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Two-state transition between molten globule and unfolded states of acetylcholinesterase as monitored by electron paramagnetic resonance spectroscopy

(protein folding/denatured forms/equilibrium)

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ABSTRACT **Cys-231 of *Torpedo californica* acetylcholinesterase (EC 3.1.1.7) was selectively labeled with the mercury derivative of a stable nitroxyl radical. In 1.5 M guanidinium chloride, this conjugate exists in a molten globule state (MG), whereas in 5 M denaturant, it is in an unfolded state (U). The transition between the two states is reversible. In the MG, the label is highly immobilized, whereas in the U, it is almost freely rotating. The clearly distinct electron paramagnetic resonance (EPR) spectra of the two states permits the study of this transition. Upon elevating the guanidinium chloride concentration, a decrease in the EPR signal of the MG occurs concomitantly with an increase in the U signal, the total intensity of the EPR spectra remaining constant. This behavior is characteristic of a two-state transition. The thermodynamic characteristics of this transition (ΔG^0 and m), whether estimated directly from the EPR data or from both CD and fluorescence data analyzed by assuming a two-state scheme, are in good agreement.**

Recent evidence suggests that a two-state model (1, 2) is inadequate to describe protein folding and unfolding. Under certain conditions, an intermediate state between the fully folded native state (N) and the fully unfolded state (U) may exist (3–5). This intermediate state has been named molten globule state (MG) (6). MG is rather compact and demonstrates a significant amount of secondary structure, although lacking the tertiary structure of the native protein. Such a compact state seems to be an obligatory intermediate en route from the nascent polypeptide synthesized on the ribosome to the fully folded native protein (7). While it is generally believed that destruction of the unique native conformation, whether by transition to MG or to U, is indeed a two-state (all-or-none) transition (4, 5, 8, 9), the description of the MG ⇋ U transition is still controversial. It has been theoretically suggested that, depending on their amino acid compositions, proteins may undergo this transition either by gradual expansion of the polypeptide chain or as a two-state process (10). Others have claimed that it must occur through gradual swelling of MG (11).

Similar controversy also exists with respect to the experimental data. Exclusion chromatography suggested that the guanidine hydrochloride (Gdn·HCl)-induced MG ⇋ U transition of bovine α-lactalbumin is two state (12). However, an NMR study, published at the same time, presented evidence that the urea-induced MG ⇋ U transition of the same protein is noncooperative (13). While calorimetry is considered a method of choice for characterization of a given transition (2, 8, 14), it runs into difficulties for the MG ⇋ U transition, since the excess heat absorption and change in heat capacity measured seem very small (15).

The methods most commonly employed for monitoring transitions in proteins, namely, CD and intrinsic fluorescence (16), produce information about the average state of a protein and are not capable of resolving states coexisting in solution. Obviously, a method allowing simultaneous observation of distinct protein states coexisting at equilibrium would be a powerful tool for characterizing the MG ⇋ U transition. Size-exclusion chromatography can permit separation of such coexisting species (12, 17); however, this method requires that the rate of interconversion is slow on the time scale of the chromatographic procedure (5, 17). Application of NMR to monitor the MG ⇋ U transition seems promising, since it permits, in principle, simultaneous observation of signals corresponding to MG and U species. However, no separation between MG and U signals was observed for bovine α-lactalbumin in the transition region as a result of fast exchange on the NMR time scale (13).

Nitroxyl radicals provide spin labels for proteins whose mobility displays high sensitivity to conformational change (18). Changes in mobility of the bound probe are reflected in concomitant changes in field position and line width (19). Such spectral shifts are usually rather large, $>10^7$ sec^{-1} (in frequency units), whereas rates of interconversion between MG and U are in the range of 1–10^3 s^{-1} (5, 20), which is slow on an electron paramagnetic resonance (EPR) time scale. Their spectral resolution should thus be feasible.

We have shown (21) that a stable nitroxyl radical can be covalently attached to a single cysteine residue, Cys-231, of *Torpedo californica* acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7; AcChoEase). We here use this approach for selective modification of AcChoEase with the thiol-specific spin-labeled organomercurial 2,2,5,5-tetramethyl-4-(2-chloromercuriphenyl)-3-imidazoline-1-oxyl (HgR). By using EPR spectroscopy, we demonstrate the coexistence of MG and U under equilibrium conditions and show that the MG ⇋ U transition induced by Gdn·HCl is described by a two-state model.

MATERIALS AND METHODS

Chemicals. AcChoEase was the dimeric (G_2) glycosylphosphatidylinositol-anchored form purified from electric organ of *T. californica* by affinity chromatography subsequent to solubilization with phosphatidylinositol-specific phospholipase C (22). AcChoEase concentration [expressed as the concentration of active sites, assuming a subunit molecular weight of 65,000 (22)] and activity were determined as described (21).

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Abbreviations: N, native state; U, unfolded state; MG, molten globule state; AcChoEase, acetylcholinesterase; HgR, 2,2,5,5-tetramethyl-4-(2-chloromercuriphenyl)-3-imidazoline-1-oxyl; ANS, 1-anilino-8-naphthalenesulfonic acid; Gdn·HCl, guanidine hydrochloride; EPR, electron paramagnetic resonance; GSH, glutathione.

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HgR was synthesized as described (23). 2,2,6,6-Tetramethyl-1-piperidinyloxy, 1-anilino-8-naphthalenesulfonic acid (ANS), acetylthiocholine iodide, 4,4'-dithiodipyridine, and reduced glutathione (GSH) were obtained from Sigma. Gdn·HCl (ultra pure) was purchased from Schwartz/Mann.

Sample Preparation. Modification of AcChoEase by HgR and 4,4'-dithiopyridine was performed as described (21). Unless otherwise stated, the buffer employed was 0.1 M NaCl/50 mM sodium phosphate, pH 7.3 (buffer A). HgR was added to 0.5 mM; within 1 h at 13°C, >99% inhibition of enzymic activity had been achieved. Excess HgR was removed by gel filtration, the modified protein was concentrated, and spectral characteristics and reactivatibility were determined immediately. Samples of spin-labeled enzyme at different denaturant concentrations were prepared from a stock solution of 8 M Gdn·HCl in buffer A and equilibrated for 7 h at room temperature prior to physicochemical measurements.

Spectroscopic Measurements. CD, protein intrinsic fluorescence, and ANS fluorescence measurements were performed at 22°C (CD) and 24°C (fluorescence), as described (24). 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 AcChoEase was determined by double integration of the EPR spectrum of the label after release upon addition of GSH to 1 mM; 2,2,6,6-tetramethylpiperidinyloxy was used for calibration.

RESULTS AND DISCUSSION

AcChoEase is a homodimer of two 537-amino acid catalytic subunits, which are linked by a disulfide bridge. The three-dimensional structure of AcChoEase was recently solved (25). Each monomer contains only one free thiol group (Cys-231), chemical modification of which by a repertoire of sulfhydryl reagents results in complete inhibition of enzymic activity (21, 26). Accordingly, AcChoEase was inhibited by HgR with pseudo-first-order kinetics, as shown for several thiol reagents (21, 26). The reaction rate was used to estimate the order of the reaction with respect to HgR concentration by construction of a double logarithmic plot (21). The order of reaction calculated was 1.4 ± 0.2.

Fig. 1, trace 1, shows the EPR spectrum of AcChoEase modified with HgR (HgR-AcChoEase). It consists of two superimposed signals, one typical of a strongly immobilized radical (19) and the other typical of a sharp triplet, corresponding to a small amount (<5%) of unbound radical. Modification of SH groups in proteins by organomercurials is reversible (27). When HgR-AcChoEase is exposed to GSH, the broad EPR signal of the immobilized radical disappears, with concomitant appearance of the sharp peaks characteristic of unbound radical. Such a demodification restored enzymic activity up to 80%. The stoichiometry of modification was estimated to be ≈1.0 ± 0.2, in agreement with the inactivation kinetics. Furthermore, if the free thiol of AcChoEase is occupied by treatment with the disulfide reagent DTP, under conditions causing complete inactivation (21), no bound spin label can be detected upon subsequent exposure to HgR. These data clearly demonstrate that the spin label reacts with a single thiol group, most likely Cys-231.

Spectroscopic characteristics of HgR-AcChoEase are shown in Fig. 2. The intrinsic fluorescence spectrum is shifted ≈1 nm to the red relative to unmodified AcChoEase (Fig. 2*A*). The CD spectrum in the near UV is slightly changed, and ellipticity in the far-UV is somewhat decreased (Fig. 2*B*). Fluorescence of ANS is sensitive to changes in protein conformation (28), and we observed a large increase in ANS emission for HgR-AcChoEase relative to native enzyme (Fig. 2*C*). Cys-231 is buried within the protein, ≈8 Å

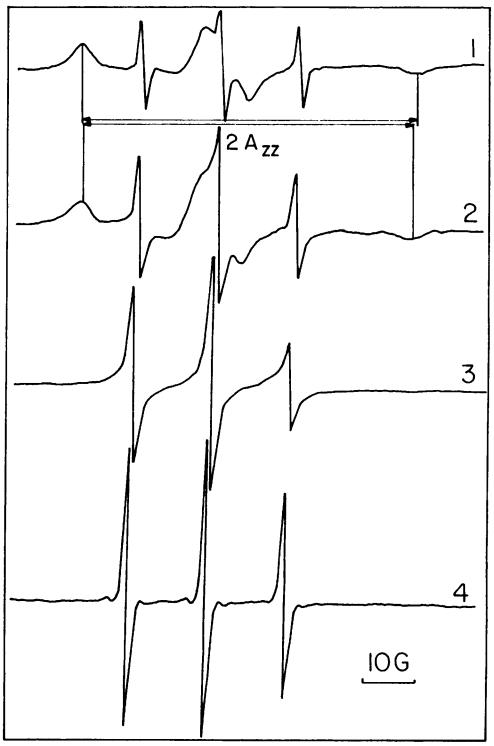

FIG. 1. EPR spectra of HgR-AcChoEase (5 μM in buffer A). Traces: 1, HgR-AcChoEase; 2, HgR-AcChoEase in 1.5 M Gdn·HCl; 3, HgR-AcChoEase in 5.0 M Gdn·HCl; 4, HgR-AcChoEase in 5.0 M Gdn·HCl after demodification by 1 mM GSH. EPR conditions: microwave power, 20 mW; modulation amplitude, 1 G (1 G = 0.1 mT). The first two traces are at gain 5 × 10^5, trace 3 is at 10^5, and trace 4 is at 5 × 10^4.

from the γ-O of the active-site serine Ser-200 (25), and is not involved in catalysis (29). The changes in the spectroscopic characteristics of AcChoEase modified by HgR (Fig. 2) presumably reflect a local perturbation in AcChoEase structure induced by the insertion of HgR. These conformational changes, in turn, must be responsible for the observed loss of enzymic activity.

The HgR-AcChoEase equilibrated in 5.0 M Gdn·HCl displays a broad intrinsic fluorescence spectrum centered at ≈352 nm (Fig. 2*A*, trace 4), typical of an unfolded protein (30). The CD spectrum in the far UV is greatly decreased, the band in the near-UV is collapsed (Fig. 2*B*, trace 4), and no binding of ANS is observed (Fig. 2*C*). Thus, in 5.0 M Gdn·HCl, HgR-AcChoEase displays spectral characteristics typical of U.¶ In 1.5 M Gdn·HCl, HgR-AcChoEase appears to be partially unfolded, since the intrinsic fluorescence emission maximum is at 343 nm, intermediate between that of N (332 nm) and of U (352 nm) (Fig. 2*A*, trace 3). Ellipticity in the far UV remains substantial at this Gdn·HCl concentration, while the CD band in the near UV is almost eliminated (Fig. 2*B*, trace 3). The HgR-AcChoEase displays high ANS binding, reflecting an exposure of hydrophobic surfaces (Fig. 2*C*). These spectral data for the HgR-AcChoEase in 1.5 M Gdn·HCl reveal the absence of tertiary structure and the retention of secondary structure elements, which provide commonly accepted criteria for MG (3–5). The elimination of the near-UV band cannot discount the possibility that domain(s) exist(s) within the protein that remain(s) native yet devoid of ellipticity in this spectral range. This possibility, however, seems to be unlikely since in the presence of 1.5 M Gdn·HCl, a complete loss of enzymic activity of unmodified

¶The spectroscopic characteristics of HgR-AcChoEase (Fig. 2) are very similar to those obtained earlier for unmodified enzyme (24).

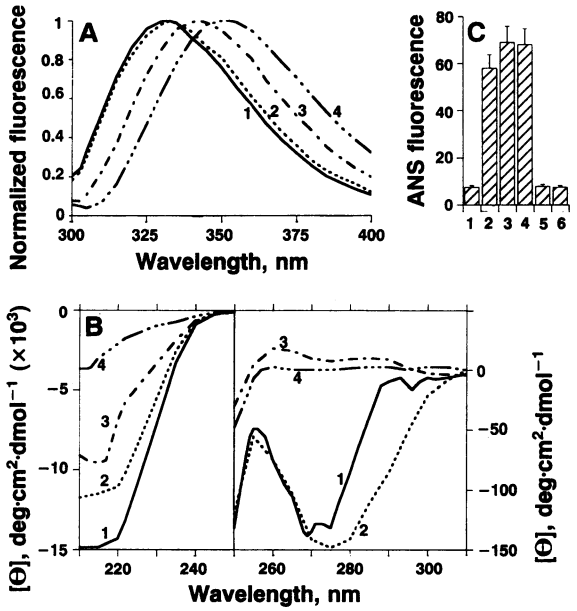

FIG. 2. Optical characteristics of AcChoEase. (*A*) Normalized intrinsic fluorescence spectra. Traces: 1, native enzyme; 2, HgR-AcChoEase; 3, HgR-AcChoEase in 1.5 M Gdn·HCl; 4, HgR-AcChoEase in 5.0 M Gdn·HCl. AcChoEase was 0.75 μM in buffer A. Excitation wavelength was 295 nm. (*B*) CD spectra in the far- and near-UV. The notation is as in *A*, but the AcChoEase concentration was 3.7 μM. (*C*) ANS binding by native AcChoEase and HgR-AcChoEase. Traces: 1, native AcChoEase; 2, HgR-AcChoEase; 3 and 4, native AcChoEase and HgR-AcChoEase, respectively, in 1.5 M Gdn·HCl; 5 and 6, native AcChoEase and HgR-AcChoEase, respectively, in 5.0 M Gdn·HCl. ANS was added 30 min prior to the fluorescence measurements, with excitation at 390 nm and emission at 490 nm. AcChoEase samples were 0.23 μM and ANS was 0.1 mM. ANS fluorescence is presented in arbitrary units.

AcChoEase is observed, indicating disruption of tertiary structure.‖

The EPR spectrum of HgR-AcChoEase in the presence of 1.5 M Gdn·HCl (Fig. 1, trace 2), which represents an MG (see above), is similar to that of the corresponding N-like state (trace 1), indicating that the spin label is highly immobilized also in MG. However, the N → MG transition is accompanied by a slight decrease in $2A_{zz}$ (1.3 G). This can be attributed to a slight increase in mobility (31) and/or a change in the polarity of the immediate environment of the label (19). A similar modest increase in mobility of spin-labeled β-lactamase in the MG, relative to N, was recently reported (32). The reduction of $2A_{zz}$ due to increased mobility is associated with a line-width increase (31), which is most evident in the high-field component. A broadening of ≈10% is observed in the spectrum of MG, indicating only a small change in mobility.

The EPR spectrum of HgR-AcChoEase in 5.0 M Gdn·HCl (Fig. 1, trace 3) (i.e., in U) is characteristic of a highly mobile radical. The asymmetric broadening, however, indicates some residual restriction of motion. Release of the label from the protein by GSH (21) produced an EPR spectrum characteristic of freely rotating radical in solution (trace 4).

The distinct EPR spectra of the MG and U states of HgR-AcChoEase make EPR a very effective tool for studying the MG ⇆ U transition. Fig. 3*A* shows the EPR spectrum as

‖No dissociation to monomers occurs as the result of chemical modification and further Gdn·HCl treatment. SDS/PAGE revealed that HgR-AcChoEase remains in its dimeric form. HPLC size-exclusion chromatography, performed in the presence of 5 M Gdn·HCl, showed that the chemically modified enzyme was eluted as a dimer.

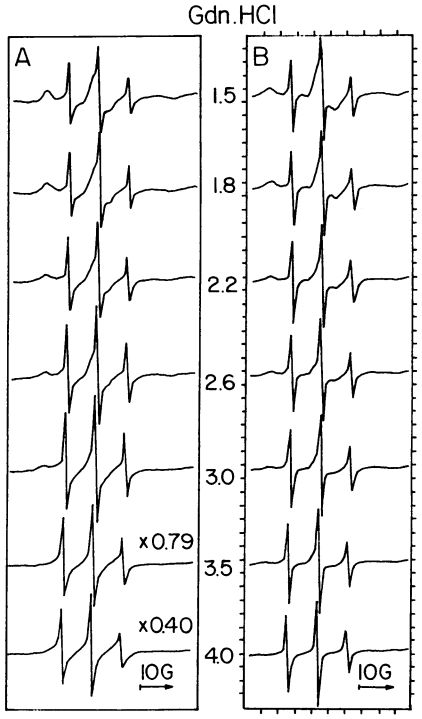

FIG. 3. Experimental (*A*) and corresponding simulated (*B*) EPR spectra of HgR-AcChoEase as a function of Gdn·HCl concentration (M). EPR conditions were as in Fig. 1. The top five traces are at gain 8×10^5, the trace at 3.5 M Gdn·HCl is at 5×10^5, and the trace at 4.0 M Gdn·HCl is at 3.2×10^5. Simulated spectra were obtained by varying the ratio of anisotropic to isotropic forms; from top to bottom, the successive values are 1:99, 1.1:98.9, 1.6:98.4, 2.2:97.8, 2.7:97.3, 3.2:96.8, and 100:0.

a function of Gdn·HCl concentration in the range 1.5–4 M. As the Gdn·HCl concentration increased, the intensity of the broad EPR signal corresponding to MG decreased and a concomitant increase in the intensity of the triplet corresponding to U was observed. At denaturant concentrations ≥4.0 M, the EPR spectra lack the signal corresponding to MG, and little, if any, change in their shape is seen. The integral intensity of the EPR signal at each Gdn·HCl concentration, obtained by double integration, was found to be constant (within an accuracy of ±25%). The line widths measured for both the low-field and high-field components of the signals corresponding to MG and to U were found to be constant throughout the transition. Furthermore, the $2A_{zz}$ value remained constant. Dilution from the high Gdn·HCl concentration resulted in rapid (namely, within the time of sample preparation) restoration of the signal corresponding to MG, with a concomitant decrease in the sharp signal corresponding to U. So, the transition, as monitored by EPR, is fully reversible. Thus, the two forms coexist throughout the transition region and their ratio varies. To substantiate this interpretation, a series of spectral simulations was performed aimed at reproducing the changes in the experimental spectra through the MG ⇆ U transition.

The simulated EPR spectra were generated by superimposing two types of spectra: The first, corresponding to U, was described by isotropic *g* and hyperfine interactions. The effect of the slightly restricted motion was accounted for by introducing different line widths for each of M_I components of the ^{14}N. The second spectrum, corresponding to MG, was taken as an anisotropic powder pattern with both *g* and *A* anisotropies (33). In this case, a single value for the line width was used for all M_I components. In both the isotropic and anisotropic spectra, the line shape was assumed to be Lorentzian. The parameters used in the simulation are shown in

Table 1. Parameters used for simulation of EPR spectra of AcChoEase

Spectrum	g_{xx}	g_{yy}	g_{zz}	A_{xx}, G	A_{yy}, G	A_{zz}, G	Δ_{-1}, G	Δ_0, G	Δ_{+1}, G
Isotropic form	2.0057	2.00570	2.0057	14.85	14.85	14.85	0.87	0.75	1.15
Powder pattern	2.0071	2.00604	2.0030	5.7	5.7	31.5	3.0	3.0	3.0

Line width of each M_I component is shown (Δ).

Table 1, and the best fit spectra are shown in Fig. 3*B*. The simulated spectra reproduce the experimental spectra well and account for the changes occurring through the transition. It should be noted that in the calculated spectra, we did not differentiate between the contributions to the isotropic signal of the highly mobile radical in the U state and of the small amount of unbound radical. From the experimental and calculated EPR results, we conclude that the MG ⇋ U transition is a two-state transition in which exchange between the two states is slow on the EPR time scale. A gradual swelling model for the MG ⇋ U transition (11) or a transition occurring within the same macroscopic state due to a redistribution of the population of its microscopic states (8) is ruled out, since a gradual change of the line width and line position throughout the transition is expected in both these cases.

The relative amounts of U (and MG) at each Gdn·HCl concentration (Fig. 4) were estimated from the amplitudes of the low-field components of both types of spectra, where the spectra obtained at 1.5 and 4.0 M Gdn·HCl are assumed to correspond to 100% MG and 100% U, respectively. The assumption that the peak amplitudes are proportional to the area and are then comparable is valid since no variation in line width was observed through the transition. The curves obtained for the MG ⇋ U transition, by using either the decrease in the low-field component for MG or the increase in the low-field component for U, coincided. The values of equilibrium constants obtained from the MG/U ratio at each Gdn·HCl concentration were used to calculate the corresponding ΔG. A plot of ΔG vs. [Gdn·HCl] was linear (Fig. 4 *Inset*), as is usual for protein denaturation (1, 16), thus permitting calculation of ΔG° and m (Table 2). Because one type of spectrum (of U) consists of very sharp lines, whereas the other (of MG) is a broad powder pattern, the method is subject to larger error at the higher Gdn·HCl concentrations. We have not used the MG/U ratios obtained from the simulated spectra to similarly calculate ΔG° and m, since the simulations did not reproduce the line shape of the experimental spectra exactly. This is due to the simple model used for calculation of the isotropic signal, which did not include motion effects.

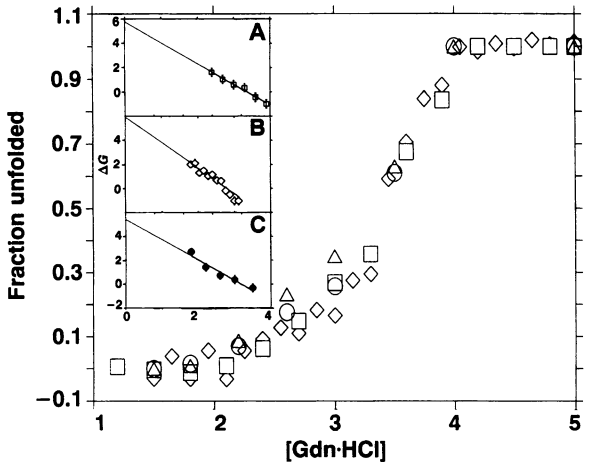

FIG. 4. Fraction of unfolded HgR-AcChoEase as a function of Gdn·HCl concentration. Δ, Relative peak intensity of the low-field component of the EPR spectrum, corresponding to MG; ○, relative peak intensity of the low-field component, corresponding to U; □, ellipticity at 222 nm; ◇, intrinsic fluorescence intensity at 320 nm (excitation at 295 nm). (*Inset*) ΔG (kcal/mol) vs. [Gdn·HCl], as derived from CD data (*A*), fluorescence (*B*), and EPR measurements (*C*). ΔG values were calculated using the formula: $\Delta G = -RT \ln(f_U/f_{MG})$ at each [Gdn·HCl] in the transition region, where f_U and f_{MG} are the fractions of protein in the U and MG states, respectively. For analysis of CD and fluorescence data, f_{MG} was estimated as $f_{MG} = 1 - f_U$. For EPR data, both f_U and f_{MG} were determined directly from the measurements. Data were fitted to the equation $\Delta G = \Delta G^\circ - m$[Gdn·HCl] (16) by weighted least-squares analysis using KALEIDAGRAPH.

To our knowledge, this is the first instance in which EPR has been used to study the equilibrium MG ⇋ U transition. It is, therefore, important to understand whether this technique reflects local or general changes in protein structure. Accordingly, we compared the results obtained by EPR with those obtained by traditional methods for analyzing the MG ⇋ U transition, CD and intrinsic fluorescence (34, 35). For HgR-AcChoEase, Fig. 5 shows the dependence of the emission intensity at 320 nm and of the ellipticity at 222 nm upon the Gdn·HCl concentration. Both curves display plateau regions at 1.2–2.1 M Gdn·HCl and 4.0–5.0 M Gdn·HCl. The transition, as monitored by both techniques, is fully reversible. Results of an analysis of these changes, making the assumption that the transition is indeed two state (1, 16), are shown in Fig. 4 and Table 2. The good agreement between the data obtained by the integral optical methods and by the local method, EPR, confirms the validity of the two-state model for describing the MG ⇋ U transition in AcChoEase and clearly shows, in this case, that the spin-label reflects the overall transition.

A Gibbs energy difference between MG and U, evaluated in the absence of Gdn·HCl, is ≈5.5 kcal/mol (1 cal = 4.184 J) (Table 2). This value is higher than that reported for analogous transitions for α-lactalbumin [1.3 kcal/mol (36)], apomyoglobin [2.1 kcal/mol (34)], and acetylated cytochrome *c* [4.5 kcal/mol (37)]. The stability of AcChoEase in MG, relative to U, is quite comparable to the overall stabilization energy for small proteins [5–15 kcal/mol (2)]. Indeed, the high stability of the MG might be somehow responsible for the observed irreversibility of a partial unfolding of N, produced either by Gdn·HCl treatment of AcChoEase (D.I.K., I.S., and L.W., unpublished work) or by reversible chemical modification of the AcChoEase with disulfides (21).

Though information on structural changes obtained by monitoring the EPR label is of an intrinsically local nature, the method has an important advantage, the possibility of resolving signals corresponding to discrete conformational states coexisting under equilibrium conditions, as we have clearly demonstrated for the HgR label attached to the free thiol of Cys-231 in native *T. californica* AcChoEase. Obvi-

Table 2. Thermodynamic parameters for the MG ⇋ U transition of HgR-AcChoEase as determined by various methods

Method	ΔG°, kcal/mol	m, kcal/(mol·M)
EPR	5.4 ± 0.7 (3)	1.7 ± 0.3 (3)
CD	5.7 ± 0.4 (2)	1.7 ± 0.2 (2)
Fluorescence	5.8 ± 0.6 (3)	1.7 ± 0.3 (3)

Data are the mean ± SD; the number of measurements is in parentheses.

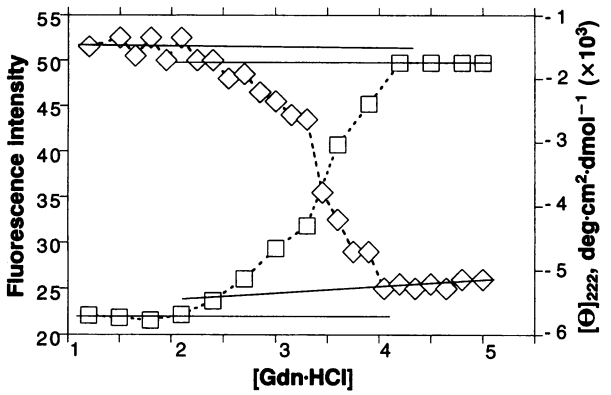

FIG. 5. Fluorescence intensity at 320 nm and ellipticity at 222 nm of HgR-AcChoEase as a function of Gdn·HCl concentration. ◇, Emission intensity (excitation at 295 nm); □, ellipticity. HgR-AcChoEase concentrations were 0.75 and 3.7 μM, respectively. The lines represent baselines (16) for MG (1.2–2.1 M Gdn·HCl) and U (4.0–5.0 M Gdn·HCl) used to construct the corresponding curves in Fig. 4. Fluorescence intensity is in arbitrary units.

ously, this approach could be extended by inserting cysteine residues, by site-directed mutagenesis, at different loci of a given protein, followed by attachment of a suitable spin label. As mentioned above, the time scale of the MG ⇆ U transition is believed to be in the millisecond range (5, 20). Thus, even if the rates of intraconversion between the two conformational states were substantially faster, EPR would still be applicable.

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