Chaperone Activity and Prodan Binding at the Self-associating Domain of Erythroid Spectrin*

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Spectrin, the major constituent protein of the erythrocyte membrane skeleton, exhibits chaperone activity by preventing the irreversible aggregation of insulin at 25 °C and that of alcohol dehydrogenase at 50 °C. The dimeric spectrin and the two subunits, α-spectrin and β-spectrin prevent such aggregation appreciably better, 70% in presence of dimeric spectrin and at an insulin:spectrin ratio of 1:1, than that in presence of the tetramer of 25%. Our results also show that spectrin binds to denatured enzymes α-glucosidase and alkaline phosphatase during refolding and the reactivation yields are increased in the presence of the spectrin derivatives when compared with those refolded in their absence. The unique hydrophobic binding site on spectrin for the fluorescence probe, 6-propionyl-2-(dimethylamino)naphthalene (Prodan) has been established to localize at the self-associating domain with the binding stoichiometry of one Prodan/both dimeric and tetrameric spectrin. The other fluorescence probe, 1-anilino-naphthalene-8-sulfonic acid, does not show such specificity for spectrin, and the binding stoichiometry is between 3 and 5 1-anilino-naphthalene-8-sulfonic acid/dimeric and tetrameric spectrin, respectively. Regions in α- and β-spectrins have been found to have sequence homology with known chaperone proteins. More than 50% similarities in α-spectrin near the N terminus with human Hsp90 and in β-spectrin near the C terminus with human Hsp90 and Escherichia coli DnaJ have been found, indicating a potential chaperone-like sequence to be present near the self-associating domain that is formed by portions of α-spectrin near the N terminus and the β-spectrin near the C terminus. There are other patches of sequences also in both the spectrin polypeptides, at the other termini as well as in the middle of the rod domain having significant homology with well known chaperone proteins.

Newly synthesized proteins fold to unique three-dimensional structures and become functionally active. It has been well established that the newly synthesized proteins require a complex cellular machinery of molecular chaperones to reach their native conformations. Molecular chaperones identify the non-native proteins, prevent them from misfolding and aggregation, bind with them, and assist in correct folding inside cells (1–5). Cells increase the expression of several classes of proteins in response to environmental stresses such as heat shock (6). The major conserved families of these heat shock proteins (Hsps) have been shown to be involved in assisting protein folding (7, 8). Molecular chaperone proteins have some common structural features. They have a domain containing a bundle of hydrophobic residues suitably located at the surface of the protein enabling it to bind lipophilic substances such as hydrophobic fluorescent probes, ANS or bis-ANS, evidenced in α-crystallin (9, 10). Moreover, many chaperones have distinct hydrophilic and hydrophobic domains and exist as micellar oligomeric aggregates like those in GroEL, TrIC, and α-casein (11–15). There are also some other properties that are typically found in molecular chaperones: (a) inhibition of protein aggregation, (b) protection of enzymes against activity loss caused by denaturation, and (c) promotion of the renaturation of denatured proteins. Numerous reports in recent literature have shown that several new chaperones have one or more of these activities in them. Among the chaperone proteins not belonging to the Hsp family, α-crystallin was first shown to have chaperone-like function in suppressing aggregation of various proteins by heat or by using other agents that induce aggregation (16). Similarly for tubulin, a ubiquitous cytoskeletal protein and the building block unit of the microtubule assembly, it was observed that the protein could also suppress both thermal and nonthermal aggregation of a number of unrelated proteins, and the negatively charged C-terminal tail plays a crucial role for its chaperone like activity (17, 18).

Spectrin is the major constituent protein of the erythrocyte cytoskeleton that forms a filamentous network on the cytoplasmic face of the membrane. It is an elongated αβ-heterodimer with large molecular mass of ~520,000 Daltons (19–23). Spectrin isoforms have been identified in a wide variety of nonerythroid cells. Nonerythroid spectrin also localizes predominantly along cellular plasma membranes (21). Spectrin binds to a wide range of ligands like hemein and protoporphyrin (24), antitumor antibiotics of aureolic acid group, chromomycin, andmithramycin (25), the local anesthetic dibucaine (26), fluorescence probes like Prodan and pyrene (27, 28), metal ions like calcium and molybdenum (29, 30), other proteins like actin (31), globin chains and hemoglobin (32–34), calmodulin (35), and other skeletal proteins like ankyrin, adducin and Band 4.1 (21, 36, 37). Association of spectrin with other proteins are essential to establish the planar network along with additional interactions of fatty acids (38) and phospholipids (39–41). The major function of spectrin is presumed to be to establish the cytoskeletal network that provides mechanical strength to cell membrane. However, its capability to interact with such wide

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1 The abbreviations used are: Hsp, human heat shock protein; ADH, alcohol dehydrogenase; ANS, 1-anilino-naphthalene-8-sulfonic acid; BSA, bovine serum albumin; DTT, dithiothreitol; Prodan, 6-propionyl-2-(dimethylamino)naphthalene.
ranges of different proteins and small hydrophobic ligands along with its wide abundance also in nonerythroid tissues suggests that spectrin participates in other cellular functions as well.

Spectrin shares some common interactive features with the proteins exhibiting chaperone activity. It has the hydrophobic binding sites for lipophilic molecules through which it can interact with the denatured proteins. Spectrin interacts with other proteins to establish the cytoskeleton and self-associate to form tetramers and higher order aggregates. Spectrin is presumably heat-stable to ensure its isolation at 37 °C. In an earlier study, we have shown that binding of denatured horse-radish peroxidase with spectrin takes place during refolding of the denatured heme enzyme leading to inhibition of enzyme activity (42). Since then we have tried to investigate in detail the chaperone properties of spectrin in its dimeric, tetrameric forms along with both the α- and β-subunits using conventional chaperone assay systems. We have compared the chaperone properties in terms of preventing the extent of nonthermal aggregation of insulin and thermal aggregation of alcohol dehydrogenase (ADH) by the spectrin derivatives and have compared the activities with that of Hsp90 with which both α- and β-spectrin showed appreciable sequence homology. We have also studied the effects of spectrin derivatives on the enzyme activity of α-glucosidase and alkaline phosphatase. Spectrin could prevent both thermal and nonthermal aggregation of ADH and insulin and increased the reactivation yield of both the enzymes after refolding from a completely denatured state. We have, on the other hand, studied the binding of two well known fluorescence probes, Prodan, and ANS, and have characterized a unique binding site of Prodan at the self-associating domain of spectrin with the binding stoichiometry of one Prodan bound to both the dimeric and tetrameric spectrin. However, ANS did not show such specificity for spectrin as observed with that of Prodan, and the stoichiometry of binding was between 3 and 5 ANS/dimeric and tetrameric spectrin, respectively. In our opinion, the unique Prodan-binding site at the self-associating domain of erythroid spectrin could be correlated with the origin of the chaperone activity in spectrin. The N-terminal residues of α-spectrin and C-terminal residues of β-spectrin have also been found to have more than 50% sequence homology with the well known chaperone protein, Hsp90.

**EXPERIMENTAL PROCEDURES**

Insulin from bovine pancreas, recombinant Hsp90 expressed in *Escherichia coli* (44). Spectrin dimers were purified by following published protocol (43). All other chemicals were of analytical grade and were obtained locally. Deionized water was doubly distilled.

**Isolation and Purification of Spectrin Dimer, Tetramer, and Its Subunits—**Clean, white ghosts from ovine blood were prepared by hypotonic lysis in 5 mM phosphate, 1 mM EDTA containing 20 μg/ml of phenylmethylsulfonyl fluoride at pH 8.0 (lysis buffer) following the procedure of Dodge et al. (44). Spectrin dimers were purified by following published protocol that is elaborated in our earlier work (45). After washing the membranes thoroughly in lysis buffer, the band 6 depleted ghosts were resuspended in 20 volumes of spectrin removal buffer (0.2 M sodium phosphate, 0.1 mM EDTA, 0.2% sodium deoxycholate, 0.1 mM phenylmethylsulfonyl fluoride, pH 8.0) and incubated at 37 °C for 30 min for purification of the dimeric spectrin. The ghost membranes, resuspended in 20 volumes of spectrin removal buffer, were dialyzed overnight against the same buffer at 4 °C for purification of the spectrin tetramer (46). The crude spectrin was collected in the supernatant after centrifugation. Both dimeric and tetrameric spectrin were then purified after concentration by 30% ammonium sulfate precipitation followed by chromatography on Sepharose CL-4B. Spectrin was stored in the buffer containing 5 mM phosphate, 20 mM KCl, 1 mM EDTA, pH 7.5 containing 0.2 mM DTT. To isolate the subunits, spectrin was first dialyzed overnight against the buffer containing 20 mM Tris-HCl, 0.1 mM EDTA, pH 8.0 and then adjusted to 3 mM urea and 0.15 mM NaCl by the addition of urea and NaCl from a concentrated stock in the same buffer. This solution was incubated for 1 h and then applied on a DEAE-cellulose column, pre-equilibrated with the same buffer. The β-spectrin came out in the flow-through, and the α-spectrin was eluted with the same buffer containing 0.5 M NaCl as described earlier (47). The subunits obtained were renatured by dialyzing first against 5 mM Tris-HCl, 0.1 mM EDTA, 4 mM β-mercaptoethanol at pH 7.4, followed by dialysis against the same buffer without the β-mercaptoethanol (48). Before all of the fluorescence experiments, the protein was dialyzed extensively against the buffer containing 10 mM Tris-HCl, 20 mM NaCl, pH 7.8, to remove DTT. The purity of the preparation was checked by 7.5% SDS-polyacrylamide gel electrophoresis under reducing and nonreducing condition. A characterization of spectrin dimer (α chain of ~280 kDa and β chain of 248 kDa) after Coomassie Blue staining. Dimeric spectrin concentration was determined spectrophotometrically using an absorbance of 10.7 at 280 nm for 1% spectrin solution, and the dimeric, tetrameric, α-spectrin, and β-spectrin concentrations were determined by the method of Lowry et al. (49).

**Binding of Prodan and ANS to Spectrin by Fluorescence Measurements—**Steady state fluorescence experiments were performed using a Hitachi F-4010 spectrofluorometer using 10-mm-path length quartz cuvettes. Prodan binding experiments were done using 0.2–0.5 μM Prodan, to which increasing amounts of spectrin were added, and fluorescence emission intensity was measured by using excitation at 360 nm with 5-nm band pass slits for both excitation and emission channels. The emission intensity was measured at 430 nm (λ_{max} and 530 nm (characteristic band of spectrin dimer (α chain of ~280 kDa and β chain of 248 kDa) after Coomassie Blue staining. Dimeric spectrin concentration was determined spectrophotometrically using an absorbance of 10.7 at 280 nm for 1% spectrin solution, and the dimeric, tetrameric, α-spectrin, and β-spectrin concentrations were determined by the method of Lowry et al. (49).

In Equations 1 and 2, ΔF is the change in fluorescence emission intensity at 470 nm (λ_{ex} = 372 nm) or the ratio I_{470/520} (λ_{ex} = 360 nm) for each point on the titration curve, ΔF_{max} denotes the same when spectrin is completely bound to Prodan, and C_{p} is the initial concentration of Prodan.

The double reciprocal plot was used for determination of ΔF_{max} and also the apparent binding constant (K_{app}) using Equation 3.

$$K_{d} = \frac{[C]_{p} - (\Delta F/ΔF_{\text{max}})C]}{C} = \frac{[C]_{p} - (\Delta F/ΔF_{\text{max}})}{C}[/(ΔF/ΔF_{\text{max}})C_{p} = C_{0}$$

Equation 1

$$C_{p}([ΔF/ΔF_{\text{max}}])^{2} = (C_{0} + C_{p})(ΔF/ΔF_{\text{max}}) + C_{0}$$

Equation 2

Equations 1 and 2, ΔF is the change in fluorescence emission intensity at 470 nm (λ_{ex} = 372 nm) or the ratio I_{470/520} (λ_{ex} = 360 nm) for each point on the titration curve, ΔF_{max} denotes the same when spectrin is completely bound to Prodan, and C_{p} is the initial concentration of Prodan.

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1/ΔF = 1/ΔF_{max} + 1/[K_{app}ΔF_{max}(C_{p} - C_{0})]

Equation 3

The linear double reciprocal plot of 1/ΔF against the input concentrations of spectrin/ANS.

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This is also valid because the quantum yield of ANS is appreciably low in aqueous buffer and only increases with increasing concentrations of AMS after binding to a reasonably higher concentration of spectrin (4 μM). In case of AMS, the reverse titration is done, and accordingly the K_{app} becomes the concentration of AMS, and C_{p} becomes the concentration of spectrin.
In this equation, \( r = C_r/C_p \), when \( C_r \) is the concentration of the spectrin-bound Prodan, and \( C_p \) is the input concentration of spectrin. The concentration of the bound Prodan \( (C_r) \) was determined by normalizing the input concentration of Prodan with \( \Delta P/\Delta F_{\text{max}} \). The Scatchard plot was obtained by plotting \( r/C_r \) against \( r \) where \( C_r \) is \( (C_r - C_p) \), and the best fit straight line through the experimental data points led to the determination of binding constant, \( K_b \), and the stoichiometry, \( n \). Similarly, the ANS binding data were also analyzed to obtain the binding constant and the stoichiometry.

The method of continuous variation was also applied to obtain reliable estimates of the binding stoichiometry of spectrin with Prodan and ANS (53). At constant temperature, 25 °C, the fluorescence intensities of Prodan-bound and ANS-bound spectrin were recorded for solutions where the concentration of both spectrin and Prodan/ANS were varied, whereas the sum of their concentration was kept constant at 1.5 \( \mu M \) for Prodan and 4 \( \mu M \) for ANS. This concentration was chosen from the binding isotherm, indicating appreciable binding (-50%) between spectrin and the ligands. The difference in the fluorescence of the ligands in absence line presence of the spectrin \( \Delta F \) was plotted as a function of the input mole fraction of spectrin. The break point in the resulting plot corresponds to the mole fraction of the ligand in the spectrin-ligand complexes. Therefore, stoichiometry is obtained in terms of spectrin-ligand \( (x_p - 1 - x_l) \), where \( x_l \) denotes the ratio of molar concentration of the protein to the total molar concentrations of spectrin and the ligands.

Both Prodan and ANS binding experiments were done with dimeric and tetrameric spectrin. The binding experiments with the spectrin subunits could not be done because of the problem of solubility of the \( \alpha \) and \( \beta \)-subunits at higher concentrations. To obtain binding estimates of Prodan and ANS for the individual subunits, fluorescence measurements were done with spectrin in 3 \( \mu M \) urea, where the individual subunits stay dissociated from the intact dimeric spectrin (47). Measurements of the steady state fluorescence polarization of both tryptophan (\( \lambda_{\text{ex}} = 295 \) nm) and the spectrin-bound ANS and Prodan also indicated binding.

**Results**

To characterize the chaperone activity, we have used insulin and alcohol dehydrogenase as the model substrates to check whether spectrin could prevent the aggregation of the two proteins. The B-chain of insulin aggregates upon reduction of the disulfide bond over a wide temperature range. Insulin aggregation was studied with time in the presence and absence of different concentrations of the spectrin dimer at 25 °C. Spectrin in all of the four dimeric, tetrameric, and \( \alpha \) - and \( \beta \)-forms prevents the aggregation of insulin at 25 °C to different extents in a concentration-dependent manner (shown in Fig. 1 for dimeric spectrin). At insulin to spectrin weight ratio of 1:0.2 aggregation is significantly prevented to ~30% and at 1:0.5 and 1:1, the aggregation of insulin was prevented up to ~45 and ~70%, respectively, after 30 min. The well known chaperone protein Hsp90, used as the positive control, at 1:1 weight ratio of insulin to Hsp90 could bring about 50% prevention, whereas BSA at 1:2 weight ratio could prevent only 5% of the extent of insulin aggregation. The dimeric spectrin and its subunits prevented aggregation of insulin with comparable efficiency. Fig. 2 illustrates the different extents of prevention of insulin aggregation by spectrin dimers, tetramers, \( \alpha \)-spectrin, and \( \beta \)-spectrin at insulin to spectrin weight ratio of 1:0.5 for all four spectrin derivatives. The efficiency of tetrameric spectrin to prevent such aggregation has been significantly lesser. At an insulin to tetrameric spectrin weight ratio of 1:1, the extent of prevention is only 20% compared with the dimer preventing insulin aggregation to 70%. ADH was used as a model substrate to study the influence of different forms of spectrin on its thermal aggregation at 50 °C.

ADH was kept at 50 °C in the absence and presence of spectrin dimer, tetramer, and its subunits, and the aggregation at different time was measured by monitoring the light scattering intensity. The inset of Fig. 2 shows the SDS-PAGE of the purified \( \alpha \)-spectrin, dimeric spectrin, and \( \beta \)-spectrin. Fig. 3 illustrates the results of the aggregation of ADH, which shows that the spectrin dimer prevents the aggregation significantly: ~40% as compared with spectrin tetramer (~8%) after 30 min at the ADH to a spectrin dimer and tetramer weight ratio of 1:0.5. It has also been shown that the \( \alpha \) - and \( \beta \)-spectrin prevent
enhanced the reactivation yield of the enzymes from those refolded in the absence of spectrin. The activity of the denatured α-glucosidase was 23% after refolding in absence of spectrin for 30 min at 25 °C compared with the same amount of native enzyme taken as 100%. When the same experiment was performed in the presence of 100 mM concentrations of the dimeric and tetrameric spectrin, the reactivation yield was increased by 50–70% over those in the absence of spectrin. The increase in the reaction yield of denatured α-glucosidase, in 8 M urea, after 30 min refolding in the presence of dimeric and tetrameric spectrin also showed concentration dependence with respect to spectrin (data not shown). In the presence of the subunits, α-spectrin and β-spectrin, the reactivation yield, however, did not show any increase. Similar observations were also made with the enzyme alkaline phosphatase denatured in 6 M guanidine hydrochloride and then refolded in the presence and absence of spectrin for 30 min at 25 °C. The reactivation yield of the denatured enzyme, after refolding in presence of spectrin dimer, tetramer, α-spectrin, and β-spectrin increased to 65, 75, 28, and 42%, respectively, each at 100 nM concentrations, from that of 21% in the absence of spectrin. Histogram representations of the reactivation yields in the presence of the spectrin subunits, dimer, and tetramer are shown in Fig. 4 for the enzymes α-glucosidase (Fig. 4A) and alkaline phosphatase (Fig. 4B).

The emission of Prodan was maximum at 520 nm in the aqueous buffer and is blue shifted to 430 nm in presence of dimeric, tetrameric, and α- and β-spectrin (27). Similar blue shift in the emission maximum was also observed in ANS to 470 nm when bound to spectrin from that at 520 nm in aqueous buffer. In both the cases the blue shifts were associated with large enhancements in the emission intensity with spectrin both in its tetrameric and dimeric form. The spectrin subunits also showed such enhancement, however to a smaller extent, in the fluorescence intensity of ANS and Prodan. The binding of ANS and Prodan were also reflected in large increase in the fluorescence polarization of ANS and Prodan in presence of dimeric, tetrameric, and α- and β-spectrin (27). The representative binding isotherms of Prodan and ANS are shown in Fig. 5 as the plots of ΔF/ΔF_{max} against the different concentrations of tetrameric and dimeric spectrin along with the same for dimeric spectrin incubated in 3 M urea where the spectrin subunits dissociate (Fig. 5A). Fig. 5B shows the same plots of ΔF/ΔF_{max} against the increasing concentrations of ANS in presence of 4 μM dimeric spectrin, tetrameric spectrin, and dimeric spectrin incubated in 3 M urea. The double reciprocal plots of 1/ΔF against 1/[C_{PL}] for Prodan and 1/[ANS] were extrapolated to ordinate to evaluate ΔF_{max} (not shown). We have also done binding studies of ANS and Prodan with dimeric spectrin in 3 M urea and compare the binding affinities of the spectrin subunits with those of the intact dimeric and tetrameric spectrin. The dissociation constants of

| Proteins          | Insulin aggregation | ADH aggregation |
|-------------------|---------------------|-----------------|
| Dimeric spectrin  | 45                  | 40              |
| Tetrameric spectrin| 26                  | 8.5             |
| β-Spectrin        | 54                  | 69              |
| α-Spectrin        | 59                  | 88              |
| Hsp90 (1:1)       | 50                  |                 |
| BSA (1:2)         |                     | 5               |

The effects of the spectrin, in different forms, were also studied with the enzymes α-glucosidase and alkaline phosphatase during refolding from completely unfolded state. Spectrin, both in its dimeric and tetrameric form and the two subunits, summarized the data on the percent extent of prevention of aggregation of insulin and ADH, at a weight ratio of 1:0.5, by different spectrin derivatives, BSA at 1:2 and Hsp90 at a 1:1 weight ratio.

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Prodan binding to spectrin dimer and tetramer are 0.43 ± 0.1 and 0.74 ± 0.2 μM, respectively and 3.0 ± 0.3 μM, about an order of magnitude larger, in spectrin dimer incubated with 3 M urea. However, the same for ANS binding to spectrin dimer and tetramer are 22.7 ± 2.3 and 38.6 ± 1.3 μM, respectively, when the same for the spectrin dimer incubated with 3 M urea is 33 ± 1.2 μM, comparable with that of the tetramer. The insets in Fig. 5 also show the representative Scatchard plots for Prodan and ANS binding to the dimeric spectrin. The binding dissociation constants of dimeric and tetrameric spectrin with Prodan and ANS, evaluated in the three different methods, are summarized in Table III. The method of continuous variation was also applied to obtain reliable estimates of the binding stoichiometry of spectrin with Prodan and ANS, evaluated in the three different methods, are summarized in Table III. The method of continuous variation was also applied to obtain reliable estimates of the binding stoichiometry of spectrin with Prodan and ANS, evaluated in the three different methods, are summarized in Table III. The method of continuous variation was also applied to obtain reliable estimates of the binding stoichiometry of spectrin with Prodan and ANS. At a constant temperature, 25 °C, the fluorescence intensities of spectrin-bound and ANS-bound spectrin were recorded for solutions where the concentration of both spectrin and Prodan/ANS were varied, whereas the sum of their concentration was kept constant at 1.5 μM for Prodan and 4 μM for ANS. Fig. 6 shows the Job’s plots of spectrin binding to Prodan and ANS both in their dimeric and tetrameric forms. The binding stoichiometry of Prodan and ANS to both dimeric and tetrameric spectrin has

**Table II**

Fluorescence polarization of spectrin tryptophans, spectrin-bound Prodan, and ANS at 25 °C

| Spectrin    | Polarization | Trp | Prodan | ANS |
|-------------|--------------|-----|--------|-----|
| Dimer       | 0.18 (0.04)  | 0.27 (0.03) | 0.25 (0.02) |
| Tetramer    | 0.176        | 0.359 | 0.29   |
| α-Spectrin  | 0.175        | 0.223 | 0.202  |
| β-Spectrin  | 0.176        | 0.276 | 0.238  |

Prodan binding to spectrin dimer and tetramer are 0.43 ± 0.1 and 0.74 ± 0.2 μM, respectively and 3.0 ± 0.3 μM, about an order of magnitude larger, in spectrin dimer incubated with 3 M urea. However, the same for ANS binding to spectrin dimer and tetramer are 22.7 ± 2.3 and 38.6 ± 1.3 μM, respectively, when the same for the spectrin dimer incubated with 3 M urea is 33 ± 1.2 μM, comparable with that of the tetramer. The insets in Fig. 5 also show the representative Scatchard plots for Prodan and ANS binding to the dimeric spectrin. The binding dissociation constants of dimeric and tetrameric spectrin with Prodan and ANS, evaluated in the three different methods, are summarized in Table III. The method of continuous variation was also applied to obtain reliable estimates of the binding stoichiometry of spectrin with Prodan and ANS. At a constant temperature, 25 °C, the fluorescence intensities of spectrin-bound and ANS-bound spectrin were recorded for solutions where the concentration of both spectrin and Prodan/ANS were varied, whereas the sum of their concentration was kept constant at 1.5 μM for Prodan and 4 μM for ANS. Fig. 6 shows the Job’s plots of spectrin binding to Prodan and ANS both in their dimeric and tetrameric forms. The binding stoichiometry of Prodan and ANS to both dimeric and tetrameric spectrin has
been summarized in Table IV. All three independent methods yielded binding stoichiometry of one Prodan/both dimeric and tetrameric spectrin. However, the stoichiometry for ANS was 3 for the dimeric and 5 for the tetrameric spectrin. The binding stoichiometry for the spectrin subunits, in 3 mM urea, was comparable with those obtained from the dimeric spectrin.

Appreciable sequence homology of α-spectrin and β-spectrin were observed with other established chaperone proteins (e.g. heat shock proteins, human Hsp70 and Hsp90, and E. coli Hsp40 and Hsp60) and other chaperone proteins (e.g. human nucleophosmin, clusterin, α-synuclein, calreticulin, tubulin, and α-crystallin). Pairwise alignment exercise has been done for α-spectrin and for β-spectrin with all those chaperone proteins. More than 50% similarities have been found in α-spectrin near the N terminus with human Hsp90 and in β-spectrin near the C terminus with human Hsp90 and E. coli DnaJ. Fig. 7 shows such alignments of Hsp90 with the N-terminal sequence of α-spectrin (Fig. 7A) and C-terminal sequence of β-spectrin (Fig. 7B). This study indicates the presence of potential chaperone-like sequence near the self-associating domain of erythroid spectrin. In addition there are other patches of sequences in both the spectrin polypeptides at the other termini as well as in the middle with significant homology with other chaperone proteins (data not shown). Doubling the GAP extension penalty showed alignments with ~40% sequence homology at both the N terminus of α-spectrin and C terminus of β-spectrin, indicating the possibility of having putative chaperone domains in spectrin.

**DISCUSSION**

Spectrin is the major constituent protein of the erythrocyte cytoskeleton that forms a filamentous network on the cytoplasmic face of the membrane by providing a scaffold for a variety of proteins (58). The interaction of the spectrin-based protein network with the cytoplasmic membrane controls the elasticity of the bilayer membrane. The inherent flexibility of spectrin is believed to be a major factor that contributes to the elastic deformability displayed by red cells during their passage through the circulatory system. Several blood diseases are associated with erythrocyte deformations and defects in spectrin, e.g. various types of hereditary hemolytic anemia involve mutations in spectrin (59–61). A spectrin-based network has recently been implicated as a membrane protein-sorting machine (62) and in the maintenance of Golgi structure and organization (63). In addition, spectrin has been reported to be involved in the maintenance of dynamic (phase state) asymmetry in erythrocyte membranes (64) and to have a chaperone-like activity with denatured heme proteins as the substrate (42).

Spectrin is a large dimeric amphiphilic protein 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 N and C termini toward the ends of the rods. These heterodimers associate head-to-head to form 200-nm tetramers and higher order oligomers. The primary sequence of spectrin is comprised of a series of contiguous motifs called “spectrin repeats” (typically 106-amino acid repeating sequences) that are characteristic of all members of the spectrin family of proteins (65). In addition to these features, spectrin exhibits additional structural motifs. These include an actin-binding domain, a pleckstrin homology domain, a Src homology 3 domain, and a calmodulin-like domain. These structural features allow spectrin to take part in a number of physiological events through protein-protein interactions. The ability of spectrin to expand and contract has been attributed to its modular structure made of repeats (66).

The hydrophobic binding site in spectrin is crucial because 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 (27). 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 (28). In this paper, we have tried to correlate the chaperone activity of erythroid spectrin with its unique Prodan binding activity localized at the self-associating domain. Regions in α- and β-spectrins have been found to have sequence homology with known chaperone proteins. The sequences have >50% similarity and >30% identity that are reduced to 40% and ~25%, respectively, by doubling the GAP extension penalty. This indicates that there can be putative chaperone-like domains in spectrin, and they can be located near the N terminus of α-spectrin and the C terminus of β-spectrin, respectively. However, considering the known three-dimensional structures of spectrins (three-helix bundle and rodlike shape) and that of the chaperones (mostly globular), it is unlikely that spectrin will have structural homology with the chaperones. This has been also observed with the tubulin, which has a tubular structure and is known to have chaperone like activity (17, 18).

Small Hsps have been found in all of the organisms studied so far, and they share conserved regions in the C-terminal part of the protein, whereas the N-terminal part is quite divergent in sequence and length, ranging from 12 to 40 kDa in different organisms (67, 68). The conserved C-terminal domain exhibits high sequence homology to the family of α-crystallins, which constitute a major part of the eye lens and both small Hsps and α-crystallins form large oligomeric complexes (69). The subunit stoichiometry varies substantially and complex sizes in the range of 150–800 kDa have been found for different members of the small Hsp family (69). The chaperone activity and binding of the fluorescent probe bis-ANS to a hydrophobic domain have been exhibited by Atp11p, a soluble protein of mitochondria (70). Recently it was observed that a subfragment produced by the removal of 67 residues from the N terminus of recombinant Atp11p retains its full chaperone activity (71). An important acidic neuronal protein of 140 amino acids, α-synuclein exhibits chaperone function with the N-terminal region of the protein binding the substrate protein to form high molecular weight complex and the C-terminal acidic tail solubilizing the large oligomeric complex during the chaperone function (72–74). α-Casein abundant in mammalian milk, having no well defined secondary and tertiary structures, exists in
nature as a micellar aggregate and can prevent aggregation of a variety of unrelated proteins and enzymes against stress conditions (15).

The mechanism of this chaperone action is presumably through preferential binding of the substrate proteins in their non-native or denatured states at the hydrophobic binding site. The binding step is the key step, common to all other known molecular chaperones. To investigate the hydrophobic binding site of spectrin to which the denatured substrate binds, we have chosen the spectrin-specific fluorescence probe Prodan (27, 75). Our results show that Prodan binds with stronger affinity with the spectrin dimer as compared with the tetramer (Table III). A similar trend is followed by another well-known fluorescence probe, ANS, however with about 100-fold weaker affinity from that of Prodan. The binding stoichiometry of Prodan to both dimeric and tetrameric spectrin has always been 1 (Table IV), revealing the Prodan-binding site to localize at the self-association site of the spectrin dimer. On the other hand, the ANS binding stoichiometry varied between 3 and 5 depending on the dimeric or tetrameric state of spectrin. The Job’s plot, shown in the Fig. 6, clearly indicates the difference in binding stoichiometry between Prodan and ANS (Table IV).

Insulin and alcohol dehydrogenase have been used as substrates to study the chaperone-like activity of many proteins including α-crystallin, tubulin, and Hsps. Aggregations of insulin have been shown to suppress in the presence of tubulin, α-crystallin, and Hsp90 (17, 76–78). Our result shows that both human and ovine erythroid spectrin in its dimeric form could prevent aggregation of insulin at 25 °C and of ADH at 50 °C more efficiently than tetrameric spectrin (Table I). The potentials of the two spectrin subunits, in this respect, are more or less comparable with that of the dimeric spectrin (Fig. 2). The suppression of the insulin aggregation was concentration-dependent with respect to all of the spectrin derivatives used, in this work. Although BSA, used as a nonspecific control, could not even prevent 5% of insulin aggregation at an insulin to BSA ratio of 1:2, Hsp90 on the other hand, used as the positive control, could prevent 50% of aggregation at an insulin to Hsp90 weight ratio of 1:1. However, the spectrin derivatives, e.g. the dimeric spectrin, α-spectrin, and β-spectrin, showed better efficiency in preventing the aggregation of insulin B chain to more than 70% at a 1:1 weight ratio of insulin to spectrin (Table I). All of the spectrin derivatives could prevent the thermal aggregation of ADH following the same trend as observed in the case of insulin aggregation (Fig. 3). The potentials of the dimeric spectrin and the two subunits to prevent aggregation of both insulin and ADH better than that of the tetrameric spectrin, actually present in the intact membrane skeleton indicate against the chaperone-like function of spec-
trin inside the erythrocytes. However, under different pathophysiological conditions, when the spectrin-based skeletal integrity is altered, the chaperone activity of dimeric spectrin could be useful in interacting with non-native and denatured proteins (34).

Our results also show that spectrin binds to unstable intermediates of the enzymes \(a\)-glucosidase and alkaline phosphatase in their unfolding or refolding pathways. The lost enzymatic activity is thus regained to the considerable extent in presence of the dimeric, tetrameric, \(a\)-spectrin, and \(\beta\)-spectrin when compared with those refolded in their absence. This behavior is very similar to some of the well studied molecular chaperones. We have also studied the effects of dimeric spectrin on the reactivation of other enzymes, e.g. horseradish peroxidase (42), malate, lactate dehydrogenase, and carbonic anhydrase, and in all cases spectrin could increase the reactivation yield of those enzymes (not shown). In the reactivation of denatured \(a\)-glucosidase and alkaline phosphatase, however,
both the dimeric and tetrameric spectrin showed comparable chaperone efficiency over those of α-spectrin and β-spectrin (Fig. 4). More than 50% sequence similarities of the N-terminal regions of α-spectrin and the C-terminal regions of β-spectrin found with human Hsp90 further indicated the chaperone potential of erythroid spectrin originating from its self-associating domain (Fig. 7). In addition, there are patches of sequences in both of the spectrin polypeptides at the other termini as well as in the rod domain, also showing significant homology with other chaperone proteins, e.g. E. coli GroEL, Hsp70, and Hsp90, indicating that spectrin performs its chaperone function using structural domains other than the more potent self-associating domain.

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