetylcholinesterase reveals that this hydrophobic sequence is largely devoid of tertiary structure and is localized primarily on the surface of the protein. It is suggested that this hydrophobic sequence is aligned parallel to the surface of the vesicle membrane, with nonpolar residues undergoing shallow penetration into the bilayer.

Keywords: acetylcholinesterase; hydrophobic peptide; liposome; molten globule; protein–lipid interaction

Acetylcholinesterase (AChE) is a cell-surface enzyme whose principal biological role is to terminate impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (Barnard, 1974). In the *Torpedo* electric organ, a major part of the AChE is a hydrophobic dimer of two catalytic subunits (Bon & Massoulié, 1980; Viratelle & Bernhard, 1980; Witzemann, 1980), which displays solubility characteristics typical of integral membrane proteins. This dimer is a member of the family of glycoposphatidylinositol-anchored proteins

(Low, 1989), and the hydrophobic characteristics of the native dimer are entirely due to the fatty acid chains of the diglyceride moiety of a single phosphatidylinositol residue that is attached, via an oligoglycan extension, to the COOH-terminus of each subunit (Mehlert et al., 1993). Cleavage of this anchor by a PI-specific phospholipase C of bacterial origin, with concomitant removal of the diglyceride, produces a water-soluble form of the dimer that does not interact with liposomes (Futerman et al., 1985).

We recently showed that the native water-soluble AChE dimer, prepared by enzymic cleavage of the phosphatidylinositol (PI) anchor followed by purification by affinity chromatography, can be converted to partially unfolded species displaying many of the features of the “molten globule” (MG) state (Dolginova et al., 1992; Kreimer et al., 1994a; Weiner et al., 1994). Such species, which can be produced by chemical modification of a buried cysteine residue (Dolginova et al., 1992; Kreimer et al., 1994a), by mild denaturation (Eichler et al., 1994), or by oxidative stress (Weiner et al., 1994), can exist for several hours

Reprint requests to: Lev Weiner, Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel; e-mail: cilev@weizmann.weizmann.ac.il.

Abbreviations: AChE, acetylcholinesterase; PI, phosphatidylinositol; PIPLC, PI-specific phospholipase C; MG, molten globule; ANS, 1-anilino-8-naphthalene sulfonate; N, native; DMPC, dimyristoylphosphatidylcholine; DTP, 4,4'-dithiodipyridine; Gdn-HCl, guanidine hydrochloride; TMA-TEMPO, 4-trimethylammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide; HgR, 2,2,5,5-tetramethyl-4-(2-chloromercuri-phenyl)-3-imidazoline-1-oxyl; EPR, electron paramagnetic resonance.

under physiological conditions, without aggregating. The criteria for their assignment as MG or MG-like species are a shift to the red in the intrinsic fluorescence emission peak; a large decrease in molar ellipticity in the near UV, together with a much smaller decrease in molar ellipticity in the far UV; greatly increased binding of the amphiphilic probe 1-anilino-8-naphthalene sulfonate; and heightened susceptibility to trypsinolysis, all relative to the fully folded native state. The definition of the MG state is controversial, and different research groups attribute different meanings to the term (see, for example, Griko et al., 1994; Ewbank et al., 1995; Okazaki et al., 1995). In the present paper, we use the term *molten globule* to refer to compact states of AChE that preserve substantial secondary structure and lack most of the tertiary structure of the native enzyme. This usage does not imply precise structural identity of MG preparations of AChE obtained by different means; namely mild denaturation, chemical modification, or oxidative stress (Eichler et al., 1994; Kreimer et al., 1994a; Weiner et al., 1994).

The MG state is currently assumed to serve as an intermediate in protein folding both in vitro and in vivo (Kuwayama, 1989; Gething & Sambrook, 1992; Bychkova & Pritsyn, 1993). Conversely, some native proteins assume the MG state when subjected to mild denaturing conditions. It has been suggested that the MG state not only serves as a folding intermediate in protein biosynthesis in vivo but also may be a state in which proteins are translocated across or inserted into biological membranes (Bychkova et al., 1988; Bychkova & Pritsyn, 1993). Thus, insertion of the toxin colicin A into membranes proceeds through a transition of its pore-forming domain from the native (N) to the MG state, which is induced by low pH (van der Goot et al., 1991; Muga et al., 1993). Similar data have been presented for colicin Ia (Mel & Stroud, 1993) and for a fragment of colicin E1 (Shin et al., 1993). The outer membrane protein Omp A of *Escherichia coli* spontaneously refolds from its unfolded form upon addition of preformed lipid vesicles and inserts into the vesicle membranes (Surrey & Jähnig, 1992). It has also been observed that deep penetration of a portion of the Sec A protein of *E. coli* into small unilamellar vesicles is promoted by the partial unfolding induced by increased temperature (Ulbrandt et al., 1992). Finally, it has been shown that native rhodanese that retains the presequence responsible for translocation into the mitochondrion does not bind to liposomes, whereas partially folded rhodanese, obtained by dilution of a solution of urea-unfolded enzyme, does interact with liposomes (Zardeneta & Horowitz, 1992; Mendoza et al., 1993). Although these experimental results support involvement of the MG state in translocation through biological membranes, they do not offer direct experimental evidence to support this possibility.

In the colicin fragment-liposome system, incorporation of protein into the lipid bilayer occurs concomitantly with transition of native protein to an MG state (van der Goot et al., 1991; Mel & Stroud, 1993). In the experiments performed with rhodanese and Omp A, incorporation into liposomes is concomitant with refolding (Surrey & Jähnig, 1992; Zardeneta & Horowitz, 1992; Mendoza et al., 1993). Neither of these paradigms investigates directly the interaction of a well-defined MG with a lipid bilayer.

As mentioned above, the MG states of *Torpedo* AChE produced by various chemical and physicochemical manipulations are stable for several hours under physiological conditions (Dolginova et al., 1992; Kreimer et al., 1994a). They were thus an

attractive experimental system for investigating the physicochemical parameters of the MG-liposome interaction, which could serve as a useful model for MG-plasma membrane interactions in vivo. In this paper, we describe the interaction of MG states of *Torpedo* AChE with small unilamellar vesicles prepared from dimyristoylphosphatidylcholine (DMPC). A repertoire of physicochemical techniques was used to monitor and characterize this interaction. Furthermore, trypsinolysis of the bound MG followed by microsequencing provided evidence that attachment involves a unique hydrophobic sequence that undergoes insertion into the lipid bilayer.

Results

Interaction of AChE with DMPC liposomes

To produce an MG state, two physicochemical treatments were employed: exposure of AChE to guanidine hydrochloride (Gdn-HCl) and chemical modification of AChE by 4,4'-dithiodipyridine (DTP; see the Materials and methods). The MG forms of AChE so obtained were designated MG_{Gdn} and MG_{DTP}, respectively. The binding to liposomes of AChE in an MG state was investigated by a flotation-gradient centrifugation technique, which permits separation of free AChE and AChE-liposome complexes (Futerman et al., 1985). Under the conditions employed, liposomes (together with adhering AChE) float to the top of the gradient, whereas unbound AChE remains at the bottom. Figure 1 shows that *Torpedo* AChE that has undergone treatment to produce an MG state, in this case MG_{Gdn}, binds well to DMPC liposomes, whereas native AChE fails to interact. The protein/vesicle molar ratio was ~7–8; that is, seven to eight subunit dimers bound per vesicle. The number of vesicles was estimated on the assumption that one lipid molecule occupies ~0.7 nm² of vesicle surface (Small, 1986), and from the hydrodynamic dimensions of the vesicles as determined from our light-scattering measurements (~500-Å diameter). The weight ratio of protein to lipid was calculated to be about 0.2. Such a protein-lipid ratio was achieved

Fig. 1. Flotation-gradient centrifugation profiles of native AChE and MG_{Gdn} incubated with DMPC liposomes. Flotation gradients were constructed as described in the Materials and methods. Native AChE and MG_{Gdn} (2–3 mg/mL) were incubated for 15 min with DMPC liposomes (10 mg/mL) at 25 °C before loading onto the gradients. Liposomes floated to the top of gradient (i.e., to the left of the figure), as measured by monitoring phosphorus. ○, phosphorus; ●, native AChE; △, MG_{Gdn}.

within 15 min under the experimental conditions employed, and it did not increase upon further incubation for up to 4 h, even at a ratio of 20 dimers per vesicle. Similar experiments performed using MG_{DTP} yielded similar results (not shown), but the number of subunit dimers bound per vesicle was larger, ~12.

The interaction of MG_{Gdn} with large unilamellar vesicles was also tested. After incubation for 1 h at 28 °C, only traces of protein were associated with the liposome fraction. After 3 h of incubation, the weight ratio of protein to lipid was calculated to be about 0.05. This value is much lower than those obtained after much shorter incubations with small vesicles and is in agreement with observations from other laboratories, which suggest that the curvature of the bilayer membrane may play a role in governing insertion (Greenhut et al., 1986; Silversmith & Nelsestuen, 1986).

The MG state is characterized by exposure of hydrophobic residues on the surface, as revealed by increased fluorescence of 1-anilino-8-naphthalene sulfonate (ANS; Semisotnov et al., 1991), and this is also true for *Torpedo* AChE (Dolginova et al., 1992; Kreimer et al., 1994a, 1994b). Our finding that AChE in an MG state was bound to DMPC vesicles, whereas native AChE was not, suggested that interaction was primarily hydrophobic. To obtain direct experimental support for this possibility, we studied the interaction of MG_{Gdn} with DMPC liposomes at different concentrations of NaCl, and analyzed the preparations obtained using sucrose gradients made up at the same ionic strength. We found that binding of AChE to liposomes is not substantially affected by the salt concentration, essentially identical sucrose gradient profiles being obtained at 10 mM and 1 M NaCl (data not shown). Thus, electrostatic forces do not play a major role in the MG_{Gdn}-DMPC interaction.

Almost no free protein was observed if either MG_{Gdn} or MG_{DTP} was added to liposomes at a stoichiometry of one dimer per vesicle. Such samples, essentially devoid of free MG, were used in the following investigations.

Because it has been reported that α -lactalbumin, at acid pH—where it may undergo a N $\rightarrow$ MG transition (Bychkova & Ptit-syn, 1993)—induces fusion of phospholipid vesicles (Kim & Kim, 1986), the effect of MG_{Gdn} on the size of DMPC vesicles was investigated by quasi-elastic light scattering. The diameter of control vesicles was 500 ± 10 Å, and that of vesicles treated with MG_{Gdn} was 560 ± 10 Å. This small change could be attributed to a slight increase in size due to attachment of a small number of dimers to the surface of the vesicle, but it ruled out any major change such as vesicle fusion or aggregation.

Magnetic resonance studies of the influence of bound AChE on the DMPC vesicles

To understand whether interaction of AChE in the MG state with liposomes induces changes in the lipid bilayer, we studied the effect of MG_{Gdn} on the melting curve of DMPC vesicles, which reflects the transition from the ordered to the fluid state of the fatty acid chains of DMPC (Grant & McConnell, 1974). The effect on this transition of incorporating MG_{Gdn} into the DMPC liposomes was studied by incorporating a small amount of spin-labeled cholesterol into the liposomes (Hubbell & McConnell, 1971; Boggs et al., 1981). The parameter of electron paramagnetic resonance spectra, h_{+1}/h_0 , is sensitive to the state of the alkyl chains of the lipid bilayer. It can, therefore, be used to monitor the ordered $\rightarrow$ fluid phase transition. Figure 2A shows

Fig. 2. Effect of MG_{Gdn} on the melting curve of DMPC vesicles. Vesicles composed of DMPC and of spin-labeled cholesterol (molar ratio 100:1) were prepared as described in the Materials and methods. To investigate the effect of MG_{Gdn} on such vesicles, a vesicle suspension (10 mg/mL in buffer 1) was mixed with an equal volume of MG_{Gdn} (0.7 mg/mL in buffer 1). (A) EPR signal of spin-labeled vesicles at 20 °C; at this temperature, the EPR signal for the MG_{Gdn}-vesicle complex is identical (not shown). (B) The spectral parameter h_{+1}/h_0 , where h_{+1} and h_0 are the intensities of the low-field and central components, respectively, of the EPR signal of spin-labeled cholesterol, is plotted as a function of temperature. ●, control liposomes; △, liposomes treated with MG_{Gdn}. EPR conditions: microwave power, 20 mW; modulation amplitude, 1 G.

a representative electron paramagnetic resonance (EPR) spectrum at 20 °C for DMPC vesicles labeled by 17- β -hydroxy-4',4'-dimethylspiro(5- α -androstane-3,2-oxazolidin)-3'-yloxy (spin-labeled cholesterol), and Figure 2B compares the temperature dependence of h_{+1}/h_0 for control vesicles and for vesicles into which MG_{Gdn} had been incorporated. We could not measure this ratio at temperatures higher than 32 °C owing to pronounced aggregation of the protein in the MG state at such temperatures. Thus, the plateau value of h_{+1}/h_0 at higher temperatures could not be measured, and the melting temperature of the membrane could not be determined accurately. Nevertheless, incorporation of MG_{Gdn} into the DMPC liposomes consistently shifted the melting curve 3 ± 1 °C toward a lower temperature, as observed in three independent experiments.

Perturbation of the lipid bilayer induced by incorporation of a polypeptide or protein may induce leakiness of lipid vesicles (Weinstein et al., 1981). We therefore examined the effect of incorporating MG_{Gdn} on the permeability of the DMPC vesi-

cles. If 4-trimethylammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (TMA-TEMPO) is trapped in lipid vesicles at a high concentration (e.g., 0.1 M), it displays a greatly broadened EPR signal owing to Heisenberg exchange interactions between unpaired electrons. As the probe escapes from the vesicles, the line width decreases and the amplitude of the EPR signal increases concomitantly, owing to dilution of the spin label as it is released into the bulk phase (Humphries & McConnell, 1974). Use of this technique allowed us to show that, as expected, there was almost no leakage from control DMPC vesicles in the presence or absence of native AChE. If vesicles were preloaded with TMA-TEMPO and then exposed to MG_{Gdn} , transient efflux of TMA-TEMPO occurred for several minutes after addition of the protein, resulting in release of 30–40% of the probe (Fig. 3), after which little further efflux occurred. Similar results were obtained with MG_{DTP} . Complete recovery of the TMA-TEMPO signal was obtained upon addition of Triton X-100. These results are in agreement with our observation that formation of the protein-liposome complex occurs within minutes, as monitored by the flotation gradient technique. Thus, either relatively large pores open concomitantly with fusion, but the lipid bilayer reseals almost completely within minutes, or else some of the vesicles are damaged irreversibly and release all their probe, whereas others remain intact.

Natural abundance ^{13}C NMR was used to analyze which structural elements of the lipid bilayer were perturbed by the interaction with MG_{Gdn} . Figure 4 shows the ^{13}C NMR spectra of control DMPC vesicles (Fig. 4A) and of DMPC vesicles in the presence of MG_{Gdn} (Fig. 4B). Upon addition of AChE at a 1:1 molar ratio (that is, one subunit dimer of AChE per liposome), the signal for the $COCH_2$ group of glycerol was found to shift ~ 13 Hz upfield, that for the CHO of glycerol was found to shift ~ 5 Hz upfield, and that for the $POCH_2$ group of choline was found to shift ~ 6 Hz downfield. No significant chemical shift was observed in the $(CH_2)_n$ signal of the fatty acid alkyl chains. These data show clearly that incorporation of the MG induces

Fig. 3. Effect of MG_{Gdn} on leakage of TMA-TEMPO from DMPC vesicles. Native AChE or MG_{Gdn} were added to a suspension of DMPC vesicles preloaded with 0.1 M TMA-TEMPO (dimer:vesicle ratio 7:1). Leakage of TMA-TEMPO was monitored by measuring the intensity of the EPR signal as a function of time at 25 °C. The maximum EPR signal intensity, corresponding to 100% leakage, was determined by adding Triton X-100 solution (final concentration 0.1%) to the vesicle suspension. Δ , control vesicles; $\circ$, vesicles treated with native AChE; $\bullet$, liposomes treated with MG_{Gdn} . EPR conditions were as in Figure 2.

Fig. 4. Natural abundance ^{13}C -NMR spectra of DMPC vesicles in the presence and absence of MG_{Gdn} . The concentration of lipid in the vesicle suspension was 30 mg/mL, and the dimer/vesicle ratio in the vesicle-protein complex was 1:1. In both cases, the vesicles were suspended in buffer containing 30% D_2O , in order to observe a deuterium signal for the field-frequency lock. Sample temperature was maintained at 28 °C. Chemical shifts are from the sodium salt of trimethylsilyl propionic acid. (A) Control vesicles. (B) Vesicles treated with MG_{Gdn} .

a perturbation in the head group region of the bilayer. Furthermore, the observation of a single chemical shift for each of these head group carbons suggests fast exchange, on an NMR time scale, between annular and free lipid molecules. Thus, this phenomenon, which is well documented for various membrane proteins (Chapman & Benga, 1984), is also found for AChE in the MG state in contact with the lipid bilayer.

Effect of the lipid bilayer on the bound protein

Comparison of the CD spectra in the far UV of MG_{Gdn} free in solution and complexed with DMPC vesicles revealed only slight differences. Similar data were obtained with MG_{DTP} (Fig. 5). Because no substantial changes were observed in the ellipticity at 222 nm, which is ascribed primarily to α -helices, we concluded that little change in α -helix content had taken place. Measurements of intrinsic protein fluorescence revealed that binding of

Fig. 5. CD spectra in the far UV of native AChE, MG_{Gdn} , and MG_{DTP} , in the presence and absence of DMPC liposomes. Protein concentration, 400 $\mu\text{g/mL}$; dimer/vesicle ratio, 1:1. $\blacktriangle$, native AChE; $\triangle$, native AChE in the presence of DMPC vesicles; $\blacklozenge$, MG_{DTP} ; $\lozenge$, MG_{DTP} in the presence of DMPC vesicles; $\bullet$, MG_{Gdn} ; $\circ$, MG_{Gdn} in the presence of DMPC vesicles.

both MG_{Gdn} and MG_{DTP} to the DMPC vesicles was accompanied by a 1-nm blue shift in the emission maximum from 340 to 339 nm (not shown). This shift could be due either to an overall reorganization of the environment of the indole chromophores, induced by the interaction with the liposome, or to insertion of one or a small number of tryptophan residues into a more hydrophobic environment, presumably the lipid bilayer. Overall, the two spectroscopic techniques do not indicate a major structural change as a result of complexing of the MG-AChE with the lipid bilayer.

Increased susceptibility to proteolytic digestion is one of the features of the MG state (Bychkova & Ptitsyn, 1993). We showed earlier that the MG state of *Torpedo* AChE produced by chemical modification is much more susceptible to trypsinolysis than native AChE (Dolginova et al., 1992). Here, this approach was used to compare the proteolytic susceptibility of a spin-labeled MG-AChE preparation either free in solution or complexed with DMPC vesicles. Labeling was carried out with 2,2,5,5-tetramethyl-4-(2-chloromercuriphenyl)-3-imidazoline-1-oxyl (HgR), and the preparation was preincubated at 36 °C for 1 h (see the Materials and methods) to ensure complete transformation to an MG state (Kreimer et al., 1994a). As expected (Kreimer et al., 1994b), the EPR spectrum of the MG state so produced (MG_{HgR}) is typical of an immobilized radical, whereas peptide fragments generated after 5 h of trypsinolysis display almost freely rotating spin label (Fig. 6A). It was thus possible to monitor the kinetics of trypsinolysis of MG_{HgR} by EPR spectroscopy. When MG_{HgR} was complexed with DMPC vesicles, it became somewhat less susceptible to proteolysis (Fig. 6B), but the decrease in the rate of digestion was not pronounced. This suggests that the HgR-containing segment—that is, the proteolytic fragment of AChE containing residues 222–242, including Cys 231, which is modified by HgR—is not in direct contact with the bilayer.

Separation and characterization of a liposome-associated polypeptide fragment

A difference in susceptibility to trypsinolysis between free MG_{Gdn} and that complexed with DMPC vesicles similarly can

Fig. 6. Effect of trypsinolysis in the presence or absence of DMPC vesicles on the EPR spectrum of spin-labeled AChE in an MG state. AChE was modified with HgR and converted to MG_{HgR} by heating for 1 h at 36 °C (see the Materials and methods). (A) MG_{HgR} before (upper trace, gain 8×10^5) and after (lower trace, gain 1×10^5) trypsinolysis for 5 h at room temperature. (B) Time course of trypsinolysis as followed by monitoring the increase in intensity of the EPR signal. $\bullet$, MG_{HgR} in solution; $\circ$, MG_{HgR} incorporated into DMPC vesicles. EPR conditions as in Figure 2.

be distinguished by SDS-PAGE (Fig. 7, lanes 2 and 3). In the preparation complexed with the liposomes, a pronounced band of apparent mass ~ 5 kDa appears after 2 h of trypsinolysis (lane 4) and is still present after 10 h (lane 5). Because this band is not observed in the absence of liposomes (lane 6), it most likely represents a segment of the protein that is sequestered in the lipid bilayer and is, therefore, protected against tryptic attack. Lane 7 shows the pattern of membrane-protected fragments of AChE incorporated into the vesicle; these were obtained by separating the liposomes from the rest of the tryptic digest by gel filtration on Sephacryl S-1000 (see the Materials and methods). Several heavier, weakly stained bands can be seen, but the principal component, obtained reproducibly, is at 5 kDa. This band was excised from the gel, and sequencing revealed the N-terminal sequences EKKPQELIDV and KPQELIDVEW, corresponding to sequences in the *T. californica* enzyme starting, respectively, at Glu 268 and Lys 270 (Schumacher et al., 1986). The apparent molecular weight of 5 kDa suggests that the tryptic fragments in question correspond to sequences of 48 and 46 amino acids, respectively, stretching from Glu 268 or Lys 270 to Lys 315. The question arises why this particular part of the polypeptide chain

Fig. 7. SDS-PAGE of MG_{Gdn} after trypsinolysis in the presence or absence of DMPC vesicles. Samples were incubated with trypsin (1 or 5%) in buffer 1 at 22 °C. Aliquots were removed at appropriate times. SDS-PAGE was performed on Tricine gels under nonreducing conditions (see the Materials and methods). Lane 1, MG_{Gdn} ; lane 2, MG_{Gdn} after exposure to trypsin (1%) for 30 min; lane 3, MG_{Gdn} complexed with DMPC vesicles after exposure to trypsin (1%) for 30 min; lane 4, MG_{Gdn} complexed with DMPC vesicles after exposure to trypsin (5%) for 2 h; lane 5, MG_{Gdn} complexed with DMPC vesicles after exposure to trypsin (5%) for 10 h; lane 6, MG_{Gdn} alone after exposure to trypsin (5%) for 10 h; lane 7, polypeptides retained in vesicles after exposure of MG_{Gdn} complexed with DMPC vesicles to trypsin (5%) for 10 h.

is protected and, presumably, is interacting with the lipid bilayer. It should also be noted that this polypeptide contains one bond, Arg 289–Phe 290, which should, in principle, be susceptible to tryptic cleavage. Visual inspection reveals that the polypeptide contains a hydrophobic stretch of 35 amino acids, from Leu 274 to Met 308. A hydropathy plot, made according to Kyte and Doolittle (1982) but employing a window of 7 (Eisenberg et al., 1984), reveals four significant hydrophobic sequences in *Torpedo* AChE (sequences a through d in Fig. 8). One of these sequences, that of peptide c, Leu 274–Met 308, is included in the residual polypeptide adhering to DMPC vesicles after trypsinolysis. This sequence is located primarily on the surface in the native protein (Fig. 9), whereas peptides a and d are partially buried, and peptide b is almost completely buried (not shown). Furthermore, peptide c contains 35 amino acids and is substantially longer than peptides a, b, and d, which contain 22, 22, and 27 amino acids, respectively. It can also be seen from Figure 9 that the hydrophobic sequence within Glu 268–Lys 315 is largely devoid of secondary structure.

Discussion

The water-soluble dimer of *Torpedo* AChE, generated from the glycosylphosphatidylinositol (GPI)-anchored form by solubilization with PI-specific phospholipase C (PIPLC), does not interact with liposomes (Futerman et al., 1985). In our present study, we show that conversion of the dimer to an MG, either by physicochemical means or by chemical modification, leads to its interaction with the lipid bilayer. Tryptic digestion followed by SDS-PAGE and sequencing provided strong evidence that this

Fig. 8. Hydropathy plot of *Torpedo* AChE. The sequence was analyzed using the GCG Package (subprogram plot structure and peptide structure) (Genetic Computer Group, Inc., Madison, WI, 1991). Hydrophobic sequences detected by this procedure are: a, residues 17–38; b, 138–159; c, residues 274–308; d, residues 429–455. The complete amino acid sequence of fragment c is shown over the hydropathy plot.

was due to the interaction of a single polypeptide with the liposome, and that the peptide in question, Glu 268–Lys 315, contained the most extended hydrophobic sequence in the protein, namely Leu 274–Met 308. Our findings raise two principal questions: (1) What is the nature of the conformational rearrangement that permits this specific interaction to occur? (2) What is the nature of the interaction of the polypeptide sequence in question with the lipid bilayer?

As already mentioned, Figure 9 shows that polypeptide c lies mainly on the surface of the protein in the native state, but that a small segment, Ser 291–Ile 296, threads under another sequence, Ser 235–Arg 244, which is primarily α -helical. One might expect a surface polypeptide to be hydrophilic. In fact, as pointed out by Branden and Tooze (1991), such polypeptides are often amphiphilic, with the hydrophilic residues pointing outward and the hydrophobic residues oriented primarily toward the interior. This is indeed the case for the polypeptide in question in the native state (Sussman et al., 1991). Thus, both its topology and the orientation of its hydrophobic residues preclude interaction of

Fig. 9. Three-dimensional structure of native AChE showing the localization of the peptide Glu 268–Lys 315 isolated after tryptic digestion of MG_{Gdn} complexed with DMPC vesicles. C_{α} carbons are color-coded in magenta, and Glu 268–Lys 315 is shown as a green ribbon. Residues Leu 274 and Met 308, which are the first and last amino acids in the hydrophobic sequence identified on the basis of the hydropathy plot, are also marked.

peptide c with the lipid bilayer when AChE is in its native conformation. Whether the MG state retains the tertiary fold of the native protein or undergoes substantial rearrangement to a different conformation or ensemble of conformations is the subject of controversy (Baldwin, 1991; Ptitsyn, 1992). However, our recent findings concerning the rearrangement of disulfide bonds in *Torpedo* AChE in the MG state (Eichler et al., 1994) render this controversy irrelevant, because, whatever its average conformation, the *Torpedo* AChE dimer in the MG state has enough flexibility to permit the sequence in question to make contact with the surface of the liposome.

It was already noted that the peptide that we have isolated and sequenced contains a substantially longer hydrophobic sequence than the other three hydrophobic peptides that we identified on the basis of hydropathy plots, being approximately the same length as the hydrophobic stretch shown to be responsible for insertion of cytochrome b_5 into the lipid bilayer (Bendzko et al., 1981). It has been suggested that the insertion sequence of cytochrome b_5 is highly structured and must have a compact conformation (Ozols, 1989). Nevertheless, its fold and orientation with respect to the bilayer are still controversial, despite the considerable research effort devoted to this problem (Ladokhin et al., 1992).

The amino acid sequence of the incorporated peptide contains only one Trp residue, Trp 279, which is within the hydrophobic stretch. It is likely that this tryptophan is responsible for the 1-nm blue shift observed in the maximum of intrinsic fluorescence, which would be caused by its insertion into a more hydrophobic environment. It cannot be excluded, however, that this small shift may reflect an overall reorganization of the environment of all fourteen Trp indole groups within the AChE monomer due to its interaction, in the MG state, with the DMPC vesicle.

Both the Chou-Fasman (Chou & Fasman, 1974) and Garnier-Osguthorpe-Robson (Garnier et al., 1978) algorithms indicated that peptide c had little tendency to form secondary structure. Furthermore, various attempts to produce models in which the polypeptide formed transmembrane helices (Lemmon & Engelman, 1994) or β -hairpins (Brown, 1993) were unsuccessful. It is also difficult to conceive how structures such as β -barrels

(Weiss et al., 1991) or α -helical bundles (Lemmon & Engelman, 1994) could arise, because the size of the individual subunits (approximate dimensions 50 Å) would produce severe steric constraints. The most attractive possibility appears to involve alignment of the polypeptide parallel to the surface of the liposome, with only shallow penetration into the hydrophobic region of the lipid bilayer. Such a possibility is in agreement with our own NMR data, and Gilbert and Baleja (1995), also on the basis of NMR measurements, have suggested such an interaction with cell membranes of a membrane-binding peptide from the C2 domain of factor VIII. Pouny et al. (1992) similarly provided evidence, based both on susceptibility to proteolysis and on spectroscopic studies employing fluorescent analogs, that the antimicrobial polypeptide dermaseptin displays relatively shallow penetration into the lipid bilayer. Figure 10 illustrates a plausible mode of interaction of the hydrophobic portion of peptide c with the lipid bilayer, in which most of the hydrophobic side chains penetrate the bilayer, whereas all the charged residues (seven negative and one positive) face outward.

As already mentioned, it has been suggested that proteins may be translocated across or inserted into membranes in the MG state (Bychkova et al., 1988). Christensen and Pain (1994) have suggested that "adsorption to the membrane requires interaction of the membrane surface with sites on the protein that are more readily accessed from the partially-folded than from the native protein." Thus, our MG preparations of AChE may serve as a simple model for studying this initial phase. Interaction of colicin A with cell membranes is believed to follow an "umbrella" model in which initial absorption is followed by slow, rate-limiting insertion of central helices into the membrane interior, and it has been suggested that this process involves an MG intermediate (van der Goot et al., 1991). Interaction of the channel-forming fragment of colicin E1 with neutral bilayers occurs preferentially at low pH, indicating that this interaction is hydrophobic rather than electrostatic (Shin et al., 1993). Thus, our data may mimic the first stage of this process, in which the hydrophobic face of the polypeptide is oriented parallel to the membrane surface.

Our findings may be of general relevance to the possible modes of anchoring of membrane proteins. Until recently, the

Fig. 10. Model showing possible interaction of peptide c (Leu 274–Met 308) with the lipid bilayer. The model was constructed manually, using Insight II (Biosym Technologies, 1993). Charged residues are color coded in yellow and other residues in blue. The bilayer lies below the dashed line.

only accepted modes of anchoring were via transmembrane peptide sequences (Lemmon and Engelman, 1994), or by covalent linkage to a lipid anchor, as is the case for native form of our *Torpedo* AChE dimer (Mehlert et al., 1993). The recent work on the 3D structure of prostaglandin H₂ synthase-1 (Picot et al., 1994) revealed a bona fide membrane protein that lacks either of these features. This raises the possibility that anchoring may occur via the type of "surface binding" we have described. Thus, our experimental system may serve as a model for studying this type of interaction.

Materials and methods

Materials

Torpedo electric organ was obtained from Marinus, Inc. (Long Beach, CA). DTP, Tris, Tricine, TPCK-treated trypsin (Type XIII), and soybean trypsin inhibitor (Type I-S) were from Sigma (St. Louis, MO), and spin-labeled cholesterol was from Syva (Palo Alto, CA). Gdn-HCl, ultrapure grade, was purchased from Schwarz/Mann Biotech (Cleveland, OH); TMA-TEMPO from Molecular Probes (Junction City, OR); and DMPC from Avanti Polar Lipids, Inc. (Alabaster, AL). HgR was synthesized as described (Volodarsky & Weiner, 1983). AChE was purified from electric organ tissue of *T. californica* by affinity chromatography after solubilization with PIPLC (Sussman et al., 1988).

Assay methods

AChE concentrations were determined and enzymatic activity measured as described (Kreimer et al. 1994a). Phospholipid concentrations were determined by phosphorus analysis (Bartlett, 1959).

Preparation of AChE in MG states

Gdn-HCl-induced unfolding of AChE was performed as follows: $1-3 \times 10^{-5}$ M AChE in 150 mM NaCl/10 mM Tris-HCl, pH 7.2 (buffer 1), was mixed, under stirring, with the appropriate amount of 8 M Gdn-HCl to a final concentration 1.2 M. After 2 h, Gdn-HCl was removed by gel filtration on a Bio-Gel P6 column (12×0.85 cm). The MG form of AChE so obtained was designated MG_{Gdn}.

Modification of AChE by DTP was performed as described (Dolginova et al., 1992). Unbound DTP was removed by gel filtration on Bio-Gel P6. The MG form of AChE so obtained was designated MG_{DTP}.

Modification of AChE by HgR was performed as follows: A 10^{-2} M HgR solution in acetonitrile was mixed, under stirring, with $1-3 \times 10^{-5}$ M AChE in buffer 1. The final concentration of the spin label was 10^{-4} M. After incubation for 1 h at 36 °C, to ensure complete conversion to an MG state similar to that produced directly by modification with DTP (Kreimer et al., 1994a), unbound spin label was removed by gel filtration on Bio-Gel P6. The MG form of AChE so obtained was designated MG_{HgR}.

Preparation of liposomes

Small unilamellar phospholipid vesicles were prepared by sonication. Briefly, dry DMPC (2–4 mg) was dissolved in 0.2 mL

CHCl₃/MeOH (2:1). The solvents were evaporated under a stream of nitrogen, and the lipid was put under vacuum for 1 h and then resuspended in 0.2 mL of buffer 1 by vortex mixing. This dispersion was then sonicated for 10–30 min in a bath-type sonicator (G 1125SP1 sonicator, Laboratory Supplies Co. Inc., New York, NY) until clear. Spin-labeled vesicles were prepared similarly, using a ratio of DMPC to spin-labeled cholesterol of 100:1. Electron microscopy, using negative staining with uranyl acetate, revealed a population of unilamellar vesicles of diameter 300–500 Å, in the range to be expected for unilamellar vesicles (Papahadjopoulos & Miller, 1967).

Large unilamellar vesicles were prepared as described (Hope et al., 1985). A dried film of lipid was hydrated in buffer 1 and dispersed by vortexing. The lipid suspension was freeze-thawed 5 times and extruded 10 times through two polycarbonate membranes with a 0.1-μm pore diameter (Nuclepore Corp., Pleasanton, CA).

Leakage measurements

Leakage experiments were performed according to Humphries and McConnell (1974). TMA-TEMPO-containing vesicles were prepared in 50 mM NaCl/10 mM Tris-HCl containing 100 mM spin label. The nonencapsulated TMA-TEMPO was removed from the liposome suspension by gel filtration on a Sephadex G-50 column (27×1 cm) equilibrated in buffer 1. Leakage of TMA-TEMPO from the vesicles at room temperature was followed by monitoring the central component of the EPR spectrum, using a Bruker ER200 D-SRC EPR-spectrometer. The maximal EPR signal intensity, corresponding to 100% leakage, was determined by adding Triton X-100 to a final concentration of 0.1%.

Binding of AChE to liposomes

To investigate the binding of AChE to liposomes, a flotation-gradient centrifugation technique was used (Futerman et al., 1985). Samples were prepared by mixing 100 μL of the appropriate AChE sample (2–3 mg/mL) with 100 μL of a DMPC liposome suspension (10 mg/mL lipid), both in buffer 1. The mixture was adjusted to a final volume of 0.7 mL by adding 2 M sucrose in buffer 1, to yield a final sucrose concentration of 1.2 M. This sample, in 1.2 M sucrose, was layered over a cushion of 0.5 mL of 2 M sucrose. A 0.5-mL aliquot of buffer 1 was then layered over the 1.2-M sucrose layer. The step gradients so obtained were centrifuged in an SW 50.1 rotor (50,000 rpm, 7 h, 4 °C) using a Beckman L8-70 centrifuge. After centrifugation, 0.2-mL fractions were collected and assayed for protein and phospholipid. Liposome concentration was calculated from the phosphorus content, on the assumption that the vesicles remain spherical and constant in lipid content. Protein concentration was determined by measuring intrinsic fluorescence, exciting at 280 nm, and measuring emission at 340 nm.

Optical spectroscopy

Absorption spectroscopy was performed in a Kontron Uvikon 940 spectrophotometer. Fluorescence measurements used a Shimadzu RF-540 spectrofluorometer. CD spectra were recorded in a Jasco J-500C spectropolarimeter, at 22 °C, using a cuvette with a path length of 0.2 mm. Spectra represent the av-

erage of five scans and are corrected to the baseline for the corresponding buffer. Data are expressed as the mean residue ellipticity, $[\Theta]$ (deg cm² dmol⁻¹).

Light scattering measurements

The sizes of AChE molecules and DMPC vesicles were measured by quasi-elastic light scattering (Berne & Pecora, 1976), using an apparatus constructed in-house (Kam et al., 1981). A Spectra Physics 165-4 argon laser was employed that generated an incident beam at 514.5 nm. A scattering angle of 90° was used. Thin-walled cylindrical cells (outer diameter 10 mm) were employed, and measurements were carried out at concentrations of 0.2–1.0 mg/mL protein and 0.5–2.0 mg/mL lipid, in buffer 1, at 22 °C. All samples were filtered before measurements, using 0.22 µm pore diameter Durapore filters (Millipore Corporation, Bedford, MA).

Tryptic digestion

An aliquot (0.3 mL) of MG_{Gdn} (1 mg/mL in buffer 1) was mixed with a 0.3-mL aliquot of a DMPC vesicle suspension (15 mg/mL in buffer 1). The protein/liposome ratio thus obtained was calculated to be approximately one dimer per liposome. After 5 min preincubation, trypsin (5% w/w of AChE, in buffer 1) was added, and digestion was carried out at 22 °C. Aliquots were removed at appropriate intervals, and proteolysis was terminated by addition of soybean trypsin inhibitor (2:1 w/w); samples were then frozen until taken for SDS-PAGE as described below. For exhaustive trypsinolysis, digestion was performed for 10 h at room temperature, and a fresh aliquot of trypsin was added after 5 h.

SDS-PAGE

SDS-PAGE was done according to Schagger and von Jagow (1987), using 16.5% separating, 10% spacing, and 4% stacking gels, with 0.2 M Tris, pH 8.9, as the anode buffer, and 0.1 M Tris/0.1 M Tricine, pH 8.25, as the cathode buffer, under non-reducing conditions. Before application to gels, samples were delipidated by chloroform-methanol extraction (Wessel & Flügge, 1984) and dissolved by boiling in sample buffer (2% SDS/10% glycerol/62.5 mM Tris chloride, pH 6.8).

Sequencing of polypeptides incorporated into the lipid bilayer

To characterize the polypeptide fragment(s) that remained bound to the liposome after trypsinolysis, the digest was passed over a Sephacryl S-1000 column (27 × 1 cm). The liposome fraction (together with adhering polypeptide fragments) came through in the excluded volume, whereas unbound polypeptides were retarded. The liposome fraction was pooled and delipidated (Wessel & Flügge, 1984), and residual polypeptides were analyzed by SDS-PAGE. Polypeptide bands selected for sequencing were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and processed for microsequencing (Matsudaira, 1989). Sequencing was performed on an Applied Biosystems model 475A sequencer equipped with an on-line model 120A phenylthiohydantoin analyzer, in the Biological Services Unit of the Weizmann Institute.

NMR measurements

Natural abundance ¹³C NMR spectra were recorded in 5-mm sample tubes on a Bruker AMX400 spectrometer operating at 161 MHz. Radiofrequency pulses of 7 µs were used, with a recycling time of 0.5 s. The liposomes were suspended in buffer containing 30% D₂O. The sample temperature was maintained at 28 °C. Chemical shifts are in ppm from the sodium salt of trimethylsilyl propionic acid. Chemical shifts of phospholipid carbons were assigned according to Hamilton and Small (1981) and adopting their nomenclature.

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