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Analysis of the Major Polypeptides of Spectrin by Tryptic Digestion

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The two major polypeptides of erythrocyte membrane spectrin have been isolated by preparative polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The tryptic peptide maps of the two polypeptides have been prepared by thin-layer chromatography and electrophoresis. Radioactive peptides have been prepared by ^{14}C-carboxymethylation and chloramine T-catalysed ^{125}I iodination. Maps of both sets of peptides demonstrate a marked similarity between the two parent polypeptides.

Key words: spectrin, fractionation, trypsin digestion, peptide mapping

The term "spectrin" was originally applied [1] to the protein extracted from erythrocyte ghosts when it was believed that the extract was homogeneous. It is now known that the extract contains three major polypeptides, a pair with molecular weights between 200,000 and 250,000 (P1 and P2 in the terminology of Fairbanks, Steck, and Wallach [2]) and the third with a molecular weight of 43,000. This third component has been identified as actin [3], although it is slightly different from muscle actin [4], and the term "spectrin" is now confined to the two high-molecular-weight chains which probably exist as a P1 + P2 dimer or tetramer in the intact membrane [5–9].

The chemical nature of these two large polypeptides is not yet fully understood and we here report on the peptide maps obtained after trypsin digestion of the polypeptides. Maps show that P1 and P2 share many common peptides, which suggests that they share methylation, or with ^{125}I by iodination under chloramine T catalysis. In both cases the maps show that Pa and P2 share many common peptides, which suggests that they share certain common amino acid sequences: a conclusion consistent with their similar amino acid compositions [10–12] the similarity of their N terminal amino acids [13, 14], and their immunological cross-reactivity [15]. The maps of the two polypeptides also possess peptides unique to themselves, indicating that the two chains are not identical.

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EXPERIMENTAL

Preparation of Ghosts

Fresh cattle (Bos taurus) blood was washed three times with isotonic saline to remove white cells and plasma. The erythrocytes were haemolysed in 5 volumes of 5.0 mM phosphate buffer (pH 6.0) and the ghosts washed several times in this buffer until the supernatant was virtually free of haemoglobin. The remaining haemoglobin was almost completely removed from the ghosts by washing repeatedly in 5.0 mM phosphate buffer at pH 7.5. The final trace of haemoglobin was removed by freezing the ghosts at -20° over night and washing once more in the buffer at pH 7.5 [16].

EDTA Extraction

The ghosts were pelleted by centrifugation at 18,000 g for 20 min and extracted with 4 volumes of 0.5 mM ethylenediaminetetracetic acid (EDTA), at pH 7.5, for 1.5 h at 18°.

Polyacrylamide Gel Electrophoresis

Analytical sodium dodecyl sulfate (SDS) gel electrophoresis was carried out using the buffer system of Fairbanks, Steck and Wallach [2]. The gels, of 7% (w/v) acrylamide and 0.1% bisacrylamide, were polymerised in glass tubes with an internal diameter of 4 mm using ammonium persulphate and N,N,N^1,N^1-tetramethylethylene diamine as catalysts. Protein samples were prepared by addition of dithioerythritol to 20 mM and SDS to 5%, heating at 100° for 5 min, and a final addition of urea to 8.0 M. The gels were electrophoresed for 2 h at 5 mA per gel (10 V/cm).

Preparative electrophoresis was performed in a vertical slab gel of the same composition (200 × 200 × 5 mm) with the same buffer, which was continuously recirculated between the two buffer chambers. P1 and P2 were sufficiently separated after 72 h at 7 V/cm.

Proteins were recovered from the gel by cutting the gel slab into 5 mm horizontal strips and macerating each strip in 10 ml 5 mM sodium bicarbonate, 0.5 mM EDTA, 0.5% SDS. After 16 h the gel was filtered off on a stainless steel sieve and the extract centrifuged at 100,000 g for 1 h to remove small pieces of gel. A second 16-h extraction with 5 ml of bicarbonate yielded a second batch of protein indistinguishable from the first. By this process 70% of a radioactive protein was recovered from a gel.

Carboxymethylation

The method was adapted from Harris and Perham [17]. The protein sample (10 ml EDTA extract at 1 mg/ml) was dialysed over night against 50 mM Tris/HCl, pH 8.0, at 4°; 50 μCi of iodo-(2-^{14}C)-acetic acid (10–25 mCi/ mmole, Radiochemical Centre, Amersham) in 0.5 ml water was added to the protein (12 μCi were used for each protein fraction obtained by preparative gel electrophoresis). Solid urea was added to 8.0 M after 30 min at 18° and 0.1 ml β-mercaptoethanol after a further 90 min; 60 min later the mixture was put to dialyse at 4° for 24 h against three changes of 50 mM Tris/HCl to remove diffusible radioactive material. After dialysis 30 mg of nonradioactive iodoacetic in 0.5 ml water (10 mg for the smaller slab fractions) was added, followed by urea to 8.0 M and dithioerythritol to 20 mM. After 90 min at 18° the mixture was dialysed over night in the cold against the buffer used for trypsinisation.

Purification of the ^{14}C Carboxymethyl Peptides

The peptides were concentrated to 1.0 ml by rotary flash evaporation and passed through a Sephadex G50 column equilibrated with water. Radioactivity was confined to a retarded component which was collected, acidified by the addition of formic acid to 0.1 M, and applied to a sulphoxyethyl (SE) Sephadex column equilibrated with 0.1 M formic acid. All the radioactivity was bound to the column, which was washed with the acid, and then water, before elution of the radioactive peptides with 0.3 M ammonium hydroxide. The peptides were concentrated to a small volume for thin-layer chromatography.

Iodination of Proteins

The method was adapted from Hunter and Greenwood [18]. A 1 mg/ml solution of protein (1 ml) was dialysed against 0.2 M phosphate buffer (pH 7.5) containing 0.5 mM EDTA and 0.1% SDS. Then 1 mCi of ^{125}I (Iodine-125, carrier free, Radiochemical Centre, Amersham) was added followed by 10 μl chloramine T solution (5 mg/Ml). The reaction was stopped after a few seconds by 100 μl sodium metabisulphite solution (2.4 mg/ml) and 100 μl of potassium iodide solution (1 mg/ml). Low-molecular weight radioactivity was removed by an overnight dialysis against 0.5 mM EDTA and finally by an over night dialysis against 10 mM Tris/HCl buffer (pH 8.0).

Trypsinisation

The samples in the 10 mM Tris/HCl buffer (pH 8.0) were trypsinised for 24 h at 37° by the addition of 100 μl of a 1 mg/ml solution of pig trypsin (Miles Laboratories, Ltd) and a second batch after 6 h. Pig trypsin has a very low chymotrypsin activity.

Peptide Mapping

The tryptic digest was fractionated on silica thin-layer plates (Polygram SIL N-HR, Camlab, Cambridge). The mixture was chromatographed in the first dimension in ethanol/ammonia (70:30, v/v) and electrophoresed in the second dimension for 4 h at 15 V/cm in a pyridine/acetic acid/water (5:50:945, v/v) solvent at pH 3.5. The radioactive peptides were finally located by autoradiography.

RESULTS

Peptide maps of tryptic digests of spectrin, P1 and P2 labeled with either ^{14}C carboxymethyl side chains or ^{125}I iodination demonstrate the similarity, but not identity, of the two chains. Many peptides are common to both polypeptides but some are peculiar to P1 and others confined to P2. Some common spots differ in intensity between the two polypeptides. The identification of common spots is based on our interpretation of these maps and similar maps made from replicate preparations. Comparable constellations of spots can be seen, but in complicated iodinated maps the possibility of coincidence of nonidentical peptides must be countenanced (Figs 1–3). The presence of the same peptides in maps of both proteins cannot be due to cross-contamination, for analytical gels of the separated P1 and P2 show no cross contamination. Coomassie blue staining would reveal a contamination of less than 10%, and the similarities between the maps could not result from such low levels of contamination. The presence of peptides peculiar to either of the two fractions is additional evidence against cross-contamination.

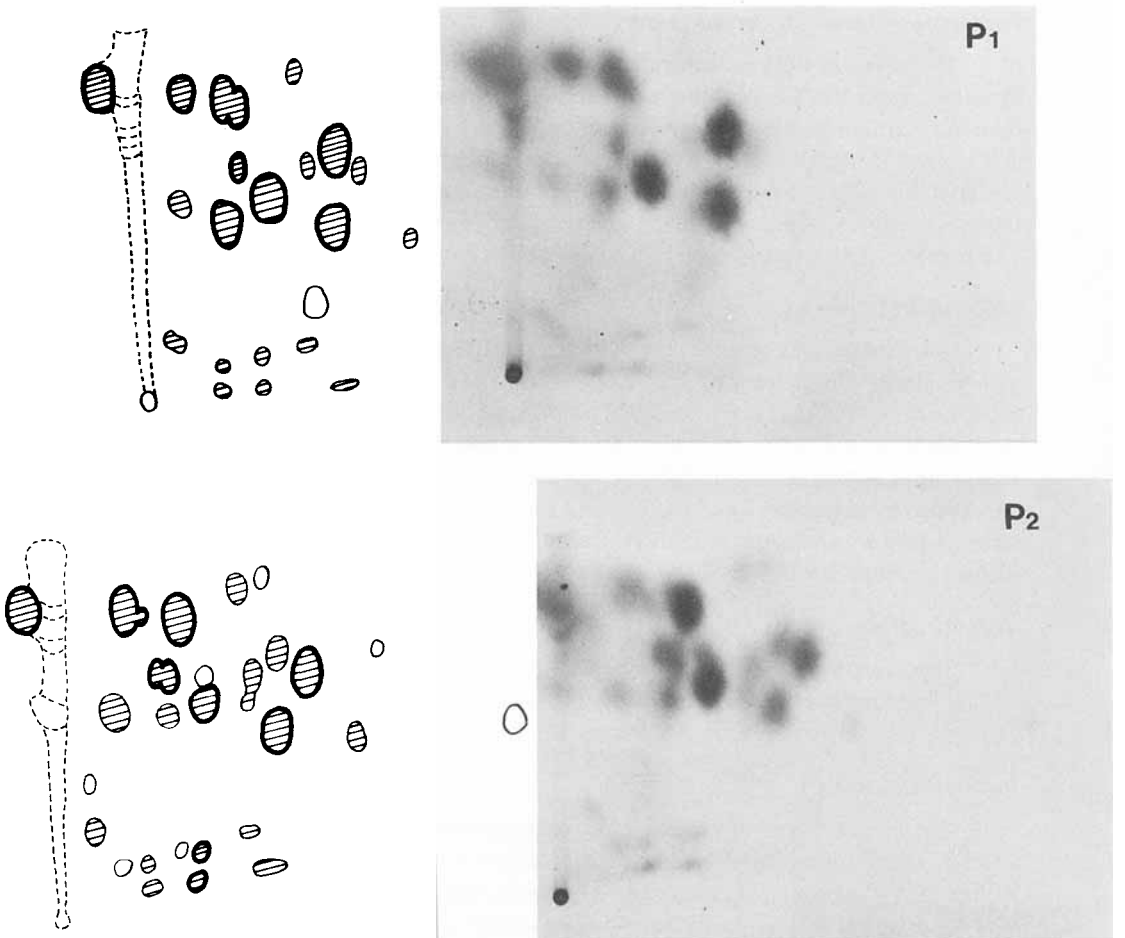

Fig 1. A comparison of the ^{14}C-carboxymethylated peptides in tryptic digests of purified P1 and P2. The maps were prepared by chromatography in ethanol/ammonia followed by electrophoresis in pyridine/acetic acid/water (pH 3.5) in the second dimension. Weak spots in the autoradiographs are represented by thin lines in the line drawings. In the map of P1, peptides also present in P2 are indicated with cross-hatching, and conversely P1 peptides are marked in the map of P2. (Figures 1–3 reproduced with the permission of the editors of the Biochemical Journal from Dunn, Kemp, and Maddy [11]).

No proteolytic activity can be detected in the EDTA extract using standard protein substrates such as azocoll or azoalbumin, but a slow breakdown of P1 and P2 over a period of weeks at 4° may be observed by SDS electrophoresis. Fresh material showing no sign of degradation was always used for the mapping. Maps of polypeptides which had not been digested with trypsin were devoid of radioactivity except at the origin. Cattle red cell ghosts are much less prone to proteolysis from white cells than are those of humans.

The number of peptides (20–25) in the carboxymethyl maps is consistent with the polypeptides being single chains of very approximately 200,000 daltons. (Assuming an average molecular weight of 120 for amino acids, a residue present at 1 mole % would generate around 20 peptides from a 240,000-dalton polypeptide.)

DISCUSSION

Peptide maps of trypsin digests of carboxymethylated and iodinated proteins show the close similarity of P1 and P2. This similarity has previously been inferred from immunologic data, N-terminal analyses, and the amino acid compositions of these same fractions, but the immunology and N-terminal content also led to the conclusion that P1 and P2 were heterogeneous. The relative simplicity of the present maps, particularly carboxymethylated maps, confirm the more widely held belief that P1 and P2 are single polypeptides of high molecular weight.

The immunologic data are ambiguous. Bjerrum et al [15] raised antisera against spectrin which contained antibodies that reacted with P1 and P2, and the P1 and P2 antibodies cross-reacted with each other but not with any other membrane proteins. By contrast the antisera raised by Sheetz, Painter, and Singer [19] against spectrin contained antibody against P1 which did not react with P2.

Comparison of P1 and P2 by their N-terminal end amino acids showed that each had several N-termini and that they both contained the same mixture of N terminal ends [13, 14]. The multiplicity of N termini was found when the usual precautions were taken to minimise proteolytic degradation and the analyses were performed on high-molecular-weight fractions which comigrated with bands P1 and P2 in freshly prepared ghosts, ie, the same type of preparation as used in the present report. The multiplicity of ends is inconsistent with the generally held view that the two fractions consist of single high-molecular-weight polypeptide chains. This apparent anomaly could arise from a limited exopeptidase activity which does not change the molecular weights enough to be detected by gel electrophoresis, or, conceivably, the SDS does not remove some tightly bound small peptides from the long chains [20]. A third possibility which has not been excluded is the formation of covalent cross-links between chains under the influence of the transaminase

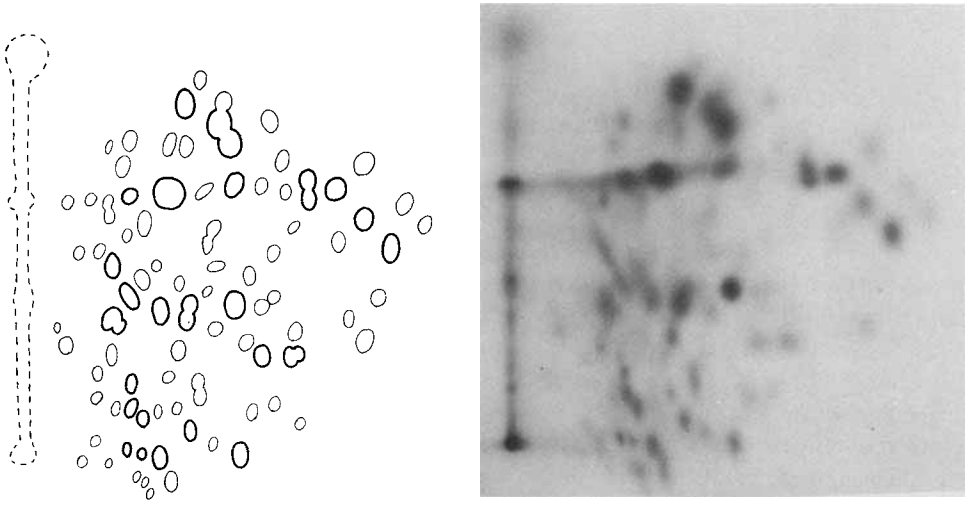

Fig 2. The tryptic map of ox spectrin labeled with ^{125}I with chloramine T catalysis. The map was prepared by chromatography in ethanol/ammonia followed by electrophoresis in pyridine/acetic acid/water (pH 3.5) in the second dimension. Weak spots in the autoradiograph are represented by thin lines in the line diagram.

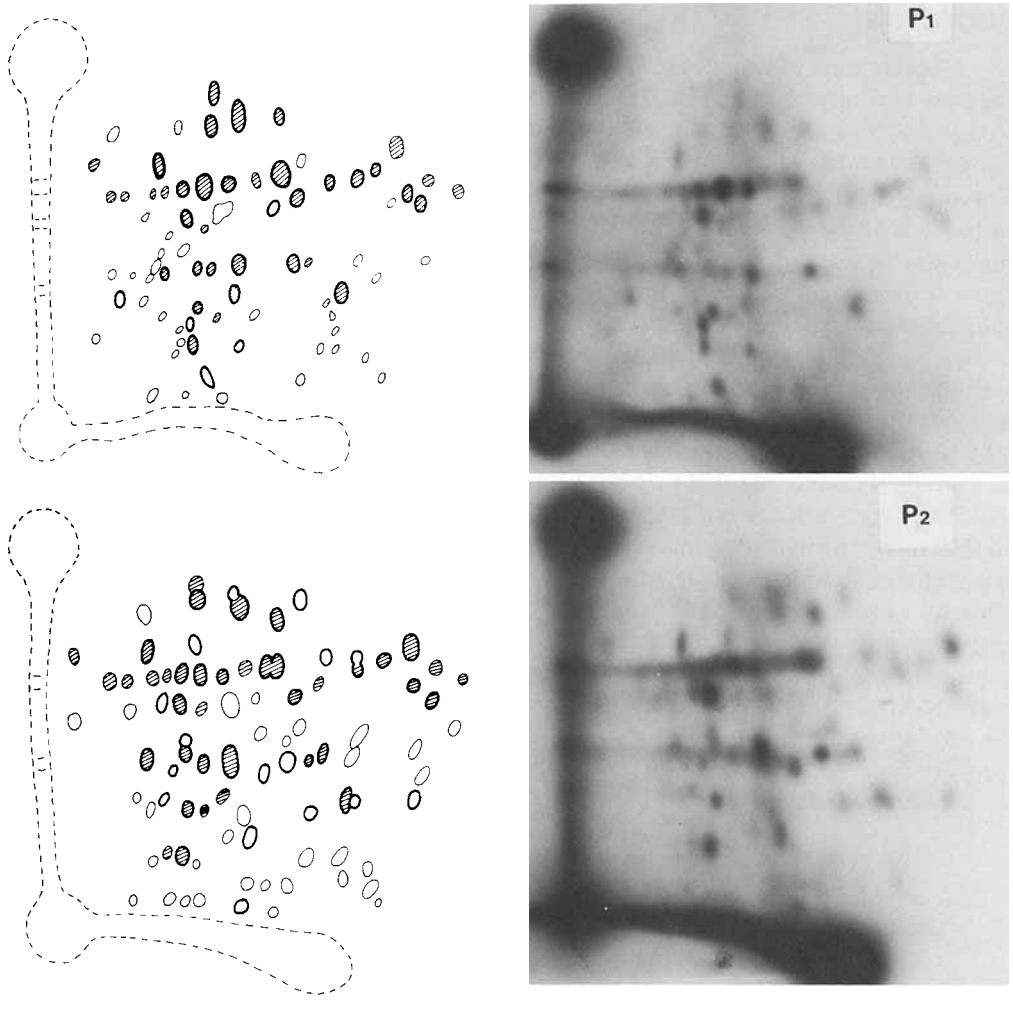

Fig 3. A comparison of the tryptic digests of ^{125}I-iodinated P1 and P2. The large radioactive area spreading from the origin in the second dimension is an iodinated product of the polyacrylamide gel used during the preparation of P1 and P2. In the line drawing of P1, peptides also present in P2 are indicated with cross-hatching and conversely P1 peptides are marked in the map of P2.

present in erythrocytes [21]. Whatever the true explanation for the identical results obtained from both P1 and P2, they point to the similarity of the two polypeptides.

Similarity of the amino acid compositions of P1 and P2 have been reported by three laboratories [10–12]. Too much significance cannot be attached to similarity of amino acid composition, but both fractions possess the high leucine and glutamate contents that characterise the unfractionated spectrin.

The biologic significance of the similarities between the two chains is not obvious. Fukui, Nachbar, and Salton [22] observed common peptides shared by several different proteins extracted from Micrococcus lysodeikticus membranes and concluded that these were derived from segments of proteins which, in vivo, were buried in the lipid hydrocarbon domain and were similar in structure because they all served the same function of

anchoring the proteins into the membrane. This explanation is unlikely to apply to the spectrin polypeptides, as spectrin is an extrinsic protein. Another explanation arises out of the model proposed by Ralston [9] for the organization of spectrin in the membrane. Ralston has suggested that the P1 + P2 dimer consists of one P1 chain and one P2 chain at 90° to each other and that these dimers associate as four-armed tetramers. These tetramers are then assembled, with the involvement of actin, into a continuous two-dimensional array on the inner surface of the membrane. It might be argued that the maintenance of symmetry in such an array is achieved by the similarity of the two constituent polypeptide chains. However, the similarities between the chains that have been revealed by the peptide mapping must not be allowed to overshadow the differences. In addition to their differing size and the differences between the peptide maps, P2 can be phosphorylated [23–25] and its phosphorylation affects cell shape [26, 27] and its interaction with actin [4].

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