# Chemical Modification of *Torpedo* Acetylcholinesterase by Disulfides: Appearance of a "Molten Globule" State†

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ABSTRACT: Modification of *Torpedo californica* acetylcholinesterase (AChE) both by bis(1-oxy-2,2,5,5-tetramethyl-3-imidazolin-4-yl) disulfide (biradical) and by 4,4′-dithiopyridine, via a thiol–disulfide exchange reaction, was monitored by EPR and optical spectroscopy, respectively. Incubation with these reagents caused complete loss of enzymic activity. Treatment with glutathione of AChE modified by either of the two disulfides led to rapid release of the bound reagent with simultaneous regeneration of the single free thiol group of the enzyme. However, no concomitant recovery of catalytic activity was observed. SDS–PAGE showed that both the modified and demodified enzymes retained their structure as a disulfide-linked dimer. Circular dichroism revealed that modification of AChE by the disulfide agents with or without demodification by glutathione led to a complete disappearance of the ellipticity in the near-UV and to a much smaller decrease in ellipticity in the far-UV. The CD spectra observed are typical of the "molten globule" state of proteins. 1-Anilino-8-naphthalenesulfonate binding measurements and an enhanced susceptibility to trypsinolysis supported the supposition that chemical modification had transformed native AChE to a "molten globule".

Acetylcholinesterase (AChE)[1] is an externally-oriented membrane-bound enzyme whose principal physiological role is termination of neuronal transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (Barnard, 1974; Quinn, 1987). Many studies concerning the structural and functional properties of AChE have been performed with enzyme purified from the electric organic of the electric ray *Torpedo* since this tissue is a rich source of AChE (Silman & Futerman, 1987).

In AChE purified from *Torpedo californica*, there are six cysteines involved in intrachain disulfide formation, one cysteine, close to the COOH-terminus, which forms an intersubunit disulfide, and yet another, $Cys^{231}$, whose thiol group remains free (MacPhee-Quigley et al., 1986). Whereas the cysteines involved in disulfide bond formation are conserved, $Cys^{231}$ is unique to *Torpedo* AChE (Gentry & Doctor, 1991).

It was recently shown that *T. californica* AChE can be inhibited by a repertoire of sulfhydryl reagents (Steinberg et al., 1990). The kinetics of inhibition were pseudo-first-order, as would be expected from inhibition of a single class of sites, most likely the single thiol group of $Cys^{231}$ alluded to above; the kinetics of inhibition were, however, much slower than expected for reaction of the various sulfhydryl reagents tested with a free thiol group. Edrophonium, a quaternary ion which is a powerful competitive inhibitor of AChE, strongly retarded the rate of irreversible inhibition of the *Torpedo* enzyme by two of the sulfhydryl reagents tested. These data, taken together, suggest that $Cys^{231}$ is located in the interior of the protein molecule, in proximity to the active site (Steinberg et al., 1990). The recent solution of the three-dimensional structure of *Torpedo* AChE showed that these conclusions were basically correct. The catalytic triad is located close to the bottom of a deep and narrow gorge leading down to the active site from the surface of the catalytic subunit, and the thiol group of $Cys^{231}$ is buried within the protein ca. 8 Å from the $O_\gamma$ of the active-site serine, $Ser^{200}$ (Sussman et al., 1991).

The existence of a free thiol group, close to the active site, within the interior of the protein, suggested its value as a site for insertion of a "reporter" group. Such a "reporter" might provide information concerning its immediate environment, and about the effect on this environment of various perturbants, such as quaternary ligands and denaturants. Typical reporter groups would include fluorescent labels (Lakowicz, 1983) and spin-labels (Morrisett, 1976; Chignell, 1984). Although fluorescent probes have been used extensively to study the active site of AChE, whether in the form of noncovalent inhibitors (Mooser et al., 1972; Shinitzky et al., 1973) or incorporated into organophosphates (Berman et al., 1980; Amitai et al., 1982), little use has been made of spin-labels and probes in studies on AChE. Spin-labeled bisquaternary ligands were used to study their interaction with *Torpedo* AChE (Wee et al., 1976), and an organophosphate containing a monocyclic spin-label was used to label the active-site serine of AChE from electric eel (Morrisett & Broomfield, 1972). The mobility of the spin-label in the organophosphoryl conjugate was high, indicating that immobilization of the radical was not extensive, whereas the EPR spectrum of AChE labeled with bisquaternary spin probes was typical for highly immobilized radicals and these ligands were effective inhibitors of AChE ($I_{50}$ = 0.2–3 μM).

It is well-known that disulfides participate in thiol–disulfide exchange reactions with thiols (Brocklehurst, 1979; Creighton, 1989), and this was most probably the basis for the slow inhibition of *Torpedo* AChE by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), since the same reagent reacted rapidly and

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[1] Abbreviations: AChE, acetylcholinesterase; biradical, bis(1-oxy-2,2,5,5-tetramethyl-3-imidazolin-4-yl) disulfide; DTP, 4,4′-dithiodipyridine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSH, glutathione; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy; NEM, *N*-ethylmaleimide; ANS, 1-anilinonaphthalene-8-sulfonate; CD, circular dichroism; Gdn-HCl, guanidine hydrochloride.

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stoichiometrically with the denatured protein (Steinberg et al., 1990).

The stable spin-label reagent biradical, in which two nitroxyl groups are linked by a disulfide bond, has been shown to take part in thiol–disulfide reactions with the SH–groups of proteins and low molecular weight thiols (Khramtsov et al., 1989; Weiner et al., 1991). It seemed, therefore, ideally suited for use as a spin-label directed to the thiol group of $Cys^{231}$ in *Torpedo* AChE. In the following, we present a study of the reversible modification of *Torpedo* AChE by biradical, and employ a number of physicochemical techniques to characterize the products of modification and demodification. By use of circular dichroism, ANS binding, and susceptibility to proteolysis, we provide evidence that chemical modification of $Cys^{231}$ has produced a "molten globule" state of the enzyme.

## MATERIALS AND METHODS

### *Materials*

Acetylcholinesterase was purified from electric organ tissue of *Torpedo californica* by affinity chromatography subsequent to solubilization with phosphatidylinositol-specific phospholipase C (Sussman et al., 1988). The ratio $OD_{280}/OD_{260}$ for the purified enzyme was >1.6.

Biradical (RS–RS) and monoradical (RS) were synthesized as described previously (Khramtsov et al., 1989). 4,4′-Dithiodipyridine was purchased from Sigma (St. Louis, MO). Stock solutions of the disulfides (0.1–0.5 M) were prepared in acetonitrile or in DMSO, and stored at −20 °C.

The stable radical TEMPO and *N*-ethylmaleimide were purchased from Aldrich (Milwaukee, WI), and [$^3$H]acetylcholine iodide was from New England Nuclear (Boston, MA). ANS (magnesium salt), acetylthiocholine iodide, DTNB, TPCK-treated trypsin, bovine serum albumin, and glutathione were all purchased from Sigma. Gdn-HCl (Ultra Pure) was from Schwurz/Mann Biotech (Cleveland, OH).

### *Methods*

*Modification of AChE by Disulfides and with NEM.* One milliliter of *Torpedo* AChE [$10^{-5}$–($3 \times 10^{-5}$) M in 50 mM sodium phosphate/0.1 M NaCl, pH 7.0] was treated with either 50 μL of 0.1 M biradical in acetonitrile or 50 μL of 0.02 M DTP in either acetonitrile or DMSO. The final concentrations of the disulfides in the reaction mixture were 5 mM for biradical and 1 mM for DTP. NEM was diluted in acetonitrile (0.1 M) and added to the reaction mixture to a final concentration of 5 mM. After 4–5 h of incubation at room temperature, when ca. 70–80% inhibition of AChE activity had been achieved, unbound reagent was removed by gel filtration on a Bio-Gel P4 column which had been preequilibrated with 0.1 M NaCl/50 mM phosphate, pH 7.0. The eluant was monitored at 280 nm, and the protein peak was concentrated in a Centricon-30 microconcentrator to 0.3–0.5 mL. The samples of modified enzyme obtained were stored at 4 °C until use.

*Glutathione Treatment of Modified AChE.* Glutathione was added in the same buffer to the modified enzyme [$10^{-5}$–($5 \times 10^{-5}$) M protein] to a final concentration of 1 mM thiol in the reaction mixture followed by incubation for 30 min. Free GSH was removed by gel filtration on Bio-Gel P4. The repeated modification with biradical, after GSH treatment, was performed in the same way as the initial modification, and excess free radical was again removed by gel filtration.

*Kinetic Measurement of AChE Inactivation by Disulfides.* The reaction mixture contained $10^{-7}$ M AChE and a 1–10 mM concentration of the appropriate disulfide, either biradical or DTP, in 0.1 M NaCl/50 mM phosphate, pH 7.0, and incubation was at room temperature. During incubation, aliquots were diluted 100-fold into the same phosphate buffer with addition of 0.01% gelatin, and AChE activity was measured either radiometrically (Johnson & Russell, 1975) or spectrophotometrically using acetylthiocholine as substrate (Ellman et al., 1961).

*Electron Paramagnetic Resonance Measurements.* EPR spectra were measured on a Varian E-12 spectrometer operating at X-band frequency with 100-kHz magnetic field modulation at room temperature in a 200-μL flat cell. The concentration of radical covalently bound to AChE was determined by double-integration of the EPR spectra, using TEMPO for calibration.

*ANS Binding Measurements.* Aliquots of 5 mM ANS in acetonitrile were added to the solutions of AChE previously modified with biradical or exposed to various concentrations of Gdn-HCl. After 10 min, ANS fluorescence was monitored, exciting at 390 nm and measuring the emission at 490 nm. The AChE concentration was ca. 4 μM, and ANS was added in 25-fold molar excess.

*Determination of Protein Concentration.* Protein concentrations were measured by a microplate modification of the Bradford method (Bradford, 1976). The concentration of AChE is expressed as the concentration of active sites, assuming a subunit molecular weight of 65 000 (Sussman et al., 1988).

*Tryptic Digestion of Native and Biradical-Modified AChE.* Native or modified AChE (1 mg/mL in 50 mM phosphate buffer, pH 7.0) was treated with trypsin (1% w/w) at room temperature. Aliquots were removed at appropriate intervals and frozen until taken for SDS–PAGE as described below.

*Electrophoresis.* Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on 5–15% (w/v) acrylamide gradient slab gels [according to Futerman et al. (1985)] employing a Bio-Rad minigel system. The gels were stained with Coomassie brilliant Blue R.

*Optical Spectroscopy.* Absorption spectroscopy was performed using a Kontron Uvicon 940 spectrophotometer. Fluorescence measurements utilized a Shimadzu RF-540 spectrofluorometer. Circular dichroism spectra were recorded with a Jasco J-500C spectropolarimeter, in 1-mm or 1-cm path-length cuvettes, at room temperature. Data are expressed as the mean residue ellipticity, $[\theta]_{222nm}$ (deg cm$^2$ dmol$^{-1}$).

## RESULTS AND DISCUSSION

*Kinetics of AChE Inhibition by Disulfides.* It was shown earlier (Steinberg et al., 1990) that a number of sulfhydryl reagents are able to inhibit *Torpedo* AChE, and it was suggested that the reaction of the free thiol group of $Cys^{231}$ with these reagents is responsible for the observed inhibition. The spin-label biradical (for its chemical formula, see Figure 3A, inset) has been shown to participate in thiol–disulfide exchange reactions with the sulfhydryl groups of proteins (Khramtsov et al., 1989); since DTNB was one of the reagents which inhibited *Torpedo* AChE, we were encouraged to examine the possibility of a similar reaction between biradical and AChE. It may be assumed that such an exchange reaction between a disulfide (RS–SR) and the free SH–group of $Cys^{231}$ of *Torpedo* AChE would occur as

$$\text{RS–SR} + \text{AChE–SH} \rightarrow \text{AChE–S–SR} + \text{RSH} \quad (1)$$

In the process, the biradical molecule should, accordingly, be converted into two monoradical species: one being a mixed

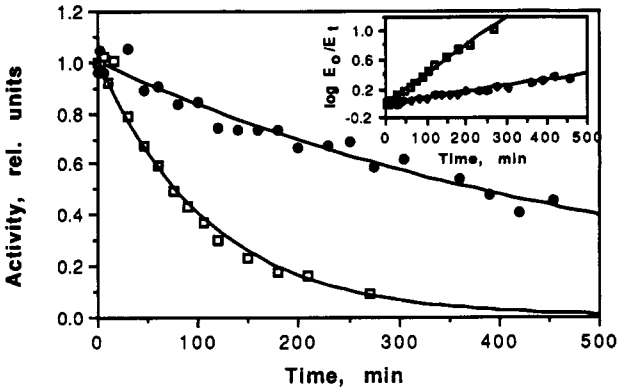


FIGURE 1: Kinetics of inactivation of acetylcholinesterase by biradical and by DTP. AChE ($10^{-7}$ M) was incubated with 6 mM biradical (●) and with 2.5 mM DTP (□) in 50 mM phosphate buffer, pH 7.0, at room temperature, and enzyme activity was monitored as a function of time. Inset: Semilogarithmic plot of the same kinetic data. ($E_0$, activity at time zero; $E_t$, activity at time $t$).

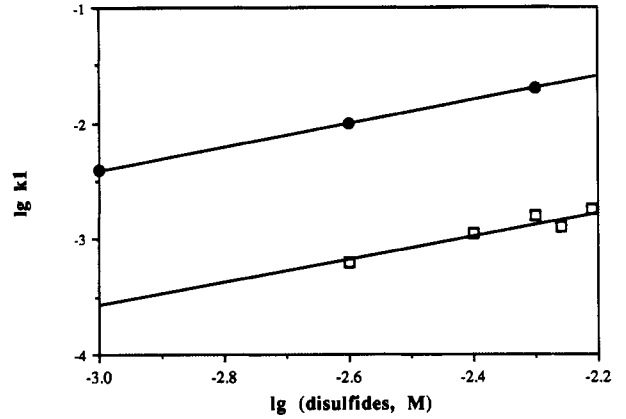


FIGURE 2: Apparent order of the inactivation reaction with respect to disulfide concentration. Pseudo-first-order rate constants for inactivation of AChE by biradical and DTP were calculated from data similar to those presented in Figure 1 (inset) which were obtained at various concentrations of the two disulfides. The plots show a double-logarithmic plot of the rate constants vs the concentration of reagent. (□) Biradical; (●) DTP.

disulfide with the thiol group of $Cys^{231}$ and the other a free thiol. In parallel, we examined the modification of AChE by another disulfide reagent, 4,4'-dithiodipyridine, which served as an internal control for biradical in our study. We first measured the kinetics of inactivation of AChE by both these disulfides. For calculations of the kinetic constants, we used an approach suggested previously (Levy et al., 1963). Figure 1 shows that both biradical and DTP inhibit AChE activity with pseudo-first-order kinetics, as was shown earlier (Steinberg et al., 1990) for DTNB as well as for a number of other sulfhydryl reagents. The rate constant measured for inactivation by DTP is approximately the same as those observed for monobromobimane and *N*-ethylmaleimide and considerably higher than that measured for DTNB. The rate constant for biradical was intermediate between those observed for DTP and DTNB. Monoradical (final concentration, 5 mM) had no effect on AChE activity under the same conditions. The order of reaction with respect to the disulfide concentration (Figure 2) was determined from the slopes of curves 1 and 2 in Figure 1 (inset) according to Levy et al. (1963). For both disulfides, the order of reaction calculated was 1 ± 0.1, which is consistent with inactivation by reaction of 1 mol of disulfide with 1 mol of AChE active sites. Taking into account the high specificity of the thiol–disulfide reaction (Ziegler, 1985), it may be assumed that thiol–disulfide exchange occurs

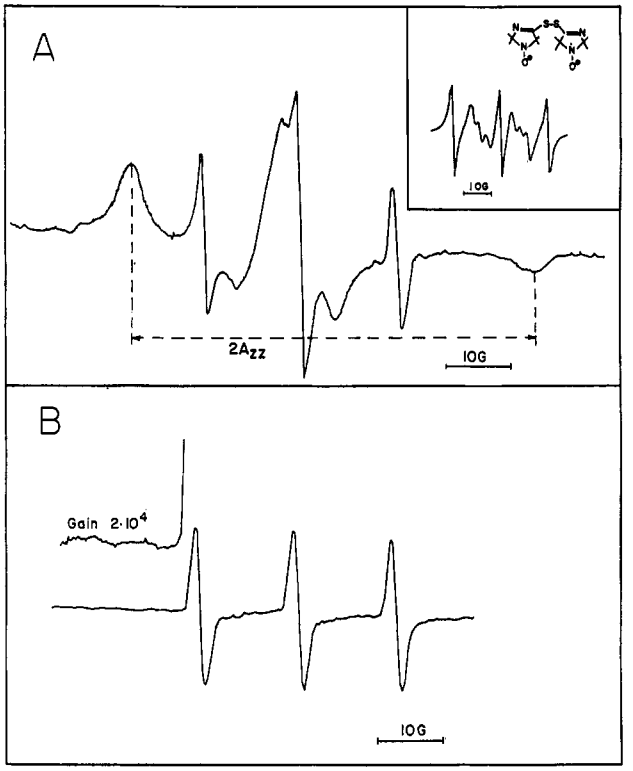


FIGURE 3: EPR spectrum of the AChE–biradical conjugate. Modification was performed as described under Materials and Methods. The concentration of conjugate was $0.24 \times 10^{-5}$ M in 50 mM phosphate buffer/0.1 M NaCl, pH 7.0. (A) EPR conditions: microwave power, 20 mW; modulation amplitude, 2 G; gain, $2 \times 10^4$. Inset: EPR spectrum of $10^{-4}$ M biradical in 10 mM phosphate buffer, pH 7.5. EPR conditions: microwave power, 20 mW; modulation amplitude, 1 G; gain $10^3$. (B) EPR spectrum of conjugate 5 min after addition of 1 mM glutathione. EPR conditions were the same as in (A), but the gain was $3.2 \times 10^3$.

between the disulfides and the –SH group of $Cys^{231}$, to yield a mixed disulfide with both biradical and DTP.

*EPR Labeling of AChE.* Figure 3A shows the EPR spectrum of AChE inhibited with biradical. Such a spectrum ($2A_{zz} = 64$ G) is typical for an immobilized radical (Morrisett, 1976). The spectrum of unbound biradical in aqueous solution is given in the inset. A signal corresponding to a small amount of unbound monoradical (less than 5%) could be detected. This might be due either to a contribution from weakly immobilized spin-label bound covalently or noncovalently to the enzyme or to a small amount of free monoradical. The stoichiometry calculated for the immobilized free radical, $n = 0.92 \pm 0.2$ per subunit, is in good agreement with modification of the single free thiol of $Cys^{231}$.

Addition of a 1–2-fold molar excess of reduced glutathione to the modified enzyme transformed the EPR spectrum (Figure 3B). The broad peak, corresponding to the covalently bound spin-label, disappeared completely, and a large increase was seen in the magnitude of the sharp peaks which are characteristic of the unbound monoradical. This may be ascribed to thiol–disulfide exchange between the reduced glutathione and the monoradical–AChE mixed disulfide. Under the experimental conditions employed, release of radical from the enzyme is quite fast, occurring within minutes. It may be noted that measurement of the increase in concentration of unbound radical, using the EPR spectrum of a TEMPO solution of known concentration for calibration, allowed us to make an independent estimate of the degree of modification of AChE. By this method, too, it was found that the degree of modification approached 1 mol of biradical/mol of subunit.

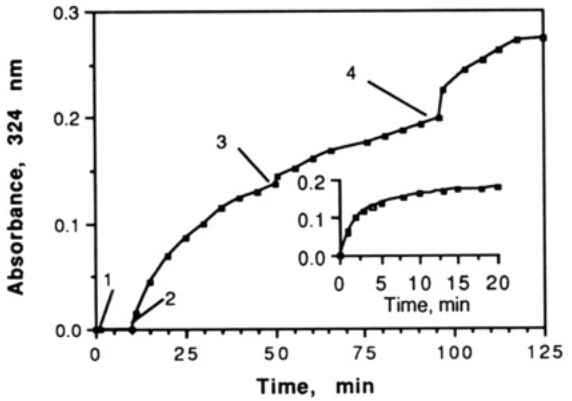


FIGURE 4: Titration with glutathione of DTP-modified AChE. The concentration of the DTP–AChE conjugate was $0.19 \times 10^{-4}$ M in 50 mM phosphate buffer/0.1 M NaCl, pH 7.0. Arrows show the times at which GSH was added to final concentrations in the reaction mixture of $10^{-5}$ (1), $5 \times 10^{-5}$ (2), $10^{-4}$ (3), and $3 \times 10^{-4}$ M (4). Inset: Addition of 1 mM GSH to $1.1 \times 10^{-5}$ M DTP–AChE conjugate.

*Modification of AChE by DTP.* As in the case of AChE modified with biradical (Figure 3B), we were able to demonstrate spectrophotometrically the release of the free thiol from the 4-thiopyridone–AChE mixed disulfide by addition of GSH as a result of the thiol–disulfide exchange reaction. This was done after excess DTP, as well as the 4-thiopyridone produced in the initial modification, had been removed by gel filtration. Figure 4 presents the results of titration of the AChE–4-thiopyridone conjugate with increasing concentrations of GSH. One can see the complete release of free thiol from mixed disulfide after addition of a 5-fold molar excess of GSH over AChE concentration (Figure 4). The inset of Figure 4 shows the fast kinetics of appearance of free 4-thiopyridone after addition of a 100-fold molar excess of GSH to the conjugate. The progress of the reaction was followed spectrophotometrically by monitoring the appearance in solution of the monosulfide at 324 nm (Grassetti & Murray, 1967). If we assume that the residual activity (approximately 15%) could be attributed only to the unmodified enzyme, we can evaluate the concentration of modified SH– groups of AChE, and there was good agreement between the estimated concentration of the conjugate ($0.85 \times 10^{-5}$ M) and the amount of 4-thiopyridone released ($0.92 \times 10^{-5}$ M).

*Properties of AChE Demodified by GSH.* The data presented above showed clearly that both monoradical and 4-thiopyridone were released almost quantitatively upon addition of excess GSH. We assumed, therefore, that the inactivation of AChE would also be reversed concomitantly. This was not, however, the case. GSH treatment failed to produce any substantial recovery of AChE activity. This is in contrast to the recovery of 90% of the control activity upon similar treatment with GSH of NADPH–cytochrome P-450 reductase which had been previously modified with and inactivated by biradical (Yelinova et al., 1989). In control experiments, we have shown that subjection of nonlabeled enzyme to the same procedures utilized in the modification studies, viz., separation from excess free radical by gel filtration and concentration in the Centricon microconcentrator, does not diminish enzymic activity by more than 10%. Furthermore, neither GSH alone nor monoradical has any effect on AChE activity.

Another possibility considered was that either the initial modification or the ensuing demodification had produced an inactive monomeric form of AChE. SDS–PAGE did not reveal that any significant change had occurred as a result of these procedures. After treatment with both biradical and

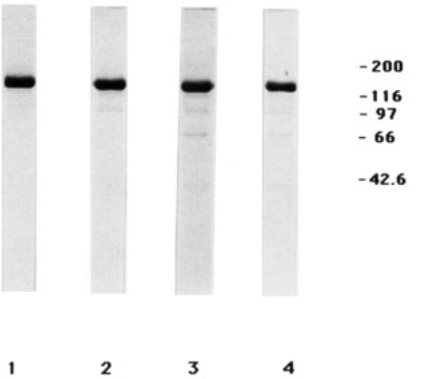


FIGURE 5: SDS–PAGE of the DTP–AChE and biradical–AChE conjugates. Lane 1, native AChE; lane 2, biradical–AChE conjugate; lane 3, biradical–AChE conjugate after demodification with GSH; lane 4, DTP–AChE conjugate. The positions of molecular weight markers are indicated, with molecular weights expressed $\times 10^{-3}$.

DTP, most of the AChE migrates as a dimer, with a small amount of monomer also present, whether in the control, after modification, or subsequent to demodification (Figure 5). After treatment with either of the two disulfides, we observed the appearance of a small band with a mobility intermediate between those of the dimer and the monomer. Although the origin of this band is unclear, its amount is too small to account for our observations.

Another possible reason we considered for the irreversible inactivation observed was the formation of a new intersubunit disulfide bridge. We determined, by titration with DTNB subsequent to denaturation in 6 M Gdn-HCl (Steinberg et al., 1990), the number of free sulfhydryl groups in the modified enzyme both before and after demodification with GSH. The modified sample, the enzymic activity of which had been inhibited 80%, possessed ca. 14% of the free SH groups of the control sample. After demodification with GSH, the number of free SH groups approached 80% of the value for the native enzyme, strongly supporting the hypothesis that most of the SH groups are free after GSH treatment.

We have also shown that demodified enzyme can take part in the thiol–disulfide exchange reaction with biradical a second time, again demonstrating the presence and availability of the free SH groups in the demodified enzyme. Thus, demodified AChE was freed of excess GSH by gel filtration on Bio-Gel P4, and reacted with biradical under the same conditions as had been employed for the native enzyme. The EPR spectrum of AChE which had been relabeled with biradical subsequent to demodification was similar to that presented for modification of native AChE in Figure 3A. Similarly, addition of a 100-fold molar excess of GSH to remodified enzyme produces a spectrum corresponding to free monoradical, similar to that presented in Figure 3B (data not shown). Using the same approach described above, we determined the degree of modification of AChE by measuring the peak intensity of the unbound radical generation by GSH treatment. It was found that ca. 0.8 mol of monoradical had been bound per mole of catalytic subunit.

As already mentioned, release of the low-molecular-weight thiol, upon addition of GSH, was rather rapid, occurring on a time-scale of minutes, as compared to a time-scale of hours required for inactivation of AChE by the corresponding disulfides, even though, in both cases, a similar reaction, viz., thiol–disulfide exchange, was occurring. Since the polar disulfide DTNB reacts very slowly with native AChE (Steinberg et al., 1990), it may be concluded that interaction with biradical or DTP changes the structure of the protein and thus leads to increased accessibility of the buried SH– group

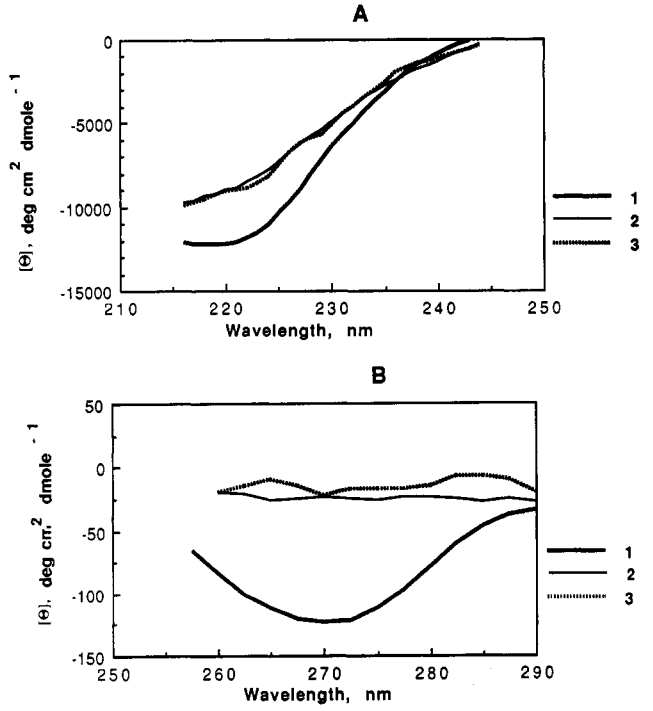


FIGURE 6: CD spectra of native AChE and of the biradical–AChE conjugate. (A) Native (1), biradical–AChE conjugate (2), and biradical–AChE conjugate demodified with GSH (3). Protein concentrations were 2.3, 2.7, and 0.73 $\mu$M, respectively, in 50 mM phosphate buffer, pH 7.0. (B) CD spectra of the same samples in the near-UV. Protein concentrations were 6.6, 2.4, and 0.73 $\mu$M, respectively, in 50 mM phosphate buffer, pH 7.0.

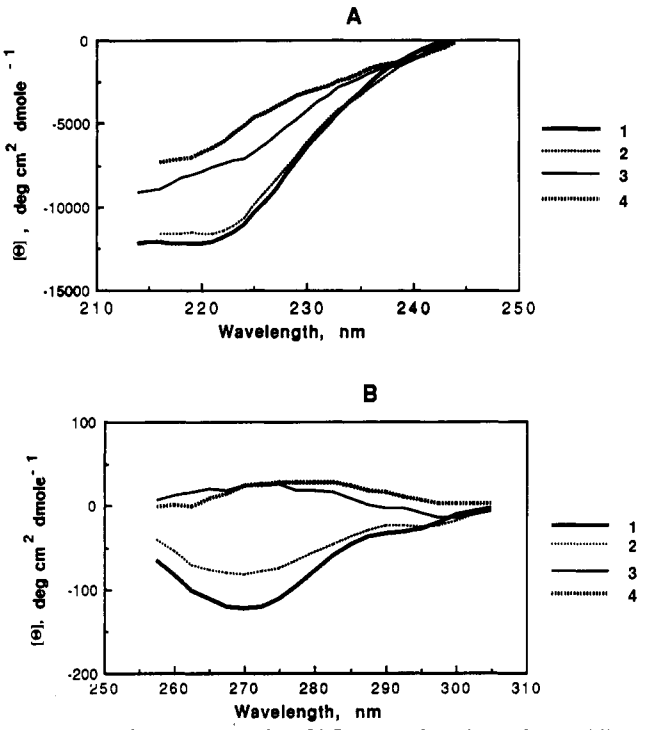


FIGURE 7: CD spectra of AChE as a function of guanidine hydrochloride concentration. (A) CD spectra were measured at protein concentrations of 6.6 $\mu$M in 50 mM phosphate buffer, pH 7.0. (1) 0 M Gdn-HCl; (2) 0.6 M Gdn-HCl; (3) 1 M Gdn-HCl; (4) 3 M Gdn-HCl. (B) CD spectra of the same samples in the near-UV. Protein concentrations were 2.3 $\mu$M, in 50 mM phosphate buffer, pH 7.0.

of Cys[231] (Sussman et al., 1991) to polar molecules such as glutathione and DTNB.

*CD Studies of Native and Modified AChE.* To study the possible influence of modification of AChE by biradical and by DTP on its structure, we have used circular dichroism (CD), a technique which is very sensitive to the structure of proteins (Steinberg et al., 1989; Imbert et al., 1990; Takrama & Graves, 1991). CD measurements revealed that the disulfide-modified enzyme differs significantly from the native enzyme. The broad peak of ellipticity in the region of 270 nm disappears completely, and does not reappear upon addition of GSH (Figure 6B). The ellipticity in the far-UV also decreased upon modification with the disulfide reagents and, as at 270 nm, did not revert to the "native" spectrum upon addition of GSH (Figure 6A). It is known that Gdn-HCl, a commonly used denaturant, can influence the CD spectra of proteins (Morrisett & Broomfield, 1971; Batra & Uetrecht, 1990; Wetterau et al., 1991). As shown in Figure 7A, Gdn-HCl gradually reduces the ellipticity in the vicinity of 220 nm as its concentration is increased, and there is a complete collapse of the CD spectrum in the near-UV region (Figure 7B). Comparison of the spectrum of modified AChE presented in Figure 6 with the series of curves presented in Figure 7A,B suggests that modification by biradical has produced a degree of unfolding comparable to that produced by 0.6–1 M Gdn-HCl (Figure 7). An estimation of the $\alpha$-helical content of our samples was performed according to Batra et al. (1990), and is presented in Table I. It is worth mentioning that the $\alpha$-helical content of the native enzyme, as estimated from our data, is in good agreement with the figures estimated by others (Manavalan et al., 1985; Sussman et al., 1991).

The CD data presented above suggest that the chemically modified AChE (as well as the enzyme which had been subsequently demodified with reduced glutathione) possesses a structure which has lost almost all its tertiary structure, as judged by the very low ellipticity in the near-UV, while maintaining a substantial part of its secondary structure, as judged by the relatively small change in ellipticity in this region. Modification with DTP produced CD changes similar to those produced by biradical (not shown). It was earlier shown that chemical modification of an essential cysteine sulfhydryl group in rhodanese triggered an increased protein flexibility and increased exposure of hydrophobic surfaces (Horowitz & Criscimagna, 1988).

Table I: Mean Residue Ellipticity at 222 nm of Native AChE, of AChE Modified with Biradical, and of AChE at Various Guanidine Hydrochloride Concentrations

| sample | $[\theta_{222}]$ (deg cm$^2$ dmol$^{-1}$) | $\alpha$-helix (%) | % of control |
|---|---|---|---|
| acetylcholinesterase | −12100 | 33 | 100 |
| AChE in 0.6 M guanidine | −11586 | 32 | 97 |
| AChE in 1 M guanidine | −7459 | 20 | 61 |
| AChE in 3 M guanidine | −6122 | 16 | 48 |
| AChE modified with biradical | −8594 | 23 | 70 |
| AChE modified with biradical and demodified with GSH | −8953 | 24 | 73 |

Changes in the CD spectrum of the type which we have observed are a principal criterion for ascribing a "molten globule" structure to a protein (Dolgikh et al., 1981; Goto et al., 1990; Christensen & Pain, 1991; Ewbank & Creighton, 1991). We sought, therefore, additional experimental evidence to support such a possibility.

The fluorescence of the amphiphilic probe ANS increases upon binding to hydrophobic parts of proteins; the intensity of ANS fluorescence is significantly higher for the "molten globule" state than both for the native and for the fully unfolded states (Semisitnov et al., 1987; Goto & Fink, 1989; Ptitsyn et al., 1990). Figure 8 shows that modification of AChE with biradical results in a substantial (ca. 10-fold) increase in ANS

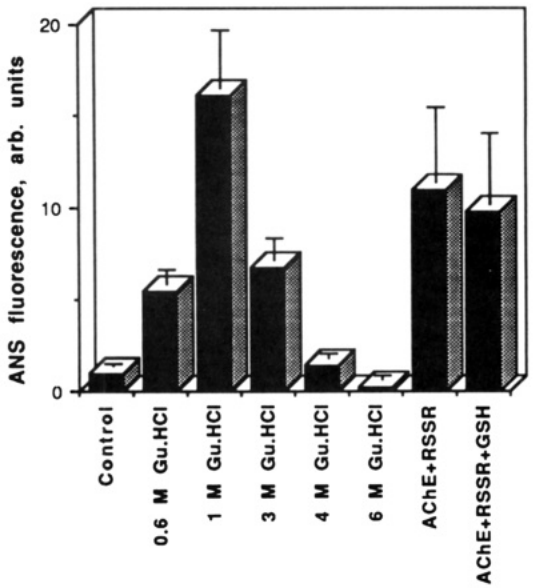


FIGURE 8: ANS binding by native, denatured, and modified AChE. ANS fluorescence of the various samples was measured employing $\lambda_{ex}$ = 390 nm and $\lambda_{em}$ = 490 nm. The protein concentration was 4 $\mu$M, and ANS was present in 25-fold molar excess. Measurements were made after 10-min incubation with ANS at room temperature. The data are presented as the relative fluorescence intensity for (from left to right) native AChE, AChE exposed to increasing concentrations of Gdn-HCl, biradical-modified AChE, and biradical-modified AChE after demodification with reduced glutathione. Data points are mean ± SD of three experiments.

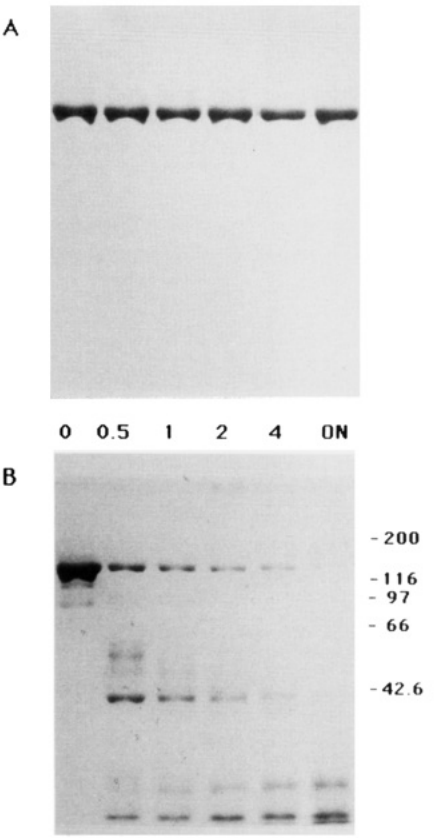


FIGURE 9: Time dependence of tryptic digestion of native and biradical-modified AChE. Samples were incubated with trypsin at room temperature, as described under Materials and Methods; aliquots were removed at appropriate times (0; 0.5 h; 1 h; 2 h; 4 h; ON) and frozen until taken for electrophoresis. SDS–PAGE was performed under nonreducing conditions as described under Materials and Methods. The positions of molecular weight markers are indicated, with molecular weights expressed × $10^{-3}$. (A) Native AChE; (B) biradical-modified AChE.

fluorescence, which is maintained after demodification. If the ANS fluorescence of the unmodified enzyme is measured as a function of the concentration of Gdn-HCl, maximum fluorescence is obtained in ca. 1 M Gdn-HCl. (The effect of Gdn-HCl was not due to its ionic strength since NaCl in a concentration range of 10 mM–6 M had no effect on the ANS fluorescence of AChE.) Similar results to those which were obtained with biradical were also obtained by modification with DTP (not shown).

It has been reported that proteins in the "molten globule" state display enhanced susceptibility to proteolytic attack as compared to the native protein (Martin et al., 1991). Figure 9 shows a comparison of the tryptic digestion of native and biradical-modified AChE. Under the experimental conditions employed, native AChE is almost completely stable to trypsinolysis, whereas scission of the treated enzyme can be observed as early as 30 min and digestion is complete within several hours.

The "molten globule" state is produced in various ways; these include thermal denaturation, acidification, exposure to moderate concentrations of denaturing agents, or removal of metal ions by chelating agents (Dolgikh et al., 1981; Christensen & Pain, 1991; van der Goot et al., 1991). We have shown, in the case of *Torpedo* AChE, that it can be produced also by chemical modification of a buried sulfhydryl group and that the effect is not reversed by demodification. Both biradical and DTP modify AChE by a thiol–disulfide exchange reaction (eq 1). As mentioned already, *Torpedo* AChE can be modified by a variety of sulfhydryl reagents, including the alkylating agent *N*-ethylmaleimide (Steinberg et al., 1990). Incubation of $5 \times 10^{-5}$ M AChE with 5 mM NEM for 5 h at room temperature resulted in ca. 80% inhibition of enzymic activity. *Torpedo* AChE so modified by NEM possesses a CD spectrum very similar to those produced by modification with the disulfides: almost complete collapse of the CD spectrum in the near-UV and a much smaller decrease in ellipticity (ca. 30%) in the far-UV (not shown). We observed also a large increase [(10.6 ± 1.3)-fold compared to native AChE] in ANS fluorescence in the presence of NEM-treated AChE, and increased susceptibility to trypsinolysis of the NEM conjugate, relative to native AChE, similar to that seen for enzyme modified by disulfides (not shown). These data indicate that NEM-modified enzyme, too, exists as a "molten globule". The "molten globule" state can thus appear as a result of chemical modification of AChE by various SH reagents.

As already mentioned, demodification by glutathione of AChE previously treated with biradical or DTP failed to restore the enzymic activity, and the demodified enzyme still remained a "molten globule". It is known that proteins in the "molten globule" state display a tendency to aggregate due to increased exposure of hydrophobic surfaces (Jaenicke, 1991; Christensen & Pain, 1991). Recent experiments have shown that modification of AChE by biradical leads to partial aggregation which persists after demodification (Dolginova et al., unpublished results). This aggregation might explain the failure of demodified AChE to recover enzymic activity, and we are currently investigating this issue. It will also be of interest to explore the possibility that misfolded AChE can be restored to its active state by the use of molecular chaperones (Gething & Sambrook, 1992), whether in the demodified state or even when modified by an irreversible SH reagent.

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