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Irreversible thermal denaturation of *Torpedo californica* acetylcholinesterase

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Abstract

Thermal denaturation of *Torpedo californica* acetylcholinesterase, a disulfide-linked homodimer with 537 amino acids in each subunit, was studied by differential scanning calorimetry. It displays a single calorimetric peak that is completely irreversible, the shape and temperature maximum depending on the scan rate. Thus, thermal denaturation of acetylcholinesterase is an irreversible process, under kinetic control, which is described well by the two-state kinetic scheme $N \xrightarrow{k} D$, with activation energy 131 ± 8 kcal/mol. Analysis of the kinetics of denaturation in the thermal transition temperature range, by monitoring loss of enzymic activity, yields activation energy of 121 ± 20 kcal/mol, similar to the value obtained by differential scanning calorimetry. Thermally denatured acetylcholinesterase displays spectroscopic characteristics typical of a molten globule state, similar to those of partially unfolded enzyme obtained by modification with thiol-specific reagents. Evidence is presented that the partially unfolded states produced by the two different treatments are thermodynamically favored relative to the native state.

Keywords: acetylcholinesterase; differential scanning calorimetry; irreversible denaturation; molten globule; thiol-disulfide exchange; two-state kinetic model

Folding of many small globular proteins is a cooperative process in the course of which only two states, the fully unfolded state, U, and the native state, N, are significantly populated. In the case of large proteins, which are generally believed to contain several domains (Jaenicke, 1991; Garel, 1992), folding has generally been considered to be cooperative within each domain, the only species populated in the course of either folding or unfolding being combinations of completely folded and completely unfolded domains (Privalov, 1982; Brandts et al., 1989; Garel, 1992). Evidence is accumulating, however, that another state, intermediate between N and U, can exist. This is a compact state that lacks the unique tertiary structure of the native protein but possesses substantial secondary structure. This state has been named the molten globule (MG) state (Kuwajima, 1989; Kim & Baldwin, 1990; Ptitsyn, 1992). The MG state is considered to serve as an intermediate on the pathway from the nascent polypeptide chain to the fully folded native protein in vivo (Gething & Sambrook, 1992). For most proteins, the MG state is unstable under physiological conditions and readily converts to the N state in vitro (Ptitsyn, 1992).

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Because the definition of the MG state is controversial (see, for example, Griko et al., 1994; Ewbank et al., 1995; Okazaki et al., 1995), in the present paper this term is used to refer to compact states of acetylcholinesterase (AChE) that preserve substantial secondary structure and lack most of the tertiary structure of the native enzyme. We have shown recently that exposure of a native dimeric form of *Torpedo* AChE to various treatments generates long-lived partially unfolded species displaying many of the characteristics of the MG state. Such partially unfolded species are generated by exposure to low concentrations of denaturant, e.g., 1.2–2 M Gdn·HCl (Kreimer et al., 1994b; Weiner et al., 1994), as a result of chemical modification of a single nonconserved residue, Cys^{231} (Dolginova et al., 1992; Kreimer et al., 1994a), by a repertoire of thiol-specific reagents, and under conditions of oxidative stress (Weiner et al., 1994). An important property of AChE observed in these experiments is that the states so generated are maintained even when the perturbing agent is removed, e.g., if Gdn·HCl is removed by dialysis or by dilution (Eichler et al., 1994; Kreimer et al., 1994b) or if chemical modification is reversed (Dolginova et al., 1992; Kreimer et al., 1994a). Aggregation often prevents refolding of MG species (Kiefhaber et al., 1991). We have shown, however, that the MG species of AChE are stable for many hours without aggregating (Kreimer et al., 1994a; Weiner et al., 1994).

Differential scanning calorimetry (DSC) is the most useful technique for characterizing thermal stability of proteins in

terms of their thermodynamic characteristics, and permits analysis of thermal transitions in proteins containing several domains (Privalov, 1982; Brandts et al., 1989). Description of DSC data in terms of equilibrium thermodynamics requires that denaturation be a reversible equilibrium process (Privalov, 1979). Thermal denaturation of many proteins is, however, irreversible; as a consequence, such thermodynamic functions as entropy and Gibbs free energy cannot be derived directly from the DSC trace (Freire et al., 1990). For such cases, it is necessary to use a more sophisticated approach that involves analysis of DSC traces obtained at different scan rates. This approach takes into account the kinetics of the irreversible transition (Lumry & Eyring, 1954; Sanchez-Ruiz et al., 1988; Conejero-Lara et al., 1991b; Lepock et al., 1992; Milardi et al., 1994). The irreversibility of thermal denaturation of AChE is well documented (Wu et al., 1987; Görne-Tschelnokow et al., 1993). In the present study, we utilize DSC to characterize the thermal unfolding of AChE, and demonstrate that this irreversible process can be described by a two-state kinetic model. We further show that partially unfolded species produced by thermal denaturation are very similar to the MG state(s) produced by chemical modification of AChE (Dolginova et al., 1992; Kreimer et al., 1994a).

Results and discussion

DSC of AChE

DSC of AChE in buffer A at a scan rate of 59.6 K/h results in a transition with temperature maximum (T_m) centered at ca. 45 °C (Fig. 1). The transition is calorimetrically irreversible, because no thermal effect is observed upon reheating. No dependence of the shape of the peak on the AChE concentration was found, at the above scan rate, in the range of 6–15 μM. At 59.6 K/h and 6 μM AChE, the denaturation enthalpy was found to be 398 kcal/mol, and values of 388 and 405 kcal/mol were obtained at 10 μM and 15 μM AChE, respectively. No pronounced dependence of the denaturation enthalpy on the scan rate was observed, a mean value of 383 ± 24 kcal/mol being obtained in the range of 12.9–86.7 K/h at 6 μM AChE. These data argue against an effect of intermolecular aggregation on the DSC curves obtained. Inspection of the DSC curve shown in Figure 1 reveals an asymmetry in the shape of the peak that might arise from two overlapping transitions. This would be a reasonable possibility for AChE, which is a large protein and could, in principle, be composed of several domains (Privalov, 1982; Garel, 1992). We analyzed this possibility by application of successive annealing procedures (Shnyrov et al., 1984; Shnyrov & Mateo, 1993; Shnyrov, 1994). Thus, AChE was first heated in the microcalorimeter cell to a temperature of 42 °C, which would be close to the maximum for a putative first transition. The sample was cooled and then heated to 70 °C. The second run revealed that the only effect of the first run was to decrease the peak intensity by ca. twofold, and that there was no change in T_m nor any effect on the shape of the curve (not shown). This experiment rules out the possibility of overlapping independent transitions. Thus, at a scan rate 59.6 K/h, AChE demonstrates a single thermal transition within a narrow temperature range.

A similar asymmetry of the DSC peak has been observed for several proteins that undergo irreversible thermal transition, including thermolysin, cytochrome *c* oxidase, carboxypeptidase B, phosphoglycerate kinase, G-actin, and 8-kDa sea anemone cytotoxin (Sanchez-Ruiz et al., 1988; Morin et al., 1990; Conejero-Lara et al., 1991a, 1991b; Galisteo et al., 1991; Le Bihan & Gicquaud, 1993; Zhadan & Shnyrov, 1994). In all these cases, the behavior of the systems was described as a thermal denaturation process under kinetic control. For proteins undergoing thermal denaturation under kinetic control, the temperature at which C_p is maximal, viz. T_m, is dependent upon the scan rate. We considered it likely, therefore, that the observed irreversible thermal denaturation of AChE is also under kinetic control; indeed, DSC of AChE reveals a dependence of T_m on the scan rate (Fig. 2, continuous lines). We assumed that the pro-

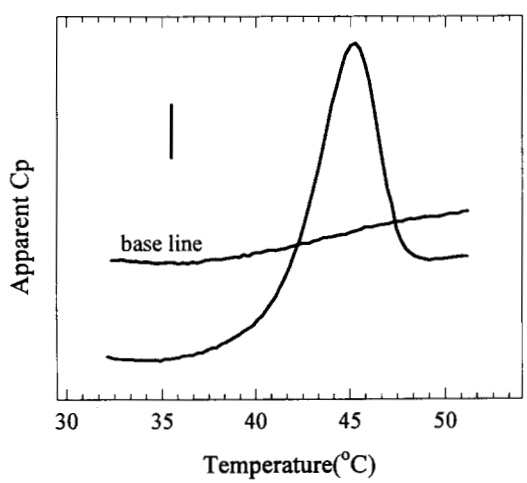

Fig. 1. Original calorimetric recording of the apparent heat capacity of *Torpedo* AChE as a function of temperature. The baseline represents the curve obtained with buffer in both cells of the calorimeter, and the experimental curve shown is for 6.2×10^{-6} M AChE in buffer A, at a scan rate of 1 K/min. The vertical bar represents 20 cal K^{-1} mol^{-1}.

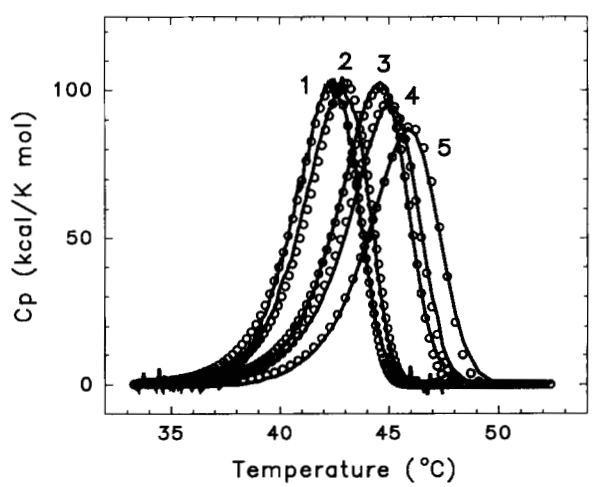

Fig. 2. Temperature dependence of excess heat capacity for AChE as a function of scan rate. Experimental conditions as in Figure 1. Scan rates were, respectively, 12.9 (1), 20.4 (2), 45.3 (3), 59.6 (4), and 86.7 K/h (5). Lines display the experimental data and points show the fit obtained by use of Equation 5.

cess could be described by the simplest two-state kinetic model (Sanchez-Ruiz et al., 1988). This model considers the irreversible transition of the native state (N) to a denatured state (D), according to the scheme $N \xrightarrow{k} D$, as a first-order reaction with a rate constant, k, that changes with temperature according to the Arrhenius equation. In a system behaving according to this scheme, the rate of increase in the concentration of D (and of the corresponding decrease in the concentration of N) is determined by the free energy of activation (Lepock et al., 1992). In other words, the rate of accumulation of D is determined by the conformation of the transition state, and the properties of the final state, D, will have no effect on the rate of transition.

If thermal denaturation of AChE follows this model, the activation energy of the process (E_A) can be calculated in several ways, and agreement between the various values thus obtained will support the validity of the model (Sanchez-Ruiz et al., 1988). Mathematical elaboration of the results of DSC measurements leads to four different ways for calculating the activation energy, E_A, of the kinetic process (Sanchez-Ruiz et al., 1988).

1. The temperature dependence of the first-order rate constant, k, can be obtained from the following equation:

$$k = v\Delta C_p/(Q_t - Q), \tag{1}$$

where v (K/min) stands for the scan rate, ΔC_p is the excess heat capacity, Q_t is the total heat of the process (proportional to the area below the thermogram), and Q is the heat evolved up to a given temperature (the total area below the thermogram between the initial temperature and a given temperature, T). Figure 3A shows Arrhenius plots of the DSC experiments presented in Figure 2 (continuous lines). The value of E_A obtained from analysis according to Equation 2 is 129 ± 8 kcal/mol.

2. The temperature dependence of heat evolution is given by the equation:

$$\ln\{\ln[Q/(Q_t - Q)]\} = E_A/R(1/T_m - 1/T), \tag{2}$$

where T_m is the temperature at the maximum of the heat capacity curve and R is the gas constant. The slope of plots of $\ln\{\ln[Q_t/(Q_t - Q)]\}$ versus $1/T$ should give $-E_A/R$; as can be seen from Figure 3B, plotting the results of five independent DSC runs (Fig. 2) yields very similar slopes, corresponding to E_A = 138 ± 10 kcal/mol.

3. The effect of the scan rate on T_m is given by the equation:

$$v/T_m^2 = (A_0 R/E_A)\exp(-E_A/RT_m), \tag{3}$$

where A_0 is the Arrhenius frequency factor. E_A can be obtained by linear fitting of the dependence, $\ln(v/T_m^2)$, versus $1/T_m$, and such fitting (Fig. 3C) yields a value of 118 ± 10 kcal/mol for E_A.

4. The activation energy can be calculated directly from the equation:

$$E_A = eRT_m^2\Delta C_p^{max}/Q_t, \tag{4}$$

where ΔC_p^{max} is the heat capacity at the trace maximum and e stands for the base of the natural logarithm. Such a direct calculation yields a value E_A = 134 ± 6 kcal/mol.

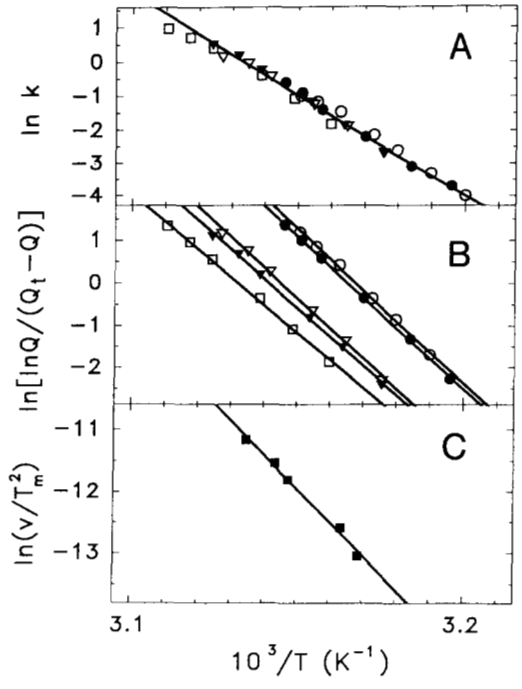

Fig. 3. A: Arrhenius plots for the rate of thermal denaturation obtained at five different scan rates: ○, 12.9; ●, 20.4; ▽, 45.3; ▼, 59.6; □, 86.7 K/h. **B:** Dependence of $\ln\{\ln[Q/(Q_t - Q)]\}$ on $1/T$ at different scan rates, plotted using the same symbols employed in A. **C:** Plot of $\ln(v/T_m^2)$ versus $1/T_m$. Here the abscissa actually represents $1/T_m$.

To further support the validity of the proposed kinetic model, we fitted the experimental curves to the theoretical equation, which, in addition to these four methods, describes quantitatively the thermal dependence of heat capacity, C_p^{ex}, in the scanning calorimetry experiment, for a system that behaves in accordance with a two-state kinetic model (Conejero-Lara et al., 1991b):

$$C_p^{ex} = eC_p^{max}\exp[E_A(T - T_m)/R(T_m)^2] \times \exp\{-\exp[E_A(T - T_m)/R(T_m)^2]\}. \tag{5}$$

The results of fitting the experimental curves (traces) to theoretical curves generated by use of Equation 5 (symbols), employing E_A values in the range of 128–142 kcal/mol, are shown in Figure 2. The theoretical curves are practically superimposed upon the experimental traces, confirming that thermal denaturation of AChE follows the kinetic model proposed.

The results obtained by the five different methods of calculation, corresponding to Equations 1–5, are in agreement, yielding similar values for E_A, with an overall average value of 131 ± 8 kcal/mol.

Aggregation and thiol–disulfide exchange in the process of thermal denaturation of AChE

Thermal denaturation of proteins is often accompanied by aggregation, which is mostly due to exposure of hydrophobic surfaces in the partially unfolded state (Zale & Klibanov, 1986). In the case of *Torpedo* AChE, description of the process of ther-

mal unfolding might be further complicated by the occurrence of thiol–disulfide exchange of the free sulfhydryl group of Cys^{231} with both intrasubunit and intersubunit disulfide bonds, which has been shown to occur in the presence of guanidine hydrochloride (Gdn·HCl) (Eichler et al., 1994). Sucrose gradient centrifugation reveals that thermally denatured AChE has a tendency to aggregate, albeit at a modest rate (Fig. 4). Thus, after 3 min at 45 °C, which results in complete (>99%) and irreversible inactivation, <20% of the denatured enzyme can be found in a ca. 10S peak, most likely corresponding to a tetrameric species produced by oligomerization of the original G_2 form. After 10 min, this tetramer, together with heavier species, accounts for ca. 50% of the total protein. Even after 2 h at 45 °C, a measurable amount, 5–10% of the total, still migrates at the position of the dimer on the sucrose gradient. It should be noted that the sucrose gradient experiments were performed at a concentration of 23 μM AChE, which is substantially higher than in most DSC experiments. This notion provides support for our contention that DSC traces are not substantially affected by aggregation.

As mentioned above, we recently found that Gdn·HCl-unfolded AChE undergoes spontaneous intramolecular thiol–disulfide exchange (Eichler et al., 1994). This results in appearance of various novel species with a nonnative profile of disulfides, including species in which the intersubunit disulfide bridge has been eliminated. These latter species behave as monomers when subjected to SDS-PAGE under nonreducing conditions, and the rate of their appearance is correlated with the thiol–disulfide exchange process. Figure 5 shows that thermal denaturation, at 45 °C, also results in thiol–disulfide interchange. Thus, SDS-PAGE under nonreducing conditions reveals additional protein bands, including some that migrate at the position of monomers (Fig. 5, lanes 2–4); modification of Cys^{231} by 2,2,5,5-tetramethyl-4-(2-chloromercuriphenyl)-3-imidazoline-1-oxyl (HgR) (Kreimer et al., 1994a), which precludes thiol–disulfide exchange (Eichler et al., 1994), abolishes this phenomenon (Fig. 5, compare lanes 5 and 6). SDS-PAGE under reducing conditions reveals a single polypeptide species corresponding to the catalytic subunit monomer, demonstrating that thermal denaturation had not resulted in cleavage of the polypeptide chain (not shown). The data presented are very similar to those seen upon Gdn·HCl-induced unfolding. Thiol–disulfide exchange is already substantial after thermal denaturation for as little as 1 min (lane 2). At 3 min (lane 3), the principal component, accounting for ca. 70% of the total protein, still migrates at the same position as the control dimer. However, a series of somewhat heavier bands can be detected, as well as a doublet migrating approximately at the position to be expected for nonreduced monomer (Eichler et al., 1994); as in the case of Gdn·HCl denaturation, this doublet accounts for ca. 5–10% of the total protein applied to the gel. Upon continued thermal denaturation, the doublet still remains substantial, but, after 10 min, little of the original dimer remains, and large amounts of very heavy species accumulate close to the origin (lane 4).

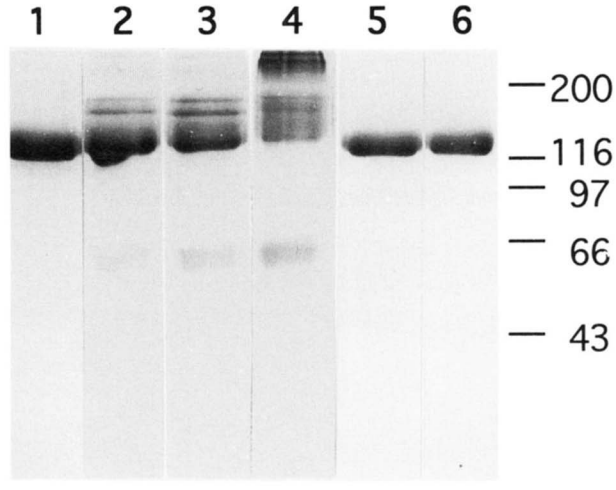

Fig. 5. SDS-PAGE of AChE before and after thermal denaturation. Electrophoresis was performed as described in the Materials and methods, employing nonreducing conditions. Lane 1, native AChE; lane 2, after heating at 45 °C for 1 min (18% residual activity); lane 3, 45 °C for 3 min (<1% residual activity); lane 4, 45 °C for 10 min; lane 5, AChE modified by HgR; lane 6, as in lane 5, but after heating at 45 °C for 10 min. Staining was with Coomassie Brilliant Blue R.

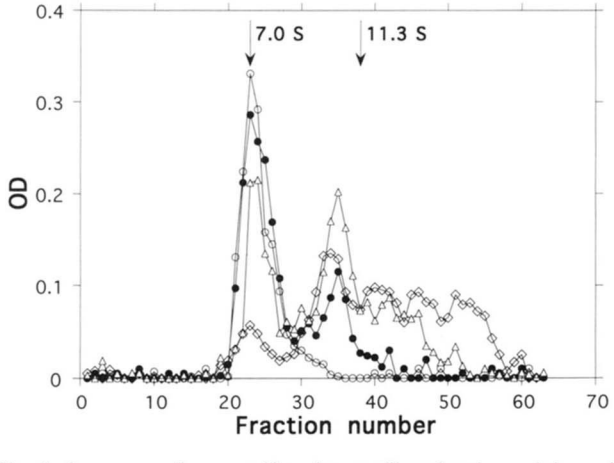

Fig. 4. Sucrose gradient centrifugation profiles of native and thermally denatured AChE. ○, Native AChE; ●, AChE denatured by heating at 45 °C for 3 min; △, AChE denatured by heating at 45 °C for 10 min; ◇, AChE denatured by heating at 45 °C for 2 h. Arrows mark the positions of catalase (11.3S) and of the activity peak of native AChE (7.0S), which served as markers.

Kinetics of thermal inactivation of AChE

Figure 6 shows the time dependence of loss of catalytic activity, caused by thermal inactivation, in the temperature range of the transition detected by DSC. As can be seen, experimental points can be fitted well to the first-order kinetic scheme. This scheme was found to be applicable to description of the thermal denaturation of AChE in a concentration range of 0.05–1.5 μM, which permitted us to conclude that the process of thermal inactivation is not significantly affected by aggregation. The rate constants obtained, k_i, were used to construct an Arrhenius plot (Fig. 6, inset). The activation energy thus obtained is 121 ± 20 kcal/mol, in satisfactory agreement with the value obtained from the DSC experiments. Thus, this independent experimental approach supports the conclusion already reached from the DSC measurements, viz. that thermal denaturation of AChE can be described by a two-state kinetic model, and that

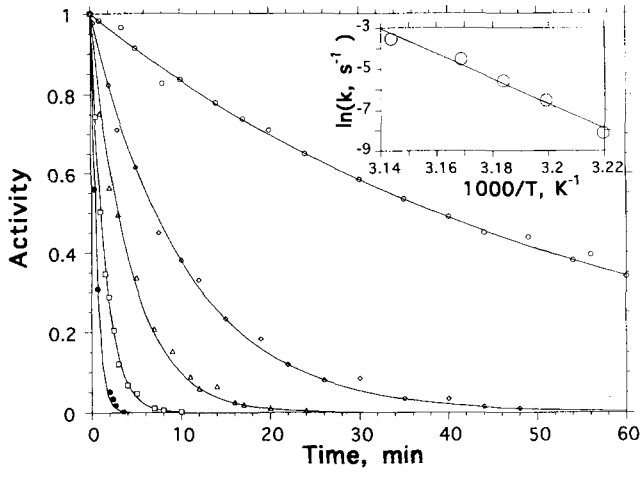

Fig. 6. Kinetics of loss of AChE activity as a function of temperature. Aliquots (20 μL) of AChE (1×10^{-5} M) in buffer A were added to 500 μL of the same buffer pre-equilibrated at the desired temperature. Aliquots (10 μL) were withdrawn at appropriate times, diluted into ice-cold buffer, and assayed for enzymic activity 1 h later. Kinetics were analyzed by fitting the inactivation curve to a first-order rate equation: $a_t/a_0 = \exp(-k_i t)$, a_0 being the activity at time zero and a_t that at time t. ●, 45.0 °C; □, 42.5 °C; △, 41.0 °C; ◇, 39.5 °C; ○, 37.5 °C. **Inset:** Arrhenius plot of the first-order rate constants obtained from the experimental data.

only two states, native (N) and denatured (D), are populated in the denaturation process.

Spectral characteristics of thermally denatured AChE

As shown above, AChE, which had been thermally denatured for 3 min at 45 °C, although it had apparently undergone some thiol–disulfide exchange, contains only small amounts of aggregates, and the bulk of the protein migrates to the position of the dimer upon SDS-PAGE under nonreducing conditions. Nevertheless, for spectroscopic characterization of the thermally denatured AChE, we collected a pure fraction corresponding to nonaggregated AChE (fractions 21–28 from the sucrose gradient displayed in Fig. 4) and applied spectroscopic techniques that we had used earlier to characterize partially unfolded species of AChE produced by other procedures (Dolginova et al., 1992; Kreimer et al., 1994a, 1994b; Weiner et al., 1994). Experimental approaches commonly employed to assign a given partially unfolded conformation of a protein as MG include: CD spectroscopy in the far and near UV, intrinsic protein fluorescence, NMR spectroscopy for characterization of the packing of the side chains, fluorescence of the amphiphilic probe 1-anilino-8-naphthalenesulfonic acid (ANS), analysis of the dimensions of the protein and its tendency to aggregate, susceptibility to proteolytic attack, and loss of biological function of the native protein (Kuwajima, 1989; Ptitsyn, 1992; Christensen & Pain, 1994). In order to characterize the various partially unfolded states of AChE we applied all these criteria, except for NMR spectroscopy, which cannot be readily applied to the AChE dimer due to its large molecular weight, 130,000. For purposes of comparison, the spectral characteristics of thermally denatured AChE are shown alongside those of unfolded AChE in 5 M Gdn·HCl (Kreimer et al., 1994b; Weiner et al., 1994) and of the partially unfolded state produced by chemical modification of AChE with the disulfide 4,4′-dithiodipyridine (DTP) (Dolginova et al., 1992; Kreimer et al., 1994a). It can be seen that the spectral characteristics of the state produced by thermal treatment are very similar to those obtained for chemically modified AChE and very different from those of the N and U states (Fig. 7). Thus, thermally denatured AChE retains substantial ellipticity in the far UV but little ellipticity in the near UV (Fig. 7A), and there is a ca. 6-nm red-shift in intrinsic fluorescence relative to native AChE, substantially less than the 20-nm red-shift observed for the unfolded state (Fig. 7B). These spectroscopic data indicate a severe perturbation in tertiary structure, together with substantial retention of secondary structural elements, a characteristic

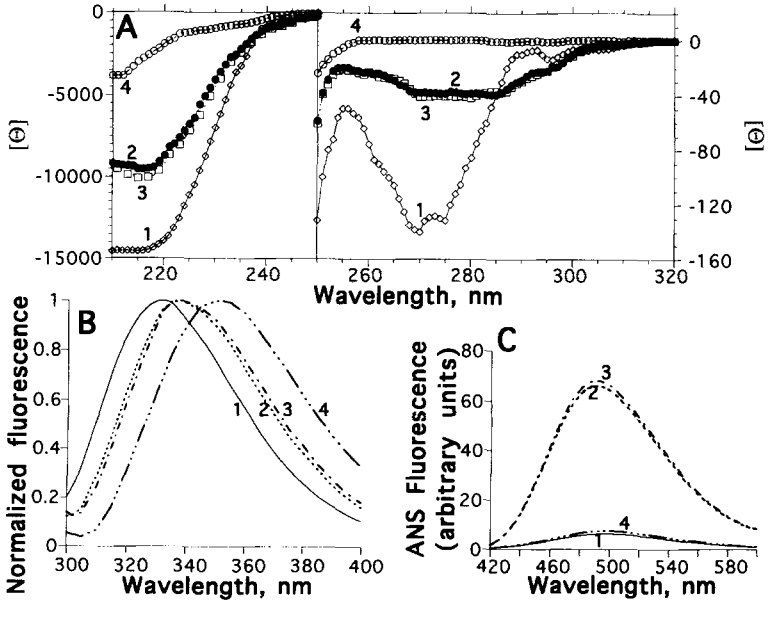

Fig. 7. Effect of thermal inactivation upon the spectroscopic characteristics of AChE. Native AChE in buffer A was inactivated by heating at 45 °C for 3 min, and thermally denatured AChE so obtained was freed from aggregated species (see the Materials and methods) prior to spectroscopic characterization. **A:** CD spectra, in the far and near UV (AChE concentration, 4.5 μM). **B:** Normalized intrinsic fluorescence emission spectra (AChE concentration, 1 μM). **C:** ANS fluorescence emission spectra (AChE concentration, 0.1 μM; ANS concentration, 0.1 mM). 1, Native AChE; 2, thermally inactivated AChE; 3, AChE inactivated by chemical modification with DTP (see the Materials and methods); 4, AChE unfolded in 5 M Gdn·HCl.

feature of the MG state (Kuwajima, 1989; Ptitsyn, 1992; Christensen & Pain, 1994). A large increase in the fluorescence of the amphiphilic probe ANS, accompanied by a ca. 10-nm blue-shift in its emission maximum, is produced by its binding to thermally denatured AChE, whereas ANS displays little or no affinity for either native or unfolded AChE (Fig. 7C). This increase in ANS fluorescence reflects exposure of hydrophobic surfaces and, again, is characteristic of the MG state (Kuwajima, 1989; Ptitsyn, 1992; Christensen & Pain, 1994). Furthermore, upon sucrose gradient centrifugation, the thermally denatured AChE migrates with a sedimentation constant not significantly different from that of the native enzyme (ca. 7S), as is also shown to be the case for chemically modified AChE (Kreimer et al., 1994a); it is, therefore, compact, as would be expected for a MG. Both the thermally denatured species (Fig. 4) and chemically modified AChE (Kreimer et al., 1994a) have a tendency to aggregate. The rate of aggregation is, however, slow enough in both cases to permit characterization of these species prior to aggregation. Thermally denatured AChE is devoid of enzymic activity, just as is the chemically modified AChE both prior to and after demodification (Dolginova et al., 1992; Kreimer et al., 1994a). Finally, both the thermally denatured and chemically modified compact states are susceptible to tryptic digestion, displaying detectable cleavage within as little as 30 min under our experimental conditions and complete digestion within 5 h, whereas no traces of proteolysis are detectable upon similar treatment of native AChE for up to 12 h (data not shown).

In both chemically modified and thermally denatured AChE, there is some residual ellipticity in the near UV that is lacking in samples unfolded in 5 M Gdn·HCl (Fig. 8A). This ellipticity might arise from the presence of residual fully folded native domains in both thermally denatured and chemically modified AChE, as has been shown recently to be the case for a fragment of staphylococcal nuclease lacking its 13 C-terminal amino acids (Griko et al., 1994). If this were the case, an additional heat capacity peak, corresponding to thermal unfolding of such a putative domain, would be expected in the DSC experiment (Griko et al., 1994). However, neither thermally denatured AChE nor enzyme chemically modified by either the disulfide, DTP, or $HgCl_2$ displays a heat capacity peak (not shown). Consequently, it is most unlikely that any native domain is retained in either chemically modified or thermally denatured AChE. Thus, thermal denaturation, like chemical modification, completely transforms the entire AChE dimer, containing 537 amino acid residues in each subunit, to a partially unfolded state. This state, as described above, satisfies the criteria necessary and sufficient to be defined as a MG state. Our observation that thermally denatured AChE displays residual structural elements and compactness is not surprising, because such behavior is well documented for many thermally denatured proteins (Tanford, 1968; Evans et al., 1991; Sosnick & Trewhella, 1992; Shortle, 1993; Seshadri et al., 1994).

Denaturation of AChE, either by chemical modification or by heating, is irreversible; i.e., no restoration of the structural characteristics or of the catalytic activity of native AChE is observed upon demodification of chemically modified enzyme (Dolginova et al., 1992; Kreimer et al., 1994a) or upon restoration to the ambient temperature of the heat-denatured enzyme, as shown in the present study as well as by Wu et al. (1987) and Görne-Tschelnokow et al. (1993). Thus, although the partially unfolded species of AChE can exist without aggregating for up to 2 days under physiological conditions, reversion to the native state cannot be detected. We earlier considered two explanations for this behavior (Kreimer et al., 1994a). One would involve kinetic trapping of AChE in the denatured state under physiological conditions (for reviews, see Matthews, 1993; Baker & Agard, 1994; Eder & Fersht, 1995). In such a case, even though the native enzyme might be at a lower free energy level than the denatured, a high free energy barrier would retard reversion of the denatured enzyme to the native conformation to such an extent that no recovery would be observable on the time scale of the experiment. The most impressive example of such kinetic trapping is provided by α-lytic protease lacking the pro region, which fails to refold within 1 month, although restoration of the native conformation, with concomitant recovery of proteolytic activity, occurs within minutes upon addition of the pro region (Baker et al., 1992). The authors suggested that the pro region permits folding to occur at a measurable rate by lowering the free energy barrier separating the denatured state from the thermodynamically more favored native state. Similar findings have been presented for carboxypeptidase Y (Sørensen et al., 1993) and subtilisin (Eder et al., 1993; Shinde et al., 1993). However, AChE lacks a pro region (Maulet et al., 1990), and kinetic trapping of AChE in the MG state must be due to some other reason.

The alternative possibility that we have considered is that the MG state of AChE is more favored thermodynamically than the N state under physiological conditions, the free energy difference between the two states being estimated to be >5.5 kcal/mol (Kreimer et al., 1994a). Such a situation has been claimed to exist in the case of influenza virus hemagglutinin, where, as discussed by Baker and Agard (1994), the native state is kinetically trapped at neutral pH (see also Hinds & Levitt, 1995); upon acidification, it goes to a lower energy state, which is more thermostable than the native form even at neutral pH (Ruigrok et al., 1988). We earlier argued, based on our chemical modification studies using both organomercurials and disulfides (Kreimer et al., 1994a), that the MG state of AChE produced by chemical modification, whether or not it is subsequently demodified, is energetically favored relative to the N state. Our justification for this was that modification with mercurials initially produces a state that can be described as "native-like" on the basis of its spectral characteristics, and is separated from the N state by a low energy of activation. Nevertheless, this state is spontaneously converted to the state produced by chemical modification with disulfides (Kreimer et al., 1994a). Because our experimental data suggest that the state produced by chemical modification is very similar to that produced by thermal treatment, we can argue by analogy that the native state is also metastable with respect to the thermally denatured state.

No spontaneous inactivation of *Torpedo* AChE is observed at room temperature, but irreversible thermal denaturation of AChE is already observable at 35 °C (Fig. 2). Extrapolation of the Arrhenius plot for the temperature dependence of the rate constant for thermal denaturation (Fig. 3A) yields a first-order rate constant, $k = 1.66 \times 10^{-6}$ min^{-1}, corresponding to an estimated free energy barrier, $\Delta G\ddagger$, of 27.7 kcal/mol at 25 °C. Such a high-energy barrier between the native and denatured states would correspond to a half-life of 290 days for denaturation of native AChE at room temperature. The analogous value of $\Delta G\ddagger$ obtained from an Arrhenius plot for the inactivation

of AChE, measured over a narrower temperature range (Fig. 6, inset) and using the same extrapolation procedure, yields $\Delta G\ddagger$ = 26.9 kcal/mol at room temperature and a corresponding half-life of 87 days. It should be noted that these estimates hold true irrespective of whether or not the MG state of AChE is in a kinetic trap at room temperature.

Assuming that the MG state of AChE is indeed at a lower free energy level than the N state, how can it serve as an intermediate on the pathway from the fully unfolded nascent polypeptide to the fully folded native enzyme? We note that the form of *Torpedo* AChE that we are studying is a homodimer in which the two subunits are connected by a disulfide bond linking the Cys^{537} residues at the C-terminus of each subunit (Gibney et al., 1988). For each subunit, formation of the disulfide bond between subunits can be considered as a posttranslational modification. It is thus possible that, although each individual subunit polypeptide folds to a stable N state corresponding to a global energy minimum, the dimer is not at such an energy minimum. Preliminary results (D.I. Kreimer, L. Varon, I. Silman, L. Weiner, in prep.) show that in the MG state there is much stronger noncovalent interaction between the two subunits of the dimer than in the N state. These data provide support for the notion that intersubunit forces may be responsible for the MG state being thermodynamically favored relative to the N state in *Torpedo* AChE.

Materials and methods

Materials

AChE was the dimeric (G_2) glycosyl-phosphatidylinositol-anchored (GPI-anchored) form purified from electric organ tissue of *Torpedo 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). ANS (magnesium salt), DTP, and 5,5′-dithiobis(2-nitrobenzoic acid) were obtained from Sigma (St. Louis, Missouri). Gelatin was from Merck (Darmstadt, Germany). All other reagents were of analytical grade or higher.

Assay methods

AChE concentrations were determined either spectrophotometrically, taking ϵ^{280}(1 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 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 in the physicochemical studies 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 essentially 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 utilizing such samples were performed only after preincubation for >12 h at room temperature, so as to decrease the population of metastable, nativelike species of mercurial-modified AChE produced initially, which are amenable to reactivation, to levels of less than 1% (Kreimer et al., 1994a).

DSC measurements

DSC was performed in a MicroCal MC-2 differential scanning microcalorimeter (MicroCal Inc., Northampton, Massachusetts) with cell volumes of 1.22 mL, interfaced with an IBM-compatible personal computer. Scanning rates of 12.9–86.7 K/h were employed. 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 being loaded into the reference cell. An overpressure of 2 atm of dry nitrogen was maintained over the solutions in the cells throughout the scans to prevent any degassing during heating. The experimental calorimetric traces were corrected for the effect of instrument response time using the procedure of Lopez-Mayorga and Freire (1987) and were corrected for the calorimetric baseline and for determination of the enthalpy of the transitions by using a sigmoidal baseline as described by Takahashi and Sturtevant (1981). The excess molar heat capacity was calculated assuming the molecular mass of *Torpedo* AChE to be 130,000. Data were analyzed and plotted employing the Windows-based software package Origin, supplied by MicroCal Inc., and the curve-fitting option of SigmaPlot software.

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 60 fractions of ca. 80 μL were collected and assayed for protein concentration and enzymic activity. Native G_2 AChE from *T. californica* (7.0S) and catalase (11.4S) served as markers. Sucrose gradient centrifugation was also used to prepare the samples for spectroscopic characterization of thermally denatured AChE. The peak of thermally denatured AChE (3 min at 45 °C), sedimenting at 7S in the gradient, was identified by assaying protein concentration. Fractions from the peak were pooled, sucrose was removed by gel filtration on a Bio-Gel P6 column, and eluted protein was concentrated in a Centricon-30 microconcentrator. All sucrose gradient experiments were performed by employing preparations of 23 μM AChE in buffer A.

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. Spectra represent the average of five to eight scans and are corrected to the baseline for the corresponding buffer.

Intrinsic fluorescence measurements

Intrinsic fluorescence of AChE was 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.

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. ANS fluorescence was measured using an excitation wavelength of 390 nm. Measurements were performed in a Shimadzu RF-540 spectrofluorometer at 25 °C.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed on 7.5% gels, under either reducing or nonreducing conditions. The former were achieved by including 5% β-mercaptoethanol in the sample buffer. Staining was with Coomassie Brilliant Blue R. Relative amounts of protein in individual bands were estimated by scanning the stained gels employing a Molecular Dynamics 300A computing densitometer.

Tryptic digestion of native and partially unfolded AChE

Native or partially unfolded AChE was produced either by incubation for 3 min at 45 °C or by chemical modification by DTP as described above (AChE concentration 1 mg/mL in all cases). The AChE was then treated with trypsin (1% w/w) at room temperature and fractions were withdrawn at appropriate intervals, frozen, and analyzed by SDS-PAGE as described earlier (Dolginova et al., 1992; Weiner et al., 1994).

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