Biochimica et Biophysica Acta 905 (1987) 100–108
Elsevier

BBA 73779

Heat-induced dissociation of human erythrocyte spectrin dimer into monomers

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(Received 17 March 1987)
(Revised manuscript received 3 August 1987)

Key words: Spectrin; Subunit interaction; Thermal dissociation; Erythrocyte membrane; Membrane skeleton

Human erythrocyte spectrin heated above 49 °C could be separated into two fractions by DEAE-Toyopearl column chromatography at room temperature. The first fraction eluting with the salt gradient was predominantly the α subunit, indicating a heat-induced dissociation of the spectrin $\alpha\beta$ dimer into monomers. The second fraction, obtained with 0.5 M NaOH after salt elution, consisted of high-moleclar-weight proteins in addition to α and β subunits, which were visualized by gel electrophoresis with sodium dodecyl sulfate. The isolated β subunit when heated above 48 °C could also be separated into two fractions by column chromatography. About 30% of the protein eluted with the salt solution and the rest of the proteins were in the alkali eluate in which high molecular weight protein bands also appeared, indicating a heat-induced aggregation of the β subunits. Almost all the isolated α subunit, however, eluted out with the salt solution, even though the subunit was heated at 52 °C. Studies of the binding of subunits to inside-out vesicles indicate that the isolated β subunit was denatured irreversibly by heating; on the other hand, the α subunit kept its binding ability after heating above 50 °C. These findings are attributed to the heat-induced dissociation of the spectrin molecules into α and β subunits at 49–50 °C, and eventual aggregation of the denatured β subunits.

Introduction

Spectrin is a major structural protein of the human erythrocyte membrane skeleton, and is located along the inner surface of the membrane, maintaining the shape and deformability of the red blood cell [1,2]. It is well established that the spectrin molecule is a dimer composed of two nonidentical subunits referred to as α (240 kDa) and β (220 kDa), and that the subunits are joined together by side-to-side association of parallel monomers [3]. This model was originally conceived on the basis of the observation on polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS) in which the ratio of protein intensity of α to β bands was always approx. 1 for crude and purified spectrin fractions, and also for erythrocyte ghosts [4,5]. Under appropriate conditions, however, spectrin shows various molecular states, $(\alpha\beta)_2$ tetramer and $(\alpha\beta)_n$ oligomers which are associated by a head-to-head interaction of $\alpha\beta$ dimers [6–8].

The effect of heating on parameters of the protein structure of spectrin studied as CD, scanning calorimetry [9], fluorescence polarization [10] and trypsin susceptibility [11] indicated that clear structural transitions occurred at temperatures around 49 °C. These results should be very im-

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluorophosphate.

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portant in understanding the role of spectrin in the membrane skeleton, since the shape and rigidity of red blood cells change drastically at temperatures around 49°C [12,13].

We recently reported that he fluorescence anisotropy of spectrin in 3 M urea, under which condition the spectrin molecule dissociated to subunits, was almost the same as that of spectrin at 49°C without urea [10]. Furthermore, the fluorescence quenching of tryptophan residues in spectrin with acrylamide in 3 M urea was also the same as that at 49°C without urea (unpublished result).

Taken together, these results suggest that the spectrin molecule dissociates into the subunits upon heating at 49°C. In this paper we provide direct evidence of this heat-induced dissociation of the spectrin molecule.

Experimental procedures

Preparation of spectrin and spectrin subunits

Spectrin was prepared from fresh blood drawn from volunteers in Sapporo medical College by previously reported methods [10]. Spectrin subunits were isolated from purified spectrin fraction by the method of DEAE-cellulose column chromatography with 3 M urea as previously reported [10]. The protein concentration was determined by the method of Lowry et al. [14].

Column chromatography of spectrin and spectrin subunits

The column chromatography was performed either at room temperature or at various temperatures in a water bath (thermal column chromatography). The spectrin or spectrin subunits in 120 mM NaCl/1 mM EDTA/0.5 mM PMSF/0.5 mM DFP/1 mM β-mercaptoethanol/20 mM Tris-HCl (pH 8.0) was heated at various temperatures for 5 min in a water bath, and the sample solution was cooled down immediately after heating by standing on ice. The heated protein samples (0.5 ml of 1.2 mg/ml for spectrin and of 0.6 mg/ml for the α and β subunits) were applied to DEAE-TOYOPEARL M650 * (Toyo Soda, Japan) columns (0.24 × 7 cm) equilibrated in 20 mM Tris-HCl (pH 8.0)/1 mM EDTA (buffer A) at room temperature. First, the column was eluted with buffer A for 5 min, then with a linear gradient of NaCl between 0 and 0.6 M in buffer A for 15 min at 1 ml/min, which was programmed by an FPLC system (Pharmacia). Thermal column chromatography of the spectrin was performed as follows. The same size of DEAE-Toyopearl M650 column was set in a water bath and washed by buffer A with 0.18 M NaCl for 10 min at 1 ml/min. To equilibrate at the appropriate temperature, the washing buffer was passed through a long spiral polyethylene tube (1.5 m in extended length and 0.7 ml volume) connected to the column. Then, the spectrin solution (0.3 ml of 1.2 mg/ml) under the same conditions described above was injected into the spiral tube to incubate at an appropriate temperature for 5 min. The column system including the spiral tube was eluted with buffer A plus 0.18 M NaCl for 5 min, then eluted with a linear salt gradient of NaCl between 0.18 and 0.8 M in buffer A at 1 ml/min. It was confirmed that the temperature of the elution buffer was equilibrated by passing through the spiral. The suspension of ion-exchange gel and all solutions were degassed before column chromatography. When the amount of protein elutes from the column had decreased appreciably, the column was washed with 0.5 M NaOH to release the adhering proteins. Fractions of 0.5 ml were collected at room temperature, and the peak fraction of protein (0.5 ml) was dialyzed overnight against 3% SDS/2 M urea/2 mM EDTA/1 mM β-mercaptoethanol/60 mM Tris-HCl (pH 6.9). Then 150 μl of the sample was used for SDS-polyacrylamide gel electrophoresis analysis after boiling for 5 min unless otherwise stated in the legends to the figures.

Binding of heated spectrin and the subunits to inside-out vesicles

After the extraction of spectrin at 37°C from erythrocyte membranes, pelleted inside-out vesicles were allowed to stand in 10 vol. excess of the spectrin extraction solution [15] for 20 h on ice to extract residual spectrin. The pellets obtained by centrifugation at 16000 rpm for 25 min were washed once with the cold spectrin extraction solution by a second centrifugation. The resulting

* Toyopearl-gel is identical to Fracto-gel TSK of E. Merk or E.M. Science.

pellets were washed twice with the binding assay buffer of 1 mM $CaCl_2$/1 mM $MgCl_2$/120 mM KCl/10 mM NaCl/0.1 mM DFP/0.1 mM PMSF/20 mM Tris-HCl (pH 8.0) by centrifugation at 10000 rpm for 10 min. The inside-out vesicles suspended in the binding assay buffer (protein content was 3.5 mg/ml) were used for the assay. Spectrin or its subunit in 120 mM NaCl/1 mM EDTA/0.1 mM DFP/0.1 mM PMSF/20 mM Tris-HCl (pH 8.0) (20 μg proteins in 20–30 μl) were incubated at various temperatures for 5 min in polyethylene tubes (1.5 ml capacity); then the samples were cooled down on ice. These preheated proteins were incubated with inside-out vesicles (0.4 mg of membrane protein) in 0.3 ml of the binding assay buffer at 30 °C for 1 h. The free and membrane-bound proteins were separated by centrifugation of 30 min at 15000 rpm (TOMY MC-15A, Japan) after adding a further 0.3 ml of the cold binding assay buffer. The pellets were dissolved in SDS solution directly for SDS-polyacrylamide gel electrophoresis analysis. Neither the heated spectrin nor any subunit was pelleted by the centrifugation procedure described above. Controls were always confirmed by parallel manipulations without inside-out vesicles.

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed by the procedure of Laemmli [16] using a 2 mm thick slab gel with 6.8% and 3.7% acrylamide (Bio-Rad) for separating and stacking gel, respectively. The gel was stained with Coomassie blue and densitometric patterns of the gels were obtained with a dual-wavelength scanner (Shimadzu CS-910).

Results

The preheated spectrin was applied to a DEAE-Toyopearl column and eluted at room temperature as described in Experimental procedures. The elution patterns monitored by absorbance at 280 nm displayed two major peaks, at retention times of 1.5 min and about 15 min, as shown in Fig. 1. The first peak at 1.5 min, however, contained no proteins as confirmed by SDS-polyacrylamide gel electrophoresis and staining with Coomassie blue or silver. Excess PMSF and DFP

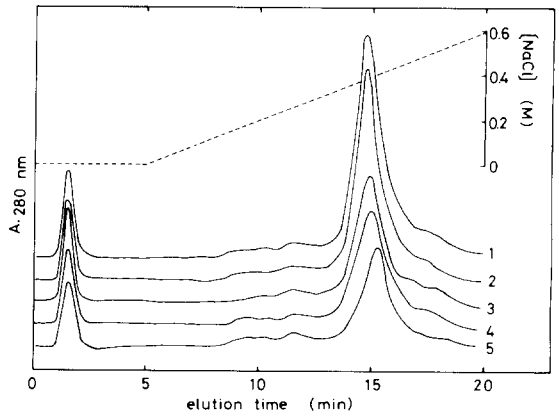


Fig. 1. Profiles of elution of heated spectrin from a DEAE-Toyopearl column. Purified spectrin fractions (0.5 ml of 1.2 mg/ml) in 120 mM NaCl/1 mM EDTA/0.5 mm PMSF/0.5 mM DFP/1 mM β-mercaptoethanol/20 mM Tris-HCl (pH 8.0) were heated at various temperatures for 5 min, then applied to the column. The traces 1–5 represent heating at 40, 45, 48, 49 and 50 °C, respectively. The details of the column chromatography are described in Experimental procedures.

in the sample solution must have caused the first peak, since a control column with sample solution without spectrin gave exactly the same peak at 1.5 min. The second peak around 15 min consisted of proteins which could be visualized by SDS-polyacrylamide gel electrophoresis as described below. The elution pattern obtained with spectrin heated to temperatures lower than 45 °C (traces 1 and 2 in Fig. 1) displayed a protein peak at 14.5 min with a high protein yield (about 95%). Nonheated spectrin eluted out at the same position of 14.5 min, though the yield was somewhat lower (about 80%). Presumably, the oligomers of spectrin hardly eluted from the column. When the spectrin was heated above 48 °C, however, the protein peak appeared at a retention time longer than 15 min, with a considerable decrease in protein yield (about 40%) as shown by traces 3–5 in Fig. 1. The changes in protein yield estimated from the area of elution patterns as a function of temperature are shown in Fig. 3. It decreased steeply between 45 and 50 °C, indicating that a large quantity of protein adhered to the column when the sample was heated above 50 °C.

The protein composition of these peaks, as analyzed by SDS-polyacrylamide gel electrophoresis and densitometry of the protein bands, is shown in Fig. 2. This clearly shows that the β subunit

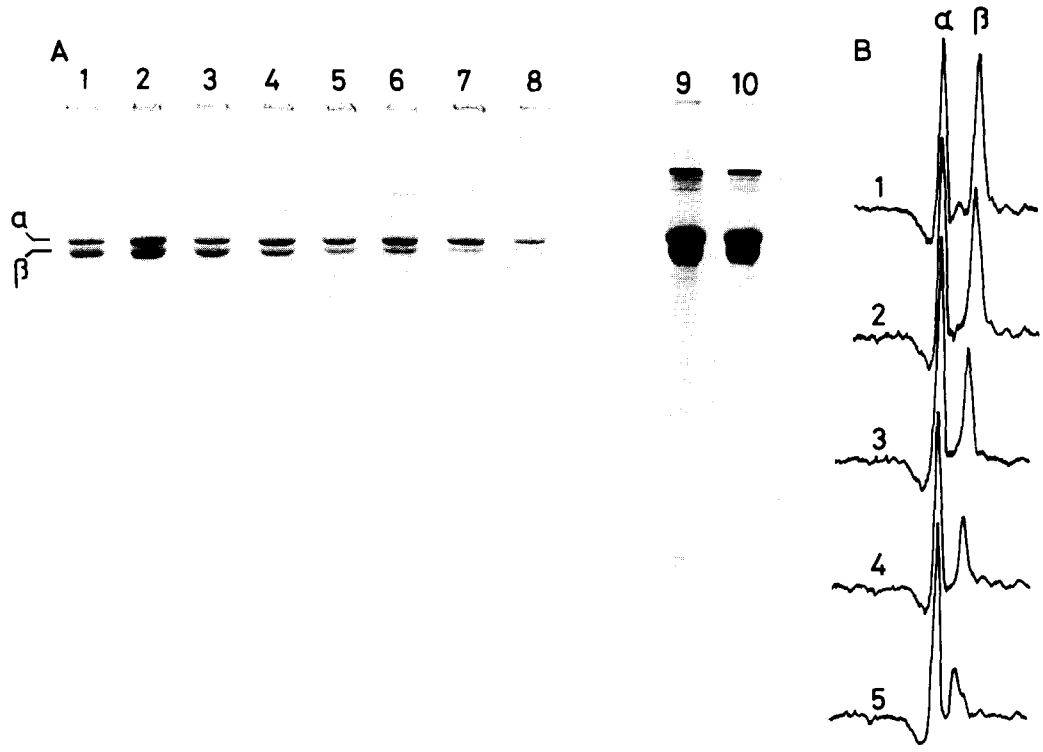


Fig. 2. Protein compositions in chromatographed fractions of heated spectrin analyzed by SDS-polyacrylamide gel electrophoresis. In panel A, lanes 1–8 are the chromatographed fractions of spectrin heated at 30, 40, 45, 48, 49, 50, 52 and 54 °C, and lanes 9 and 10 are the alkali washing fractions after salt elution of the spectrin heated at 50 and 52 °C, respectively. In panel B, traces 1–5 are densitometric patterns in the regions of the α and β subunits on the gel of the chromatographed fractions of spectrin heated at 30, 45, 48, 49 and 50 °C, respectively.

disappeared when spectrin was heated above 48 °C (lanes 5–8 of panel A in Fig. 2). The ratios of protein intensity of β to α subunit bands on the gel calculated from the area of the densitometric patterns (typical patterns are shown in Fig. 2B) were plotted against temperature. As shown in Fig. 3, the ratio decreased steeply between 45 and 50 °C. For the spectrin heated above 48 °C, as already described, a large quantity of proteins could not be recovered from the column by salt elution, even when the column was eluted with 1 M NaCl. The adhering proteins in the column could be removed only by eluting with 0.5 M NaOH. The protein composition in the fraction obtained by alkali washing after salt elution is shown in lanes 9 and 10 in Fig. 2. It should be noted that, in addition to the bands of α and β subunit, a high-molecular-weight protein band can be seen at the top of the separating gel.

Thermal column chromatography of spectrin

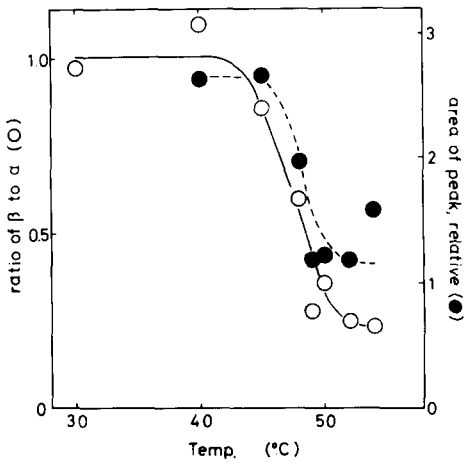


Fig. 3. Changes in subunit compositions and protein yield in the chromatographed fraction of the heated spectrin. The ratio of β to α (○) in the chromatographed fraction is estimated from the area of the densitometer profile shown in the Fig. 2. The protein yield for heated spectrin is represented as the area of the peak eluted from DEAE-Toyopearl column with the gradient of salt solution (●).

with a DEAE-Toyopearl column was performed to examine whether the change in the ratio of β to α subunits described above occurs during the heating step or not. The elution conditions of the thermal column chromatography were changed a little from those of Fig. 1 to obtain the best protein yield at high temperature, as described in Experimental procedures. The elution pattern also displayed two major peaks, as described, and the protein peak appeared at 12.4 min at 30 °C and 14.3 min at 49 °C, respectively. As shown in Fig. 4, the ratio of β to α subunits analyzed by SDS-polyacrylamide gel electrophoresis and densitometries of the protein bands also decreased remarkably between 45 and 50 °C, as seen also in Fig. 3. The protein yield, however, decreased only gradually with increasing temperature from 30 to 50 °C, and proteins adhered to the column could hardly be recovered, even with 0.5 M NaOH.

The isolated α and β subunits were also heated at various temperatures and applied to DEAE-Toyopearl columns at room temperature. Some of the typical elution patterns are shown in Fig. 5. They also show two major peaks, but the first one at 1.5 min has no proteins, as already described. The α subunit eluted at 15.2 min with high yield (about 90%) in both samples heated at 45 °C or 50 °C (traces 1 and 2 in Fig. 5). The β subunit when heated at 45 °C eluted at 15 min with high yield (trace 3, Fig. 5), but the protein peak shifted to a longer retention time (15.4 min) with a poor protein yield (30–40%) when the subunit was heated at 49 °C as shown in trace 4 in Fig. 5. The SDS-polyacrylamide gel electrophoresis patterns of these peaks are shown in Fig. 6, and the changes in protein yield as function of the temperature are shown in Fig. 7. For the α subunit, heating seems to have little effect on either the SDS-gel pattern and protein yield over the range 40 to 52 °C. The yield of α subunit when heated slightly (30 °C) or not at all was always low. This might be due to polymorphic forms of the isolated α subunit at low temperature [10]. The protein yield of β subunit decreased remarkably when it was heated above 48 °C, suggesting that a considerable amount of protein (about 70%) adhered to the column. The lanes 7–9 of Fig. 6B show the protein compositions in the fraction obtained by eluting the column with 0.5 M NaOH after salt elution. It

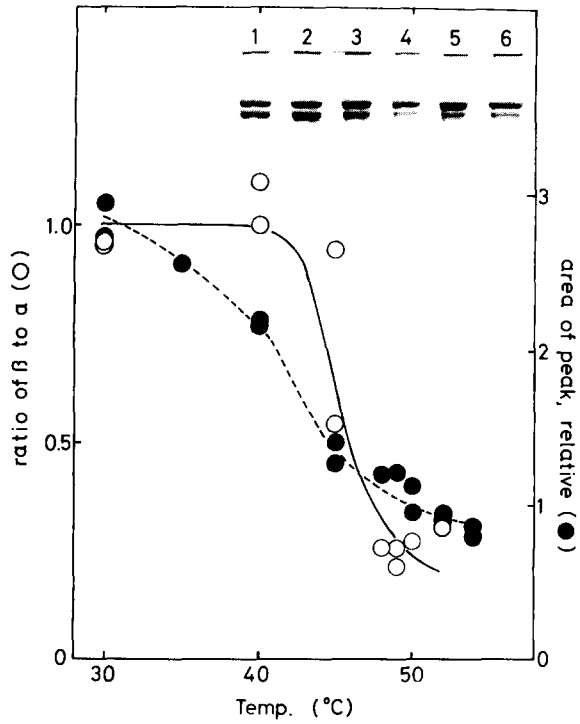
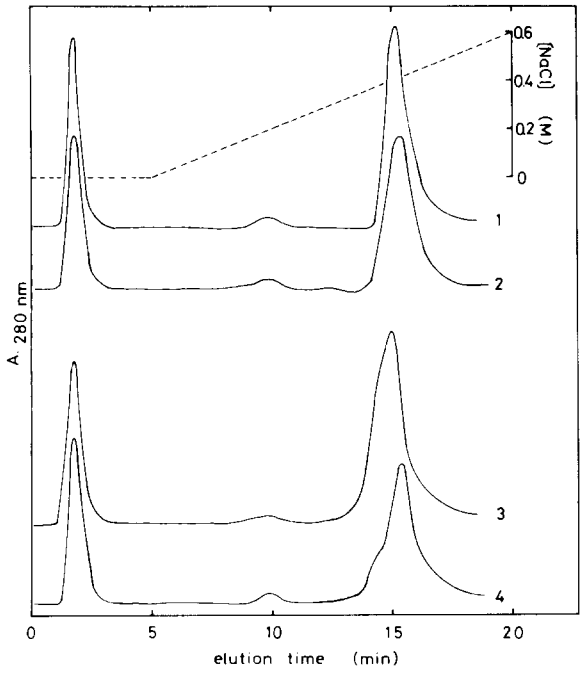


Fig. 4. Changes in subunit compositions and the protein yield in the chromatographed fraction of the spectrin at various temperatures. The thermal column chromatography of spectrin with DEAE-Toyopearl was performed as described in Experimental procedures. Lanes 1–6 in the inset are typical SDS-polyacrylamide gel electrophoresis patterns of the protein fractions obtained at 30, 40, 45, 48, 49 and 50 °C, respectively. In this case, appropriate quantities of protein samples precipitated with 6% trichloroacetic acid/0.026% deoxycholate and dissolved in SDS were applied to the gels, because protein peaks from the column at high temperatures were extremely broad. The ratio of β to α (○) in the chromatographed fraction is estimated from the area of the densitometric profile of α and β subunits on the gels shown in the inset. The protein yield of spectrin (●) is represented as the area of the peak eluted from the column with the salt gradient at the temperature denoted.

should be noted that high-molecular-weight protein bands can be seen at the top of the separating gel as well as the heated spectrin shown in Fig. 2.

To estimate the binding of preheated spectrin and its α and β subunits to inside-out vesicles, the protein composition of vesicles which had been incubated with these preheated proteins was analyzed by SDS-polyacrylamide gel electrophoresis, since the binding of spectrin to the membrane should be one of important functions of this molecule. In Fig. 8 A–C are shown densitometer scans



scans of those gels in region where spectrin should be seen. The area of the densitometric patterns of inside-out vesicles incubated with spectrin and its α and β subunits preheated at 30° C increased considerably (compare trace 2 with 1 in each panel A–C, of Fig. 8), indicating the binding of these proteins. Therefore, the extent of binding of the proteins to inside-out vesicles could be estimated from the increment of the area obtained by subtracting the area of trace 1 from trace 2. As

Fig. 5. Elution profiles from DEAE-Toyopearl column of the heated α and β subunit. The isolated α and β subunit (0.5 ml of 0.6 mg/ml) in 120 mM NaCl/1 mM EDTA/0.5 mM PMSF/0.5 mM DFP/1 mM β-mercaptoethanol and 20 mM Tris-HCl (pH 8.0) were heated at various temperatures for 5 min, then applied to the column. The traces 1 and 2 were obtained with the α subunit heated at 45 and 50° C, and traces 3 and 4 with the β subunit heated at 45 and 49° C, respectively. The details of the column chromatography are described in Experimental procedures.

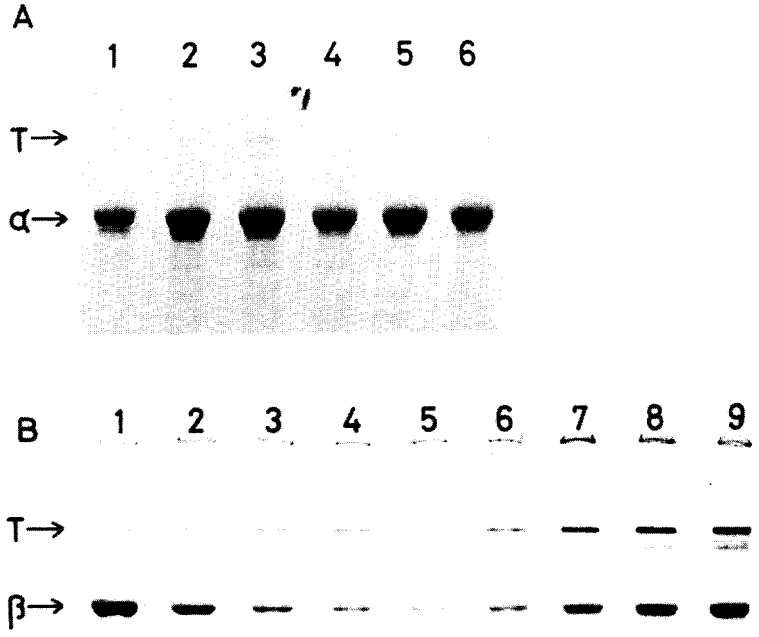


Fig. 6. SDS-polyacrylamide gel electrophoresis for the chromatographed fractions of the heated α and β subunits. In panel A, lanes 1–6 are salt-eluted fractions of the α subunit heated at 30, 45, 48, 49, 50 and 52° C, respectively. In panel B, lanes 1–6 are salt-eluted fractions of the β subunit heated at 30, 45, 48, 49, 50 and 52° C, and lanes 7–9 are alkali-washed fractions of the β subunit heated at 49, 50 and 52° C, respectively. The arrows α, β and T indicate the positions at which nonheated α and β subunit normally run, and the top of separating gel, respectively.

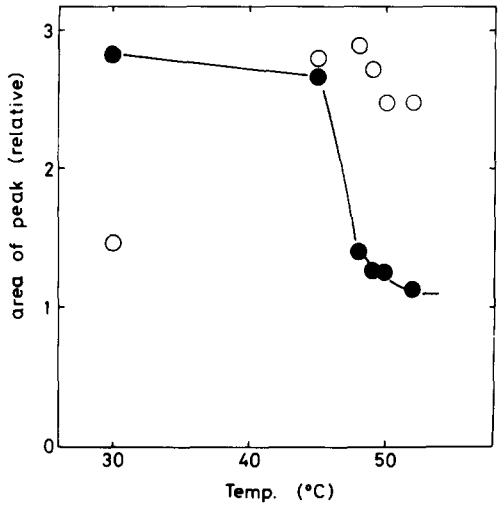


Fig. 7. Changes in the protein yields for α and β subunits in chromatographed fractions. The protein yields for the heated α (○) and β (●) subunits are represented as the area of peaks eluted from DEAE-Toyopearl column with the salt gradient.

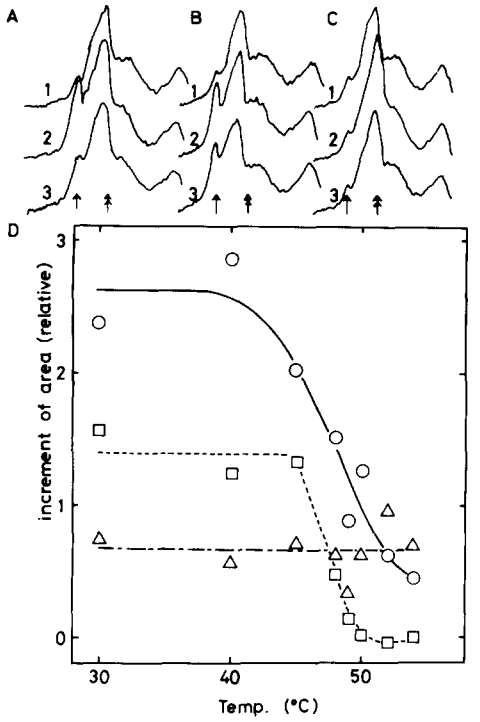


Fig. 8. The binding of heated spectrin and its α and β subunits to inside-out vesicles. In panels A–C, are shown the densitometer traces from SDS gels of inside-out vesicles incubated with spectrin (A), α subunit (B) and β subunit (C) preheated at 30 °C (2) and 50 °C (3), and traces 1 in A–C are inside-out vesicles without incubation. Control α and β subunits migrated on the gel to the positions denoted by arrows for α and double-headed arrows for β, respectively. The binding of spectrin (○), α subunit (△) and β subunit (□) to inside-out vesicles as a function of the preheating temperatures is shown in panel D. The quantities of proteins binding were estimated as follows. For instance, the binding of spectrin to inside-out vesicles caused a increase in the area of the densitometric scan as described above. Consequently, a increment of the area obtained after subtracting the area of trace 1 from that of trace 2 should reveal the quantities of spectrin binding. It is reasonable to assume that the increments of area are in proportion to the quantities of the proteins binding.

shown in Fig. 8D, it is very interesting that the binding of α subunits to inside-out vesicles is independent of preheating temperatures; however, both spectrin and the β subunits lose binding ability, depending on temperature, between 45 and 50 °C. It is noted that the β subunits heated at over 50 °C lose the binding ability completely (in panel C, trace 3 can be superimposed on trace 1); on the other hand, a small amount of spectrin heated at 50 °C still retains its binding ability (compare trace 3 with 1, especially at the α subunit region in panel A). The quantities of protein binding in the case of spectrin and its β and α subunits preheated at 30 °C are about 20, 14 and 7 μg per mg protein of inside-out vesicles, respectively.

Discussion

When the human erythrocyte spectrin was preheated above 48 °C, the ratio of β to α subunit in the fraction chromatographed by DEAE-Toyopearl column decreased, falling to 0.25 depending on the temperature, as shown in Figs. 2 and 3. Almost the same result was obtained as shown in Fig. 4 when spectrin was applied and eluted at above 48 °C with the thermal column chromatography. These findings indicate directly that the spectrin molecule dissociates into subunits upon heating, and that the dissociated α subunit is unable to reassociate with the dissociated β subunit, even after the temperature is lowered. Therefore, the fraction in which the α subunit was dominant could be separated, and this is further supported by the result that the elution pattern of spectrin heated at 49 °C (trace 4 in Fig. 1) is similar to that of the isolated α subunit (trace 1 and 2 in Fig. 5).

The heat denaturation curve of spectrin mea-

by loss of binding to inside-out vesicles is similar to the result reported previously [17]. It is quite interesting that the isolated β subunit denatures irreversibly upon heating above 45 °C, though the isolated α subunit still displays binding ability standing on ice after heating above 50 °C (Fig. 8). The nonspecific binding of proteins to inside-out vesicles might have little effect on our analysis, because the β subunit heated above 50 °C, which was a control as a heat-treated protein, did not show any significant binding to inside-out vesicles (Fig. 8).

The results of column chromatography at room temperature with β subunit heated above 49 °C suggest at least three kinds of denatured forms of β subunit. About 30% of the β subunits which eluted out with salt solution comprised one of the denatured forms of the β subunit, because the retention time increased as compared to native β subunit (compare tace 4 to 3 in Fig. 5). The remaining 70% of the denatured β subunits adhered to the column. In that fraction there might be yet two more kinds of denatured states, one able to dissociate into a 220 kDa polypeptide but the other not dissociating in SDS solution, as shown in lanes 7–9 of Fig. 6B. Presumably, the β subunits might form multiple types of aggregate. Consequently, β subunits dissociated from spectrin molecules by heating should also form multiple types of aggregate. Here, we interpret what happens when spectrin is heated above 49 °C as follows. The spectrin molecules dissociate into α and β subunits by heating above 49 °C, and irreversible denaturation of dissociated β subunits leads to the formation of multiple types of aggregate which incorporate the dissociated α subunits nonspecifically into the protein clusters. In the spectrin solution preheated above 49 °C, however, a small amount of the α subunit still remains in a free form which can bind to inside-out vesicles as shown in trace 3 of Fig. 8A. The protein yield from the column for spectrin preheated at above 49 °C (about 40%) is lower than the value of 60% which has been predicted from the yield of isolated subunits (90% of α and 30% of β). Therefore, the formation of clusters of the denatured β subunit might be stimulated by incorporation of the α subunit. The change in protein yield shown in Fig. 3 and the yield of 40% are consistent with the results obtained by the precipitation technique [18]. It is difficult to explain the change in protein yield from thermal column chromatography shown in Fig. 4. Presumably, the hydrophobic portion of the protein which was exposed by heating might cause nonspecific adsorption of protein to the ion-exchange resin.

At temperatures above 37 °C, the spectrin dimer became the dominant species [7,19], though the spectrin preparations used here were a mixture of $\alpha\beta$ dimer, $(\alpha\beta)_2$ tetramer and $(\alpha\beta)_n$ oligomers [10]; therefore, the critical temperatures for dissociation of spectrin $\alpha\beta$ dimer into monomers is discussed here.

It is quite interesting that the change in ratios of β to α shown in Fig. 3 bore striking resemblance to the change in fluorescence anisotropy [10], different scanning calorimetry and the CD spectrum [9] of spectrin as a function of temperature. Based on the findings described here, the changes in those parameters for secondary and tertiary structures of protein between 45 and 50 °C might be caused by the dissociation of spectrin molecules. The changes in all those parameters, therefore, were partial [9,10] and irreversible, since dissociated β subunits undergo irreversible denaturation. The fluorescence anisotropy of tryptophan residues in the β subunit displayed a remarkable increase at approx. 48 °C, suggesting that segmental motion in the vicinity of tryptophan residues was restricted by the formation of aggregates [10].

Striking morphologic changes in the normal erythrocytes occurred at 47–50 °C [12], at which temperature spectrin dimers dissociated into monomers, as described above. In hereditarily abnormal erythrocytes, however, heat-induced morphologic changes occurred at a temperature lower than that of normal cells [20,21], and the transition temperature of CD spectrum and heat-sensitive solubility which might reveal the heat-induced dissociation of spectrin dimers also shift to lower temperature [18,22]. Furthermore, in those abnormal cells the spectrin is defective in dimer–dimer self association [23,24]. Since a modified structural domain in the α subunit which is concerned with α–β association was obtained from patients with hereditary pylopoikilocytosis [11,25], one might infer that a specific interaction between

α and β subunit should be essential for the normal heat stability and self-association of spectrin dimers. It is reasonable to consider that favorable arrangements of α and β chains at the head end allow the formation of spectrin tetramer or oligomers which are regarded as functional units in the membrane skeleton [8,21,26]. Similarly, other funcitonal sites on the spectrin dimer, such as the tail end, where spectrin interacts with band 4.1 protein and actin and with ankyrin [27,28], should be in a favorable position to build up the meshwork of the membrane skeleton. It is emphasized that all those arrangements of the multiple functional sites on the spectrin dimer must be regulated by a specific type of side-to-side interaction between α and β subunits.

Acknowledgment

We wish to thank miss H. Iwamizu for typing the manuscript.

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