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Two partially unfolded states of *Torpedo californica* acetylcholinesterase

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Abstract

Chemical modification with sulfhydryl reagents of the single, nonconserved cysteine residue Cys^{231} in each subunit of a disulfide-linked dimer of *Torpedo californica* acetylcholinesterase produces a partially unfolded inactive state. Another partially unfolded state can be obtained by exposure of the enzyme to 1–2 M guanidine hydrochloride. Both these states display several important features of a molten globule, but differ in their spectroscopic (CD, intrinsic fluorescence) and hydrodynamic (Stokes radii) characteristics. With reversal of chemical modification of the former state or removal of denaturant from the latter, both states retain their physicochemical characteristics. Thus, acetylcholinesterase can exist in two molten globule states, both of which are long-lived under physiologic conditions without aggregating, and without either intraconverting or reverting to the native state. Both states undergo spontaneous intramolecular thiol-disulfide exchange, implying that they are flexible. As revealed by differential scanning calorimetry, the state produced by chemical modification lacks any heat capacity peak, presumably due to aggregation during scanning, whereas the state produced by guanidine hydrochloride unfolds as a single cooperative unit, thermal transition being completely reversible. Sucrose gradient centrifugation reveals that reduction of the interchain disulfide of the native acetylcholinesterase dimer converts it to monomers, whereas, after such reduction, the two subunits remain completely associated in the partially unfolded state generated by guanidine hydrochloride, and partially associated in that produced by chemical modification. It is suggested that a novel hydrophobic core, generated across the subunit interfaces, is responsible for this noncovalent association. Transition from the unfolded state generated by chemical modification to that produced by guanidine hydrochloride is observed only in the presence of the denaturant, yielding, on extrapolation to zero guanidine hydrochloride, a high free energy barrier (ca. 23.8 kcal/mol) separating these two flexible, partially unfolded states.

Keywords: acetylcholinesterase; differential scanning calorimetry; guanidine hydrochloride; molten globule; protein folding; thiol-disulfide exchange

Recent evidence suggests that a two-state model (Tanford, 1968; Privalov, 1979) is inadequate to describe protein folding and unfolding. Under certain conditions, intermediate states between the native (**N**) state and fully unfolded (**U**) state can be significantly populated (Kuwajima, 1989; Kim & Baldwin, 1990; Ptitsyn, 1992; Christensen & Pain, 1994). One such intermediate state has been called the molten globule (**MG**) state (Ohgushi & Wada, 1983). It has been proposed that such a state serves as an intermediate in the process of protein folding in vivo (Gething & Sambrook, 1992; Bychkova & Ptitsyn, 1993; Fink, 1995). The possibility that proteins may be translocated through membranes in such a partially unfolded state, and that it may be preferentially recognized by chaperones, are topics that are under active discussion (Bychkova & Ptitsyn, 1993; Christensen & Pain, 1994). Various research groups have attributed rather different meanings to the term **MG** (see, for example, Griko et al., 1994b; Ewbank et al., 1995; Okazaki et al., 1995). It is, however, generally agreed that **MG** refers to a rather compact state, displaying a significant amount of secondary structure, but lacking the unique packing of side chains characteristic of the **N** state (Kuwajima, 1989; Ptitsyn, 1992; Fink, 1995). We will adopt this broad and general usage in the following.

The extent of retention of secondary structural elements, the degree of expansion relative to **N**, and the flexibility of **MG** vary substantially from protein to protein (Alexandrescu et al., 1993; Christensen & Pain, 1994; Redfield et al., 1994; Ptitsyn, 1995). Moreover, recent studies have described more than one **MG** state

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for a given protein. Thus, distinct **MG**s have been described for apo-myoglobin (Loh et al., 1995; Nishii et al., 1995), apo-α-lactalbumin (Okazaki et al., 1995), and β-lactamase (Ptitsyn, 1995). The issue of whether the polypeptide chain in the **MG** preserves the overall tertiary fold of the native protein (Peng & Kim, 1994; Redfield et al., 1994), or represents an ensemble of fluctuating conformations (Creighton & Ewbank, 1994), is the subject of active discussion (Baldwin, 1991; Ptitsyn, 1992, 1995; Chan et al., 1995; Fink, 1995).

We have shown recently that a dimeric form of *Torpedo* acetylcholinesterase (AChE), in which the two subunits are linked by a disulfide bond close to the carboxyl-terminus (MacPhee-Quigley et al., 1986), is in an **MG** state at ca. 1–2 M guanidinium hydrochloride (Gdn·HCl), whereas at 5–6 M Gdn·HCl, it is in a **U** state (Eichler et al., 1994b; Kreimer et al., 1994b; Weiner et al., 1994). **MG** states of *Torpedo* AChE are also produced by chemical modification of a single buried nonconserved cysteine, Cys^{231}, by various thiol-specific reagents (Dolginova et al., 1992; Kreimer et al., 1994a) and by thermal denaturation (Kreimer et al., 1995). An important property of the **MG** states so generated is that they are maintained even when the perturbing agent is removed, e.g., if Gdn·HCl is removed by dialysis or by dilution (Eichler et al., 1994b), or if chemical modification is reversed (Dolginova et al., 1992; Kreimer et al., 1994a). The **MG** species of AChE obtained in this way are stable for hours to days without aggregating (Kreimer et al., 1994a; Weiner et al., 1994). A detailed physicochemical comparison of the **MG** state(s) produced by treatment with Gdn·HCl and by chemical modification, and of their intraconversion, has not, however, been performed.

In the following, we demonstrate that the **MG** state produced by Gdn·HCl-treatment of *Torpedo* AChE ($\mathbf{MG_{Gdn}}$) differs in its spectroscopic and hydrodynamic characteristics from the **MG** state produced by chemical modification ($\mathbf{MG_{Chem}}$), as well as in its behavior on differential scanning calorimetry (DSC). We further show that, in the absence of the denaturant, transition from $\mathbf{MG_{Chem}}$ to $\mathbf{MG_{Gdn}}$ is a very slow process, indicating that a high energy barrier separates these two flexible, partially unfolded states. We also provide evidence that non-native interactions between subunits stabilize these **MG** states.

Results

Spectral and hydrodynamic characteristics of AChE

Both specific chemical modification of a single nonconserved cysteine residue, Cys^{231}, and exposure to low concentrations of Gdn·HCl produce severe perturbation of the native structure of *Torpedo* AChE (Dolginova et al., 1992; Kreimer et al. 1994a, 1994b; Weiner et al., 1994). Figure 1 shows the effect of both treatments on the CD spectra in the far and near ultraviolet (UV). Table 1 summarizes the CD, intrinsic fluorescence, quasielastic light scattering (QELS), and 1-anilino-8-naphthalenesulfonic acid (ANS)-binding parameters for partially unfolded AChE preparations obtained by treatment with 1.5 M Gdn·HCl and by chemical modification with the thiol-specific disulfide 4,4′-dithiopyridine (DTP) or with mercuric chloride ($HgCl_2$). The corresponding parameters for native AChE and for the **U** state obtained in 5 M Gdn·HCl are shown for comparison. These partially unfolded states are characterized by a greatly reduced ellipticity in the near UV relative to that of native AChE. The ellipticities at 222 nm and the corresponding estimates of α-helical content, calculated according to Batra et al. (1990), suggest that the secondary structure of native AChE is largely retained, whereas the CD spectra in the near UV indicate that its tertiary structure is largely destroyed. The emission maximum of intrinsic fluorescence is shifted to the red, indicating perturbation of the hydrophobic environment of tryptophan residues, but less than in the **U** state. ANS fluorescence is greatly enhanced relative to both the **N** and **U** states of the enzyme, demonstrating similar exposure of hydrophobic surfaces in the various partially unfolded states. QELS data show that the partially unfolded species are compact, with a Stokes radius ca. 10–20% larger than that of the **N** state. In addition, in contrast to the native enzyme, the partially unfolded states of AChE, whether produced by chemical modification or by Gdn·HCl-treatment, are susceptible to trypsin (Dolginova et al., 1992; Weiner et al., 1994). These findings, taken together, lead us to conclude that the partially unfolded states under consideration can all be described as **MG** states on the basis of commonly accepted criteria (Kuwajima,

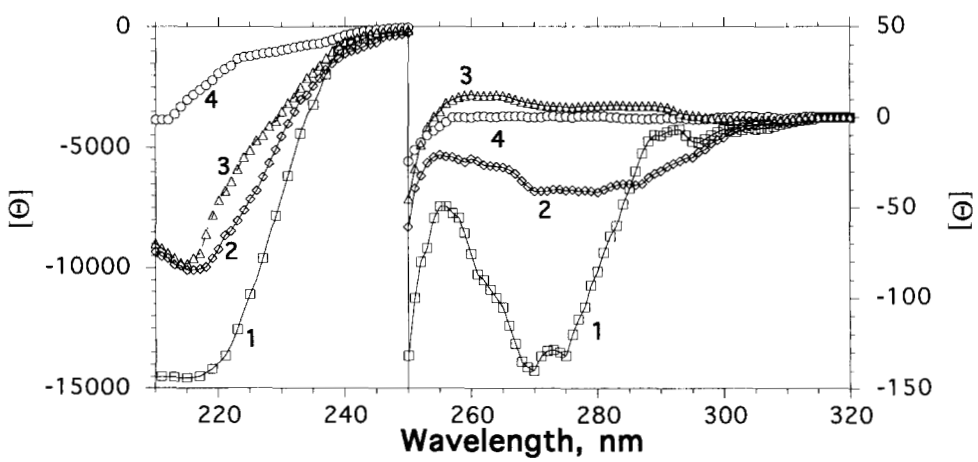

Fig. 1. CD spectra in the fa- and near UV of *Torpedo* AChE in **N**, $\mathbf{MG_{Chem}}$, $\mathbf{MG_{Gdn}}$, and **U**. 1, Native enzyme (**N**); 2, DTP-modified AChE ($\mathbf{MG_{Chem}}$); 3, DTP-modified AChE in 1.5 M Gdn·HCl ($\mathbf{MG_{Gdn}}$); 4, DTP-modified AChE in 5 M Gdn·HCl (**U**). Samples in 1.5 M and 5 M Gdn·HCl were pre-equilibrated for 2 h at room temperature, and CD spectra were measured at the same Gdn·HCl concentrations. In all cases, the final AChE concentration was 3.1 μM in buffer A.

Table 1. *Spectroscopic and hydrodynamic characteristics of native AChE and AChE after various treatments*

Sample[a]	State	$[\Theta^{222}]$ (deg cm^2 dmol^{-1})	α-helix[b] (%)	$[\Theta^{270}]$ (deg cm^2 dmol^{-1})	λ_{max}[c] (nm)	I_{ANS}[d] (arb.un.)	R_S (Å)
Native AChE	**N**	−13,000 ± 500	35.6	−140 ± 10	332.5 ± 0.5	8 ± 3	49 ± 2
AChE in 0.1 M Gdn·HCl (control)	**N**	−12,500 ± 500	34.2	−140 ± 10	333.0 ± 0.5	12 ± 5	49 ± 2
AChE in 1.2 M Gdn·HCl	$\mathbf{MG_{Gdn}}$	−6,500 ± 700	17.5	+10 ± 5	341.0 ± 1.0	62 ± 5	58 ± 2
AChE in 0.1 M Gdn·HCl after 1.2 M Gdn·HCl	$\mathbf{MG_{Gdn}}$	−6,300 ± 700	16.9	+10 ± 5	339.0 ± 1.0	68 ± 8	59 ± 2
DTP-modified AChE	$\mathbf{MG_{Chem}}$	−8,500 ± 700	23.1	−45 ± 10	337.5 ± 1.0	66 ± 8	54 ± 2
DTP-modified AChE demodified with GSH	$\mathbf{MG_{Chem}}$	−8,700 ± 700	23.6	−45 ± 10	337.5 ± 1.0	65 ± 8	ND[e]
$HgCl_2$-modified AChE	$\mathbf{MG_{Chem}}$	−9,000 ± 700	24.5	−45 ± 10	338.0 ± 1.0	68 ± 8	52 ± 2
AChE in 5 M Gdn·HCl	**U**	−1,600 ± 300	<0.1	0 ± 5	352.0 ± 1.5	8 ± 3	110 ± 5

[a] All measurements on AChE preparations in Gdn·HCl were performed after 2 h preincubation at room temperature under the appropriate conditions. All values displayed were derived from 3–6 independent experiments and are expressed as mean ± SD.
[b] The α-helical content, F_h, calculated from the equation, $F_h = -([\Theta^{222}] + 260)/35{,}740$ (Batra et al., 1990).
[c] Wavelength of the emission fluorescence maximum upon excitation at 295 nm.
[d] Intensity of ANS fluorescence measured at 490 nm.
[e] Not determined.

1989; Jaenicke, 1991; Ptitsyn, 1992; Christensen & Pain, 1994; Fink, 1995).

Close inspection of Figure 1 and Table 1 reveals, however, significant differences in the ellipticities at 222 and 270 nm, as well as in hydrodynamic characteristics, between the **MG** states produced by chemical modification and those produced by Gdn·HCl. The **MG** state(s) generated by chemical modification were designated as $\mathbf{MG_{Chem}}$ and those obtained by Gdn·HCl treatment as $\mathbf{MG_{Gdn}}$. Chemically modified AChE retains substantial ellipticity in the near UV; even after demodification by treatment with reduced glutathione (GSH), the shape of the CD band being similar before and after demodification (not shown). In contrast, AChE equilibrated directly in 1.2–2.1 M Gdn·HCl, or indirectly by dilution of AChE pre-equilibrated at 5 M Gdn·HCl (**U** state), displays a small positive ellipticity in the near UV, similar to that obtained for AChE in 5 M Gdn·HCl.

The $\mathbf{MG_{Gdn}}$ preparations whose CD spectra are shown in Figure 1 were obtained subsequent to chemical modification with DTP, whereas those for which spectroscopic parameters are summarized in Table 1 were obtained by direct exposure to Gdn·HCl. We earlier showed that $\mathbf{MG_{Gdn}}$ can undergo spontaneous thiol-disulfide exchange, which can be prevented by chemical modification of the free thiol group of Cys^{231} (Eichler et al., 1994b). It should be noted that the spectroscopic characteristics of $\mathbf{MG_{Gdn}}$ preparations were indistinguishable whether or not Cys^{231} had been blocked. This was true also for $\mathbf{MG_{Gdn}}$ preparations that had been obtained by dilution from high Gdn·HCl concentrations (in which the protein would be in the **U** state).

The apparent Stokes radius of $\mathbf{MG_{Gdn}}$, calculated from the QELS measurements, is ca. 20% larger than that of the native enzyme, whereas that of $\mathbf{MG_{Chem}}$ is only slightly larger, but both the **MG** preparations and **N** AChE are much more compact than the **U** state (Table 1). Increases in the radius of gyration for the **MG** state relative to the **N** state, reported for various proteins, range from <10% (Ptitsyn, 1992) to as much as 32% (Kataoka et al., 1995). Our calculation of the Stokes radius assumes the AChE dimer to be spherical. Because X-ray crystallography reveals that the native dimer has dimensions of ca. 125 × 50 × 50 Å (Sussman et al., 1991), the difference in Stokes radius between $\mathbf{MG_{Chem}}$ and $\mathbf{MG_{Gdn}}$ might well reflect a difference in shape rather than in size (Gast et al., 1992).

Differential scanning calorimetry

The differences in spectral and hydrodynamic characteristics between $\mathbf{MG_{Chem}}$ and $\mathbf{MG_{Gdn}}$ prompted us to compare them by DSC. To avoid possible complications arising from spontaneous thiol-disulfide exchange (Eichler et al., 1994b; Kreimer et al., 1995), $\mathbf{MG_{Gdn}}$ was prepared from chemically modified AChE. No thermally induced cooperative transition is observed for $\mathbf{MG_{Chem}}$, whether produced by chemical modification with DTP (Fig. 2, trace 3) or with $HgCl_2$ (Fig. 2, trace 4). However, equilibration of the $HgCl_2$-modified AChE in 1.8 M Gdn·HCl results in the appearance of a peak on the thermogram (Fig. 2, trace 2) centered at 57.5 °C. This temperature maximum is ca. 12 °C higher than that of the single irreversible thermal peak observed for the native enzyme at the same scan rate (Fig. 2, trace 1). $\mathbf{MG_{Gdn}}$ displays a completely reversible transition, characterized by a parameter of cooperativity, viz. the ratio of the calorimetric enthalpy (ΔH_{cal} = 135 kcal/mol) to the van't Hoff enthalpy (ΔH_{vH} = 136 kcal/mol), of 0.99, which serves as an indication of a two-state transition (Lumry et al., 1966). $\mathbf{MG_{Chem}}$ has a tendency to aggregate at the temperature of thermal denaturation of the **N** state, as could be seen by sucrose gradient centrifugation. Thus, the only conclusion that we can draw from the DSC traces obtained is that the native domain structure does not appear to be preserved in $\mathbf{MG_{Chem}}$ (Kreimer et al., 1995).

Fluorescence titration of the MG states with inhibitors of AChE

Conversion of native AChE to either $\mathbf{MG_{Chem}}$ or $\mathbf{MG_{Gdn}}$ reduces enzymic activity to ≤0.01% of the initial value, which is our detection limit (Kreimer et al., 1994a). This strongly argues that the active center is completely disrupted in both **MG** states. A unique structural feature of AChE is a deep and narrow gorge to which a substantial number of amino acid residues contribute, which are drawn from widely separated sequences of the polypep-

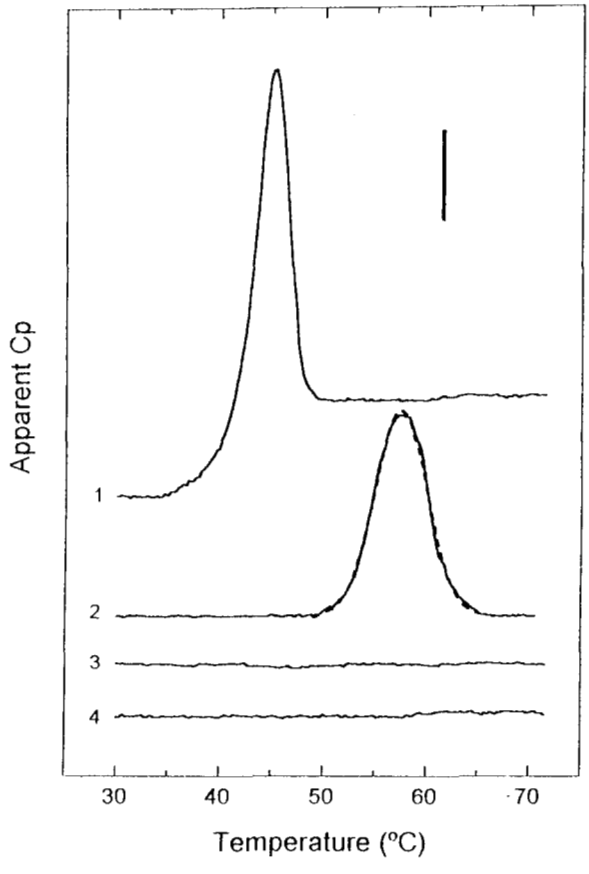

Fig. 2. Original calorimetric recordings of $\mathbf{MG_{Chem}}$ and $\mathbf{MG_{Gdn}}$ states of AChE. 1, Native AChE; 2, $HgCl_2$-modified AChE equilibrated in 1.8 M Gdn·HCl in buffer A ($\mathbf{MG_{Gdn}}$); 3, DTP-modified AChE ($\mathbf{MG_{Chem}}$); 4, $HgCl_2$-modified AChE ($\mathbf{MG_{Chem}}$). AChE concentration was ca. 5 μM. Solid lines represent experimental data after subtracting the instrumental base line; dashed line was generated by fitting the experimental data to a two-state model. Vertical bar, 5 kcal/mol. Scan rate was 59 K/h. Curves are displaced along the ordinate for clarity.

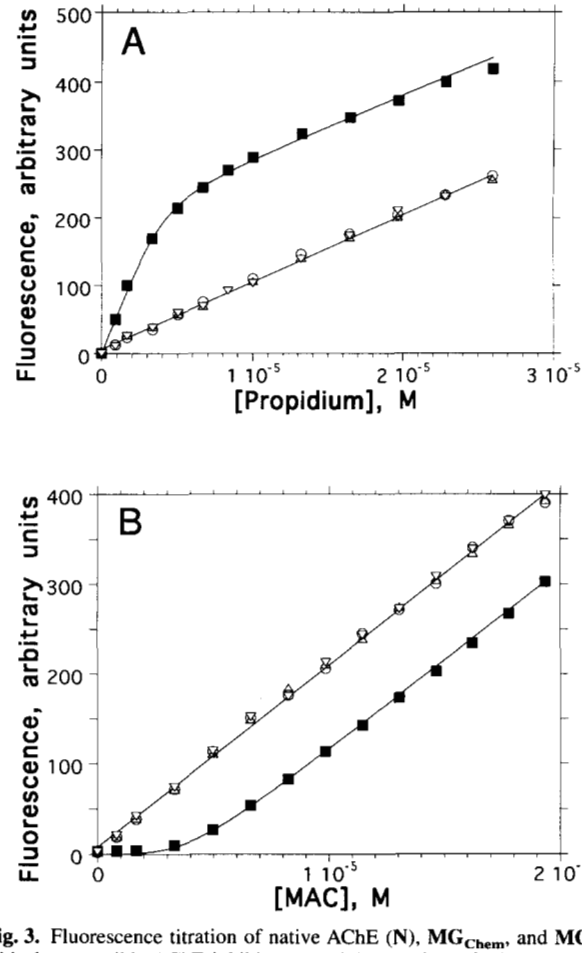

Fig. 3. Fluorescence titration of native AChE (**N**), $\mathbf{MG_{Chem}}$, and $\mathbf{MG_{Gdn}}$ with the reversible AChE inhibitors propidium and MAC. **A:** Propidium-titration of ■, native (**N**) AChE; ○, buffer A; △, DTP-modified AChE subsequent to removal of modifying agent ($\mathbf{MG_{Chem}}$); ▽, AChE equilibrated in 1.8 M Gdn·HCl subsequent to removal of the denaturant ($\mathbf{MG_{Gdn}}$). **B:** Titration of the same samples with MAC. Symbols are the same as in A. AChE concentration was 2 μM. Solid lines were generated by fitting the experimental data to Equation 4, as described in Materials and methods.

tide chain (Sussman et al., 1991). The active site is located at the bottom of this gorge. Two fluorescent probes, propidium and *N*-methylacridinium (MAC), both of which are potent reversible inhibitors of AChE (Mooser et al., 1972; Taylor & Lappi, 1975), display high affinity for two different binding sites located, respectively, on the rim and at the bottom of the gorge, which are at least 10 Å apart (Eichler et al., 1994a). We considered it worthwhile to check whether the $\mathbf{MG_{Chem}}$ and $\mathbf{MG_{Gdn}}$ retained any capacity to bind these ligands, so as to clarify whether the secondary structural elements preserved in these **MG** states retained a native-like tertiary fold (Baldwin, 1991; Ptitsyn, 1992, 1995; Chan et al., 1995).

Fluorescence titration of native AChE with propidium (Fig. 3A) yields an association constant of $(3.2 \pm 0.6)\cdot 10^6\ M^{-1}$, well in the range of reported values (Taylor & Lappi, 1975). Titration curves for $\mathbf{MG_{Gdn}}$ and $\mathbf{MG_{Chem}}$ are indistinguishable from those obtained for buffer alone (Fig. 3A). The results obtained with MAC are very similar (Fig. 3B). Again, the association constant obtained for MAC with native AChE, $(3.8 \pm 0.7)\cdot 10^6\ M^{-1}$, is similar to values reported earlier for *Electrophorus* AChE (Mooser et al., 1972). To estimate the decrease in affinity of the two ligands for the **MG** preparations of AChE, we generated theoretical titration curves corresponding to various decreases in the values of the association constants. Analysis of the curves so generated (not shown) reveals that, within accuracy of our measurements, the association constants of both propidium and MAC for $\mathbf{MG_{Gdn}}$ and $\mathbf{MG_{Chem}}$ are reduced at least 500-fold.

EPR spectra of spin-labeled MG_{Chem} and MG_{Gdn}

Modification of Cys^{231} of *Torpedo* AChE by two different spin-labeled sulfhydryl reagents, biradical and 2,2,5,5-tetramethyl-4-(2-chloromercuri-phenyl)-3-imidazolin-1-oxyl (HgR), converts AChE to $\mathbf{MG_{Chem}}$ (Dolginova et al., 1992; Kreimer et al., 1994a, 1994b); further treatment, with 1.5 M Gdn·HCl, of AChE so modified yields $\mathbf{MG_{Gdn}}$. The upper trace in Figure 4 shows the electron paramagnetic resonance (EPR) spectrum of AChE modified with HgR. It is composed of two superimposed triplet signals: one is a broad signal, typical of a strongly immobilized radical; the other is a sharp triplet, and corresponds to a small amount (<5%) of unbound radical. Only minor changes in the EPR spectrum can be observed upon conversion of $\mathbf{MG_{Chem}}$ to $\mathbf{MG_{Gdn}}$ by addition of the denaturant (trace 2). However, a drastic change in the EPR

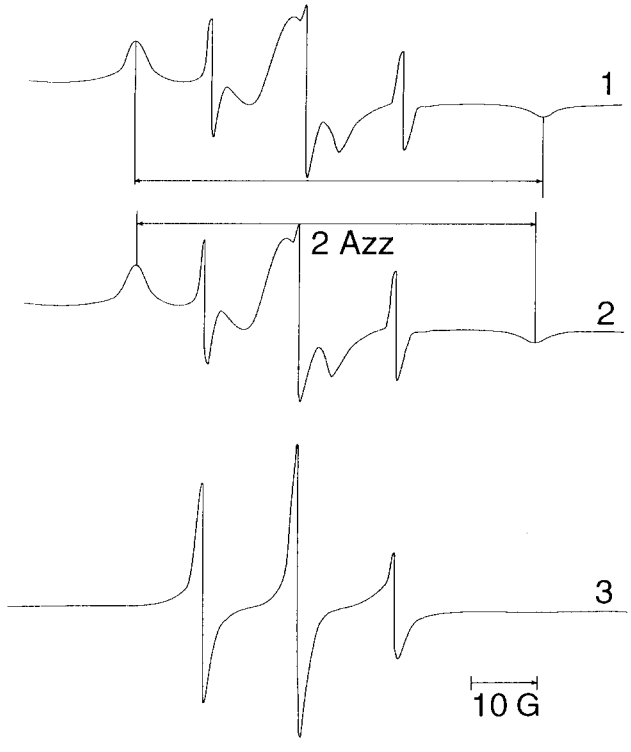

Fig. 4. EPR spectra of spin-labeled AChE in $\mathbf{MG_{Chem}}$, $\mathbf{MG_{Gdn}}$, and **U**. AChE was labeled with biradical as described in Materials and methods, directly yielding $\mathbf{MG_{Chem}}$. $\mathbf{MG_{Gdn}}$ and **U** were obtained from $\mathbf{MG_{Chem}}$ by equilibration in 1.5 and 5 M Gdn·HCl, respectively, and the EPR spectra were measured at the same Gdn·HCl concentrations. In all cases, the concentration of the conjugate was ca. 16 μM. Trace 1, $\mathbf{MG_{Chem}}$; trace 2, $\mathbf{MG_{Gdn}}$; trace 3, **U**. EPR conditions: microwave power, 20 mW; modulation amplitude, 1 G; traces 1 and 2 are at gain 5×10^5; trace 3 is at gain 10^5.

spectrum occurs on raising the Gdn·HCl concentration to 5 M, which produces the **U** state (trace 3). The broad EPR signal of the immobilized radical disappears, with concomitant appearance of the sharp peaks characteristic of a freely rotating radical, which is still, however, slightly restricted relative to the unbound radical in solution. Thus, the spin label is strongly immobilized in both $\mathbf{MG_{Chem}}$ and $\mathbf{MG_{Gdn}}$; in order to detect any possible difference in mobility, a more careful comparison will be required, employing, for example, the EPR saturation transfer technique (Hyde, 1978; Marsh & Horváth, 1989).

Thiol-disulfide exchange in the MG states

We found recently that AChE treated by Gdn·HCl undergoes spontaneous thiol-disulfide exchange, which results in the appearance of novel species on SDS-PAGE, including monomeric species arising from participation of the intrachain disulfide bond in the exchange process (Eichler et al., 1994b). The rate of appearance of these monomeric species, as visualized on SDS-PAGE, may serve as a qualitative criterion for monitoring the kinetics of the thiol-disulfide exchange process (Eichler et al., 1994b). In view of the apparent similarity of $\mathbf{MG_{Chem}}$ and $\mathbf{MG_{Gdn}}$, it was of interest to see whether thiol-disulfide exchange would occur at a similar rate in the two preparations.

HgR-modified AChE was first converted to either $\mathbf{MG_{Chem}}$ or $\mathbf{MG_{Gdn}}$ (see Materials and methods), and the HgR, which protects the thiol group of Cys^{231}, was removed by excess GSH in order to initiate spontaneous thiol-disulfide exchange. Control experiments using EPR demonstrated that such exposure to GSH released >95% of the HgR within the time of sample preparation (see above and Materials and methods). Monomer is hardly noticeable initially, for either type of **MG**, on removal of HgR by GSH on ice. After 3 min at 31 °C, however, this band is consistently visible in $\mathbf{MG_{Chem}}$ and significantly more pronounced in $\mathbf{MG_{Gdn}}$ (Fig. 5A,B), and the rate of the exchange reaction for $\mathbf{MG_{Gdn}}$ is higher than for $\mathbf{MG_{Chem}}$ (Fig. 5C). After 3 h, when the plateau level in accumulation of monomeric species is achieved, $\mathbf{MG_{Chem}}$ and $\mathbf{MG_{Gdn}}$ display a qualitatively similar distribution of species on SDS-PAGE (Fig. 5C, inset). The appearance of monomer was not the result of reduction of preexisting disulfides by the GSH treatment employed to remove the bound HgR. This possibility could be excluded because, if GSH treatment was preceded by blockage of the free sulfhydryl of Cys^{231} using the alkylating agent *N*-ethylmaleimide (NEM), no appearance of monomers was observed.

Transition of MG_{Chem} to MG_{Gdn}

As seen in Figure 1 and Table 1, $\mathbf{MG_{Chem}}$ retains substantial negative residual ellipticity in the near UV, whereas $\mathbf{MG_{Gdn}}$ displays a very small positive ellipticity close to that of the **U** state. Any spontaneous conversion of $\mathbf{MG_{Chem}}$ to a species resembling $\mathbf{MG_{Gdn}}$ would thus be expressed as a reduction in its residual ellipticity in the near UV. By this criterion, $\mathbf{MG_{Chem}}$ is stable for at least 3 h at room temperature (see Fig. 6, traces 1 and 4). No pronounced aggregation of $\mathbf{MG_{Chem}}$ occurs within this period, but more prolonged incubation at room temperature results in the appearance of aggregated species (Kreimer et al., 1994b). The rate of aggregation is increased at higher temperatures (Kreimer et al., 1995), and this hampers observation of the $\mathbf{MG_{Chem}} \rightarrow \mathbf{MG_{Gdn}}$ transition. With the addition of 0.4 M Gdn·HCl to $\mathbf{MG_{Chem}}$ produced by DTP, however, collapse of the band in the near UV occurs within 3 h at room temperature, resulting in a weak positive band characteristic of $\mathbf{MG_{Gdn}}$ (Fig. 6, traces 2 and 3). Even at this low concentration of denaturant, the CD band in the near UV is practically identical to that obtained by direct equilibration of native AChE in 1.2–2.1 M Gdn·HCl (Table 1). Similar exposure of native AChE to 0.4 M Gdn·HCl for 3 h leads to <20% decrease in ellipticity at 270 nm.

No conversion of $\mathbf{MG_{Gdn}}$ back to $\mathbf{MG_{Chem}}$ is observed within 24 h after removal of Gdn·HCl by either gel filtration or dilution, because no reappearance of ellipticity in the near UV can be detected after such a procedure, whether $\mathbf{MG_{Gdn}}$ is produced directly from native AChE or from DTP-modified enzyme. It should be emphasized that QELS measurements reveal that little or no aggregation occurs within this time period.

Another spectroscopic characteristic that differs between $\mathbf{MG_{Chem}}$ and $\mathbf{MG_{Gdn}}$ is the intrinsic fluorescence (Fig. 7A). Although the shift in the emission maximum is not very pronounced (Table 1), we made use of the fluorescence parameter *R* (where *R* is the ratio of the emission intensities at 320 nm and 375 nm), which is sensitive to the conformational state of a protein (see Jiang & London, 1990; Van Dael et al., 1993, and Materials and methods), to monitor the $\mathbf{MG_{Chem}} \rightarrow \mathbf{MG_{Gdn}}$ transition at various Gdn·HCl concentrations. Figure 7B shows that the value of this parameter is 1.60 ± 0.02 for DTP-modified AChE. With the addition of Gdn·HCl, *R* decreases, approaching values ranging from 1.22 ± 0.03 at 0.4 M

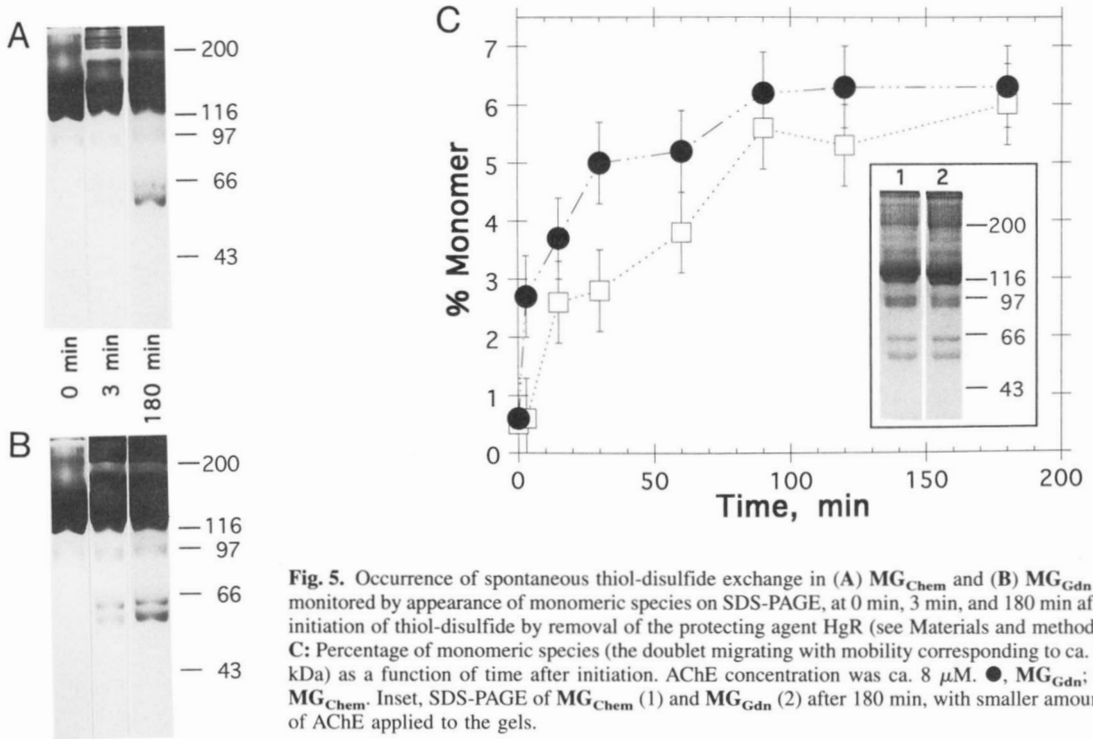

Fig. 5. Occurrence of spontaneous thiol-disulfide exchange in (**A**) $\mathbf{MG_{Chem}}$ and (**B**) $\mathbf{MG_{Gdn}}$ as monitored by appearance of monomeric species on SDS-PAGE, at 0 min, 3 min, and 180 min after initiation of thiol-disulfide by removal of the protecting agent HgR (see Materials and methods). **C:** Percentage of monomeric species (the doublet migrating with mobility corresponding to ca. 60 kDa) as a function of time after initiation. AChE concentration was ca. 8 μM. ●, $\mathbf{MG_{Gdn}}$; □, $\mathbf{MG_{Chem}}$. Inset, SDS-PAGE of $\mathbf{MG_{Chem}}$ (1) and $\mathbf{MG_{Gdn}}$ (2) after 180 min, with smaller amounts of AChE applied to the gels.

Gdn·HCl to 1.11 ± 0.03 at 1.4 M Gdn·HCl. The rate of the transition increases with the Gdn·HCl concentration, and the plateau value of R, attained at 3 h, displays a linear dependence on the Gdn·HCl concentration (Fig. 7C, open squares). Dilution of a sample of $\mathbf{MG_{Gdn}}$ obtained at 1.4 M Gdn·HCl to a lower concentration of denaturant results in an immediate (within the time of sample preparation) change in R to a value very similar to that observed by direct addition to $\mathbf{MG_{Chem}}$ of Gdn·HCl at the final concentration (Fig. 7C, closed circles). Extrapolation of the dependence of R versus [Gdn·HCl] to zero Gdn·HCl yields an R value of 1.29 ± 0.05, which is significantly lower than that for $\mathbf{MG_{Chem}}$. Various spectral characteristics of proteins, which remain within the same conformational state, change with the concentration of denaturant (Pace, 1986). These fluorescence data suggest, therefore, that at Gdn·HCl concentrations in the range of 0.4–1.4 M, the same state, viz. $\mathbf{MG_{Gdn}}$, is achieved, and that no reversion to $\mathbf{MG_{Chem}}$ is observed on dilution to low concentrations of Gdn·HCl, as was shown also by the CD measurements.

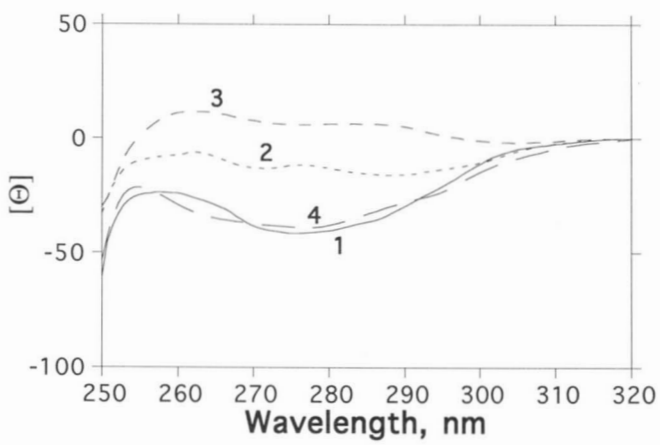

Fig. 6. CD spectra in the near UV of $\mathbf{MG_{Chem}}$ before and after addition of 0.4 M Gdn·HCl to promote conversion to $\mathbf{MG_{Gdn}}$. 1, 4, DTP-modified AChE in buffer A at room temperature, immediately after sample preparation and after 180 min, respectively. 2, 3, DTP-modified AChE in buffer A containing 0.4 M Gdn·HCl at room temperature, after 50 and 180 min, respectively. AChE concentration was 3.8 μM in buffer A.

Kinetic studies of unfolding (and refolding) of many proteins show that the logarithm of the rate constant of unfolding (refolding), k, displays a linear dependence on the concentration of the denaturant (Kuwajima et al., 1989; Matouschek et al., 1990; Schreiber & Fersht, 1993). In our case, all experimental curves (Fig. 7B) can be fitted by a single exponent, permitting us to determine rate constants for the $\mathbf{MG_{Chem}} \rightarrow \mathbf{MG_{Gdn}}$ transition at a given concentration of denaturant, and to plot log(k) versus [Gdn·HCl] (Fig. 7D). The plot shows that linear approximation of the dependence is rather poor, as has been found to be the case in several other experimental systems (see, for example, Ikeguchi et al., 1986; Kuwajima et al., 1989; Matouschek et al., 1994). We adopted, therefore, a quadratic expression that Matouschek et al. (1994) had shown to describe accurately the dependence of log k on denaturant concentration for urea-induced unfolding of barnase mutants. In our case, too, use of this expression yields a very good fit (r = 0.998), and extrapolation to zero Gdn·HCl yields a putative half-life for $\mathbf{MG_{Chem}}$ of ca. 13 h in the absence of denaturant (Fig. 7D). Together these data show that $\mathbf{MG_{Chem}}$ is separated from $\mathbf{MG_{Gdn}}$ by a high free energy barrier and that $\mathbf{MG_{Gdn}}$ seems to be the thermodynamically preferred state under physiologic conditions.

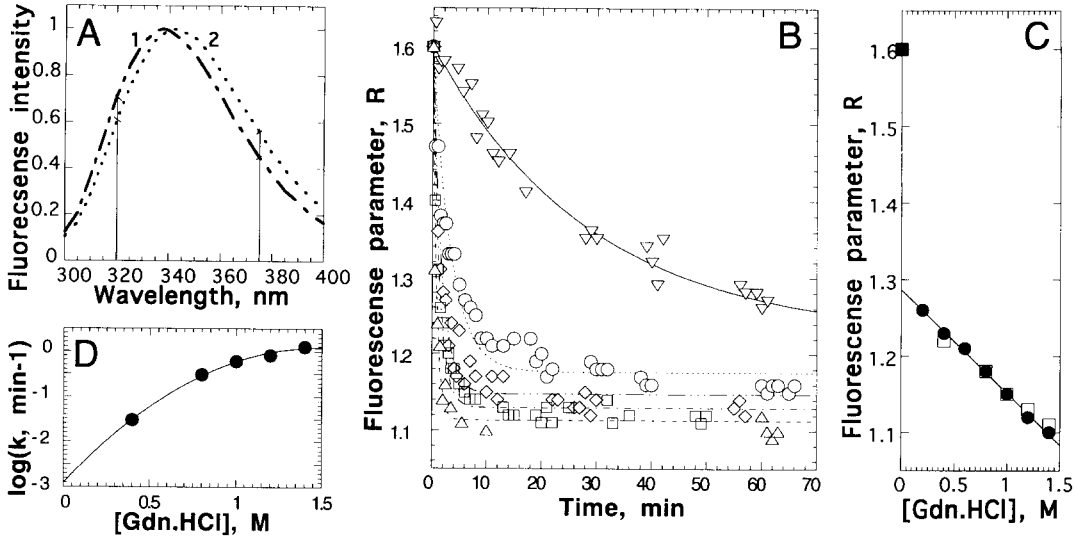

Fig. 7. Kinetics of the $MG_{Chem} \rightarrow MG_{Gdn}$ transition at various Gdn·HCl concentrations, as monitored by changes in the fluorescence parameter, *R*. **A:** Fluorescence emission spectra of DTP-modified AChE (MG_{Chem}) and the same preparation equilibrated with 1.2 M Gdn·HCl (MG_{Gdn}). Excitation was at 280 nm. Trace 1, MG_{Chem}; trace 2, MG_{Gdn}. **B:** Time-dependence of *R*, observed subsequent to addition at zero time of Gdn·HCl to final concentrations of △, 1.4 M; □, 1.2 M; ◇, 1.0 M; ○, 0.8 M; ▽, 0.4 M. Curves were generated as described in Materials and methods; **C:** *R* as a function of Gdn·HCl concentration. *R* was measured 3 h after addition to MG_{Chem} of Gdn·HCl to the same concentrations as in B (□); alternatively, *R* was measured for samples of MG_{Gdn} equilibrated in 1.4 M Gdn·HCl, and diluted to the appropriate final Gdn·HCl concentration (●); ■, denotes the value of *R* for freshly prepared MG_{Chem} in the absence of Gdn·HCl; **D:** Dependence of the logarithm of the rate constant, *k*, for the $MG_{Chem} \rightarrow MG_{Gdn}$ transition upon the Gdn·HCl concentration. The line represents a second-order polynomial fit of the experimental points.

Interaction between subunits of AChE in the MG states

We analyzed the affinity between subunits in the native and partially unfolded states by reducing of the intersubunit disulfide bond by treatment of AChE with dithiothreitol (DTT) at room temperature. Under the experimental conditions used, sucrose gradient centrifugation revealed that native AChE so treated displays a peak corresponding to monomer, ca. 4.5 S (Fig. 8A). Nonreduced MG_{Gdn} and MG_{Chem} display 7 S peaks, i.e., migrate at the same position as the native dimer, in agreement with the QELS data (Table 1). In contrast to the case for native AChE, no monomeric species is observed on DTT treatment of MG_{Gdn} (Fig. 8C). DTT treatment of MG_{Chem} yields only ca. 30% monomer (Fig. 8B). It would be plausible to ascribe these results to incomplete reduction by DTT of the intersubunit disulfide in MG_{Gdn} and MG_{Chem}. This is not the case, however, because AChE, whether native or partially unfolded, with SDS-PAGE in the absence of reducing agent, subsequent to exposure to DTT, and followed by its removal and subsequent treatment with excess NEM, migrates as a monomer, with <5% residual dimer observed (not shown). Our data thus suggest that the bulk of MG_{Gdn} is physically associated as a dimer, despite the absence of an interchain disulfide linkage, under conditions in which the native dimer dissociates, and that the same is true, to a lesser extent, for MG_{Chem}.

Discussion

We have shown above that *Torpedo* AChE can exist in two partially unfolded states. Both these states, viz. that produced by chemical modification and that produced by Gdn·HCl, possess several important characteristics of the MG state (Kuwajima, 1989; Jaenicke, 1991; Ptitsyn, 1992; Christensen & Pain, 1994; Fink, 1995). However, they also differ significantly in their spectroscopic and hydrodynamic characteristics, as well as displaying very different DSC scans. Both partially unfolded states are stable for many hours under physiologic conditions, without either interconverting or undergoing pronounced aggregation, and neither of them reverts to the N state. Thus, the two states are separated both from each other and from the native state by high energy barriers. It is important to emphasize that these differences between MG_{Chem} and MG_{Gdn} cannot arise due to reshuffling of disulfide bonds at low Gdn·HCl concentrations, because identical results are obtained if reshuffling is prevented by chemical modification of Cys^{231} (Eichler et al., 1994b).

Our spectroscopic measurements show that MG_{Chem} retains some negative residual ellipticity in the near UV, whereas MG_{Gdn} displays a very small positive ellipticity close to that of the U state. The position of the emission maximum for MG_{Gdn} is shifted slightly more to the red than that of MG_{Chem}. In addition, the CD spectra in the far UV suggest that MG_{Chem} retains more α-helical structure than MG_{Gdn} (Fig. 1; Table 1). It was claimed recently that a native domain was preserved within a partially unfolded fragment of staphylococcal nuclease, despite the small size of this protein, and that this native domain was responsible for the two-state character of the transition of this species to a fully unfolded state as observed by DSC (Griko et al., 1994b). Thermal unfolding of native AChE reveals a single peak with the maximum at 45 °C (Kreimer et al., 1995). It is unlikely that MG_{Chem} retains any native domain structure, because it lacks a calorimetric peak in the temperature range of the $N \rightarrow MG_{Chem}$ transition (Fig. 2). Furthermore, visual inspection of the 3D structure of AChE (Sussman et al., 1991) does not reveal any obvious independent domains. Therefore, it seems unlikely that the residual ellipticity is due to the presence of an intact domain within MG_{Chem}. This finding corroborates our previous conclusion that chemical modification

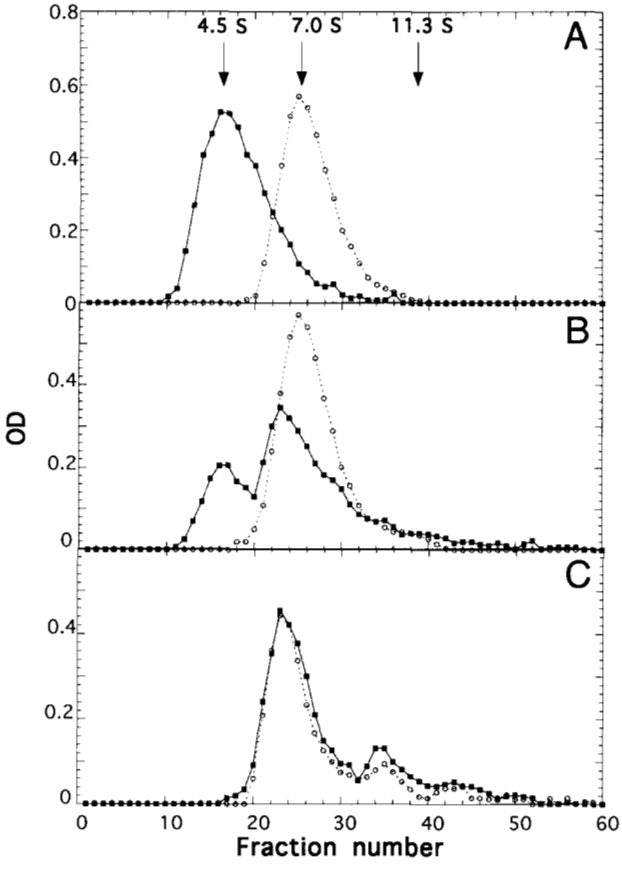

Fig. 8. Sucrose gradient centrifugation of native (**N**) AChE, $\mathbf{MG_{Chem}}$, and $\mathbf{MG_{Gdn}}$ after reduction of the intersubunit disulfide bond. **A: N. B:** $\mathbf{MG_{Chem}}$. **C:** $\mathbf{MG_{Gdn}}$. All three samples were centrifuged either before (○) or after (■) exposure to 15 mM DTT in buffer A for 1 h at room temperature. Arrows indicate positions of AChE monomer (4.5 S), AChE dimer (7.0 S), and the catalase marker (11.3 S).

transforms the *whole* AChE dimer, containing 537 amino acid residues in each subunit, to a partially unfolded state (Dolginova et al., 1992; Kreimer et al., 1994a). It seems more plausible that in $\mathbf{MG_{Chem}}$ some of the secondary structural elements are organized via residual tertiary interactions. This implies that $\mathbf{MG_{Chem}}$ may resemble the "highly-ordered" type of **MG** described recently, whereas $\mathbf{MG_{Gdn}}$ may represent a more disordered **MG** (Redfield et al., 1994).

$\mathbf{MG_{Chem}}$ displays no detectable transition to $\mathbf{MG_{Gdn}}$ under physiologic conditions over a period of a few hours, implying that a high free energy barrier separates these states. The $\mathbf{MG_{Chem}} \rightarrow \mathbf{MG_{Gdn}}$ transition is observed only in the presence of Gdn·HCl (Figs. 6, 7A). Extrapolation of the logarithm of the transition rate to zero concentration of denaturant yields a free energy barrier as high as 23.8 kcal/mol. As already mentioned, this dependence was not linear, and could be fitted well by a quadratic expression (Fig. 7C). Deviation from linearity in such plots was observed earlier for α-lactalbumin and lysozyme (Ikeguchi et al., 1986; Kuwajima et al., 1989), for barnase mutants (Matouschek et al., 1990), and for barstar (Schreiber & Fersht, 1993). Such a deviation suggests that the transition does not fit a two-state model, and that there must be at least one intermediate (Ikeguchi et al., 1986; Matouschek et al., 1990). Thus, the $\mathbf{MG_{Chem}} \rightarrow \mathbf{MG_{Gdn}}$ transition may also occur via one or more intermediate states. We cannot exclude the possibility that the deviation from linearity reflects a change in the nature of the transition state (Matouschek et al., 1994). The free energy barrier for the $\mathbf{MG_{Chem}} \rightarrow \mathbf{MG_{Gdn}}$ transition approaches those estimated for transitions between partially unfolded and native states of several proteases lacking their pro sequences (see Baker & Agard, 1994; Eder & Fersht, 1995; and references therein). However, in our case, the high energy barrier separates *two partially unfolded* states. The rate of the transition might be expected to increase at higher temperatures, but pronounced aggregation of $\mathbf{MG_{Chem}}$ occurs under such conditions (Kreimer et al., 1995). This precludes direct observation of the transition, in particular in DSC experiments.

We considered the possibility that Gdn·HCl may be directly involved in stabilization of $\mathbf{MG_{Gdn}}$, because it has been reported that Gdn·HCl can stabilize both native (Mayr & Schmid, 1993) and partially unfolded states of various proteins (Hagihara et al., 1993; see also Haynie & Freire, 1993, for recent theoretical analysis of effects of denaturants on partially unfolded states). We found, however, that $\mathbf{MG_{Gdn}}$ from which Gdn·HCl had been almost completely removed by gel filtration followed by extensive dialysis, displays essentially the same spectroscopic and hydrodynamic characteristics as in the presence of denaturant. We cannot, however, exclude the possibility of a tight complex of AChE with a small number of molecules of Gdn·HCl.

Two different **MG** states have been described for apomyoglobin, which have been characterized accurately by CD and NMR spectroscopy (Loh et al., 1995; Nishii et al., 1995). In one of these, only three of the eight α-helices of the native protein are retained, whereas in the other, an additional α-helix is retained. These **MG** states are in equilibrium with each other and with the **U** and **N** states. In the case of AChE, the large molecular weight of the dimer (130 kDa) does not permit characterization by NMR, but our CD observations suggest that $\mathbf{MG_{Gdn}}$ and $\mathbf{MG_{Chem}}$ differ in helical content (Table 1). However, the two are in equilibrium neither with each other nor with **N**, the only freely reversible transition being the $\mathbf{MG_{Gdn}} \leftrightarrow \mathbf{U}$ transition. The simplest scheme describing the relationship between the states of AChE that we have generated is thus $\mathbf{N} \rightarrow \mathbf{MG_{Chem}} \rightarrow \mathbf{MG_{Gdn}} \leftrightarrow \mathbf{U}$. The only transition permitting equilibrium thermodynamic analysis yields the free energy of stabilization of $\mathbf{MG_{Gdn}}$ relative to **U**, viz. 5.6 kcal/mol (Kreimer et al., 1994b), a value similar to that obtained for the more stable of the two **MG** states of apomyoglobin (Loh et al., 1995).

The cooperativity of the transition from the native state to a denatured state is well-established and has its origin in the destruction of the unique structure of the entire protein molecule in its native conformation (Tanford, 1968; Privalov, 1979; Karplus & Shakhnovich, 1992). It is not, a priori, obvious that the $\mathbf{MG} \leftrightarrow \mathbf{U}$ transition should be cooperative, because the **MG** state is already partially unfolded and the unique packing of side chains of the **N** state no longer exists. Studies of this transition employing a number of different proteins and experimental techniques have yielded diverse data, reporting either the presence or absence of such a cooperative transition (see, for example, Ogasahara et al., 1993; Hamada et al., 1994) and, in the case of two proteins, α-lactalbumin (Shimizu et al., 1993; Uversky, 1993; Griko et al., 1994a; Freire, 1994) and apomyoglobin (Bismuto & Gaetano, 1994; Griko & Privalov, 1994; Nishii et al., 1995), conflicting results (for a recent review, see Fink, 1995). Chan et al. (1995) consider that three criteria should be satisfied in order to firmly establish that a transition is cooperative, viz. coexistence of both states in the transition region, observation of a thermal transition by DSC that satisfies

the criterion of unity for the van't Hoff-to-calorimetric enthalpy ratio (Lumry et al., 1966), and coincidence of the sigmoidal curves obtained by independent spectroscopic methods. We showed earlier that Gdn·HCl-induced unfolding of spin-labeled AChE in the $\mathbf{MG_{Gdn}}$ state could be described by a two-state model, and our experimental data (Kreimer et al., 1994b) satisfied the first and third criteria mentioned by Chan et al. (1995). We considered it, therefore, important to use DSC to see whether the thermal transition of $\mathbf{MG_{Gdn}}$ could also be described by a two-state model.

In our study of the dependence of the $\mathbf{MG} \leftrightarrow \mathbf{U}$ transition on the Gdn·HCl concentration, HgR-modified AChE was employed so that EPR spectroscopy could be used to monitor the transition (Kreimer et al., 1994b). In our present study, we wished to block Cys^{231} with an organomercurial, so as to prevent reshuffling of disulfide bridges (Eichler et al., 1994b). Sucrose gradient centrifugation revealed, however, that HgR-modified AChE undergoes substantial aggregation within 10 min at 45 °C. In contrast, $HgCl_2$-modified AChE treated with Gdn·HCl, which displays the spectroscopic and hydrodynamic characteristics of $\mathbf{MG_{Gdn}}$, and is practically indistinguishable by these criteria from HgR-modified AChE treated with Gdn·HCl, does not show a strong tendency to aggregate. We therefore chose to employ $HgCl_2$-modified AChE for our DSC analysis. The calorimetric peak of $\mathbf{MG_{Gdn}}$ so prepared was found to be completely reversible and described well by a two-state model (Fig. 2). The fact that $\mathbf{MG_{Gdn}}$ displays cooperative thermal unfolding, as found by DSC, together with our earlier direct observation, by means of EPR, of the two-state character of Gdn·HCl-induced unfolding of $\mathbf{MG_{Gdn}}$, in combination with the demonstration of a coincidence of the $\mathbf{MG} \leftrightarrow \mathbf{U}$ transition curves obtained by CD and fluorescence techniques (Kreimer et al., 1994b), demonstrates clearly that $\mathbf{MG_{Gdn}}$ does indeed unfold cooperatively. Thus, the $\mathbf{MG} \leftrightarrow \mathbf{U}$ transition of AChE satisfies all three experimental criteria discussed by Chan et al. (1995).

As already mentioned, the *Torpedo* AChE dimer consists of two large (65 kDa) subunits, linked by a disulfide bridge. DSC studies of proteins composed of independent domains, or of subunits that unfold independently, even at the same temperature, may display more than one calorimetric peak and/or a cooperativity parameter ($\Delta H_{cal}/\Delta H_{vH}$) larger than one (Privalov, 1982). However, cases have also been reported in which multisubunit proteins display one peak and a cooperativity parameter approaching unity (Gitelson et al., 1991; Filimonov et al., 1993; Tamura et al., 1994), suggesting that they unfold as a single cooperative unit. The shape of the single calorimetric peak, as well the cooperativity parameter, indicate that $\mathbf{MG_{Gdn}}$, although much larger, behaves similarly. This behavior implies strong interaction between the two subunits. Our sucrose gradient centrifugation data clearly showed that reduction of the intersubunit disulfide of native *Torpedo* AChE produced dissociation of the two subunits. Similar reduction of the disulfide bond of $\mathbf{MG_{Gdn}}$ did not result in appearance of a monomeric peak, indicating that there are strong interactions between the subunits in $\mathbf{MG_{Gdn}}$, with $\mathbf{MG_{Chem}}$ displaying intermediate behavior (Fig. 8). It would, of course, be desirable to compare the parameters of cooperativity for thermal denaturation of **N** and $\mathbf{MG_{Gdn}}$. The irreversibility of thermal denaturation of **N** (Kreimer et al., 1995) precludes such a comparison.

Two hypotheses have been offered, which are not mutually exclusive, to account for the cooperativity of the $\mathbf{MG} \leftrightarrow \mathbf{U}$ transition in those cases in which it has been observed. According to one hypothesis, the native tertiary *fold* is retained in the **MG** state (Ptitsyn, 1992; Peng & Kim, 1994) and the cooperativity of unfolding would reflect destruction of this fold (Ptitsyn, 1995). According to the second hypothesis, secondary structural elements are preserved in the **MG** state due to costabilization within the protein molecule, which would presumably be due to hydrophobic interactions between these elements (Baldwin, 1991; Freire, 1994). Such hydrophobic clusters of secondary structural elements, whether possessing or devoid of specific organization (Baldwin, 1991), could form a hydrophobic core (Dobson, 1992; Chyan et al., 1993; Hagihara et al., 1993). Destruction of such an entity might well display cooperativity (Freire, 1994; Dill & Stigter, 1995).

With respect to the first hypothesis, we attempted to seek evidence for the presence of a tertiary fold in both $\mathbf{MG_{Gdn}}$ and $\mathbf{MG_{Chem}}$. One way to obtain information concerning possible tertiary interactions in a polypeptide chain is to assess its capacity to form specific disulfide bonds (Clarke & Fersht, 1993; Creighton & Ewbank, 1994; Peng & Kim, 1994). We used an approach (Eichler et al., 1994b) that monitored spontaneous thiol-disulfide exchange in our two **MG** states; we found evidence that species with a non-native profile of disulfide bonds appear in both states. Careful chemical analyses will be needed to characterize the species so produced, but we used the appearance of monomeric species on SDS-PAGE (Eichler et al., 1994b) as a diagnostic tool for monitoring the time-course of the process. Occurrence of such reshuffling does not support the notion that the native tertiary fold is preserved in either $\mathbf{MG_{Chem}}$ or in $\mathbf{MG_{Gdn}}$. Reshuffling appears, however, to be faster for $\mathbf{MG_{Gdn}}$ than for $\mathbf{MG_{Chem}}$. This may reflect differences in both structure and *flexibility* between the two **MG** states.

Another possible approach to assess possible retention of a native tertiary fold is to seek residual affinity for ligands capable of recognizing the **N** state. If such affinity were to be detected, it would suggest that a specific fold recognizing the ligand was partially retained in the **MG** state, or was readily reorganized with the addition of the ligand. Thus, high affinity of an **MG** state of subtilisin for both Ca^{2+} and for the pro region was reported recently; binding of either of these "ligands" induced refolding to the native state, suggesting that the native fold either preexisted or reformed upon binding (Eder & Fersht, 1995). Our failure to detect any affinity of either $\mathbf{MG_{Gdn}}$ or $\mathbf{MG_{Chem}}$ for two specific ligands, MAC and propidium, which bind to AChE at two different sites, clearly shows that the native fold, at least in the proximity of the active-site gorge, is not preserved. Thus, neither of our two experimental approaches provides any support for the presence of a native fold in either $\mathbf{MG_{Chem}}$ or $\mathbf{MG_{Gdn}}$. Our data cannot, however, exclude the possibility that a *non-native* fold is formed in $\mathbf{MG_{Gdn}}$ that is responsible for the cooperativity of its unfolding that we observed both in the present study and earlier (Kreimer et al., 1994b).

With respect to the possible existence of organized hydrophobic clusters within either $\mathbf{MG_{Chem}}$ or $\mathbf{MG_{Gdn}}$, as already postulated for certain experimental systems (see above), we suggest that such a core, composed of hydrophobic clusters organized across the interface between the two subunits of the dimer, is formed in $\mathbf{MG_{Gdn}}$. This assumption is supported both by the DSC data and by the fact that strong non-native interactions maintain subunit association even when the disulfide link is destroyed. We speculate that the unexpectedly slow conversion of $\mathbf{MG_{Chem}}$ to $\mathbf{MG_{Gdn}}$ is driven hydrophobically (Dill, 1990) by interaction between clusters, preexisting within each subunit, to produce the common hydrophobic core. The novel structural entity, once formed, would be thermodynamically favored relative to both $\mathbf{MG_{Chem}}$ and **N**. This

would explain the stability of $\mathbf{MG_{Gdn}}$ on removal of denaturant, and the fact that the thermal transition to the **U** state, observed by DSC at ca. 57 °C, occurs at a temperature 12° higher than thermal denaturation of the native enzyme (Kreimer et al., 1995).

Our data present clear evidence that both $\mathbf{MG_{Chem}}$ and $\mathbf{MG_{Gdn}}$ are compact, flexible, and essentially devoid of any elements of the native tertiary fold. It might have been expected, therefore, that there would be a low energy barrier between $\mathbf{MG_{Chem}}$ and $\mathbf{MG_{Gdn}}$, and that the spontaneous transition from $\mathbf{MG_{Chem}}$ to the more thermodynamically stable $\mathbf{MG_{Gdn}}$ would occur rapidly, as is the case for the transition between two **MG** states observed for apomyoglobin (Loh et al., 1995). However, we observed a very slow rate of conversion of $\mathbf{MG_{Chem}}$ to $\mathbf{MG_{Gdn}}$. This indicates that compactness and flexibility of a partially unfolded state, per se, are insufficient for it to attain rapidly a conformation corresponding to a lower free energy level, and that folding of large proteins requires a nonrandom search mechanism.

Materials and methods

Materials

AChE was the dimeric (G_2) glycosyl-phosphatidylinositol-anchored (GPI-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).

HgR was synthesized as described previously (Volodarsky & Weiner, 1983). $HgCl_2$ was purchased from BDH Laboratory Chemical Division (Poole, England) and MAC from Molecular Probes (Junction City, Oregon). Gdn·HCl (Ultra Pure) was purchased from Schwartz/Mann Biotech (Cleveland, Ohio). ANS (magnesium salt), acetylthiocholine iodide, DTP, propidium iodide, NEM, 5,5′-dithiobis(2-nitrobenzoic acid), GSH, and DTT were obtained from Sigma (St. Louis, Missouri). Gelatin was from Merck (Darmstadt, Germany). All other reagents were of analytical grade or higher.

Assay methods

Concentrations of AChE were determined spectrophotometrically, taking $\epsilon^{280}(1\ mg/mL) = 17.5$ (Taylor et al., 1974), or colorimetrically (Bradford, 1976), using a solution of native *Torpedo* AChE of known concentration for calibration. The AChE concentration is expressed as the concentration of the dimer (molecular weight 130,000), assuming a subunit molecular weight of 65,000 (Sussman et al., 1988). AChE activity was monitored as described previously (Kreimer et al., 1994a).

Buffers

Unless otherwise stated, the buffer employed was 0.1 M NaCl/10 mM Na-phosphate, pH 7.3 (buffer A).

Modification of AChE by sulfhydryl reagents

Modification of AChE by DTP and by the mercurials $HgCl_2$ and HgR was performed as described previously (Dolginova et al., 1992; Kreimer et al., 1994a). Unbound sulfhydryl reagent was removed by gel-filtration on a Bio-Gel P6 column (1 × 7 cm). If necessary, the eluted protein was concentrated in a Centricon-30 microconcentrator. When modification of AChE was performed with mercurials, physicochemical studies using such samples were performed only after preincubation for >12 h at room temperature, to decrease to <1% the population of metastable, native-like species of mercurial-modified AChE produced initially, which are amenable to reactivation (Kreimer et al., 1994a).

Gdn·HCl-treatment of AChE

A solution of Gdn·HCl, at an appropriate concentration, in 0.1 M NaCl/50 mM Na-phosphate, pH 7.3 (obtained from a stock solution of 8 M Gdn·HCl in the same buffer), was mixed, under stirring, with a small aliquot (ca. 30% of final volume) of native or chemically modified AChE in buffer A. In some of the experiments, Gdn·HCl was removed by gel-filtration on a Bio-Gel P6 column (1 × 7 cm).

DSC measurements

DSC was performed on a MicroCal MC-2 differential scanning micro-calorimeter (MicroCal Inc., Northampton, Massachusetts) with cell volumes of 1.22 mL, interfaced with a personal IBM-compatible computer. Before measurement, samples were dialyzed for 18–24 h against buffer A, degassed under stirring, in vacuo, for 5 min, and immediately loaded into the calorimeter cell, with degassed final dialysis buffer in the reference cell. An overpressure of 2 atm dry nitrogen was maintained over the solutions in the cells throughout the scans to prevent any degassing during heating. Reversibility of thermal transitions was examined by performing a second calorimetric scan immediately after cooling subsequent to the first scan. The excess molar heat capacity was calculated assuming the molecular mass of *Torpedo* AChE to be 130,000. Experimental traces were corrected for the calorimeter baseline obtained by scanning the appropriate buffer solution in both cells of the calorimeter (Ruiz-Arribas et al., 1994). Data were analyzed and plotted using the Window-based software package, Origin, supplied by MicroCal Inc.

Intrinsic fluorescence measurements

Intrinsic fluorescence spectra were measured either in a Hitachi F-4010 spectrofluorometer, the desired temperature being maintained by circulation of water through a hollow brass cell-holder, or in a Shimadzu RF-540 spectrofluorometer at room temperature. Excitation was at 295 nm, and both monochromators were set at 2-nm slit width.

Fluorescent monitoring of the kinetics of transition in DTP-modified AChE, subsequent to addition of Gdn·HCl, was performed by rapid injection, under stirring, of 200 μL of a 5-μM solution of DTP-modified AChE in buffer A to a cuvette containing 800 μL of the same buffer combined with Gdn·HCl. The time of mixing did not exceed 10 s. An excitation wavelength of 280 nm was employed, and the slit width of the monochromators was 5 nm. The ratio of intensities at fixed wavelengths on the shoulders of the emission peak has been shown to be very sensitive to structural changes and independent of protein concentration (Jiang & London, 1990; Van Dael et al., 1993). The kinetics of the transition were, therefore, followed by monitoring emission intensities at 320 and 375 nm. Intensity measurements were taken at ca. 10-s intervals, and the values of the fluorescence parameter, $R = I_{320}/I_{375}$, so

obtained, were assumed to follow the course of the transition. Data were analyzed by fitting experimental curves to the equation:

$$R = R_{final} + \Delta R \cdot \exp(-k_{app} \cdot t), \quad (1)$$

where k_{app} (min^{-1}) is the first-order reaction rate constant, t is time (min), and ΔR and R_{final} correspond to the total change in the fluorescence parameter and to the final value of the fluorescence parameter, respectively.

Fluorescence titration with propidium and MAC

Affinity of the specific AChE inhibitors propidium and MAC for native and partially unfolded states of *Torpedo* AChE was analyzed by fluorescence titration with these two ligands of a 2-μM solution of the protein. Partially unfolded AChE was prepared by treatment with 1.8 M Gdn·HCl subsequent to removal of the denaturant as described above, or by chemical modification of AChE with DTP followed by demodification with GSH (Dolginova et al., 1992). Titration was performed in a Shimadzu RF-540 spectrofluorometer at room temperature. The slits of both monochromators were set at 5-nm slit width. In the case of MAC, the excitation and emission wavelengths were 360 nm and 460 nm, respectively, and in the case of propidium, 535 nm and 620 nm. For both ligands, the stoichiometry of binding to native enzyme was taken as one mole of ligand per catalytic subunit (Mooser et al., 1972; Taylor & Lappi, 1975). Accordingly, the titration curves were analyzed on the assumption that, at a given concentration of ligand, the fluorescence intensity can be expressed as:

$$I = \alpha \cdot (L - C) + \beta \cdot C, \quad (2)$$

where α is the intensity coefficient of the free ligand (the proportionality factor between observed fluorescence intensity and free ligand concentration obtained independently by titration of the buffer), and β is the intensity coefficient of the protein–ligand complex (the proportionality factor between the observed fluorescence intensity and the concentration of the complex obtained from the initial slope of the titration curve; this factor is negative for MAC because its fluorescence in the complex is quenched). L is the total concentration of the ligand, C is the concentration of the ligand–protein complex, and they are related by the following equation:

$$K = C/((L - C) \cdot (A - C)), \quad (3)$$

where K is the association constant and A is the concentration of binding sites. From Equations 2 and 3, the fluorescence intensity, I, can be expressed as a function of L:

$$I = \alpha \cdot \{L - [((K \cdot A + K \cdot L + 1) - ((K \cdot A + K \cdot L + 1)^2 - 4 \cdot K^2 \cdot L \cdot A)^{1/2})/(2 \cdot K)]\} + \beta \cdot [((K \cdot A + K \cdot L + 1) - ((K \cdot A + K \cdot L + 1)^2 - 4 \cdot K^2 \cdot L \cdot A)^{1/2})/(2 \cdot K)]. \quad (4)$$

The association constant was obtained by varying K to get best-fit of the curve described by Equation 4 to the experimental titration points, using the KALIDOGRAPH fitting program. To estimate the limit of detection of affinity, curves described by Equation 4 with desired values of K were generated using KALIDOGRAPH.

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. Excitation was at 390 nm, and emission was measured at 490 nm, employing a Shimadzu RF-540 spectrofluorometer at room temperature. In these experiments, emission spectra were taken after 5 min preequilibration, and no substantial changes in fluorescence occurred for several hours thereafter. Furthermore, 2–5-fold dilution of samples revealed that ANS fluorescence was roughly proportional to protein concentration. Thus, under our experimental conditions, ANS did not appear to induce aggregation.

CD measurements

CD measurements were performed in a Jasco J-500C spectropolarimeter, using 0.2-mm, 1-mm, or 10-mm pathlength cuvettes at 22 °C. The spectra represent the average of 5–8 scans and are corrected to the baseline for the corresponding buffer.

Light scattering measurements

The diffusion coefficient, D, of AChE was measured by the QELS technique using an apparatus constructed "in-house" (Kam et al., 1981). A Spectra Physics 165-4 argon laser was employed, which generated an incident beam at 514.5 nm. The experiments were performed at a scattering angle of 90 degrees. Thin-wall cylindrical cells (outer diameter, 10 mm) were employed, and measurements were performed at 1.5–20 μM AChE concentration at 22 °C. All samples were filtered prior to measurement, using 0.22-μm pore diameter Durapore filters (Millipore Corporation, Bedford, Massachusetts).

Kinetics of thiol-disulfide exchange

Spontaneous thiol-disulfide exchange was monitored in samples of HgR-modified AChE (ca. 8 μM) converted to the **MG** state by exposure to 45 °C for 3 min ($\mathbf{MG_{Chem}}$), or HgR-modified AChE preequilibrated in 1.8 M Gdn·HCl for 1 h, followed by removal of Gdn·HCl by gel-filtration ($\mathbf{MG_{Gdn}}$). Chemical modification was reversed by addition of GSH, to a final concentration of 1 mM, to ice-cold preparations of AChE, followed by incubation for 5 min on ice. Such addition of GSH results in complete reversal of the modification. Within 1 h, demodified samples (volume ca. 200 μL) were diluted 10–15-fold and reconcentrated in a Centricon, at 5 °C, and this procedure was performed three times. Such samples were then incubated at 31 °C, to allow spontaneous thiol-disulfide exchange to occur at a measurable rate (Eichler et al., 1994b). Aliquots were withdrawn at fixed times, treated with a large excess of NEM (20 mM final concentration), and subjected to SDS-PAGE. The gels were silver-stained and analyzed using a Molecular Dynamics 300A Computing Densitometer.

EPR measurements

EPR spectra were recorded at room temperature using a Bruker ER200 D-SRC EPR spectrometer operating at 9.2 GHz, with 100 KHz magnetic field modulation. The concentration of radical covalently bound to AChE was determined by double integration of the EPR spectrum of the label after release on addition of GSH to a final concentration of 1 mM (Kreimer et al., 1994b). 2,2,6,6-Tetramethylpiperidinyloxy was used for calibration.

Sucrose gradient centrifugation

Analytical sucrose gradient centrifugation was performed on 5–20% sucrose gradients made up in buffer A. When pretreatment of AChE with 15 mM DTT for 1 h at room temperature was performed to reduce the intersubunit disulfide bond, sucrose gradients were made up in buffer A containing 10 mM DTT. Centrifugation was performed in an SW 50.1 rotor for 5 h at 45,000 rpm in a Beckman L8-70 ultracentrifuge. Approximately 60 fractions of 180 μL were collected and assayed for protein concentration and enzymic activity. Native G_2 AChE from *T. californica* (7.0 S) and catalase (11.4 S) served as markers.

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