This paper is dedicated to the memory of Professor Wanda Mejbaum-Katzenellenbogen

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INTERACTION OF SPECTRIN WITH HYDROPHOBIC AGAROSES*

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Purified spectrin was found to interact strongly with hydrophobic agaroses such as Phenyl- or Octyl-Sepharose, in the presence of EDTA. From the complexes formed spectrin was eluted with ethylene glycol but not with low ionic strength solutions. The binding capacity of spectrin increased with increasing ionic strength of the equilibration buffer and showed but little dependence on its pH value. The fragments obtained by proteolysis of spectrin carried out under mild conditions were also found to bind strongly to phenylagarose, and were eluted with ethylene glycol. The fractions eluted with ethylene glycol contained two closely related polypeptides of \( M_r \) 65 000 and 60 000.

Spectrin, a high \( M_r \) protein consisting of two types of subunits, \((M_r 240\,000)\) and \((M_r 220\,000)\) is the major extrinsic protein of the erythrocyte membrane. It interacts with other extrinsic and intrinsic proteins of this membrane, and presumably also with lipids of the endofacial membrane layer (for review see [1, 2]). In previous studies [3, 4] it was found that spectrin could be labelled with hydrophobic labels such as arylisothiocyanates, both in erythrocytes and erythrocyte ghosts; this might suggest penetration of membrane lipid bilayer by some segments of the spectrin molecule.

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The results of research on the effect of spectrin on the order parameter of the acyl chain region of the membrane bilayer also seem to confirm this assumption [5]. Moreover, studies on the structure suggest that a spectrin molecule contains hydrophobic domain(s) [6, 7]; this would point to the possibility of interaction of this water-soluble, extrinsic membrane protein with hydrophobic matrices.

In the present experiments spectrin has been shown to interact strongly with either phenyl- or octyl-substituted agaroses. The possibility of isolation of hydrophobic fragments of a spectrin molecule has also been demonstrated.

MATERIALS AND METHODS

Erythrocyte ghosts were prepared according to Dodge et al. [8] from recently out-dated human blood, supplied by the Wroclaw Blood Bank. Spectrin was extracted by incubation of erythrocyte ghosts with 0.1 mM EDTA, pH 7.2, at 37°C for 30 min. The membranes were removed by centrifugation and the supernatant was concentrated by reduced pressure dialysis. About tenfold concentrated supernatant, 5 ml, was applied to the Sepharose 4B column (2 x 1.4 cm) equilibrated with 50 mM phosphate buffer, pH 7.2, containing 1 mM EDTA. The second peak containing spectrin dimer [9] was used in further experiments. The preparation isolated contained only spectrin bands 1 (α) and 2 (β), as checked by polyacrylamide gel electrophoresis in the presence of SDS.

Hydrophobic interaction chromatography was performed using Phenyl- or Octyl-Sepharose CL 4B (Pharmacia) packed (15 ml of the resin) in a column (1 x 10 cm) or glass filter G-3. The conditions of adsorption and elution are specified in legends to Fig. 1, 2, 4 and Table 1.

Proteolysis of the isolated spectrin was performed at 25°C for 90 min with TPCK1 treated trypsin (Worthington) in 10 mM phosphate buffer, pH 7.2 containing 1 mM CaCl₂, at spectrin to' trypsin ratio of 50:1. After completion of treatment with the enzyme, phenylmethane sulphonyl fluoride and acetate buffer, pH 4.9, were added to the final concentration of 200 μg/ml and 20 mM, respectively. Undigested spectrin was then salted out with ammonium sulphate at 0.3 saturation. After centrifugation, the supernatant adjusted to pH 7.2 was applied to Phenyl-Sepharose 4B column (1 x 10 cm).

From the peak fractions containing spectrin proteolytic fragments ethylene glycol was evaporated at 50°C in a drier or in a desiccator over P₂O₅. The dried samples were dissolved in 0.2 ml of 1 mM NaOH, then 0.2 ml of 5 mM phosphate buffer was added and the sample was passed through

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1 Abbreviations used: TPCK, p-tosyl-L-phenylalanine chloromethyl ketone; SDS, sodium dodecyl sulphate
a Sephadex G-25 column (1 × 25 cm). The fraction emerging from the column in the void volume was dialysed against water and lyophilized.

SDS-polyacrylamide gel electrophoresis (4 - 20% acrylamide concentration gradient) was carried out according to Laemmli [10].

Protein in the samples containing ethylene glycol was determined according to Lowry et al. [11] after dialysis (twice) against 1% SDS, 1 mM EDTA in 4 mM Tris/HCl, pH 8.0, at 4°C, or spectrophotometrically using the absorbance coefficient at 280 nm A\textsubscript{280} = 11.5 [12].

To identify the protein material separated in the polyacrylamide gradient gel, it was transferred electrophoretically [13, 14] onto nitrocellulose filters (Schleicher & Schuell, 0.45 μm), where the reaction with rabbit antispectrin serum was performed, and then the sample was treated with goat anti-rabbit IgG conjugated with horse radish peroxidase and stained with 4-chloro-1-naphthol (HRP-colour reagent, BioRad).

RESULTS AND DISCUSSION

When the purified spectrin dimer was passed through the Phenyl-Sepharose column in the presence of 1 mM EDTA and 1 M NaCl, interaction of spectrin with the resin was observed. However, it should be noted that in the absence of EDTA the interaction of spectrin both with unsubstituted agarose (Sepharose) and the substituted one (Phenyl-Sepharose) was also significant (Sikorski, A. F. & Krzywińska, A., unpublished). Thus, to exclude the “EDTA-sensitive” binding, EDTA was added to all column buffers.

Chromatography of purified spectrin on phenylagarose column is shown in Fig. 1. Since the capacity of the column was exceeded, the first peak (fractions 2 - 4) contained unbound spectrin. It is apparent that complete elution of bound spectrin could be achieved only with 100% ethylene glycol.

A similar experiment was performed with the use of Octyl-Sepharose as a binding hydrophobic bed (Fig. 2). Similarly as in the previous experiment, complete elution was achieved only with 100% ethylene glycol, although small amounts of adsorbed protein were eluted with 50% and 70% ethylene glycol. With both substituted Sepharose carriers used, attempts at elution of the adsorbed spectrin with 1 mM EDTA containing 0.5 mM phosphate, pH 7.2, were unsuccessful. To check whether the elution was complete, 8 M urea was applied to the column but no material adsorbing at 280 nm did emerge under these conditions (not shown).

The reason for partial elution of spectrin with 50 and 70% ethylene glycol is not clear (especially in the case of octylagarose), as the material applied to the column was homogeneous, and the fractions eluted at these concentrations of ethylene glycol contained spectrin alone, as checked by SDS-polyacrylamide gel electrophoresis (not shown). Thus, it seems possible to assume that some spectrin molecules underwent oligomerization and
Fig. 1. Adsorption of purified spectrin on Phenyl-Sepharose 4B column. A sample of spectrin (1.2 mg), in 2 ml of 1 M NaCl, 1 mM EDTA, 50 mM phosphate buffer, pH 7.2 was applied to Phenyl-Sepharose column (1 x 10 cm) equilibrated with the same buffer. Elution was carried out with: I, 50% ethylene glycol; II, 70% ethylene glycol in the same buffer, and III, 100% ethylene glycol

Table 1

Effect of NaCl concentration in the samples and equilibration buffer on adsorption of purified spectrin on Phenyl-Sepharose 4B column

Purified spectrin was dialysed against 0.005 M phosphate buffer, pH 7.2, containing 1 mM EDTA. Samples of 1 ml (0.6 mg protein) were adjusted to the desired salt concentration with solid NaCl. Phenyl-Sepharose column (1 x 5 cm) was equilibrated with the same buffer containing NaCl at the indicated concentration. Adsorbed protein was eluted with ethylene glycol dialysed against 1% SDS. Peak fractions were pooled and the amount of unbound and adsorbed protein was determined by the method of Lowry et al. [11].

<table>
<thead>
<tr>
<th>NaCl concentration in the sample and buffer (M)</th>
<th>0</th>
<th>0.25</th>
<th>0.5</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adsorbed spectrin (mg)</td>
<td>0.12</td>
<td>0.19</td>
<td>0.35</td>
<td>0.345</td>
</tr>
</tbody>
</table>

that the oligomers formed dissociated at lower ethylene glycol concentrations (50 and 70%).

Adsorption of purified spectrin on the phenylagarose column was affected by NaCl concentration: when the salt concentration was raised up to 0.5 M, the binding capacity of the carrier was increased as well (Table 1).
Fig. 2. Adsorption of purified spectrin on Octyl-Sepharose 4B. A sample of spectrin (3.3 mg), in 2 ml 1 M NaCl, 1 mM EDTA, 50 mM phosphate buffer, pH 7.2, was mixed with 15 ml of Octyl-Sepharose on glass filter G-3 washed with the same buffer. The unadsorbed protein was washed out with several 6-ml portions of the same buffer (I). The resin was then washed with a few 6-ml portions of: 1 mM EDTA in 0.5 mM phosphate, pH 7.2 (II); 50% ethylene glycol in the starting buffer (III); 70% ethylene glycol in the starting buffer (IV), and 100% ethylene glycol (V). Fractions no. 16 - 17 and 21 - 24 contained spectrin, as checked by SDS-polyacrylamide gel electrophoresis.

Fig. 3. Effect of pH of the buffer equilibrating Phenyl-Sepharose on spectrin binding. A sample of freshly isolated dimeric spectrin was adjusted to the indicated pH by addition of 0.1 volume of 0.5 M sodium acetate buffer (pH 5.5); 0.5 M sodium phosphate buffer (pH 6.0 - 8.0) or Tris/HCl buffer (pH 8.5 - 9.0). All samples contained 1 M NaCl and 1 mM EDTA. The column was washed with the respective buffer, then elution was carried out with 100% ethylene glycol.
pH of the spectrin sample to be adsorbed had but a slight effect on the amount of material bound to Phenyl-Sepharose (Fig. 3). Furthermore, elution with equilibration buffers, even of pH as high as 9.8 (not shown) did not result in removal of all the protein from the resin. This could be attained only with 100% ethylene glycol as an eluent. The effect of salt concentration and pH of the equilibration buffer on the adsorption of spectrin on phenylagarose point to the hydrophobic nature of this interaction. The necessity to use 100% ethylene glycol for complete elution of this protein suggests that the interaction is rather strong, as compared, e.g. to that with human serum albumin which was completely eluted with 50% ethylene glycol.

Chromatographic profiles of the limited proteolysis products of spectrin, eluted from Phenyl-Sepharose column with low ionic strength solution followed by increasing concentrations of ethylene glycol, displayed four peaks (Fig. 4A and 4B). The electrophoretic analysis of the individual peaks (not shown) revealed that the low ionic strength solution eluted a mixture of low $M_r$ fragments of spectrin. The fractions eluted with 50, 70 and 100% ethylene glycol, after evaporation of the solvent, were purified on Sephadex G-25 column and the material emerging in the void volume was analysed by gradient gel electrophoresis in the presence of SDS (Plate 1). The peak eluted with 100% ethylene glycol contained two closely related polypeptide chains of $M_r$ 65,000 and 60,000 (Plate 1). Similar results were obtained both with 50 and 70% ethylene glycol (not shown). This rather surprising result was observed also when the elution was carried out with a linear ethylene glycol concentration gradient (Sikorski, A. F. & Krzywińska, A., unpublished). In that case a very broad peak containing these fragments was observed. It should be noted that the largest amount of material was eluted with 100% ethylene glycol. The elution by 50 and 70% ethylene glycol of spectrin fragments adsorbed on octylagarose might be expected by oligomer formation, similarly as partial elution by 50 and 70% ethylene glycol of intact spectrin adsorbed on octylagarose.

To demonstrate that the isolated fragments derived from spectrin are not artifacts produced during sample processing, the reaction with antispectrin antibodies was performed (Plate 1, c - e). This specific staining also suggested that the final eluate contained only those two polypeptides.

Taking into consideration the chemical domain map of the spectrin molecule established by Speicher et al. [16] it might be assumed that the fragments isolated by hydrophobic interaction chromatography correspond to the $\beta$II domain as they have a similar relative $M_r$. On the map of spectrin domains this fragment is also heterogeneous. Although final evidence that these fragments represent the $\beta$II domain is still lacking, there are no other fragments of similar $M_r$ deriving either from $\alpha$ subunit or from other parts of $\beta$ subunit of spectrin.
Fig. 4. Chromatography of proteolysis products of spectrin on Phenyl-Sepharose. The supernatant containing proteolysis products of 50 mg of spectrin adjusted to pH 7.2 was applied to the Phenyl-Sepharose column (1 x 10 cm). Elution of the proteolysis product was carried out with: in A: I, 50% ethylene glycol, 1 mM EDTA in 50 mM phosphate buffer, pH 7.2; II, 70% ethylene glycol, 1 mM EDTA in 50 mM phosphate buffer, pH 7.2; III, 100% ethylene glycol, and IV, 8 M urea; in B, I, 1 mM EDTA in 5 mM phosphate buffer, pH 7.2; II, 50% ethylene glycol, 1 mM EDTA in 50 mM phosphate buffer, and III, 100% ethylene glycol. For details see Methods.
The isolation of stable chemical domains of spectrin appeared to be of great advantage in the studies on the primary structure of this protein and on the function of its particular segments [16]. It is to be hoped that the method for isolation of hydrophobic fragment(s) presented here will find application in the studies on the structure and function of spectrin.

REFERENCES

Plate 1. Electrophoresis of spectrin fragments isolated by hydrophobic interaction chromatography.

Electrophoresis of the material eluted with 100% ethylene glycol from phenylagarose column (cf. Fig. 4) and further purified on Sephadex G-25. Electrophoresis was carried out in SDS-polyacrylamide gel, then the gels were stained with Coomassie blue. a. $M_r$ standards: phosphorylase B (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000), and lactalbumin (14 400). b. Spectrin fragments.

Immunoblotting of polypeptides eluted from Phenyl-Sepharose column with 50% (c), 70% (d) and 100% (e) ethylene glycol, purified on Sephadex G-25 column and separated by electrophoresis in gradient gel. For details see Methods.

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