A Metastable State of *Torpedo californica* Acetylcholinesterase Generated by Modification with Organomercurials†

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ABSTRACT: Chemical modification of *Torpedo californica* acetylcholinesterase by various sulfhydryl reagents results in its conversion to one of two principal states. One of these states, *viz.*, that produced by disulfides and alkylating agents, is stable. The second state, produced by mercury derivatives, is metastable. At room temperature, it converts spontaneously, with a half-life of *ca.* 1 h, to a stable state similar to that produced by the disulfides and alkylating agents. Demodification of acetylcholinesterase freshly modified by mercurials, by its exposure to reduced glutathione, causes rapid release of the bound mercurial, with concomitant recovery of most of the enzymic activity of the native enzyme. In contrast, similar demodification of acetylcholinesterase modified by disulfides yields no detectable recovery of enzymic activity. Spectroscopic measurements, employing CD, intrinsic fluoresence, and binding of 1-anilino-8-naphthalenesulfonate, show that the state produced initially by mercurials is "native-like", whereas that produced by disulfides and alkylating agents, and after prolonged incubation of the mercurial-modified enzyme, is partially unfolded and displays many of the features of the "molten globule" state. Arrhenius plots show that the quasi-native state produced by organomercurials is separated by a low (5 kcal/mol) energy barrier from the native state, whereas the partially unfolded state is separated from the quasi-native state by a high energy barrier (*ca.* 50 kcal/mol). Comparison of the 3D structures of native acetylcholinesterase and of a heavy-atom derivative obtained with $HgAc_2$ suggests that the mercurial-modified enzyme may be stabilized by additional interactions of the mercury atom attached to the free thiol group of Cys^{231}, specifically with $Ser^{228}O\gamma$ and with the main-chain nitrogen and carbonyl oxygen of the same serine residue.

Chemical modification of acetylcholinesterase (AChE)[1] from *Torpedo californica* or *Torpedo nobiliana* with a repertoire of sulfhydryl reagents, including alkylating agents, disulfides, and organomercurials, results in complete inhibition of enzymic activity. It was proposed that chemical modification of a single nonconserved cysteine residue, Cys^{231}, is responsible for this inhibition (Steinberg et al., 1990; Dolginova et al., 1992; Salih et al., 1993). This suggestion was confirmed recently by use of a radioactive alkylating agent (Salih et al., 1993). Cys^{231} is buried within the protein, *ca.* 8 Å from $O\gamma$ of the active-site serine, Ser^{200} (Sussman et al., 1991), and is not involved in catalysis (Silman et al., 1992). Furthermore, chemical modification by two disulfides, 4,4′-dithiodipyridine (DTP) and bis(1-oxy-2,2,5,5-tetramethyl-3-imidazolin-4-yl) disulfide (biradical), although fully reversible, is not accompanied by any detectable recovery of catalytic activity (Dolginova et al., 1992). A detailed investigation revealed that modification of *T. californica* AChE, by both DTP and biradical, causes a conformational transition to a partially unfolded state characterized by greatly enhanced binding of 1-anilino-8-naphthalenesulfonate (ANS) and a substantial loss of ellipticity in the near-UV, with ellipticity in the far-UV being largely retained (Dolginova et al., 1992). Chemical modification is also accompanied by a greatly enhanced sensitivity to tryptic digestion. The partially unfolded state so generated thus displays many of the features of the "molten globule" which has been invoked, in recent years, as a partially unfolded intermediate on the pathway from the fully unfolded polypeptide chain to the fully folded "native" protein [for reviews, see Kuwajima (1989), Kim and Baldwin (1990), and Ptitsyn (1992)]. The physicochemical properties of the demodified enzyme, obtained by exposure to reduced glutathione (GSH), are very similar to those of the protein which had been chemically modified by the disulfides. Thus the

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[1] Abbreviations: AChE, acetylcholinesterase; DTP, 4,4′-dithiodipyridine; biradical, bis(1-oxy-2,2,5,5-tetramethyl-3-imidazolin-4-yl) disulfide; GSH, reduced glutathione; HgR, 2,2,5,5-tetramethyl-4-[2-(chloromercuri)phenyl]-3-imidazoline-1-oxyl; $HgAc_2$, mercuric acetate; MeHgAc, methylmercuric acetate; NEM, *N*-ethylmaleimide; MeHgCl, methylmercuric chloride; PCMS, *p*-(chloromercuri)benzenesulfonic acid; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy; ANS, 1-anilino-8-naphthalenesulfonic acid; ATCh, acetylthiocholine iodide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MTS, methanethiosulfonate; MTSEA, 2-aminoethyl methanethiosulfonate; MTSET, 2-(trimethylammonio)ethyl methanethiosulfonate; CD, circular dichroism; EPR, electron paramagnetic resonance.

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molten globule-like state produced by chemical modification is a stable state which is maintained even after chemical modification has been reversed.

Another class of reversible thiol-specific reagents are the organomercurials (Cecil & McPhee, 1959), which have also been employed to insert paramagnetic probes into proteins (Boeyens & McConnell, 1966; Weiner, 1986). In the present study, we compare the effect of chemical modifiction of *T. californica* AChE by organomercurials and alkylating agents with the effect of modification by disulfides. Whereas modification by alkylating agents produces a molten globule-like state (or states) very similar to the state(s) produced by disulfides (Dolginova et al., 1992), exposure to mercurials produces a metastable inactive species. Demodification of the mercurial-modified enzyme with GSH shortly after modification leads to regeneration of the physicochemical characteristics of the native enzyme as well as to reactivation, but with time the mercurial-modified enzyme undergoes spontaneous conversion to a state similar to that generated by modification with other sulfhydryl agents. The thermodynamic and kinetic relationships between these states are explored, and a model to explain the experimental observations is proposed.

MATERIALS AND METHODS

Materials

AChE was the dimeric (G_2) glycosylphosphatidylinositol-anchored form purified from electric organ tissue of *T. californica* by affinity chromatography subsequent to solubilization with phosphatidylinositol-specific phospholipase C (Futerman et al., 1985; Sussman et al., 1988).

2,2,5,5-Tetramethyl-4-[2-(chloromercuri)phenyl]-3-imidazoline-1-oxyl (HgR) and biradical were synthesized as described previously (Volodarsky & Weiner, 1983; Khramtsov et al., 1989). Mercuric acetate ($HgAc_2$), mercuric chloride, and methylmercuric acetate (MeHgAc) were purchased from BDH Laboratory Chemical Division (Poole, England). *N*-Ethylmaleimide (NEM) was obtained from Aldrich (Milwaukee, WI), and methylmercuric chloride (MeHgCl) was from Fisher Scientific Co. (Fair Lawn, NJ). *p*-(Chloromercuri)benzenesulfonic acid monosodium salt (PCMS), 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO), 4-(2-iodoacetamido)-TEMPO, 4-maleimido-TEMPO, 1-anilino-8-naphthalenesulfonic acid (ANS, magnesium salt), acetylthiocholine iodide (ATCh), 4,4′-dithiodipyridine (DTP), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and reduced glutathione (GSH) were all obtained from Sigma (St. Louis, MO). Iodoacetamide and gelatin were from Merck (Darmstadt, Germany). The methanethiosulfonate (MTS) derivatives, 2-aminoethyl methanethiosulfonate (MTSEA) and 2-(trimethylammonio)ethyl methanethiosulfonate (MTSET) (Akabas et al., 1992), were a gift from Drs. David A. Stauffer and Arthur Karlin, Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York. All other reagents were of analytical grade or higher.

Methods

Buffers. Unless otherwise stated, the buffer employed in the physicochemical studies was 0.1 M NaCl/50 mM sodium phosphate, pH 7.0 (buffer A).

Assay Methods. AChE concentrations were determined either spectrophotometrically, taking $\epsilon^{280}(1\ \text{mg/mL}) = 17.5$ (Taylor et al., 1974), or colorimetrically (Bradford, 1976), using native AChE for calibration. The AChE concentration is expressed as the concentration of active sites, assuming a subunit molecular weight of 65 000 (Sussman et al., 1988). AChE activity was measured spectrophotometrically, at 412 nm, using ATCh as substrate (Ellman et al., 1961), according to the following procedure: Samples of AChE were diluted appropriately into ice-cold 0.1 M NaCl/50 mM sodium phosphate, pH 7.0, containing 0.01% gelatin (dilution buffer); an aliquot of AChE in the dilution buffer was then added to a cuvette containing 3 mL of the assay mixture, *viz.*, 0.5 mM ATCh/1 mM DTNB/0.01% gelatin/0.1 M Tris-HCl, pH 7.6. The final concentration of AChE in the assay mixture was in the nanomolar range, and the detection limit was in the picomolar range. Activity was monitored in a Uvikon 940 spectrophotometer at room temperature.

Table 1: Inhibition of *Torpedo* AChE by the Thiol Reagents Employed

thiol reagent	final concn (mM)	time of incubation (h)	inhibition of AChE activity (%)
HgR	0.5−2.0	0.5−1.0	>99
$HgAc_2$	0.5−1.0	0.5	>99
$HgCl_2$	1.0	0.5	>99
MeHgCl	1.0−2.0	0.5	>99
MeHgAc	1.0−2.0	0.5	>99
PCMS	3.0−5.0	20	>99
NEM	5.0	20	95
maleimido-TEMPO	10−15	24−36	95
DTP	1.0−2.0	1.0−2.0	>99
biradical	1.0−2.0	1.0−2.0	>99
MTSEA	10−12	0.5−1.0	85
MTSET	3.0−5.0	0.5−1.0	90

Modification of AChE by Sulfhydryl Reagents. Modification of AChE by sulfhydryl reagents and demodification by GSH of chemically modified AChE were performed as described previously (Dolginova et al., 1992). The various sulfhydryl reagents employed, and the conditions used for modification, are listed in Table 1, and the structures of these reagents are shown in Figure 1. Excess sulfhydryl reagent was removed by gel filtration on a Bio-Gel P6 column (1 × 7 cm). When necessary, the eluted protein was concentrated in a Centricon-30 microconcentrator.

Reactivation of Mercurial-Modified AChE. AChE (5−50 μM in buffer A) was exposed to a final concentration of 1−3 mM of the appropriate mercurial and incubated at room temperature or on ice, until >95% inhibition had been achieved. Excess mercurial was then removed by gel filtration as described above. Alternatively, the reaction mixture was diluted 30-fold into buffer A. GSH was then added to final concentration of 2 mM. Recovery of enzymic activity was monitored spectrophotometrically, as described above.

Circular Dichroism Measurements. Circular dichroism (CD) measurements were performed in a Jasco J-500C spectropolarimeter, using 0.2-, 1-, or 10-mm path-length cuvettes at 22 °C. The spectra represent the average of five to eight scans and are corrected to the baseline for the corresponding buffer. Kinetic monitoring of changes in ellipticity in the near-UV, at 36 °C, was performed by rapid scanning (*ca.* 20 s per measurement) through the range of 270−280 nm, employing a 10-mm path-length water-jacketed cuvette. Data are expressed as the mean residue ellipticity, $[\Theta]$ (deg cm^2 dmol^{-1}).

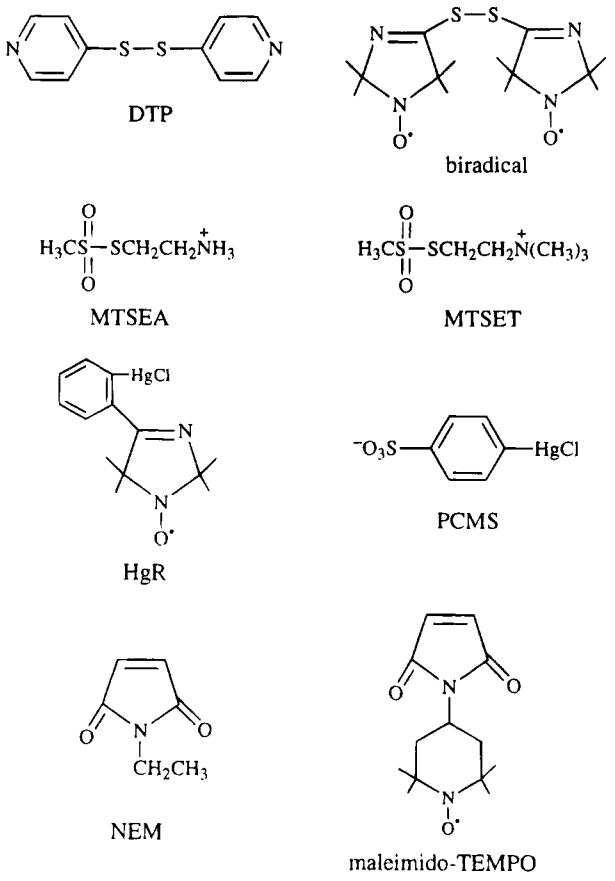

FIGURE 1: Chemical formulas of thiol reagents used for chemical modification of *Torpedo* AChE.

ANS Binding Measurements. A 10-μL aliquot of 5 mM ANS in acetonitrile was added to 500 μL of 0.23 μM AChE in buffer A. After incubation for 30 min, ANS fluorescence was measured by exciting at 390 nm and monitoring emission at 490 nm. Measurements were performed in a Shimadzu RF-540 spectrofluorometer at 26 °C.

Intrinsic Fluorescence Measurements. Intrinsic fluorescence measurements were performed in a Shimadzu RF-540 spectrofluorometer at 26 °C, employing quartz cells with a circular section of 0.5 cm. Excitation was at 295 nm, and the slit width of both monochromators was 2 nm.

Sucrose Gradient Centrifugation. Analytical sucrose gradient centrifugation was performed on 5−20% sucrose gradients made up in buffer A. Centrifugation was carried out in an SW 50.1 rotor for 5 h at 45 000 rpm in a Beckman L8-70 ultracentrifuge. Approximately 30 fractions of 170 μL were collected and assayed for protein concentration and enzymic activity. Native *T. californica* AChE (7.0 S) and catalase (11.4 S) served as markers.

EPR Measurements. Electron paramagnetic resonance (EPR) spectra were recorded in a Varian E-12 spectrometer or in a Bruker ER200 D-SRC EPR spectrometer, operating at X-band frequency with 100-kHz magnetic field modulation, at room temperature, in a 200-μL flat cell. The concentration of a radical covalently bound to AChE was determined by double integration of the EPR spectrum of freely rotating label released from the protein by addition of GSH to a final concentration of 2 mM, using TEMPO for calibration.

Crystallization. Crystals of the same dimeric form of *T. californica* AChE used for the studies in solution (Sussman et al., 1988) were grown by standard vapor diffusion techniques in hanging drops (McPherson, 1976), as described previously, with 61% saturated ammonium sulfate/360 mM sodium,potassium phosphate, pH 7.0, as precipitating agent (Sussman et al., 1991). The mercury complex was obtained by soaking the native crystals in mother liquor containing 15 mM mercuric acetate ($HgAc_2$) for 9 days at room temperature.

Table 2: X-ray Data Collection and Processing for the AChE−$HgAc_2$ Complex

no. of frames	760
oscillation range	0.25°
measured reflections	87173
merged reflections	23445
completeness	95.8%
R_{sym}	11.1%
resolution limit	2.8 Å
space group	$P3_121$
unit cell	$a = b = 110.7$ Å, $c = 135.0$ Å, $\gamma = 120°$

X-ray Data Collection, Processing, and Refinement. X-ray data (see Table 2) were collected on a Siemens/Xentronics area detector at room temperature as described earlier (Sussman et al., 1991). The data were processed using XDS (Kabsch, 1988). For refinement the program X-PLOR (version 3.1) was used (Brünger et al., 1987). The initial refinement was based on molecular replacement, using the coordinates of the native structure (Sussman et al., 1991) and omitting all water molecules. After rigid-body refinement, a heavy-atom-derivative difference map showed the location of two mercury atoms in each subunit, at distinct sites, 27.5 Å apart, appearing at intensities of 30σ and 16σ above noise level (see Figure 2). Simulated annealing was performed after the inclusion of the two mercury atoms, followed by numerous cycles of positional, occupancy, and temperature-factor refinement.

Parameters for the mercury atom, including mass, charge, and scattering factors (Cromer & Waber, 1974), were supplied to X-PLOR. For the force-field calculations, Cys^{231} was modified by removing the proton from Sγ and defining a bond between this atom and the adjacent mercury atom; the bond length (2.4 Å) was based on several cysteine−mercury complexes reported in the literature (Taylor et al., 1975; Taylor & Carty, 1977). Interactions between the two mercury atoms and other atoms of the protein were not restrained but were determined solely by the crystallographic data. A total of 94 water molecules inside the active-site gorge were included in the refinement. We also tried to fit a decamethonium molecule inside the gorge but were not able to contrive a suitable disorder model to fit the electron density satisfactorily.

RESULTS

Figure 1 shows a repertoire of sulfhydryl reagents, employed in the present study, all of which inactivate *T. californica* AChE (see Table 1). These reagents fall into three categories: disulfides, alkylating agents, and mercurials. Figure 3 shows CD spectra in the near- and far-UV for AChE modified by a representative reagent from each category, as well as for the native enzyme. We earlier reported that modification by disulfides converted AChE to a partially unfolded state (Dolginova et al., 1992), and this is confirmed by the spectrum shown for AChE modified with DTP, where

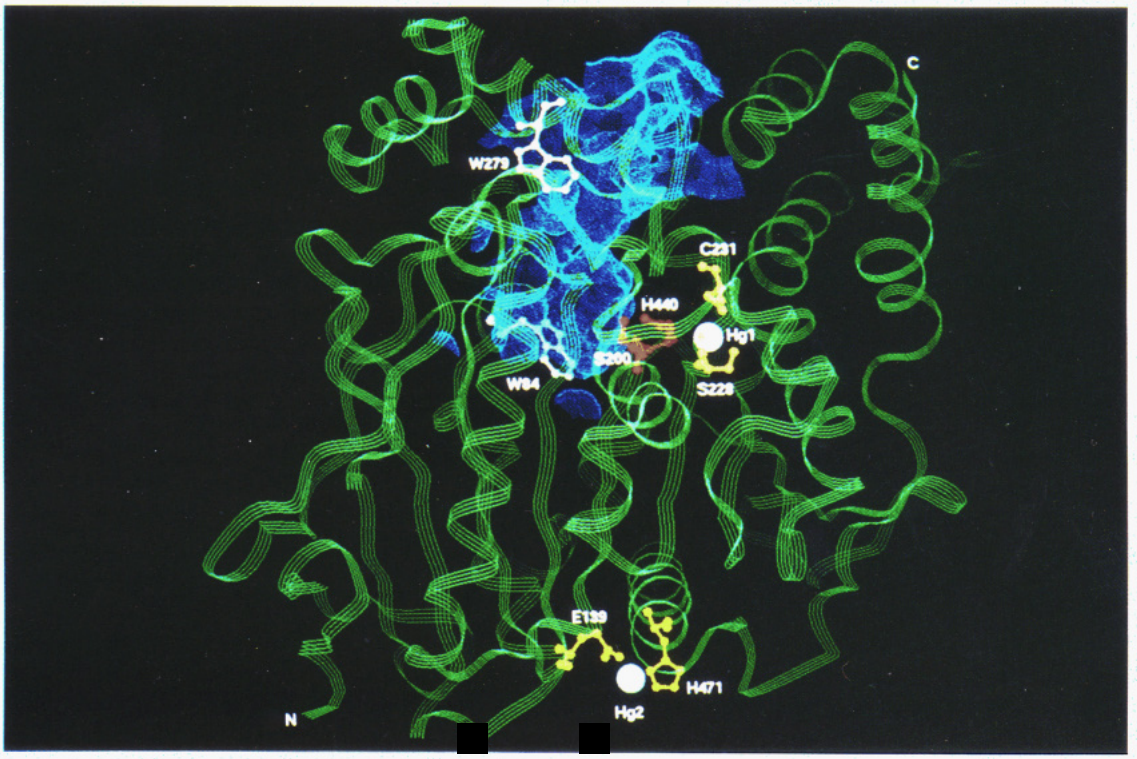

FIGURE 2: Ribbon diagram of the $HgAc_2$−AChE complex. Mercury atoms are shown as white balls and the surrounding residues (Ser^{228}, Cys^{231}, Glu^{139}, and His^{471}) as yellow balls and sticks. Ser^{200} and His^{440} of the catalytic triad are shown in red, and the solvent-accessible surface inside the aromatic gorge is depicted in blue.

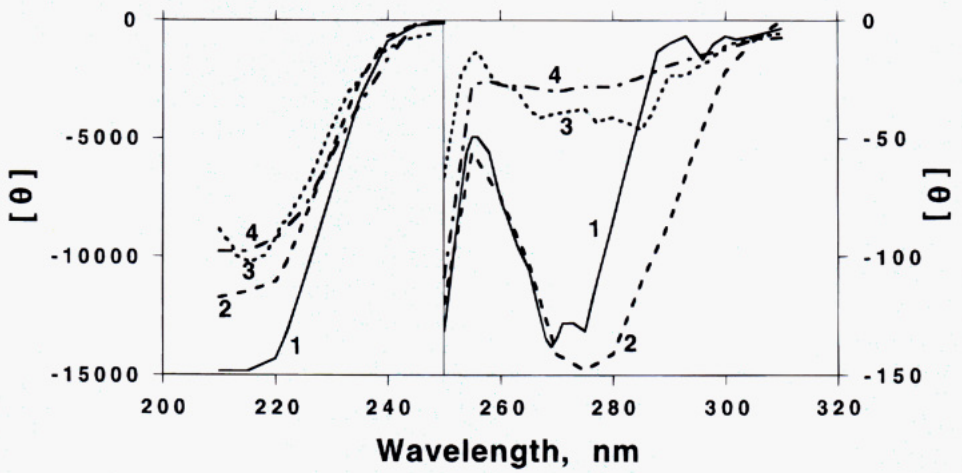

FIGURE 3: CD spectra, in the far- and near-UV, of AChE before and after modification by three thiol reagents: (1) native enzyme; (2) enzyme treated with HgR; (3) enzyme treated with DTP; (4) enzyme treated with NEM. Modification was performed as described under Materials and Methods. Protein concentrations were 4.5 μM in buffer A.

the ellipticity in the near-UV is strongly reduced, relative to native AChE, whereas the ellipticity in the far-UV is much less affected. AChE modified by the alkylating agent NEM displays a CD spectrum very similar to that obtained by modification with DTP, and the CD spectrum for AChE modified with maleimido-TEMPO is very similar (not shown). In contrast, AChE modified with the organomercurial HgR displays a CD spectrum much closer to that of native AChE, and the CD spectra for AChE modified by $HgAc_2$ and PCMS resemble that produced by modification with HgR (not shown). Figure 4 shows the normalized emission spectra for native AChE and for AChE modified by DTP, maleimido-TEMPO, and HgR. Modification by HgR produces only a 1-nm red shift in the emission maximum, which is much smaller than those produced by the other two reagents.

Whereas modification by alkylating agents is irreversible, modification by disulfides can be readily reversed, e.g., by use of GSH or dithiothreitol (Berliner et al., 1982; Dolginova et al., 1992). We earlier showed that such demodification did not regenerate any detectable enzymic activity and that the demodified enzyme retained the spectroscopic characteristics of the modified enzyme (Dolginova et al., 1992). Likewise, two methanethiosulfonate derivatives, MTSEA and MTSET (Akabas et al., 1992), reagents which also modify thiol groups in proteins reversibly (Cecil & McPhee, 1959), inhibited AChE activity irreversibly. Thus, exposure to MTSEA (10 mM in buffer A, 50 min) or to MTSET (3 mM in buffer A, 30 min) inactivated AChE 85% and 90%, respectively, at room temperature, and no detectable recovery of enzymic activity was detected within 36 h upon exposure to excess GSH.

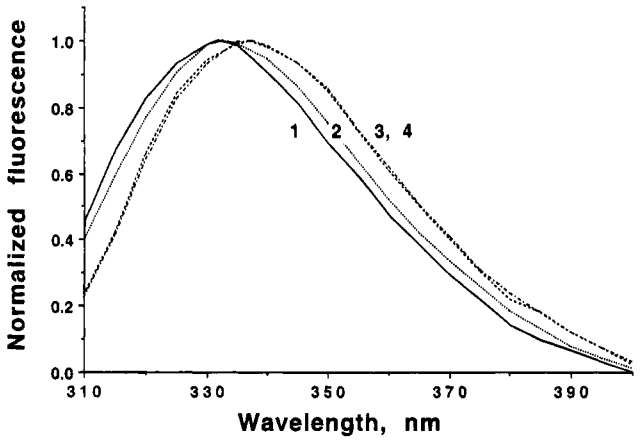

FIGURE 4: Normalized intrinsic fluorescence emission spectra of AChE before and after modification by three thiol reagents: (1) native enzyme; (2) enzyme treated with $HgAc_2$; (3) enzyme treated with DTP; (4) enzyme treated with maleimido-TEMPO. Protein concentrations were 1 μM in buffer A.

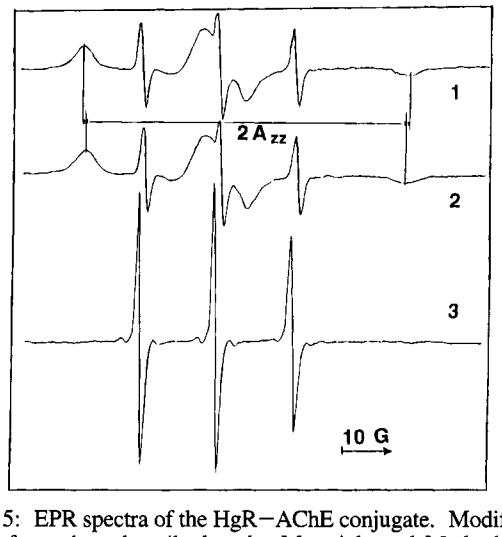

FIGURE 5: EPR spectra of the HgR−AChE conjugate. Modification was performed as described under Materials and Methods. The concentration of the conjugate was *ca.* 5 μM in buffer A. EPR conditions: microwave power, 20 mW; modulation amplitude, 1 G. The various spectra show the freshly prepared conjugate (trace 1), the conjugate after 1 h at 38 °C (trace 2), and the conjugate after exposure to 2 mM GSH (trace 3). The first two traces are at gain 5×10^5, and the lower one is at gain 5×10^4.

Modification of thiol groups in proteins by mercurials is also reversible (Cecil & McPhee, 1959). We took advantage of EPR spectroscopy to monitor the process, since this technique allows one to distinguish readily between bound and released modifying agent (Berliner et al., 1982; Khramtsov et al., 1989). The upper trace in Figure 5 shows the EPR spectrum of AChE modified with HgR. Such a spectrum is typical for an immobilized radical (Morrisett, 1976). A signal corresponding to a small amount of unbound radical (<5%) could also be detected. This might be due either to a contribution from weakly immobilized spin-label, bound either covalently or noncovalently to the enzyme, or to a small amount of free radical. When HgR-modified AChE is exposed to GSH, the broad EPR signal of the immobilized radical disappears, with concomitant appearance of the sharp peaks characteristic of the unbound radical in solution (trace 3). Release of the bound label occurs within the time of sample preparation (<30 s). The extent of modification could be estimated to be *ca.* 85% (see under Materials and Methods). Furthermore, if AChE was subjected to prior

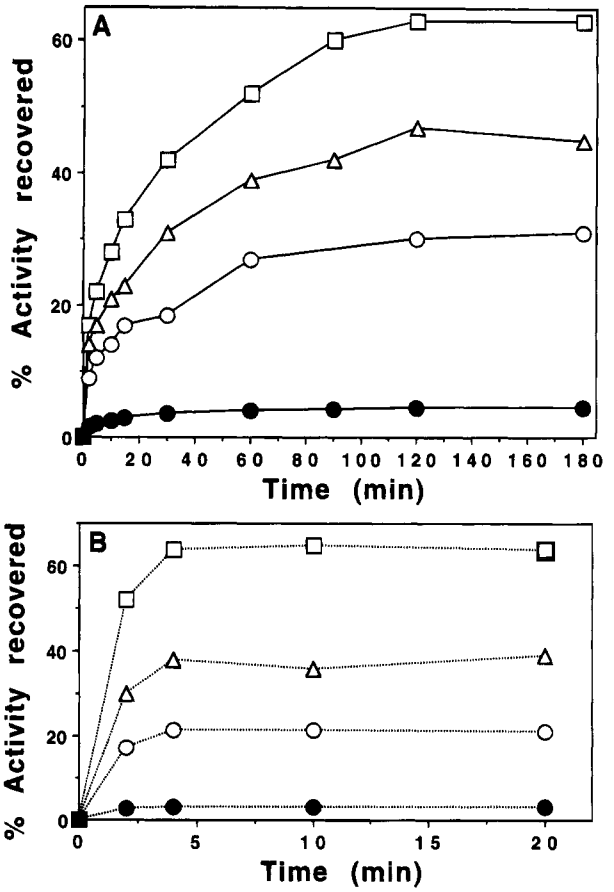

FIGURE 6: Reactivation of AChE modified by HgR (A) and $HgAc_2$ (B) as a function of time which had elapsed after modification. Modification and reactivation were performed as described under Materials and Methods, using 10 μM AChE and either 1 mM HgR or 1 mM $HgAc_2$. GSH was added at 0 h (□), 1 h (△), 2.5 h (○), and 12 h (●) after modification. The kinetics of reactivation were monitored by withdrawing aliquots for enzymic assay from each reaction mixture at the times marked on the abscissa. The ordinate denotes the percentage of activity recovered, taking the activity of an equivalent aliquot of native enzyme as 100%.

treatment with DTP, under conditions which caused complete inactivation, no bound spin-label could be detected upon subsequent exposure to HgR, clearly demonstrating that the spin-label was reacting with Cys^{231}. Conversely, little or no reaction of DTP could be detected spectrophotometrically (Dolginova et al., 1992) with AChE previously modified by HgR.

In view of the fact that the chemical modification by HgR produced only a modest perturbation of the spectral characteristics of native AChE and that demodification with GSH was quite rapid, we examined the enzymic activity of HgR-modified enzyme subsequent to exposure to GSH. Figure 6A shows that, upon addition of GSH to HgR−AChE, enzymic activity reappears, but at a rate much slower than the rate of demodification, with a $t_{1/2}$ of *ca.* 15 min. Figure 6A also shows that the extent of reactivation obtained is a function of the period of time which elapsed between completion of modification and initiation of demodification. Thus AChE treated with GSH immediately after modification could be reactivated over 60% (in some experiments up to 85%), whereas if the sample was retained for 12 h at room temperature after modification, <5% reactivation was obtained.

Such time-dependent behavior might result from aggregation of the modified enzyme. Sucrose gradient centrifugation

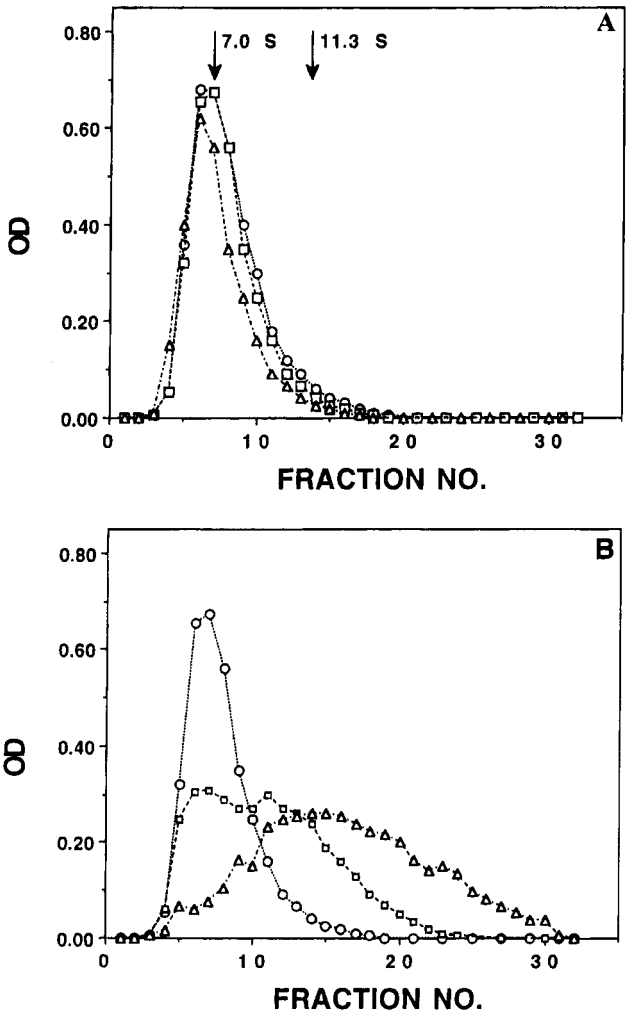

FIGURE 7: Sucrose gradient centrifugation profiles of native AChE and of AChE modified by HgR and by biradical. Modification was performed as described under Materials and Methods, using 15 μM AChE, and *ca.* 100-μL aliquots were applied to the gradients. (A) Curves: ○, native AChE; □, HgR-modified AChE 5 h after preparation; △, HgR-modified AChE 24 h after preparation. (B) Curves: ○, native AChE; □, biradical-modified AChE 5 h after preparation; △, biradical-modified AChE 12 h after preparation. The arrows mark the positions of catalase (11.3 S) and unmodified AChE (7.0 S), which served as markers.

demonstrated that HgR-modified AChE displayed no tendency to aggregate over a period of 12 h (Figure 7A). AChE modified by biradical showed some tendency to aggregate, albeit very slowly (Figure 7B).

Thus, we could assume that HgR-modified AChE undergoes a time-dependent structural change, and we attempted to characterize this process in depth.

Since HgR is a relatively bulky molecule (see Figure 1), we considered the possibility that the large substituent, attached to the buried thiol of Cys^{231}, is responsible for the time-dependent decrease in reactivatability which we observe (see Figure 6A). To evaluate this possibility, we compared the effect of five other mercurials, PCMS, $HgAc_2$, mercuric chloride, methylmercuric chloride, and methylmercuric acetate. All these compounds inactivate *Torpedo* AChE. Their removal, by GSH treatment, is accompanied by substantial reactivation, and for all five, as for HgR, the extent of reactivation decreases as a function of the time which had elapsed between modification and demodification. This is illustrated, for $HgAc_2$, in Figure 6B. Thus both the initial reversible inactivation and the subsequent time-dependent irreversible inactivation appear to result from the insertion of the mercury atom itself and are not due to disruption caused by a bulky organic substituent.

The time-dependent loss of the capacity of the mercurial-modified enzyme to be reactivated by GSH indicated that it was undergoing a further, time-dependent, conformational change subsequent to modification. Accordingly, we utilized the various spectroscopic techniques to examine mercurial-modified AChE after prolonged incubation at room temperature had yielded a preparation which had lost >99% of its capacity to be reactivated. Figure 8 compares the CD spectra of native AChE (trace 1), of AChE within 30 min of modification with $HgAc_2$ (trace 2), which could be reactivated *ca.* 60%, and of the same sample after incubation for 24 h at room temperature (trace 3), at which stage it could be reactivated <1%. Whereas AChE freshly modified with $HgAc_2$ displays high ellipticity in the near-UV, as previously shown for HgR-modified AChE (Figure 3), the prolonged incubation led to a large decrease in this parameter, producing a spectrum closely resembling that observed for AChE modified with either DTP or NEM (Figure 3). A similar pattern, although less dramatic, is observed in the far-UV.

While the intrinsic fluorescence of AChE freshly modified with HgR or $HgAc_2$ is shifted only 1 nm to the red (Figure 4), prolonged incubation produced an emission spectrum which was shifted 6 nm to the red (not shown) and was indistinguishable from those obtained after modification with DTP or NEM. Inspection of the HgR-modified AChE also revealed a time-dependent change in the EPR spectrum, which showed that the conformational change observed by CD and by fluorescence emission is accompanied by a small but significant decrease, 1.3 G, in the $2A_{zz}$ value (Figure 5). Finally, we examined the binding of ANS by mercurial-modified AChE, as monitored by enhanced fluorescence of the bound probe at 490 nm. Surprisingly, we found that ANS binding is much higher than for native AChE and is not markedly different from that observed for AChE modified by either disulfides or by alkylating agents (Figure 9).

The fact that AChE modified with mercurials could be substantially reactivated and that its spectral characteristics were not markedly different from those of the native enzyme suggested that demodification of freshly modified AChE might regenerate the spectral characteristics of the native enzyme. Figure 8 shows that modification with HgR leads to a slight increase in ellipticity in the near-UV (trace 4) and that demodification of such a freshly modified sample with GSH reverses this slight increase, producing a CD spectrum resembling that of native AChE (trace 5). Furthermore, regeneration of enzymic activity is accompanied by a decrease in ANS binding almost to the low levels obtained for native AChE (Figure 10). Similar demodification of HgR-modified AChE after overnight incubation at room temperature, i.e., of a sample with the spectral characteristics displayed in trace 3, had no effect, and the CD spectrum obtained (not shown) closely resembles trace 3. Figure 11 shows that the time−course of loss of recoverable activity, upon exposure to GSH, closely parallels the decrease in ellipticity in the near-UV.

In order to gain a better understanding of the spontaneous transition which we observed of mercurial-modified AChE from a quasi-native state to a partially unfolded state, we studied the temperature dependence of this process for AChE

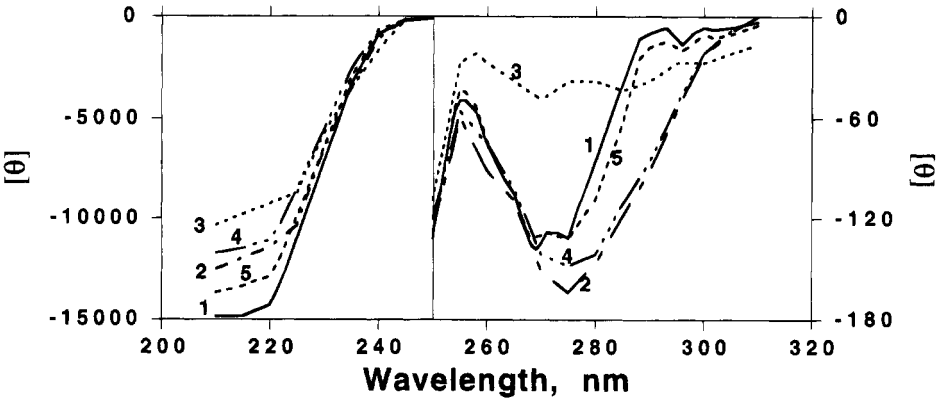

FIGURE 8: CD spectra in the near- and far-UV of AChE after modification by organomercurials: (1) native enzyme; (2) enzyme within 30 min of modification by $HgAc_2$; (3) enzyme 24 h after modification by $HgAc_2$; (4) enzyme within 30 min of modification by HgR; (5) enzyme within 1.5 h of initiation of demodification of (4) by addition of GSH. Modification with $HgAc_2$ (1 mM) was performed in buffer A at 0 °C, for 30 min, producing >99% inhibition. Modification with HgR (2 mM) was performed in buffer A at 13 °C for 1 h, also producing >99% inhibition. Demodification with GSH (2 mM) was performed for 1 h, at room temperature, resulting in recovery of ca. 60% of the initial enzymic activity.

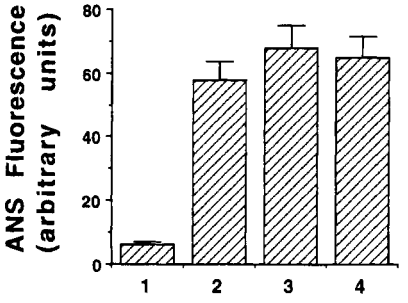

FIGURE 9: ANS binding by AChE samples exposed to various sulfhydryl reagents. ANS binding was monitored by measuring emission at 490 nm, as described under Materials and Methods. Bars: (1) native AChE; (2) AChE modified with 1 mM HgR for 30 min; (3) AChE modified with 2 mM DTP for 1 h; (4) AChE modified with 5 mM NEM for 20 h. Modification was performed in buffer A at room temperature, and in all cases, inhibition of enzymic activity was >95%.

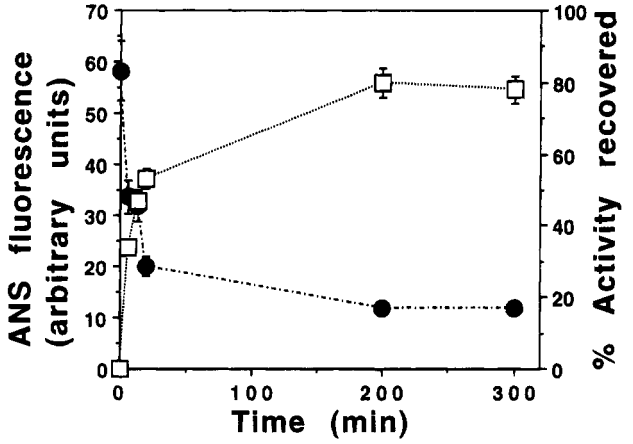

FIGURE 10: Comparison of the kinetics of reappearance of AChE activity upon addition of GSH to HgR-modified AChE with the concomitant decrease in binding of ANS. AChE (1.5×10^{-5} M) in buffer A was modified with 1 mM HgR at 13 °C for 1 h, producing >99% inhibition. The sample was immediately diluted 30-fold into 2 mM GSH in buffer A. At appropriate times, aliquots were withdrawn for measurement of both ANS binding and enzymic activity, as described under Materials and Methods. Curves: □, enzymic activity; ●, ANS fluorescence.

modified with $HgAc_2$. Figure 12 shows that the rate of the transition is temperature-dependent; the inset to this figure shows that, at each temperature, deactivation obeys first-

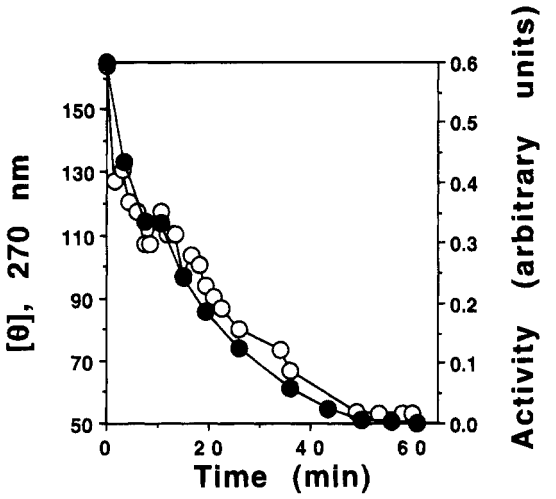

FIGURE 11: Comparison of the kinetics of loss of recoverable AChE activity subsequent to modification with $HgAc_2$ and of the parallel decrease in ellipticity in the near-UV. Native AChE was modified with $HgAc_2$ as in Figure 8. Within 30 min of modification, the sample was inserted into a water-jacketed CD cuvette (see Materials and Methods) maintained at 36 °C. In parallel to monitoring of the CD spectrum in the near-UV, aliquots were withdrawn and diluted 1:100 into 2 mM GSH in buffer A at room temperature. Enzymic activity was measured 1 h later.

order kinetics. The data so obtained allowed us to construct an Arrhenius plot and, in turn, to calculate from it the activation energy of the transition (Figure 13). The value obtained, 54 kcal/mol of catalytic subunits, was rather high and quite comparable to values of E_A reported for the unfolding of small proteins such as trypsin (67 kcal/mol; Pohl, 1968), carboxypeptidase B (64 kcal/mol; Conejoro-Lara et al., 1991), and chymotrypsin inhibitor 2, for which an activation enthalpy for unfolding of 38 kcal/mol was reported (Jackson & Fersht, 1991). We also studied the temperature dependence of the reactivation process, *viz.*, the temperature dependence of reappearance of enzymic activity upon exposure of mercurial-modified AChE to GSH. Figure 14 displays the data so obtained for AChE modified by $HgAc_2$. The kinetics of the reactivation process, which were very rapid, did not permit rigorous monitoring of its time–course. Accordingly, we used the initial slopes of the reactivation curves displayed in this figure to construct an Arrhenius plot, which yielded an apparent activation energy

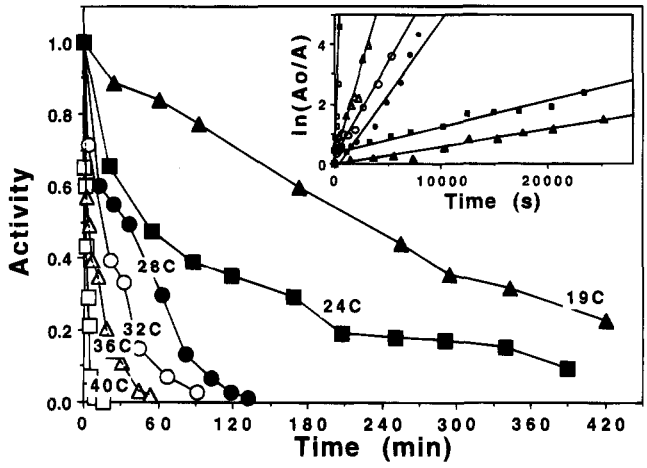

FIGURE 12: Temperature dependence of the kinetics of loss of recoverable AChE activity subsequent to modification with $HgAc_2$. Modification was performed as in the legend to Figure 8. Samples were then maintained at various temperatures, and aliquots were withdrawn at appropriate times and diluted into 2 mM GSH at room temperature, 1 h prior to assay of enzymic activity. The inset shows the same data replotted on a semilogarithmic scale.

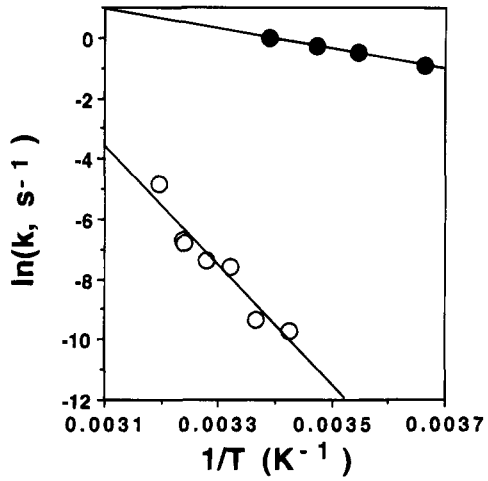

FIGURE 13: Arrhenius plots of the temperature dependence of the rates of irreversible inactivation and of reactivation by GSH of $HgAc_2$-modified AChE. Curves: ○, rates of irreversible inactivation; ●, rates of reactivation. The points are based on the experimental data presented in Figures 12 and 14, respectively.

of 6 kcal/mol (Figure 13). Since the experiments described above, on the effect of GSH on the EPR spectrum of HgR-modified AChE, showed that freely rotating free radical was generated on the time scale of sample preparation, it seems likely that the demodification step is not rate-limiting for reactivation. Thus the activation energy measured most probably represents the activation energy of refolding to the native conformation.

In considering the structure of the mercurial-modified AChE, we took note of the fact that one of the heavy-atom derivatives used to solve the phase problem, *en route* to solving the 3D structure of the enzyme, was obtained by soaking $HgAc_2$ into native crystals (Sussman et al., 1991). In fact, the chain tracing assumed that the principal Hg peak in this heavy-atom derivative was bound to Cys^{231} (see Figure 2). The fact that the mercury-modified enzyme retained a unit cell very similar to that of the native enzyme showed that this derivative could not differ markedly from the native enzyme in its overall 3D structure. Accordingly, we performed a refinement of the mercury derivative, so as to

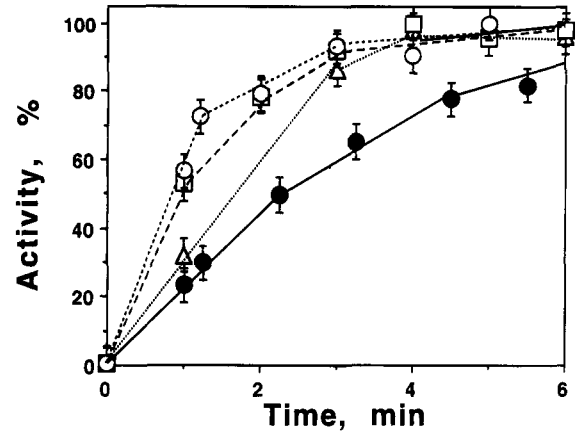

FIGURE 14: Temperature dependence of the rate of reappearance of enzymic activity upon exposure of $HgAc_2$-modified AChE to GSH. Inactivation with $HgAc_2$, at 0 °C, was performed as described in the legend to Figure 8. Demodification with GSH was also performed as described in the legend to Figure 8, but at the appropriate temperature, and aliquots withdrawn at fixed time points were assayed immediately at room temperature. Curves: ●, 0 °C, △, 9 °C; □, 15 °C; ○, 22 °C. The ordinate denotes the enzymic activity recovered, taking the maximum recovered as 100%.

compare its structure to that of the native enzyme and to ascertain the environment of the mercury atom(s) in the modified enzyme.

The final R factor of the refined crystal structure is 20.6% (R_{free} is 28.0%). The first of the two mercury atoms found in the structure (Hg_1) is situated near residues Cys^{231} and Ser^{228}, about 8 Å from Oγ of the active-site serine, Ser^{200}, as can be seen in Figure 15. The occupancy of this mercury refined to 65%. Sγ of Cys^{231} is 2.31 Å from the mercury ion, and the angle Cβ−Sγ−Hg_1 is 107°; both values are consistent with the range that is observed for Hg−S bonds in the literature (Taylor et al., 1975; Taylor & Carty, 1977). In addition, the mercury atom displays three close contacts to the hydroxyl oxygen, Oγ, and to the main-chain carbonyl oxygen and nitrogen of Ser^{228}. The bond distances for Ser^{228}Oγ, O, and N are 2.42, 2.35, and 2.27 Å, respectively (Figure 16). The 3D structures of the native enzyme and of the heavy-atom derivative obtained with $HgAc_2$, in the vicinity of Cys^{231}, are compared in Figure 16. As a consequence of the incorporation of the mercury atom, there are some changes in the interatomic distances in this region of the structure, but the overall structure of the mercury-containing derivative is very similar to that of the native enzyme (rms deviation for Cα atoms is 0.49 Å). The coordination of the mercury ion resembles an incomplete octahedron, with Sγ and N in distal positions (the angle N−Hg_1−Sγ is 165°), and the angles with O and Oγ are approximately 90°. The distortion of the angles of the octahedron can be explained by the restricted freedom of the protein backbone; there is not sufficient flexibility in the main-chain bond lengths and angles to form perfect 90° coordination angles. Significant interaction of the mercury atom with Ser^{228} is seen, which is analyzed in the Discussion.

The second mercury site, at 45% occupancy, is near His^{471} and Glu^{139}. The mercury atom in this site displays close contacts to the carboxylic oxygens of Glu^{139} and to Nδ1 of His^{471}. However, it is less tightly bound to the protein than the mercury in the first site, as indicated by the lower occupancy and by the number and nature of its surrounding atoms. Furthermore, it is situated on the outer surface of

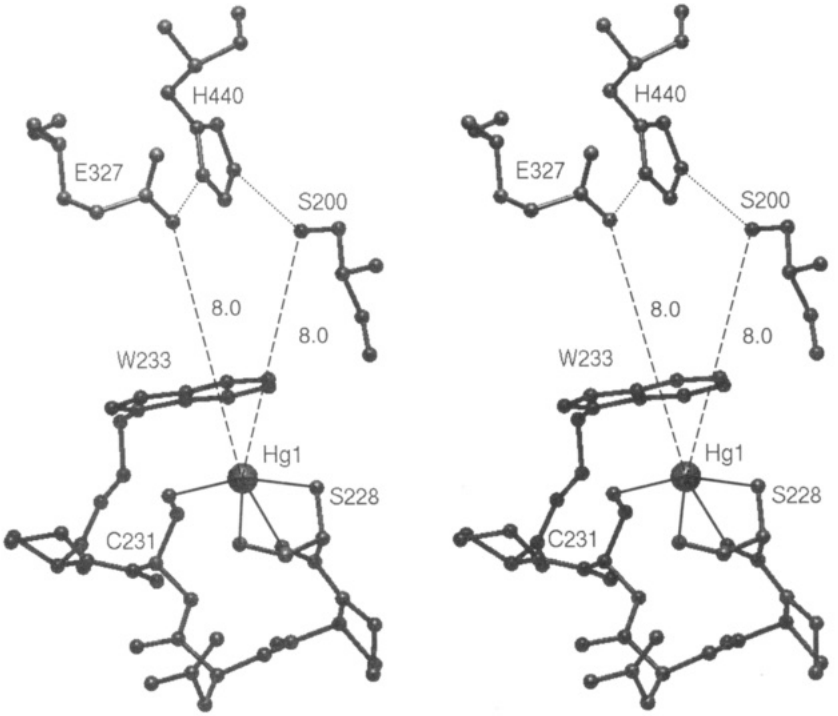

FIGURE 15: Stereoview of the position of the mercury atom, Hg_1, relative to the catalytic triad. The distances of Oγ of Ser^{200} and to Oϵ1 of Glu^{327} are both 8.0 Å. Trp^{233}, one of the aromatic residues whose rings line the gorge, lies below the plane formed by the catalytic triad and Hg_1.

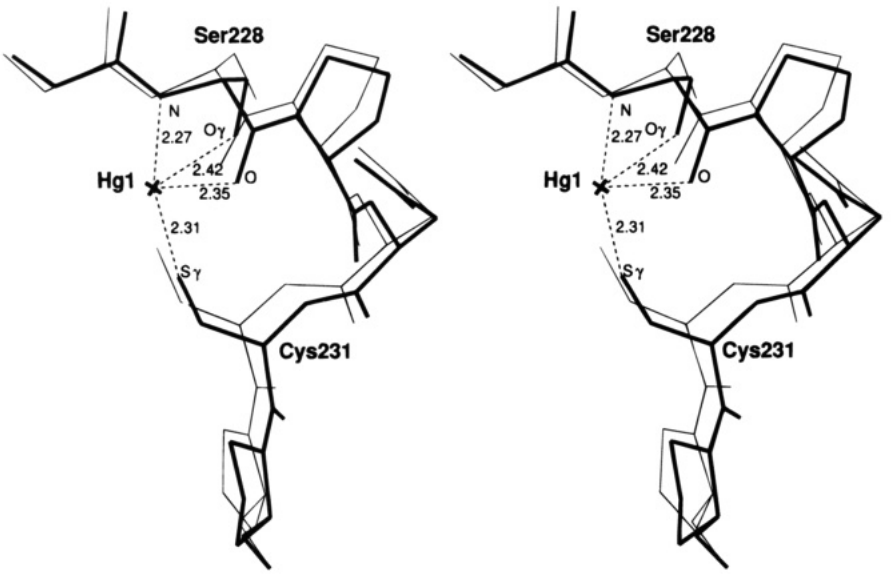

FIGURE 16: Stereo figure comparing the native structure (thin lines) to that of the mercury complex (thick lines) in the vicinity of Hg_1. The mercury atom displays close contacts to Sγ of Cys^{231} and to Oγ, N, and O of Ser^{228}.

the protein (see Figure 2) and can, therefore, be assumed not to play an important role in the experiments carried out in solution. Support for this conclusion comes also from the experiment described above, showing that stoichiometric modification of Cys^{231} by DTP precluded any attachment of the spin-label to the protein upon subsequent exposure to HgR.

DISCUSSION

We have shown above that chemical modification of AChE by various sulfhydryl reagents results in its conversion to two principal states. One of these states, *viz.*, the state produced by disulfides and alkylating agents, is stable. The second state, produced by mercury derivatives, is metastable. At room temperature, it converts spontaneously, with a half-life of *ca.* 1 h, to a stable state very similar to that produced by disulfides and alkylating agents.

The free energy of folding of proteins is generally quite small, typically 5−15 kcal/mol (Privalov, 1979), which is in the range of the free energy changes which can be produced by mutation of a single amino acid residue (Shortle, 1992; Jackson et al., 1993; Sturtevant, 1994). Moreover, it has been demonstrated that point mutation of single residues in a large fragment of staphylococcal nuclease can produce a loosely folded species resembling a molten globule (Shortle & Meeker, 1989; Gittis et al., 1993), and similar observations have been reported by Lim et al. (1992) for the λ repressor. Also interesting in the context of our study is the work of

Lu et al. (1992). These authors inserted cysteine residues in T4 lysozyme by point mutation and chemically modified the cysteines so inserted by exposure to charged mixed disulfides (e.g., cystamine). In certain cases, such a two-step procedure led to decreased stability, but their system was an equilibrium system, and the degree of destabilization could be controlled by the ratio of cystamine to cysteamine in the reaction mixture. In contrast, as already mentioned, reversible chemical modification by disulfides of the intrinsic Cys^{231} residue present in *T. californica* AChE produces irreversible unfolding and inactivation (Dolginova et al., 1992). The partially unfolded state(s) of AChE generated by chemical modification may resemble the loosely packed states generated in certain other proteins by limited site-directed mutagenesis (Shortle & Meeker, 1989; Lim et al., 1992).

The products of exposure of native AChE to either alkylating agents or disulfides display a substantial decrease in ellipticity in the near-UV, a much smaller decrease in the far-UV (Figure 3), greatly increased ANS binding (Figure 9), a red shift of *ca.* 6 nm in the fluorescence emission spectrum (Figure 4), and a tendency to aggregate (Figure 7B). All these properties suggest that modification of the native enzyme produces a state resembling a molten globule, as we had demonstrated earlier for AChE modified by disulfides (Dolginova et al., 1992). The initial state produced by modification of AChE with mercurials displays spectroscopic characteristics deviating more modestly from those of the native enzyme. Thus the CD spectrum is much closer to that of native AChE (Figures 3 and 8), the red shift in fluorescence emission is *ca.* 1 nm (Figure 4), and there is little or no tendency to aggregate (Figure 7A). If we take into account that, in solution, the spectroscopic characteristics of AChE freshly modified with mercurials approach those of the native protein and that only small changes are observed in the crystal structure upon modification with $HgAc_2$ (Figure 16), one may conclude that any structural rearrangements induced initially in the native enzyme by modification with mercurials will be of a local nature. It should be noted, however, that ANS binding by mercurial-modified AChE is almost as high as for enzyme modified by the other reagents. We do not know the nature of the ANS-binding sites thus exposed. However, binding of ANS and similar amphiphilic probes to various proteins in their native conformation is well documented, and such probes have been shown to be sensitive to conformational changes of the protein in its native state, e.g., upon binding of physiologically relevant ligands (Musci et al., 1985; Prasad et al., 1986).

The spectroscopic data, together with the capacity of the mercurial-modified AChE to be reactivated, suggest that this state is not energetically remote from the native enzyme. This was confirmed by our observation that the two states are separated by a low activation energy barrier (*ca.* 6 kcal/mol). In contrast, there is a large activation energy barrier, *ca.* 50 kcal/mol, between the initial state produced by mercurial modification and the partially unfolded state to which it is converted spontaneously, which cannot be reactivated by demodification. This latter state closely resembles the state(s) produced directly, either by reversible modification with disulfides (Dolginova et al., 1992) or by irreversible modification with alkylating agents. The fact that spontaneous conversion occurs shows unequivocally that this partially unfolded state is at a lower energy level than the initial state produced by modification with mercurials. It is likely that the partially unfolded state produced directly by alkylating agents and disulfides is at a similar energy level. It is, therefore, reasonable to ask why modification by mercurials does not lead immediately to such a low-energy state. This is a particularly cogent question since even small alkylating agents (e.g., iodoacetamide and *N*-ethylmaleimide) and small methanethiosulfonates, such as MTSEA and MTSET (Akabas et al., 1992), directly produce irreversible unfolding of the AChE molecule. In contrast, even organomercurials with bulky substituents, such as HgR and PCMS, produce, initially, the reactivatable quasi-native state of the enzyme. Thus, even though it is logical to assume that partial unfolding arises as a consequence of perturbation of the native structure by chemical modification of the buried thiol group of Cys^{231}, the mercurials seem to exert a stabilizing effect. It was for this reason that we thought it desirable to examine the environment of the Hg atom in the modified protein, and we were fortunate that X-ray data for the 3D structure of a mercury derivative of AChE, *viz.*, of an $HgAc_2$−AChE heavy-atom derivative, were already available as a result of the crystallographic studies on the enzyme (Sussman et al., 1991).

Examination of the structure of the $HgAc_2$−AChE complex shows that the changes in the atomic positions in the crystal structure, relative to the native structure, are small and seem to be restricted to the vicinity of Cys^{231}. At the resolution of the crystal structure, there is no significant change in the position of the catalytic-triad residues or of the amino acids that constitute the aromatic gorge leading to the active site. This situation is in contrast to that observed for the recently published structure of a mercury derivative of proteinase K (Müller & Saenger, 1993), in which the fully occupied mercury site has direct contacts with the His and Asp of the catalytic triad, thus displacing the His side chain by almost 2 Å. As mentioned under Results, in addition to the covalent bond to $Cys^{231}S\gamma$, the crystal structure clearly reveals that the Hg atom in the AChE−Hg complex makes close contacts with three atoms of Ser^{228} (Figure 16). It is plausible that these multiple interactions with Ser^{228} provide the means to stabilize the mercurial-modified enzyme transiently, despite the perturbation produced by the chemical modification. The mercurial-modified AChE seems, nevertheless, to be slightly less stable than the native enzyme. This is suggested by the fact that it is somewhat more susceptible to thermal denaturation. Thus $t_{1/2}$ for conversion to the irreversibly inactivated state is *ca.* 10 min at 38 °C, whereas thermal inactivation of the native enzyme takes place at a similar rate at *ca.* 42 °C, in buffer A. This suggests that enthalpic stabilization brought about by the interactions of the inserted mercury atom is accompanied by a reduction in entropy (Finkelstein & Janin, 1989; Murphy et al., 1994), resulting in a marginal overall destabilization of the AChE molecule.

The reason why AChE remains in a partially unfolded state produced by chemical modification, even after modification has been reversed, remains an open question. Fast aggregation may often prevent folding of a protein to its native conformation (Cleland & Wang, 1990; Kiefhaber et al., 1991), and we earlier raised this possibility for the molten globule-like state produced by chemical modifiction (Dolginova et al., 1992). Our sucrose gradient centrifugation data seem to exclude this possibility, since the aggregation rate,

even at an AChE concentration as high as 1 mg/mL, is rather low (Figure 7). One may conclude that demodified AChE dimers are partially denatured species, which are stable as macromolecules and not as aggregates. Two explanations may be offered for the absence of any detectable reversion of the molten globule-like state to the native state. The first explanation would involve kinetic trapping. It would assume that, even though the native enzyme might be at a lower free energy level than the molten globule, they are separated by a high free energy barrier. Examples of such kinetic trapping are the partially unfolded states of α-lytic protease (Baker et al., 1992), subtilisin (Shinde et al., 1993), and carboxypeptidase Y (Sørensen et al., 1993), which are not able to refold to the native conformation in the absence of a proregion. AChE, however, does not contain such a proregion (Maulet et al., 1990). A high energy barrier is observed in protein transitions involving isomerization of proline, *ca.* 20 kcal/mol (Brandts et al., 1975; Koide et al., 1993), and there are two proline residues, Pro^{229} and Pro^{232}, adjacent to Cys^{231}, which are both in the *trans* conformation (Sussman et al. 1991). It is possible that chemical modification of Cys^{231} "drives" one or both of these prolines to a *cis* conformation, thus providing a high energy barrier to the reverse transition. Since *Torpedo* AChE is a large protein (a dimer of two identical subunits, each of 537 amino acid residues), it is also possible that non-native domain pairing and/or intersubunit interactions may be responsible for trapping AChE in the partially unfolded state generated by chemical modification (Jaenicke, 1991; Garel, 1992).

A second explanation would be that the native state of *Torpedo* AChE does not correspond to the global free energy minimum and that the energy level of the molten globule-like state is, in fact, lower than that of the native state. This seems plausible, since the quasi-native state produced by modification with mercurials cannot be energetically very different from the native enzyme, yet it converts spontaneously to its partially unfolded counterpart. This, in turn, cannot be at a very different energy level from that of the demodified enzyme (or the analogous demodified enzyme obtained after modification with disulfides), since their spectroscopic properties are rather similar. If a mercurial-inactivated sample of AChE is allowed to stand for a period equivalent to 20 half-times of spontaneous conversion from the quasi-native state to the molten globule-like state, it may be assumed to have reached equilibrium. To estimate the amount of each of these two states present in the system at equilibrium, we made use of our observation that demodification, with GSH, of the quasi-native state leads to its complete reactivation, whereas similar demodification of the molten globule-like state produces no reactivation. The sensitivity of our assay, under the experimental conditions employed, places an upper limit of 0.01% on the recovery of the original enzymic activity upon demodification of the equilibrated system. Since no restoration of activity was observed above this limit, the ratio of the molten globule-like state to the quasi-native state must be $>10^4$. From this ratio, we can calculate that $\Delta G > 5.5$ kcal/mol, i.e., that the free energy level of the molten globule-like state is lower than that of the quasi-native state by this value. A recent theoretical paper has indeed made the point that "identification of native states with the most compact or minimum energy states may not strictly hold" (Bahar & Jernigan, 1994).

Site-directed mutagenesis is currently the main tool employed for investigation of the role of a particular amino acid residue in folding of proteins and in maintenance of their final native conformation (Shortle, 1992; Matthews, 1993, Fersht, 1993; Sturtevant, 1994). However, as pointed out by Sturtevant (1994), this powerful approach has the intrinsic limitation that it does not permit monitoring of transitions between different mutant forms. Using reversible chemical modification of a preexisting nonconserved cysteine residue in *T. californica* AChE (Dolginova et al., 1992, and the present paper), we have been able to monitor an analogous transition in AChE. The *de novo* introduction of a cysteine residue into the structure of a protein by site-directed mutagenesis, combined with its subsequent chemical modification (Ermácora et al., 1992; Lu et al., 1992; Calciano et al., 1993) and demodification, can thus be utilized to assess the role of specific structural elements in maintaining folded protein structures.

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