Journal of Fluorescence, Vol. 15, No. 1, January 2005 (©2005)
DOI: 10.1007/s10895-005-0214-x

Conformational Study of Spectrin in Presence of Submolar Concentrations of Denaturants

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Received May 13, 2004; accepted June 24, 2004

The presence of very low concentrations of the commonly used chemical denaturants, guanidinium chloride (GdmCl) and urea brought about conformational changes in the erythrocyte membrane skeletal protein, spectrin. Evidences in support of changes in the quaternary structure of spectrin have been put forward from quenching study of tryptophan fluorescence, by both steady state and time-resolved measurements, using acrylamide as the quencher. It revealed significant differences between the Stern–Volmer quenching constants (K_{SV}) and the fraction of accessible tryptophans (f_e) observed in absence and presence of GdmCl and urea concentrations below 1 M at which the association of the two subunits remains intact. The steady state anisotropy of both the spectrin tryptophans and the spectrin-bound fluorescence probe, Prodan also indicate changes in the overall flexibility of the spectrin dimer, originating from changes in the quaternary structure of spectrin. Studies on the binding of Prodan, further indicate that conformational changes also occur in spectrin near the Prodan-binding site at the terminal domain of the protein which is reflected in 3–4 fold decrease in the affinity of binding of Prodan to spectrin in the presence of GdmCl and urea compared to that observed in the absence of the denaturants. The dissociation constant (K_d) of Prodan to spectrin is 0.43 μM at 25°C.

KEY WORDS: Spectrin; protein denaturants; tryptophan; Prodan.

INTRODUCTION

Spectrin is the major constituent protein of the erythrocyte skeleton that forms a filamentous network on the cytoplasmic face of the membrane by providing a scaffold for a variety of proteins. It is a large dimeric amphiphilic protein, an elongated heterodimer with two subunits (α and β with apparent molecular masses of 280 and 248 kDa), having hydrophobic stretches in its polypeptide sequence. The two subunits are homologous with about 30% identity and are aligned in the highly elongated, antiparallel side-to-side orientation to give a flexible 100 nm rod shaped molecule with the amino and carboxy termini toward the ends of the rods. These heterodimers associate head-to-head to form 200 nm tetramers and higher order oligomers [1–8]. The largest subdomain in spectrin is called the rod domain that is the elongated backbone of each polypeptide chain. Beside the rod domain there is self-association or tetramerization domain that involves the portions of the amino terminal region of α-chain and the carboxy terminal region of the β-chain, defined as the head of dimers, the actin-binding domain at the amino terminus of the β-chain and an EF hand structural motif at the carboxyl terminus of the α-chain [4]. The α- and β-subunits consist largely of a series of 106 amino acid repeat motifs (20 and 16 complete repeats, respectively) with each repeat predicted to fold into a triple-helical bundle as determined by X-ray crystallography [5,6] and NMR [7]. The X-ray structure of a single repeat motif shows that the triple-helical bundle is stabilized both by extensive hydrophobic interactions between the interior faces of amphipathic helices and by electrostatic interactions between the charged residues on the exterior surfaces [5]. These structural features allow spectrin to take part in a number of physiological events through protein–protein

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interactions. The ability of spectrin to expand and contract has been attributed to its modular structure made of those repeat motifs [6]. The association of the subunits to form the heterodimer does not appear to impart any additional rigidity on the complex [8,9]. Moreover, studies on spectrin structure showed that part of its molecule is markedly hydrophobic, although its hydrophobicity index is considerably lower than those of actin and serum albumin [10]. Considering all the above features in erythroid spectrin, it is supposed that the conformational nature and the extent of structural rigidity or flexibility bear on its structural role in maintaining the cytoskeleton and in turn the shape of the cell. Conformational studies on spectrin indicated that the protein contains rigid and globular domains, and also a proportion of flexible, segmentally mobile conformation, which is highly hydrophobic in composition. The complete dissociation of the α- and β-chains takes place in as high as 3 M urea [11].

Urea and guanidinium chloride (GdmCl) are very frequently used as strong denaturants of proteins, which have been shown to undergo pronounced structural disintegrations at concentrations >1 M of these denaturants [12,13]. However, it is generally accepted that very low concentrations of urea and GdmCl, which are often present in the buffer during reconstitution of proteins from the completely unfolded state attained by treatment with 8–10 M urea and 4–6 M GdmCl, do not impart any alterations in their tertiary structure. We have previously shown that low, even millimolar, concentrations of GdmCl and urea can bring about structural alterations in the heme-containing enzyme, horseradish peroxidase (HRP), at the tertiary level [14,15]. In this paper, we have extended our work to study the effect of low concentrations of GdmCl and urea on the quaternary and tertiary structure of the membrane skeletal protein, spectrin. Since GdmCl is a strong electrolyte but urea is not, such a study was considered likely to offer insight into the general mechanism of denaturant–protein interaction. In a previous study, we have investigated the organization and dynamics of the functionally important tryptophan residues of erythroid spectrin and spectrin-bound Prodan (6-propionyl-2-(dimethylamino)naphthalene) in native and denatured conditions utilizing wavelength selective fluorescence approach [16]. In this work, we have investigated the effects of low concentrations of GdmCl and urea on the conformation of erythroid spectrin in presence of submolar denaturant concentrations of the two denaturants those do not cause dissociation of the two subunits. We have probed the tryptophan residues in spectrin and the spectrin specific fluorescence probe, Prodan [17] and have studied the conformational changes induced by GdmCl and urea using steady state and time-resolved fluorescence measurements. Acrylamide quenching measurements indicated changes in the quaternary structure of spectrin originating from unzipping of the two subunits, in presence of the denaturants, without causing complete dissociation.

EXPERIMENTAL

GdmCl, urea, Tris, DTT, PMSF, ultrapure acrylamide, *N*-acetyl-L-tryptophanamide (NATA), and other chemicals were obtained from Sigma. Prodan was obtained from Molecular Probes.

Spectrin dimer was purified from ovine erythrocyte ghosts following the published procedure [18]. The details of the method are elaborated in our earlier work [19]. Briefly, the clean, white ghosts were suspended in 20 volumes of spectrin removal buffer (0.2 mM sodium phosphate, 0.1 mM EDTA, 0.2 mM DTT, 20 μg/mL PMSF, pH 8.0) and incubated at 37°C for 30 min. The crude spectrin was collected in the supernatant after centrifugation. Spectrin was then purified after concentrating by 30% ammonium sulfate precipitation followed by chromatography on Sepharose CL-4B and stored in the buffer containing 5 mM phosphate, 20 mM KCl, 1 mM EDTA, pH 8.0 containing 0.2 mM DTT. Before all the fluorescence experiments the protein was dialyzed extensively against the buffer containing 10 mM Tris–HCl, 20 mM KCl, pH 7.8 to remove DTT and EDTA. Spectrin concentrations were determined spectrophotometrically using an absorbance of 10.7 at 280 nm for 1% spectrin solution [18] and also by Lowry's method [20].

Steady state fluorescence studies were performed using a Hitachi *F*-4010 spectrofluorometer. Fluorescence emission from tryptophan and Prodan was measured by using excitation at 295 and 360 nm, respectively, using 5 nm bandpass slits for both excitation and emission channels. The concentration of Prodan in dimethylformamide was determined using a molar absorbance of 18 000 at 360 nm [21]. A typical Prodan concentration of 0.5 μM, both in presence and absence of the denaturants incubated for 1 hr at 25°C, were used in all fluorescence measurements. Control baselines were subtracted for each denaturant concentration in all fluorescence measurements. Anisotropy measurements were done at an emission wavelength of 340 nm for tryptophan, 430 nm for spectrin-bound Prodan and 520 nm for free Prodan in aqueous buffer [17]. For quenching studies, acrylamide was used as the quencher of tryptophan fluorescence. The sample temperature was kept at 25°C. All measurements were done in a buffer containing 10 mM Tris–HCl, 20 mM KCl, pH 7.8 unless mentioned otherwise.

Experiments on quenching of tryptophan fluorescence were carried out by exciting at 295 nm and recording the emission intensities at 340 nm, after serial addition of small aliquots of acrylamide stock solution [15,22,23]. Corrections were applied to the observed intensities for dilution of the protein and for absorbance of the incident light by acrylamide. Spectrin (0.2 μM) was incubated with different submolar concentrations of the denaturants for 1 hr before each quenching experiment was carried out. Quenching data were analyzed by fitting to the Stern–Volmer equation

$$F_O/F_{corr} = 1 + K_{SV}[Q] \quad (1)$$

where F_O and F_{corr} are the fluorescence intensities in the absence and presence of the quencher, respectively, [Q] the quencher concentration, and K_{SV} the Stern–Volmer quenching constant. The initial linear portions of the Stern–Volmer plots were used to determine K_{SV}. The quenching data were also analyzed using a modified Stern–Volmer equation for the evaluation of the fraction of accessible tryptophans (f_e) in spectrin by the quencher [22–24].

$$F_O/(F_O - F_{corr}) = 1/(K_{SV} f_e[Q]) + 1/f_e. \quad (2)$$

Errors given by the spread in the values of K_{SV} obtained in 3–5 independent experiments at a given denaturant concentration were smaller than the uncertainties of the fitted slopes. Quenching experiments were also carried out by time-resolved fluorescence measurement when τ_o/τ is plotted against the quencher concentration, τ_o and τ are the mean lifetime values in the absence and presence of the quencher, respectively.

Fluorescence lifetimes were determined from total emission intensity decay measurements, using a time-domain fluorometer assembled in the laboratory with components from Edinburgh Analytical Instruments and EG & G ORTEC and operated in the time-correlated-single-photon-counting mode. Excitation was provided by a pulsed high-pressure 152-kPa N_2 lamp operating at 25 kHz repetition rate, the pulse profile having a full width at half maximum of 1.3 ns. The tryptophans of spectrin were excited at 295 nm and Prodan at 360 nm. The emission-decay profile was monitored at 345 nm for tryptophan and at 430 and 520 nm for spectrin-bound and free Prodan, respectively. The time-resolved measurements were performed with 0.5 μM spectrin and 0.5 μM Prodan in all experiments. And amount of 5000 photon counts were collected in the peak channel. Slits with 8 nm bandpass for excitation and 16 nm for emission channels were used. Intensity decay curves were fitted to the series

$$I(t) = \sum_i A_i \exp(-t/\tau_i) \quad (3)$$

where A_i is a preexponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime τ_1. The decay parameters were recovered using a software package supplied by Edinburgh Analytical Instruments, implementing a nonlinear least-squares iterative fitting procedure. Accepted values of the fit parameters led to values between 1 and 1.2 for the reduced χ^2 ratio. Mean lifetimes $\langle\tau\rangle$ for bi- and triexponential decays were calculated using the following equation

$$\langle\tau\rangle = \sum_i \alpha_i \tau_i \quad (4)$$

where the fractional amplitude α_i corresponding to the lifetime τ_i is given by,

$$\alpha_i = A_i \tau_i / \sum_i A_i \tau_i. \quad (5)$$

Errors in mean lifetimes quoted in this work were estimated from the uncertainties in the individual lifetimes (τ_i) emerging from the fitting process [15,19].

Fluorescence titration data to study the binding of Prodan to spectrin were analyzed by the following model independent method and the apparent dissociation constant of Prodan binding to spectrin (K_d) was determined using nonlinear curve fitting analysis using the Eq. (7). All experimental points for binding isotherm were fitted by least-square analysis using Microcal Origin software package (Version 5.0) from Microcal Software Inc., Northampton, MA. The binding stoichiometry of the Prodan–spectrin complex was estimated from the intercept of two straight lines of the nonlinear fitted plot of Δ/Δ_{max} against the ratio of the input concentrations of spectrin and Prodan.

$$K_d = [C_0 - (\Delta F/\Delta F_{max})C_0][C_L - (\Delta F/\Delta F_{max})C_0]/(\Delta F/\Delta F_{max})C_0 \quad (6)$$

$$C_0(\Delta F/\Delta F_{max})^2 - (C_0 + C_L + K_d)(\Delta F_{max}) + C_L = 0. \quad (7)$$

In Eqs. (6) and (7), Δ is the change in fluorescence emission intensity ratio $I_{520}/I_{430}(\lambda_{ex} = 360$ nm) for each point on the titration curve. Δ_{max} denotes the same when spectrin is completely bound to Prodan, C_L is the concentration of the spectrin, and C_0 is the initial concentration of Prodan. Double reciprocal plot was used for determination of Δ_{max} using Eq. (8).

$$1/\Delta F = 1/\Delta F_{max} + 1/[K_{app}\Delta F_{max}(C_L - C_0)]. \quad (8)$$

The linear double reciprocal plot of $1/\Delta$ against $1/(C_L - C_0)$ is extrapolated to the ordinate to obtain the value of Δ_{max} from the intercept [25]. The approach is based on the assumption that change of emission intensity ratio of Prodan is proportional to the concentration of the spectrin.

The Scatchard equation [26] was also used to estimate the intrinsic binding constant (K_0) and the binding stoichiometry (n):

$$r/C_f = K_0(n - r) \quad (9)$$

where $r = C_b/C_p$, when C_b is the concentration of the spectrin-bound Prodan and C_p is the input concentration of spectrin. And n is the binding stoichiometry expressed as the number of Prodan bound per spectrin dimer and K_0 is the intrinsic binding constant ($K_{app} = K_0 n$). The concentration of the bound Prodan (C_b) was determined by normalizing the input concentration of Prodan with $\Delta F/\Delta F_{max}$. The Scatchard plot was obtained by plotting r/C_f against r where C_f is $(C_0 - C_b)$ and the best-fit straight line through the experimental data points led to the determination of binding constant, K_0 and the stoichiometry, n.

RESULTS

Fluorescence emission intensity of dimeric erythroid spectrin showed marginal quenching with the addition of increasing concentrations of GdmCl and urea in the millimolar range (not shown). The presence of GdmCl and urea in the range of 10–500 mM led to ~15% quenching in the tryptophan fluorescence intensity. The maximum emission of spectrin occurred at 338 nm, which is marginally red-shifted to 340 nm in presence of 10–500 mM of GdmCl and urea. Upon denaturation in 5 M GdmCl and 8 M urea the fluorescence emission was associated with a large red shift in the emission maximum from 338 to 349 nm as reported earlier [27].

Acrylamide quenching experiments were carried out as elaborated in our earlier works, to probe conformational alterations in proteins in presence of denaturants [14–16]. The Stern–Volmer plots for spectrin in presence and absence of different concentrations of GdmCl and urea, indicated changes in the bimolecular quenching constants, K_{SV}, evaluated from the slope of the individual plots. From the modified Stern–Volmer plots (Lehrer plot) the accessibility parameter, f_e for acrylamide was evaluated. Figure 1 shows few representatives Stern–Volmer plots (Fig. 1A) and Lehrer plots (Fig. 1B) in the presence and absence of urea. Table I summarizes the data on quencher accessibility represented by f_e and the quenching constant K_{SV}, the quenching rate constant ($k_q = K_{SV}/\langle\tau\rangle$), where $\langle\tau\rangle$ is the mean lifetime, and the wavelength of maximum emission (λ_{max}) in the presence and absence of both GdmCl and urea. Similar variations of K_{SV} with low denaturant concentrations were also observed when quenching experiments were carried out by time-resolved measurements.

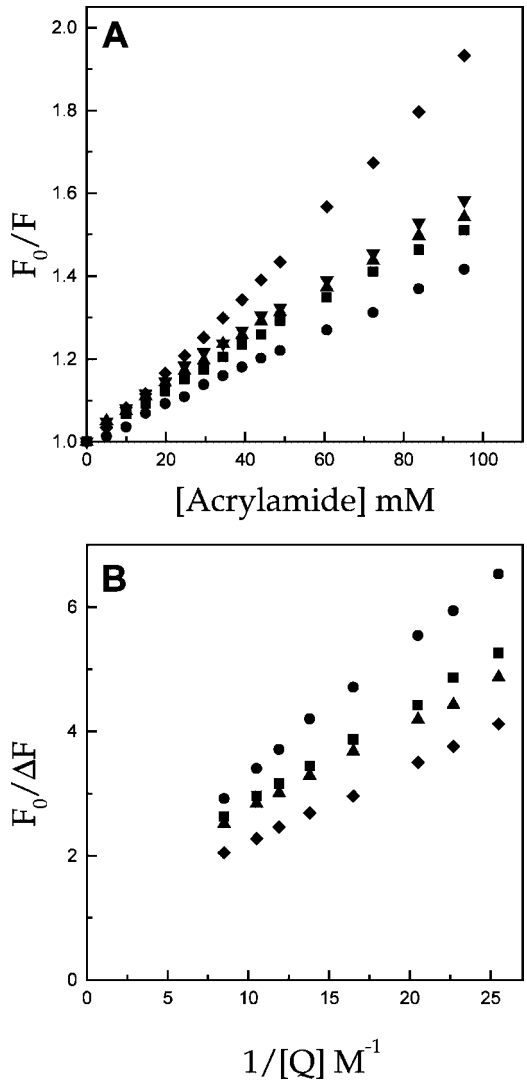

Fig. 1. (A) Stern–Volmer plot of quenching of tryptophan fluorescence of spectrin (0.2μ M) by acrylamide. The data points represent (■) for native spectrin; (●) in 50 mM; (▲) 0.5 M; (▼) 1 M urea and (♦) for denatured spectrin in 8 M urea. (B) Lehrer plots for acrylamide quenching of tryptophan fluorescence of spectrin (0.2 μM), (■) in the absence and presence of (●) 50 mM, (▲) 500 mM, and (♦) 8 M urea.

K_{SV} values were determined from the slope of the plot of τ_o/τ against the acrylamide concentrations, shown in Fig. 2. The K_{SV} came out to be $4.1 \pm 0.2\ M^{-1}$ for native spectrin that is $4.8 \pm 0.2\ M^{-1}$ obtained from steady state measurements. K_{SV} increased from $4.8 \pm 0.2\ M^{-1}$ to $5.6 \pm 0.1\ M^{-1}$ in presence of 50 mM GdmCl and on the other hand, decreased to $4.2 \pm 0.1\ M^{-1}$ in presence of urea, pointing to structural perturbations in spectrin. Similar increase from $4.1 \pm 0.2\ M^{-1}$ to $4.8 \pm 0.2\ M^{-1}$ in presence of 50 mM GdmCl and $4.7 \pm 0.1\ M^{-1}$ in presence of urea was

Table I. The Acrylamide Quenching Parameters of Spectrin Tryptophans: K_{SV}, f_e, and k_q and the Wavelength of Maximum Emission (λ_{max}) in Presence and Absence of the Denaturants

GdmCl (mM) (urea)	λ_{max} (nm)	K_{SV} (M^{-1})	k_q (M^{-1} s^{-1})	f_e (%)
Buffer	338.6	4.8 ± 0.2	1.25×10^9	67 ± 6
50	338.8 (339.0)	5.6 ± 0.1 (4.2 ± 0.1)	1.41×10^9 (1.06×10^9)	78 ± 7 (83 ± 8)
200	338.8 (339.0)	5.7 ± 0.1 (5.3 ± 0.2)	1.44×10^9 (1.34×10^9)	84 ± 6 (78 ± 7)
500	338.8 (339.4)	6.0 ± 0.2 (5.4 ± 0.2)	1.50×10^9 (1.30×10^9)	78 ± 8 (72 ± 5)
1000	339.2 (339.0)	5.5 ± 0.1 (5.7 ± 0.3)	1.39×10^9 (1.39×10^9)	84 ± 2 (75 ± 6)
5000 (8000)	351.4 (349.8)	5.1 ± 0.1 (8.6 ± 0.3)	1.56×10^9 (1.75×10^9)	100 (100)

The values in parentheses indicate those in the presence of urea.

observed in K_{SV}, evaluated from time-resolved fluorescence measurements. These changes observed in presence of GdmCl and urea were opposite in nature as indicated in the Fig. 3 that shows the plots of K_{SV} and k_q versus the concentration of both the denaturants, indicating differential effects between the electrolytic GdmCl and the nonelectrolytic urea. As a control experiment, similar quenching studies were also carried out on the model tryptophan compound, NATA. The K_{SV} value (16.5 ± 0.3 M^{-1}) remained independent of the GdmCl and urea concentration (upto 1 M) in which NATA were preincubated indicating that the changes in K_{SV} reported here are specific for spectrin.

It was earlier reported that Prodan exhibits unique fluorescence characteristics upon binding to spectrin [17]. We have used Prodan to probe structural changes in presence and absence of the two denaturants. Spectrin preincubated with different low GdmCl and urea concentrations indicated differential binding of spectrin with Prodan. The conformational changes in the vicinity of the Prodan-binding site in spectrin were reflected in the changes of fluorescence anisotropy and the dissociation constant in presence of low concentrations of GdmCl and urea. Table II summarizes the data on changes in the anisotropy and mean lifetime of spectrin tryptophans and spectrin-bound Prodan. Steady state tryptophan anisotropy in spectrin showed a large value of 0.128. In presence of very low concentration of GdmCl and urea (20–50 mM) the anisotropy of spectrin decreased to 0.118 indicating an overall structural flexibility of the spectrin dimer in presence of both denaturants. With further increase in the concentration of GdmCl and urea the anisotropy comes back to that of the native spectrin before getting drastically lowered to about 0.06 when spectrin was denatured in presence of either of 5 M GdmCl or 8 M urea. On the other hand, the steady state anisotropy of spectrin-bound Prodan shows the high value 0.198 which is drastically increased to 0.228 in presence of 50 mM GdmCl indicating the microenvironment of Prodan-binding site on spectrin to undergo conformational change in presence of 50–500 mM GdmCl, whereas in case of urea the changes in anisotropy is insignificant in this concentration range than that of the native spectrin. The mean lifetime of spectrin trytophans and the spectrin-bound Prodan also showed significant changes indicating structural alterations in the protein in the neighborhood of the Prodan-binding site in presence of only GdmCl and not urea.

Figure 4 shows two representative binding isotherms of high affinity Prodan binding to spectrin in the presence and absence of 50 mM GdmCl as a plot of $\Delta F/\Delta F_{max}$ against the concentration of dimeric spectrin. The inset of Fig. 4 shows the representative double reciprocal plots of $1/\Delta F$ against 1/[spectrin], extrapolated to the ordinate from which the value of ΔF_{max} were evaluated from the intercept. The dissociation constant (K_d) of Prodan to spectrin is 0.43 μM. In presence of 50 mM GdmCl, the dissociation constant of Prodan to spectrin is marginally increased to 0.47 μM and with increasing the concentrations to 0.5 and 1 M, the dissociation constants are further increased to 1.44–2.43 μM. However, in presence of 50 mM urea K_d increased significantly to 1.74 μM and then decreased to 0.87 μ and 1.29 μM in presence of 0.5 and 1 M urea, respectively. The binding stoichiometry of Prodan with spectrin remained one as indicated in the representative Scatchard plot in presence of 50 mM GdmCl (Fig. 5). The dissociation constants and the binding stoichiometry of Prodan to spectrin are evaluated following three independent methods of analysis and are summarized in Table III.

DISCUSSION

Guanidinium chloride and urea are commonly used for denaturation of proteins. The present work has been done with an aim to study conformational alterations in the cytoskeletal spectrin using low concentrations of the chemical perturbant where small structural changes might be reflected in its cytoskeletal integrity, altered association with proteins and differential interactions with lipids and

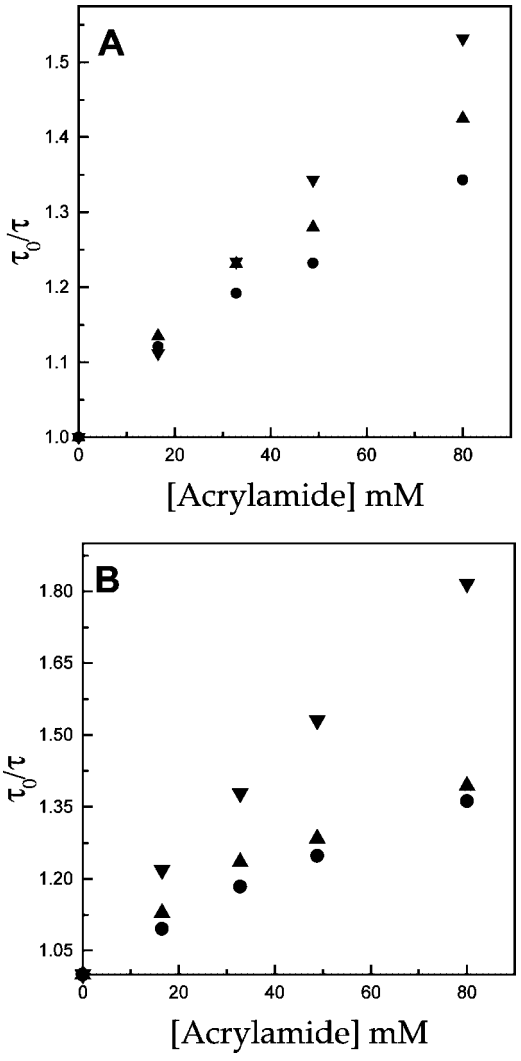

Fig. 2. (A) Stern–Volmer plot of acrylamide quenching by time-resolved tryptophan fluorescence of spectrin (0.5 M). The plot shows the ratio of mean lifetime in the absence (τ_o) and presence of different concentrations of the quencher (τ) versus the respective quencher concentrations. The data points represent (●) for native spectrin; (▲) in presence of 50 mM GdmCl and (▼) for spectrin denatured in 5 M GdmCl, respectively. (B) Stern–Volmer plot of acrylamide quenching by time-resolved tryptophan fluorescence of spectrin. The plot shows the same in presence of urea and the denatured spectrin in 8 M urea.

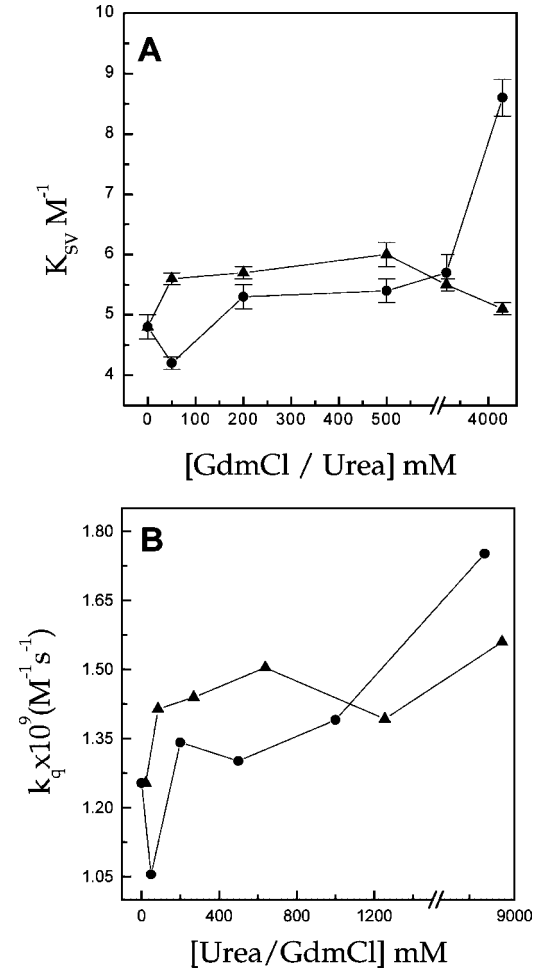

Fig. 3. (A) Plot of the Stern–Volmer quenching constant for acrylamide quenching of the spectrin tryptophan residues in presence of different concentrations of (●) urea and (▲) GdmCl. The K_{SV} value of spectrin in 8 M urea is plotted along with that at 5 M GdmCl concentration. (B) Plot of the bimolecular quenching rate constant of the spectrin tryptophan residues in presence of different concentrations of (▲) GdmCl and (●) urea.

other small hydrophobic molecules as shown earlier in case of tubulin [28]. Spectrin has a number of tryptophan residues spread over the entire length of the dimer. It is noteworthy that the 106 amino acids long repeat units in spectrin have tryptophans strongly conserved at the 45th residue and partially conserved at the 11th residue [29,30]. Some of the conserved tryptophans have been shown to promote folding of spectrin domains [31] and contribute to their thermodynamic stability [32]. The fact that tryptophans are distributed over the entire molecule and yet are localized in the same position in each domain makes them convenient intrinsic fluorescence reporter groups for monitoring conformational changes in spectrin that contribute to its elastic deformability exhibited in physiological conditions [27,33]. There are 42 tryptophans in each of the α- and β-subunits in the spectrin dimer [29,30]. Careful examination shows that there are 41 tryptophans in 23 repeat motifs in the α subunit while there are 35 tryptophans in 17 repeat motifs in the β-subunit of the spectrin dimer [16]. Taken together, the tryptophans in the repeat motifs represent more than 90% of the total tryptophans in the spectrin dimer. In addition, there are

Table II. Anisotropy and Mean Lifetime of Spectrin Tryptophans and Spectrin-Bound Prodan, in Absence and Presence of Different Concentrations Denaturants

GdmCl (mM) (urea)	Anisotropy (Prodan)	Anisotropy (Trp)	$\langle\tau\rangle$ (ns) (Prodan)	$\langle\tau\rangle$ (ns) (Trp)
Buffer	0.02 ± 0.002	0.027	1.1	2.7
Spectrin	0.198 ± 0.004	0.128 ± 0.003	4.64	3.83
50	0.228 ± 0.004 (0.204 ± 0.004)	0.118 ± 0.002 (0.119 ± 0.008)	4.84 (4.61)	3.96 (3.98)
200	0.223 ± 0.005 (0.199 ± 0.004)	0.130 ± 0.005 (0.129 ± 0.009)	4.70 (4.62)	3.96 (3.95)
500	0.227 ± 0.005 (0.203 ± 0.004)	0.134 ± 0.004 (0.126 ± 0.006)	4.78 (4.57)	3.99 (4.15)
1000	0.206 ± 0.003 (0.181 ± 0.005)	0.119 ± 0.002 (0.116 ± 0.008)	4.40 (4.53)	3.95 (4.10)
5000 (8000)	0.033 ± 0.003 (0.037 ± 0.003)	0.058 ± 0.006 (0.065 ± 0.004)	3.78 (3.82)	3.27 (4.91)

The values in parentheses indicate those in the presence of urea.

5 tryptophans in the actin-binding domain at the amino terminus and 2 tryptophans at the carboxy terminus in the β-subunit of the spectrin dimer. We have recently utilized the intrinsic tryptophan fluorescence of spectrin to monitor interaction of spectrin with micellar detergents and membrane containing phosphatidylethanolamine [19,34]. Results of the present study indicated that in presence of very low submolar concentrations of the two denaturants, the quaternary structure of spectrin changes that is reflected in the changes in Stern–Volmer quenching parameters of K_{SV}, k_q, and f_e (Table I). There are minor but definite changes in spectrin conformation also at the tertiary structural level that is revealed from the changes in the fluorescence anisotropy and the mean lifetime of spectrin tryptophans (Table II) in presence of submolar concentrations of GdmCl and urea. All these changes in the conformation of ovine erythroid spectrin, that is indistinguishable from that of human and bovine spectrin [35], also led to change in the binding affinity of the hydrophobic fluorescence probe, Prodan to spectrin.

Quenching studies of tryptophan fluorescence by acrylamide yielded a value of (4.8 ± 0.2) M^{-1} for K_{SV} in the native spectrin. In presence of 50 mM GdmCl the quenching efficiency (K_{SV}) increases markedly to (5.6 ± 0.1) M^{-1}, pointing to a possible exposure of the masked tryptophan of spectrin, due to which the quencher accessibility is greatly enhanced. The bimolecular quenching rate constant has been $(1.25 \pm 0.1) \times 10^9 M^{-1}$ s^{-1}, considerably smaller than the value of

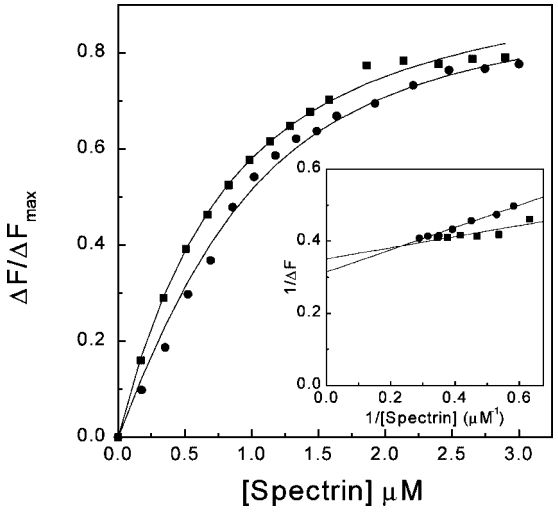

Fig. 4. Binding isotherms for the association of Prodan (0.5 μM) with the spectrin preincubated without (●) and with (■) 50 mM GdmCl in the buffer containing 10 mM Tris–HCl, 20 mM KCl at pH 7.8. The normalized increase in fluorescence ($\Delta F/\Delta F_{ax}$) is plotted against the spectrin concentrations to Prodan. Inset shows the double reciprocal plots of $1/\Delta F$ against 1/[spectrin] for Prodan (0.5 μM) in absence (●) and presence (■) of GdmCl in the same buffer. The lines drawn through the data points are fitted by least-square analysis.

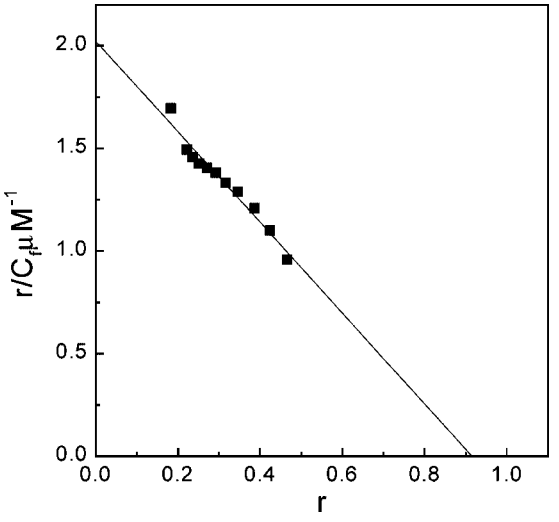

Fig. 5. Scatchard plot for the interaction of dimeric spectrin, preincubated with the 50 mM GdmCl with the Prodan (0.5 μM) in the buffer containing 10 mM Tris–HCl, 20 mM KCl at pH 7.8.

Table III. Dissociation Constant (K_d) of Spectrin-Bound Prodan in Presence of Different Concentrations of the Two Denaturants

Spectrin in	Double reciprocal plots $K_d(\mu M)$	Nonlinear plots		Scatchard plots	
		K_d (μM)	Stoichiometry	K_d (μM)	Stoichiometry
Buffer	0.47 ± 0.1	0.43 ± 0.03	1	0.38 ± 0.2	1
50 mM GdmCl (50 mM urea)	0.44 ± 0.1 (1.91 ± 0.4)	0.47 ± 0.04 (1.63 ± 0.2)	1.2 (0.4)	0.5 ± 0.2 (1.7 ± 0.3)	0.9 (1.14)
500 mM GdmCl (500 mM urea)	1.57 ± 0.3 (0.97 ± 0.2)	1.37 ± 0.2 (0.80 ± 0.1)	0.6 (0.5)	1.38 ± 0.3 (0.8 ± 0.1)	0.9 (1.1)
1000 mM GdmCl (1000 mM urea)	2.68 ± 0.4 (1.44 ± 0.2)	2.35 ± 0.2 (1.04 ± 0.1)	0.4 (0.6)	2.26 ± 0.3 (1.4 ± 0.2)	0.72 (1.9)

The values in parentheses indicate those in the presence of urea. Error estimates are SEM of 3–5 independent experiments.

$k_q(6 \times 10^9\ M^{-1}\ s^{-1})$ obtained for acrylamide quenching of free tryptophan in water [24]. The quenching rate constant increases to $(1.41 \pm 0.1) \times 10^9\ M^{-1}\ s^{-1}$ in presence of 50 mM GdmCl and decreases to $(1.06 \pm 0.1) \times 10^9\ M^{-1}\ s^{-1}$ in presence of 50 mM urea. These results also confirm that the tryptophans are in the hydrophobic regions of the protein that is indicated by lower accessibility of the tryptophans by the quencher. About 65% of the total number of tryptophans is accessible to acrylamide. Since acrylamide is a small, uncharged molecule, the most probable explanation for a low f_e of about 65% in the native spectrin is due to the fact that about 35% of the spectrin tryptophans are at the interface of the two subunits. The fraction of tryptophan residues that are accessible to the quencher increases to about 80% indicating unzipping of the spectrin subunits in presence of 50 mM GdmCl and urea (Table I). The magnitude of change in f_e is appreciable considering the fact that there are about 80 tryptophans in a spectrin dimer of which about 50 are normally accessible and the rest 30 are buried at the interface between the α- and β-subunits. The present study also indicates distinct structural differences between the unfolded spectrin when denaturation is carried out by either of 8 M urea or 5 M GdmCl. The quenching rate constant increases to $1.56 \times 10^9\ M^{-1}\ s^{-1}$ in 5 M GdmCl and $1.75 \times 10^9\ M^{-1}s^{-1}$ in presence of 8 M urea indicating structural differences in the unfolded spectrin that was denatured either by the cationic GdmCl or by the uncharged urea. A representative plot of K_{SV} and k_q versus the concentration of both the denaturants clearly shows this differential effect of GdmCl and urea (Fig. 3). The expected decrease in the $\langle\tau\rangle$ value due to exposure of tryptophans to more hydrophilic environment upon complete denaturation, supported by the red shift in the emission maximum of the denatured spectrin, was observed only in 5 M GdmCl and not in 8 M urea. In presence of 5 M GdmCl, $\langle\tau\rangle$ is found to decrease to 3.27 ns and in 8 M urea it is largely increased to 4.91 ns from that of 3.83 ns in native spectrin (Table II).

The mechanism of actions of GdmCl and urea on protein denaturation are still obscure. According to one school of thought, urea stabilizes both the native and denatured protein conformations. The larger number of denaturant binding sites, exposed in the unfolded state, favors denaturation [36–38]. Crystallographic studies of α-chymotrypsin in the presence of GdmCl and urea have shown that both denaturants bind to the surface of the folded protein, with the urea molecules (but not the charged GdmCl) also permeating the interior, occupying small cavities and somewhat perturbing the close-packed interior [39]. Since spectrin is an elongated worm-like protein, the differential effects of urea and GdmCl predominantly on the spectrin conformation take place through binding of the denaturants on the surface of the protein with the electrolytic GdmCl exerting stronger effects than the uncharged urea. A recent molecular dynamics simulation study also suggests that the preferential adsorption of urea molecules onto the charged hydrophilic residues on protein surfaces initiates the denaturation of proteins [40]. Makhatadze and Privalov have studied the interaction of urea and GdmCl with proteins using calorimetry and were able to describe the observed heat effects in terms of a simple binding model estimating 120 urea molecules bound to each molecule of native ribonuclease A and lysozyme and around 240 urea molecules were bound to each unfolded protein molecule [41]. The binding of urea to the small globular proteins bovine pancreatic trypsin inhibitor (BPTI) and PEC-60 was also investigated by NMR spectroscopy and the average binding constants were found to lie within 5–10 M^{-1} at room temperature [42]. Assuming equilibrium bimolecular binding between protein and urea, the occupancy of the binding site should be higher than 50% for urea concentrations larger than the inverse binding constant, suggesting that even submolar (10^{-2}–10^{-1}M) concentrations of urea can bind to micromolar concentration of proteins and may thus lead to structural alterations in the compact folded state.

Many of these tryptophans are at or in the vicinity of hydrophobic patches in spectrin, which can bind hydrophobic ligands such as fatty acids and phospholipids and cause quenching of tryptophan fluorescence [34,43,44]. The hydrophobic binding site in spectrin is crucial since this region is believed to facilitate interaction of spectrin with membranes. One of us has previously shown that the hydrophobic fluorescent probe, Prodan, which shows polarity-sensitive fluorescence, binds erythroid spectrin with a high affinity [17]. In addition, we have recently shown that the widely used hydrophobic fluorescent probe pyrene also binds to spectrin with high affinity and estimated the apparent dielectric constant of the binding site to be ~7 from analysis of the ratios of pyrene vibronic band intensities [45]. From the binding studies of Prodan to spectrin, the binding dissociation constant in presence of 50 mM urea, was found to increase by 4-fold over that in the absence of the denaturant. With further increase in the denaturant concentrations up to 1 M, the binding affinity was found to decrease without affecting the binding stoichiometry of one Prodan per spectrin dimer (Table III). Spectrin is a multidomain protein and the rod domain of the spectrin consists of 22 identical structural repeats in α-subunit and 17 such repeats in β-subunits of spectrin dimer. The Prodan binding stoichiometry being one confirms that the hydrophobic binding site is localized only at one of the terminal domains of the spectrin dimer.

Fluorescence anisotropy measurements offer a convenient method for obtaining information about the rotational dynamics of a fluorophore. The rotational freedom of the spectrin dimer increased in presence of low denaturant concentrations. This was indicated by the decrease in the steady state tryptophan anisotropy that went down to 0.118 from 0.128 for native spectrin in presence of very low concentration of GdmCl and urea (20–50 mM). The anisotropy decreased further to about 0.06 when spectrin was denatured in presence of either of 8 M urea or 5 M GdmCl. From an estimate of the fundamental anisotropy (r_0) for the spectrin tryptophans ranging in between 0.15 and 0.20, from Perrin equation [24], the rotational correlation times were found to be shorter by 15–30%, from that of the native spectrin of ~20 to ~15 ns in presence of 50 mM GdmCl or urea. The rotational correlation times, on the other hand, were shorter by the factor ranging from 3 to 6 from that of the native spectrin of ~20 to ~4 ns in presence of 8 M urea in its denatured state, indicating no significant change in the spectrin conformation to take place other than the change in its quaternary structure arising out of unzipping of the spectrin subunits in presence of submolar concentrations of the denaturants. The anisotropy of spectrin-bound Prodan however, increased markedly from 0.198 to 0.228 in presence of low GdmCl concentrations (Table II) and the Prodan binding affinity decreased in different submolar concentrations of the denaturants (Table III). The anisotropy of spectrin-bound Prodan, measured at 430 nm, remained unchanged at 0.198, independent of all spectrin: Prodan molar ratio of 1:1, 1:2, and 2:1 used in this study. All these data also indicate conformational changes in the vicinity of the Prodan-binding site near one of the terminal domains of spectrin. However, the major effects of low concentrations of the two denaturants, on the Trp anisotropy and the quenching parameters, are on the quaternary structure originating from a possible unzipping of the spectrin subunits.

In summary, the fluorescence quenching of spectrin tryptophans and Prodan binding studies indicate conformational changes of spectrin in the quaternary structure and in the terminal domain of the dimeric spectrin in presence of low concentrations of the commonly used denaturants GdmCl and urea, indicative of acting differentially on spectrin. The tryptophan fluorescence measurements indicated exposal of the tryptophan residues, buried at the interface of the two subunits of the native spectrin, in presence of the denaturants. Distinct changes in the fluorescence anisotropy of terminally located spectrin-bound Prodan, in presence of GdmCl, indicated immobilized microenvironment at the end of the spectrin dimer where the α- and β-subunits remain strongly associated without causing complete dissociation of the individual subunits. The origin of this quaternary structural change in presence of low millimolar concentrations of the denaturants is due to unzipping of the α- and β-subunits in the supercoiled rod domain of the spectrin dimer also altering the overall flexibility of the membrane skeletal protein and could very well take place in the cytoskeleton under minor changes of physiological conditions.

ACKNOWLEDGMENTS

We thank Dr. Soumen Basak for lending supports to perform the steady-state and time-resolved fluorescence measurements. Sibnath Ray acknowledges a Senior Research Fellowship from Council for Scientific and Industrial Research (CSIR), India.

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