PROTEOLYSIS OF SPECTRIN BY TRYPSIN AND PRONASE IN THE PRESENCE OF PHOSPHOLIPID SUSPENSIONS

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The effect of phospholipid suspensions on the proteolysis of isolated spectrin was examined by SDS-polyacrylamide gradient gel electrophoresis. Proteolysis of spectrin in the membranes by trypsin and pronase was also studied. It was found that electrophoretic patterns of spectrin fragments were influenced by the presence of the suspension prepared from phosphatidylethanolamine:phosphatidyserine (60:40) mixture and of phosphatidylcholine. Qualitative changes in the proteolytic patterns obtained after proteolysis of spectrin by pronase in the presence of phosphatidylcholine suspension were observed. The changes in the sensitivity of spectrin towards proteases result probably from changes in the accessibility of some peptide bonds upon the interaction of this extrinsic protein with phospholipids.

The erythrocyte membrane skeleton is composed mainly of spectrin and actin, and also of band 4.1 protein (for a review see e.g. refs. [1, 2]). Spectrin, a high molecular weight protein, consists of two nonidentical subunits: $\alpha$ ($M_r = 240,000$) and $\beta$ ($M_r = 220,000$) which associate with each other to form ($\alpha\beta$) 100 nm long heterodimer. Two heterodimers associate head to head to form a 200 nm long heterotetramer which is supposed to be the functional form of this protein in the membrane [3, 4]. Fragmentation of spectrin molecule into intermediate molecular weight proteolytic fragments called “chemical domains” of spectrin facilitated extensive studies on the primary structure of this protein [5, 6].

Hereditary changes in the region of $\alpha$I domain of spectrin seem to be responsible for the altered shape and deformability of erythrocytes. This defect was also reported to cause impaired association of spectrin heterodimers and instability of erythrocyte membrane skeletons ([7] and references therein).

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Ankyrin (band 2.1 protein) was found to be the main receptor site for spectrin [8]. This protein forms a complex of high affinity (affinity constant $1 \times 10^7$ M$^{-1}$ [8]) with spectrin and also with the erythrocyte membrane band 3 protein (affinity constant $1.3 \times 10^7$ M$^{-1}$ [9]) thus connecting the membrane skeleton to the intrinsic membrane domain.

There are also indications that spectrin interacts with phospholipids of the inner leaflet of membrane bilayer, playing an essential role in maintaining the asymmetrical distribution of phospholipids [10] (cf. also reviews [1, 2]). In our previous experiments it was found that spectrin in erythrocytes and in isolated membranes could be labelled with hydrophobic arylisothiocyanates [11, 12]. Moreover, spectrin was shown to diminish the order parameter of the acyl chain region as measured with the use of 5'-doxyl stearate spin label [13]. It was also found that phospholipid suspensions prepared from phosphatidylethanolamine or its mixtures with other phospholipids were able to quench the intrinsic fluorescence of spectrin [14, 15].

The aim of the present study was to compare the polypeptide patterns resulting from proteolysis of spectrin with trypsin and pronase in the absence or presence of aqueous phospholipid suspensions in order to test whether the polypeptide patterns would be changed. The proteolysis of spectrin in erythrocyte membrane was also analysed.

MATERIALS AND METHODS

Erythrocyte ghosts were isolated according to Dodge et al. [16] from recently out-dated human blood obtained from the local Blood Bank.

Spectrin dimer was isolated by 30 min extraction at 37°C with 0.1 mM EDTA (pH 7.2) of erythrocytes pre-washed with cold distilled water. Concentrated low ionic strength extract was subjected to the chromatography on Sepharose CL 4B column (1.8 × 50 cm) equilibrated with 5 mM phosphate buffer, pH 7.5, containing 1 mM NaN$_3$, 0.1 mM EDTA and 0.1 mM 2-mercaptoethanol. The second peak containing pure spectrin dimer was used for the experiments.

Phospholipid suspensions were prepared by evaporating chloroform from an appropriate volume of phospholipid solution and then shaking the content with 0.1 mM sodium phosphate buffer, pH 7.5 (the concentration of phospholipid was 2%). The suspension was heated to 60°C for 0.5 min and shaken for 15 min at room temperature. An appropriate volume of such a suspension (large multilamellar vesicles) was added to the incubation mixture to obtain the desired lipid to spectrin ratios.

Proteolysis of spectrin with TPCK$^1$-treated trypsin and pronase was

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$^1$ Abbreviations used: SDS, sodium dodecyl sulphate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TPCK, $p$-tosyl-$l$-phenylalanine chloromethyl ketone.
carried out at 25°C for 60 min. Samples containing dimeric spectrin in 0.1 M phosphate buffer, pH 7.5, with or without 1.0 M NaCl, 0.1 mM 2-mercaptopethanol and 1 mM CaCl₂ were pre-incubated with or without liposomes for 15 min. Then 10 μl of TPCK-treated trypsin (Worthington) dissolved just before use in 0.1 M sodium phosphate buffer was added to obtain trypsin to spectrin ratio of 1:100. The reaction was completed by the addition of phenylmethylsulphonyl fluoride to the final concentration of 100 μg/ml. Then 20 μl of the reducing reagent (50 mM Tris/HCl, pH 8.0, 5 mM EDTA, 12.5% SDS, 25% 2-mercaptoethanol) was added and the sample was boiled for 5 min. When proteolysis was carried out in the presence of 1 M NaCl the sample was diluted with 4 volumes of water prior to the electrophoresis and the increased volume (about 20-60 μg protein) was applied directly onto the gel. The treatment of spectrin with pronase (Sigma) was performed only at high salt concentration without the addition of CaCl₂. The pronase to spectrin ratio was also 1:100. The treatment of erythrocyte ghosts with TPCK-trypsin and pronase was also performed at 25°C for 60 min at the protease to membrane protein ratios from 1:20 to 1:100 in 0.1 M NaCl and 0.05 M sodium phosphate buffer, pH 7.5. Electrophoresis was performed in the discontinuous Laemmli [17] system, in gradient of the polyacrylamide gel concentration of 6-20% or 4-20%. Staining of polyacrylamide gels following electrophoresis was performed after the gels had been fixed in 5% sulphosalicylic acid containing 10% trichloroacetic acid and washed with 30% methanol in 10% acetic acid. Freshly prepared 0.25% Coomassie Brilliant Blue R-250 in 30% methanol and 10% acetic acid was used. The quantitation of fractions was performed by weighing the peaks cut off from the densitograms recorded at 600 nm (Pye-Unicam SP-1800). Each scanning was performed at least twice. The following molecular weight standards (Pharmacia Fine Chem.) were used: phosphorylase B - 94 000, bovine serum albumin - 67 000, ovalbumin - 43 000, carbonic anhydrase - 30 000, soybean trypsin inhibitor - 20 000, α-lactalbumin - 14 400. The proteolysis of spectrin in the membranes was visualized after transfer of the proteins separated in polyacrylamide gel electrophoresis onto nitrocellulose filter paper (Schleicher and Schuell 0.45 μm) according to Towbin et al. [18]. The reaction with rabbit anti-spectrin antibodies (serum of rabbits immunized with spectrin, diluted 1:200) and staining with goat antirabbit IgG (Bio-Rad) conjugated to horseradish peroxidase with the use of 4-chloro-1-naphtol (Bio-Rad) was performed as described previously [19].

Rabbit antispectrin antiserum was obtained by consecutive subcutaneous injections of spectrin emulsified with complete Freund's adjuvant followed by spectrin emulsified with incomplete Freund's adjuvant at multiple sites into rabbits.

Phosphatidylserine and phosphatidylethanolamine from bovine were from
Koch-Light and gave single spots in thin-layer chromatography on silica gel plates (Kieselgel 60, Merck) in chloroform: methanol: water; 65:25:4. Phosphatidylcholine (Fluka) was additionally purified according to Bangham et al. [20] and tested for purity by thin-layer chromatography as above using dimyristoylphosphatidylcholine (Avanti) as a standard.

RESULTS AND DISCUSSION

The electrophoretic patterns of polypeptides obtained after proteolysis of purified spectrin by trypsin in the presence and absence of phospholipid suspensions are presented in Plate 1. Table 1 shows the relative molecular weight values of spectrin fragments obtained in the absence (Plate 1) and in the presence of 1.0 M NaCl (Plate 2). The data for the fragments are in good agreement with the data for "chemical domains" of spectrin obtained by others [5].

Table 1

Spectrin fragments obtained as a result of limited proteolysis of spectrin by trypsin at 25°C for 60 min.

The nomenclature of "chemical domains" was taken from Speicher et al. [5].

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Proteolysis in 0.1 M phosphate buffer</th>
<th>Proteolysis in 0.1 M phosphate buffer containing 1.0 M NaCl</th>
<th>Chemical domains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( M_r )</td>
<td>( M_r \pm SD )</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1 100 000</td>
<td>84 000 ± 1 200</td>
<td>( \alpha (T100), \beta (T110) )</td>
</tr>
<tr>
<td>1</td>
<td>80 000</td>
<td>72 000 ± 600</td>
<td>( \alpha I (T80) )</td>
</tr>
<tr>
<td>1a</td>
<td>74 000</td>
<td>64 400 ± 330</td>
<td>( \beta IV (T74), \alpha I (T74) )</td>
</tr>
<tr>
<td>2</td>
<td>64 000</td>
<td>59 500 ± 350</td>
<td>( \beta II (T65) )</td>
</tr>
<tr>
<td>2a</td>
<td>45 000</td>
<td>48 100 ± 1 300</td>
<td>( \beta IV (T52) )</td>
</tr>
<tr>
<td>3</td>
<td>43 800 ± 1 600</td>
<td>40 100 ± 1 200</td>
<td>( \beta II (T46) )</td>
</tr>
<tr>
<td>3a</td>
<td>40 000</td>
<td>33 800 ± 2 100</td>
<td>( \beta IV (T41), \alpha V (T41) )</td>
</tr>
<tr>
<td>4</td>
<td>32 000 - 34 000</td>
<td>30 300 ± 1 900</td>
<td>( \beta II (T35) )</td>
</tr>
<tr>
<td>5</td>
<td>28 000</td>
<td>25 800 ± 1 600</td>
<td>( \beta IV (T28) )</td>
</tr>
<tr>
<td>6</td>
<td>22 000</td>
<td>18 500 ± 1 100</td>
<td>( \beta I (T17) )</td>
</tr>
<tr>
<td>8</td>
<td>15 800 ± 1 200</td>
<td></td>
<td>( \beta I (T12) )</td>
</tr>
</tbody>
</table>
Plate 1. SDS-polyacrylamide gel electrophoresis of spectrin fragments generated during the digestion of isolated spectrin by TPCK treated trypsin. Proteolysis was carried out in 0.1 M phosphate buffer, pH 7.5. Other conditions as in Materials and Methods. Lane (a), without additions; (b), with the addition of liposomes prepared from the mixture of phosphatidylethanolamine and phosphatidylserine (60:40) at the lipid to protein molar ratio of 1,900; (c), similarly as in (b) but the lipid to protein molar ratio was 950; (d), proteolysis in the presence of liposomes prepared from phosphatidylcholine at the lipid to protein molar ratio of 1,900; (e), similarly as (d) but the lipid to protein molar ratio was 950. The positions of the molecular weight standards are indicated (in kDa) at the right margin.

Plate 2. SDS-polyacrylamide gel electrophoresis of spectrin fragments generated in the proteolysis of isolated spectrin by TPCK treated trypsin in the presence of 1 M NaCl. Other conditions and sample content as in Plate 1 and in Materials and Methods.
The content of individual polypeptide fractions determined from the area of scanned peaks is presented in Table 2. Their content in the relation to αI domain (Mr 80 000) is also presented. In the presence of liposomes prepared from the mixture of phosphatidylethanolamine and phosphatidylinerine (60:40) changes in the relative content of several polypeptides were observed. The content of fragment(s) of Mr about 65 000 was increased. In the presence of liposomes prepared from either PE/PS mixture of phosphatidylcholine a decrease of fraction “3” (Mr 41 000) and fractions “5” and “6” taken together as well as of fraction “7” was observed. These changes may be due to the limited accessibility of certain peptide bonds to trypsin in the presence of phospholipid suspensions. The changes of the susceptibility of the basic myelin protein to proteolysis by trypsin were interpreted as indicating the penetration of phospholipid bilayer by certain protein sequences [21, 22]. However, under our experimental conditions the possibility of immobilization of trypsin on the surface of liposomes should be taken into account. There are indications of such an interaction which is, however, abolished by increased ionic strength [23]. Electrophoregrams of the products of proteolysis of spectrin in the presence of 1 M NaCl either with or without the addition of phospholipid suspensions are presented in Plate 2 and Table 3. The patterns obtained were qualitatively different from those obtained in the absence of NaCl. First of all the occurrence of the polypeptide of Mr of about 110 000 as well as the quantitative changes in other fractions were observed. Fragments of Mr of about 100 000 represent the carboxyl terminal fragment of α subunit (αT - 100) and/or amino terminal fragment of β subunit (βT - 110) [5]. The amount of αI

Table 2

Content of individual polypeptide fractions obtained on electrophoresis of spectrin fragments resulting from the proteolysis by trypsin in the presence or absence of phospholipid suspensions. Sv/Si — the ratio of the particular peak area to the area of αI domain. Average values of two independent experiments are shown. Each gel was scanned at least twice. For details see 1.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Proteolysis without liposomes</th>
<th>Proteolysis in the presence of liposomes prepared from PE/PS</th>
<th>Proteolysis in the presence of liposomes prepared from PC</th>
<th>Protein:lipid molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>Sv/Si</td>
<td>Sv/Si</td>
<td>Sv/Si</td>
<td>Sv/Si</td>
</tr>
<tr>
<td>1</td>
<td>28.9</td>
<td>35.4</td>
<td>39.1</td>
<td>41.0</td>
</tr>
<tr>
<td>2</td>
<td>1.9 0.066</td>
<td>5.9 0.17</td>
<td>2.7 0.069</td>
<td>2.7 0.066</td>
</tr>
<tr>
<td>3</td>
<td>6.6 0.23</td>
<td>5.3 0.15</td>
<td>5.4 0.14</td>
<td>4.6 0.11</td>
</tr>
<tr>
<td>4</td>
<td>8.2 0.28</td>
<td>9.0 0.25</td>
<td>8.2 0.21</td>
<td>6.1 0.15</td>
</tr>
<tr>
<td>5+6</td>
<td>16.5 0.57</td>
<td>11.9 0.34</td>
<td>11.5 0.29</td>
<td>11.3 0.28</td>
</tr>
<tr>
<td>7</td>
<td>12.5 0.43</td>
<td>10.0 0.28</td>
<td>8.4 0.21</td>
<td>4.9 0.21</td>
</tr>
<tr>
<td>8</td>
<td>25.5 0.88</td>
<td>25.1 0.71</td>
<td>23.6 0.60</td>
<td>29.6 0.72</td>
</tr>
</tbody>
</table>
Table 3

Content of individual polypeptide fractions obtained on electrophoresis of the spectrin fragments resulting from the proteolysis of spectrin trypsin in 1.0 M NaCl in the presence or absence of phospholipid vesicles.

$S_0/S_1$ — the ratio of the particular peak area to the area of $\alpha$1 domain. Other details in Materials and Methods and in legends to Table 2 and 1.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Proteolysis without liposomes</th>
<th>Proteolysis in the presence of liposomes prepared from PE/PS</th>
<th>Proteolysis in the presence of liposomes prepared from PC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% $S_0/S_1$</td>
<td>1:1 900 molar ratio</td>
<td>1:950 molar ratio</td>
</tr>
<tr>
<td>1</td>
<td>12.87 —</td>
<td>9.97 —</td>
<td>11.60 —</td>
</tr>
<tr>
<td>1a</td>
<td>8.64 0.67</td>
<td>8.79 1.07</td>
<td>9.78 0.88</td>
</tr>
<tr>
<td>2+2a</td>
<td>18.30 1.42</td>
<td>21.78 2.40</td>
<td>21.31 1.91</td>
</tr>
<tr>
<td>3+3a</td>
<td>7.90 0.60</td>
<td>12.16 1.34</td>
<td>10.91 0.98</td>
</tr>
<tr>
<td>4</td>
<td>12.20 0.95</td>
<td>10.90 1.20</td>
<td>10.44 0.93</td>
</tr>
<tr>
<td>5+6</td>
<td>13.20 1.03</td>
<td>11.89 1.31</td>
<td>11.59 1.04</td>
</tr>
<tr>
<td>7</td>
<td>15.43 1.20</td>
<td>15.41 1.69</td>
<td>16.43 1.47</td>
</tr>
<tr>
<td>8+8a</td>
<td>11.51 0.89</td>
<td>8.96 0.99</td>
<td>9.57 0.86</td>
</tr>
</tbody>
</table>

domain is also reduced, which may be due to its cleavage to the fragment of $M_r$ 74,000 [24]. Quantitative changes observed after proteolysis of spectrin in the presence of phospholipid suspensions (Plate 2 and Table 3) in the presence of 1 M NaCl were less significant than at lower ionic strength (Plate 1 and Table 2). However, an increase in the content of fragments of intermediate relative molecular weights namely of $M_r$ 62,000, 59,000, 48,000 and 44,000 was observed (Table 3). A rather unexpected result were the changes in the polypeptide patterns obtained after proteolysis in the presence of phosphatidylcholine which has been shown to interact with spectrin rather weakly as revealed by other techniques [14, 15, 25]. The existence of such an interaction could also be inferred from the observation of polypeptide patterns following the proteolysis of spectrin by pronase in the presence of this phospholipid suspension (Plate 3). When proteolysis was carried out in the presence of the suspension prepared from the mixture of phosphatidylethanolamine and phosphatidylserine the amount of undigested spectrin was increased (Plate 3, lane d). In the presence of smaller amounts of this suspension or in the absence of phospholipids large amounts of low molecular weight polypeptides and poly-peptides of $M_r$ of about 65,000 were present (Plate 3, lane b, e). In the presence of phosphatidylcholine suspension (Plate 3, lanes a and c) a substantial inhibition of proteolysis and generation of a series of fragments of intermediate molecular weight took place. The results similarly to those obtained after proteolysis of spectrin by trypsin would point to the restricted
accessibility of certain peptide bonds in the presence of phosphatidylcholine and phosphatidylethanolamine/phosphatidylserine vesicles. The possibility of interaction of spectrin with phosphatidylcholine vesicles is a rather unexpected result, as there are no data in the literature concerning the specificity of binding of this lipid to spectrin [1]. May be certain regions of spectrin bind to the vesicles without inducing large changes in spectrin conformation which would be manifested e.g. in changes of intrinsic fluorescence of spectrin. In the case of both protease preparations the immobilization of the enzyme on the surface of phospholipid vesicle by ionic interaction can be excluded because of the high salt concentration. However, the behaviour of pronase should be studied in more detail. It should be added that our preliminary results suggests a possible interaction of isolated erythrocyte membrane cytoskeletons with sonicated vesicles prepared from different phospholipids including phosphatidylcholine (Sikorski & Zieliński, unpublished).

The above presented experiments suggest that proteolytic patterns become altered in the presence of phospholipid suspensions. Thus it seemed of interest to compare the results with those obtained by proteolysis of spectrin bound in a natural membrane, i.e. the erythrocyte ghosts. The polypeptides derived from erythrocyte ghost treated with proteases following gel electrophoresis were blotted onto nitrocellulose paper and visualized by staining with anti-spectrin antibodies (Plate 4). When red cell membranes were treated
with trypsin, a considerable amount of undigested spectrin remained (Plate 4, lanes d and e). Also a higher molecular weight fragments of $M_r$ of 100 000 - 120 000 were present in substantial amounts. All three bands of this region exhibit a similar intensity of the stain in contrast to Coomassie Blue stained patterns obtained after proteolysis of purified spectrin by trypsin in the presence of high salt concentration (Plate 2 lane a). It should be noted that fragments of $M_r$ below 25 000 reacting with anti-spectrin antibodies were absent in the electrophoregrams obtained after treatment of erythrocyte membranes with both protease preparations, although such fragments derived from other membrane proteins were present in rather large quantities (Plate 4 lanes f and h).

The patterns obtained after the digestion of spectrin in the membranes by pronase are presented in Plate 4 (lanes a - c). No undigested spectrin was found and about twenty polypeptides of large to intermediate molecular weight were present. The accumulation of polypeptides of $M_r$ about 60 000 - 65 000 was observed similarly as in the case of treatment of purified spectrin with pronase. These fragments seem to be the most resistant to proteolysis by pronase (Plate 4, lane a).

When the reactivity of the antiserum was tested with erythrocyte membrane proteins separated in the SDS-polyacrylamide gradient gel no other poly-

Plate 4. Proteolysis of spectrin in erythrocyte ghosts by pronase at enzyme to membrane protein ratio of (a) 1:20, (b) 1:50, (c) and (f) 1:100; and by trypsin at enzyme to membrane protein ratio of (d) and (h) 1:50, (e) 1:100. About 30 µg of membrane protein was subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred electrophoretically onto nitrocellulose filter paper and incubated with antispectrin rabbit serum followed by goat anti-rabbit IgG conjugated to horseradish peroxidase (lanes a - c). Lanes (f) and (h) stained with Ponceau S, (g) stained with Ponceau S molecular weight standards
peptides except spectrin bands (α and β) were found to react with anti-
spectrin antibodies. No reaction of non-immune rabbit serum with erythro-
cyte membrane polypeptides was observed (not shown). The efficiency of
the transfer of the fragments was checked by staining the gel with Coomassie
Blue; only little of undigested spectrin might have been left in the gel.
Diverse reactivity of antibodies with particular fragments would also affect
the results. In the case of antibodies used in this study, no differences
in the reactivity toward individual fragments of isolated spectrin digested
by trypsin were observed (not shown).

The data discussed above indicate that the interaction of spectrin with
phospholipid vesicles and with the membrane affects the accessibility of
certain peptide bonds to proteases. It should be noted that the possibility
of interaction of spectrin with phosphatidylethanolamine vesicles has not been
reported earlier. The nature of this interaction should be further explored.

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