Isolation of Spectrin Subunits and Reassociation in Vitro

ANALYSIS BY FLUORESCENCE POLARIZATION*

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Fluorescence polarization has been used to probe the exposure of tryptophan residues of erythrocyte spectrin. A significant decrease in anisotropy occurred when spectrin was heated at temperatures ranging from 38 to 48 °C. At low concentrations of urea, these anisotropy changes shifted to lower temperatures and were minimal in concentrations of urea 3 M or greater. These findings were attributed to the stepwise unfolding of the subdomain structure of spectrin under these conditions and eventual dissociation of oligomeric spectrin to the monomer state. DEAE-cellulose column chromatography in the presence of 3 M urea confirmed this prediction and permitted isolation of pure α and β subunits of spectrin in good yields. The isolated subunits were soluble in neutral salt solutions and were readily reconstituted into high molecular weight forms that displayed "native" tryptophan fluorescence anisotropy changes and migrated as discrete oligomeric species when analyzed by non-denaturing acrylamide gel electrophoresis. The reconstituted complexes were indistinguishable from native spectrin molecules when examined by low angle shadowing and electron microscopy.

Erythrocyte spectrin is composed of two high molecular weight subunits that are joined together under native conditions through strong noncovalent associations. Studies of spectrin using circular dichroism and differential scanning calorimetry indicate that both subunits have a high α-helical content and undergo stepwise unfolding in low concentrations of denaturants or at elevated temperatures (1, 2). Attempts to isolate and analyze the structure of each individual subunit have met with limited success. Purified spectrin subunits have been isolated in the presence of SDS (3) or high concentrations of urea (4), but neither method provides a simple and reproducible way to prepare large quantities of spectrin subunits that also retain the functional properties of the native protein.

The experiments described here show that erythrocyte spectrin undergoes a reversible unfolding of the polypeptide chains of both subunits in neutral salt solutions containing 3 M urea, such that both subunits can be prepared by ion exchange chromatography. Conditions are defined for the isolation of each subunit in pure form and their subsequent reconstitution into native dimers.

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Preparation of Spectrin—Spectrin was prepared by the methods previously reported (5). Crude spectrin was precipitated by 50% saturated ammonium sulfate and dialyzed against 20 mM Tris-HCl buffer (pH 8.0), 130 mM KCl, 10 mM NaCl, 4 mM β-mercaptoethanol, and 30 mM phenylmethanesulfonyl fluoride, and applied to a Sepha-seare CL-4B column which was equilibrated with the same buffer. Tetramer and dimer fractions (6) were pooled and concentrated by a second precipitation with 50% saturated ammonium sulfate before being dialyzed in 20 mM Tris-HCl (pH 8.0) and 1 mM EDTA and stored at 4 °C.

Separation of Spectrin Subunits—Purified spectrin in 20 mM Tris-HCl (pH 8.0) and 1 mM EDTA was adjusted to 3 M urea, 0.15 M NaCl by addition of an ice-cold 6 M urea, 0.3 M NaCl solution of the same Tris-HCl-EDTA buffer and incubated for about 1 h on ice and then applied to a DEAE-cellulose (Whatman, DE32) column (1.2 × 25 mm) which was also preequilibrated with 3 M urea, 0.15 M NaCl, Tris-HCl-EDTA buffer in the cold. The column was eluted with the same solution until the first protein peak emerged, after which the concentration was changed from 0.15 to 0.4 M. A sodium chloride gradient elution (0.15–0.5 M) was also used. The flow rate was 12–14 ml/h. When the column was eluted by a NaCl gradient elution, the α subunit of spectrin was eluted by 0.18–0.20 M NaCl. A small peak tailing the first peak always appeared (see Fig. 4), but SDS-PAGE showed that the protein of this fraction was similar to that of the first main peak. Total recoveries of protein determined by the method of Lowry et al. (7) were 60 to 70%.

Reconstitution of αβ Complex—Equal quantities of α and β subunits dissolved in 3 M urea solutions were mixed and dialyzed against 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1.8 mM β-mercaptoethanol. Mixing of α and β subunits after removing urea also gave good reconstitution, provided that β-mercaptoethanol was present during the dialysis to remove the urea.

Fluorescence Measurements—Fluorescence polarization measurements were made with an SLM 4800 spectrophotometer equipped with a Hewlett-Packard 9825A minicomputer. Fluorescence from samples was automatically analyzed through two photomultipliers for parallel and perpendicular emissions. Anisotropy r is defined as

\[ r = \frac{I_p - I_l}{I_p + 2I_l} \]

where \( I_p \) and \( I_l \) are the fluorescent intensities observed parallel and perpendicular, respectively. The exiting wavelength was 295 nm for spectrin, and all fluorescence was collected through a cutoff filter above 340 nm (Scott KV 370). Fluorescence anisotropy measurements were recorded as the mean value of 6–10 readings/point. The temperature was controlled by circulating water through the cell holder.

RESULTS

Analysis of Spectrin by Fluorescence Polarization—Fluorescence polarization was chosen as a simple, rapid way to follow changes in the conformation of the spectrin molecule as a function of denaturant and temperature. Both subunits showed a decrease in fluorescence anisotropy as the temperature was increased, until at about 40 °C the anisotropy changes shifted to lower temperatures and were minimal in concentrations of urea 3 M or greater. These findings were attributed to stepwise unfolding of the subdomain structure of spectrin under these conditions and eventual dissociation of oligomeric spectrin to the monomer state. DEAE-cellulose column chromatography in the presence of 3 M urea confirmed this prediction and permitted isolation of pure α and β subunits of spectrin in good yields. The isolated subunits were soluble in neutral salt solutions and were readily reconstituted into high molecular weight forms that displayed "native" tryptophan fluorescence anisotropy changes and migrated as discrete oligomeric species when analyzed by non-denaturing acrylamide gel electrophoresis. The reconstituted complexes were indistinguishable from native spectrin molecules when examined by low angle shadowing and electron microscopy.

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temperatures below 49°C. Changes in fluorescence polarization of each of the subunits. Changes in fluorescence polarization of spectrin as a function of temperature provided the function of temperature and urea concentration in order to search for conditions that would allow separation and isolation of each of the subunits. Changes in fluorescence polarization proved to be useful in that they reflected motility of tryptophan-containing segments of the spectrin molecule at temperatures below 49°C, the temperature at which signs of irreversible change in spectrin's secondary structure occur as observed by CD measurements (1).

Measurements of the anisotropy (r) of intrinsic tryptophans of native spectrin as a function of temperature and urea concentration provided the values shown in Fig. 1. A relatively steep decrease in r occurred on heating spectrin in low ionic strength media in the temperature range of 38 to 48°C. This decrease was less if spectrin was heated in the presence of Ca2+ or Mg2+. Incubation of spectrin above 49°C produced a significant increase in r both in the presence and absence of divalent metals, although the increase in r in the presence of metals occurred at a lower temperature.

Urea had a striking effect on the changes in r of spectrin over the same temperature range. In the presence of 6.8 M urea, the fluorescence anisotropy was extremely low and dropped monotonically over a temperature range extending from 20 to 55°C (Fig. 2), a slope of change similar to that found when bovine serum albumin is incubated under the same conditions. At lower concentrations of urea, the steep transition in r described above occurred at lower temperatures, and it did not exhibit the type of transition seen without urea. The anisotropy of spectrin in 2.5 M urea at 20°C was about the same value as that of spectrin heated to 49°C without urea; both values were significantly higher than the anisotropy of spectrin in 6.8 M urea at any temperature, conditions we presume to approach those causing complete denaturation of the spectrin molecule.

Isolation of Spectrin Subunits in 3 M Urea—The decrease in anisotropy of intrinsic tryptophans of spectrin was most pronounced in the 2-4 M urea range (Fig. 3), suggesting that significant changes in secondary and tertiary structure of spectrin occurred at these relatively low concentrations of urea. Among the many explanations to account for this finding, we considered the possibility that the normally stable spectrin dimers dissociated into their individual subunits either during heating at 48°C or in 3 M urea at lower temperatures, and it was this interpretation that led us to the isolation procedure described below.

Purified spectrin preparations preincubated in 3 M urea in 0.15 M sodium chloride were fractionated by DEAE-cellulose chromatography with either a linear or stepwise salt gradient (Fig. 4) producing pure a and b subunits in preparative amounts. Preparations of both subunits were identified and assayed for purity by PAGE either in SDS or under non-denaturing conditions.

Reconstitution of Spectrin Dimers and Oligomers—Subunits...
Spectrin Subunits

Fig. 5. Polyacrylamide gel electrophoresis of spectrin subunits and reconstituted αβ complexes. A, SDS-PAGE (6.7% acrylamide). B, nondenaturing PAGE (2 to 4% gradient gel). The slower migrating zones, which from time to time appear in the pattern of native spectrin, are probably higher order aggregates.

Fig. 6. Fluorescence polarization of isolated spectrin subunits and reconstituted αβ complexes. Samples were dissolved in 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 120 mM NaCl, and 8 mM β-mercaptoethanol at 0.14 mg/ml. Native spectrin (○), purified α (△), purified β (▲), reconstituted α and β complexes (●).

Isolated subunits had approximately the same r value as native spectrin dimer at 20 °C, but this value dropped sharply in the 25-40 °C range in contrast to the more gradual fall of native spectrin at the same temperature (Fig. 6). The isolated α subunit also displayed a second transition in the 42-52 °C range and did not show the sharp increase in r at 49 °C that is characteristic of native spectrin.

Isolated β subunits had a lower anisotropy at 20 °C, and this value fell gradually over the temperature range extending from 20 to 40 °C. The anisotropy of isolated β decreased less than the α subunit, and it also displayed an increase in anisotropy at approximately 46 °C similar to that seen in native spectrin at 49 °C. In striking contrast to the anisotropy values of the individual subunits, when the two subunits were mixed together in similar proportions the fluorescence properties of the reconstituted forms appeared essentially identical with that obtained with native spectrin (Fig. 6). The mobility of mixtures of α and β subunits on nondenaturing acrylamide gels also reflected the changes seen by fluorescence; such preparations produced electrophoretic patterns that were indistinguishable from native spectrin (Fig. 5).

Electron Microscopy of Reconstituted Spectrin Subunits—Isolated α subunits of spectrin displayed polymorphic forms when analyzed by low angle rotary shadowing and electron microscopy (Fig. 7A). These varied from simple rope-like structures approximately 1000 Å in length to complex aggregated ringlets. Subunits of β, examined at the same protein concentration, appeared largely as single strands also approx-

of spectrin prepared in 3 M urea remain soluble in neutral salt solutions after removal of urea, and they regain their α-helical conformation when analyzed by circular dichroism. Both subunits display features that distinguish them from native spectrin dimers. The β subunit migrated as a single band on nondenaturing PAGE (Fig. 5) slightly ahead of the native spectrin dimers. In contrast, the α subunit migrated anomalously, frequently appearing as several indistinct bands (Fig. 5) that we assume to be different aggregation states, since such preparations displayed single sharp bands migrating as α subunits when analyzed by SDS-PAGE.

The individual subunits also displayed striking differences in their intrinsic tryptophan fluorescence when analyzed over the same temperature range described above (Fig. 6). Isolated α subunits had approximately the same r value as native spectrin dimer at 20 °C, but this value dropped sharply in the 25-40 °C range in contrast to the more gradual fall of native spectrin at the same temperature (Fig. 6). The isolated α subunit also displayed a second transition in the 42-52 °C range and did not show the sharp increase in r at 49 °C that is characteristic of native spectrin.

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Spectrin Subunits

Fig. 7. Electron micrographs of rotary shadowed spectrin subunits and reconstituted αβ complexes.
A, α; B, β; C, α and β. Magnification × 75,000.

DISCUSSION

These results show that fluorescence polarization can be used to study changes in tertiary and quaternary structure of the spectrin molecule induced by either heat or urea or a combination of the two. Unfolding of both subunits, as measured by increased mobility of tryptophans, occurs in neutral salt solutions of spectrin heated over a 40–49 °C range. These changes are modified by the presence of divalent cations and sucrose, both reduce the degree of unfolding at a given temperature, and urea which enhances it in a predictable manner. Anisotropy values of spectrin fall to their lowest levels at 49 °C when spectrin is heated in neutral salt solutions. This is the same temperature at which spectrin begins to lose a significant amount of α-helical conformation as measured by circular dichroism (1). This temperature is also the midpoint of the “A-transition” as defined by differential scanning calorimetry (1). Spectrin undergoes an increase in anisotropy of tryptophan fluorescence above 49 °C, at higher temperatures in the presence of sucrose, which is likely to be due to immobilization of tryptophans due to aggregation of loops of polypeptides that have been thermally denatured. Increasing concentrations of urea reduce or entirely abolish this effect. The interpretation of these anisotropy data is complicated by the fact that the values we record represent some average value for tryptophan mobility throughout both subunits of the spectrin molecule.

Urea causes a predictable increase in tryptophan mobility (decreased anisotropy) at all temperatures, but changes caused by urea in the 2–4 M concentration range are particularly interesting. Spectrin incubated in this concentration range shows the steepest increase in tryptophan mobility, an effect that occurred at 20 °C and was reversible. These results suggested that the spectrin subunits might be dissociated into monomers in 3 M urea, and experiments using DEAE-cellulose chromatography indicated that this was the case. These results confirm earlier studies (2) in which it was shown that comparable amounts of urea prevented the intermolecular chemical cross-linking of spectrin subunits and also induced loss of α-helical conformation as measured by circular dichroism (10, 11).

Preparative amounts of both subunits of spectrin can be prepared by the procedure described here, but the conditions for the separation must be controlled carefully. The concentration of urea at 3 M is critical; lower concentrations will not dissociate the subunits completely, and higher concentrations of urea will cause too much unfolding of the chains and complicate their recovery. Previous attempts to isolate spectrin subunits in higher concentrations of urea resulted in low yields and subunit fractions that had aggregated when examined under nondenaturing gel conditions (4). These investigators fractionated spectrin subunits on hydroxyapatite columns, after finding that attempts to prepare spectrin subunits by ion exchange chromatography in the presence of urea proved unsuccessful.

Subunits of spectrin purified by the procedure described here were homogeneous when analyzed by SDS gel electrophoresis, but the mobility of the α subunit showed a peculiar pattern when analyzed under non-denaturing conditions. Purified β subunits migrated as a single, reasonably sharp band on nondenaturing gels but the α subunit frequently displayed a number of indistinct bands. We interpreted this multiple banding pattern to be a consequence of aggregation of α subunits under these conditions, an interpretation borne out
Spectrin Subunits

by studies of such preparations by low angle rotary shadowing and electron microscopy. Preparations of purified α subunits of spectrin exhibited a number of different worm-like forms having the dimensions and general appearance of spectrin (12) but clearly more heterogeneous in shape and size than native spectrin dimers. In contrast, purified β subunits appeared as single, noodle-like forms with the dimensions expected for an individual subunit. Mixtures of equal parts of both subunits produced forms that were indistinguishable from native spectrin dimers. Such reconstituted dimers were able to form tetramers and higher oligomers and their ultrastructure appeared indistinguishable from higher spectrin forms.

These results show that fluorescence polarization is a simple, rapid, and reproducible way to study changes in spectrin conformation and subunit associations. Both isolated spectrin subunits and reconstituted forms can be analyzed in this way. We are now in a position to study the factors that regulate the α-β associations, and, by studying hybrid forms composed of subunits from different donors, such approaches could prove to be valuable in the search for structural and functional differences of spectrin molecules that may play a role in red cell membrane skeletal disorders.

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