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IMMUNOELECTROPHORETIC HETEROGENEITY AND CROSS-REACTIONS OF INDIVIDUAL "SPECTRIN" COMPONENTS ISOLATED BY PREPARATIVE SODIUM DODECYLSULFATE–POLYACRYLAMIDE-GEL ELECTROPHORESIS

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SUMMARY

1. Each of the high molecular weight protein bands 1, 2 and 2.1 of human erythrocyte membranes defined and isolated by sodium dodecylsulfate–polyacrylamide-gel electrophoresis reveals up to five distinct components when examined by quantitative immunoelectrophoresis in 1 % Berol using rabbit antibodies raised against whole membranes.

2. Cross-reactions amongst the components of these 3 fractions can be detected.

3. Quantitative analysis shows that the immunologically identical components are present in different relative concentrations in these bands.

INTRODUCTION

The solubility of membrane proteins in sodium dodecylsulfate and the high resolution and good reproducibility of the separation obtained by electrophoresis in sodium dodecylsulfate-laden polyacrylamide make this a convenient method for analysis of such proteins [1, 2]. However, there is increasing evidence that not all proteins/peptides migrate as individual molecular entities in sodium dodecylsulfate plus reducing agents [3–8]. Certain high-molecular-weight proteins of the erythrocyte membrane, often referred to as "spectrin", resolve in sodium dodecylsulfate–polyacrylamide-gel electrophoresis as 3 distinct bands termed Bands 1, 2 and 2.1 [1] which have molecular weights of approx. 320 000, 280 000 and 240 000, respectively. Several recent studies show that these comprise multiple molecular entities, and may represent aggregates which remain undissociated in sodium dodecylsulfate [6, 8–10].

Quantitative immunoelectrophoresis is a sensitive method for detecting protein heterogeneity [11]. Because membrane proteins solubilized in sodium dodecylsulfate retain some of their antigenicity [12–14], we have been able to combine preparative sodium dodecylsulfate–polyacrylamide-gel electrophoresis with immunoelectrophoretic analyses and thus obtain further information on the molecular nature of "spectrin".

MATERIALS AND METHODS

Unless otherwise stated, we obtain all chemicals and biochemicals from Serva (Heidelberg). We isolate human erythrocyte membranes as in [15] and prepare crude "spectrin" as in [16]. We avoid proteolytic degradation as in [8]. We perform analytical sodium dodecylsulfate–polyacrylamide-gel electrophoresis as in [1] and preparative sodium dodecylsulfate–polyacrylamide-gel electrophoresis with a multislab apparatus [17] according to Knüfermann, H., Bhakdi, S. and Wallach, D. F. H. (unpublished). In all experiments we use 5 % acrylamide gels cross-linked to 2.5 % with *N,N'*-methylene-bisacrylamide. For a preparative run, we apply approx. 10 mg of membrane protein solubilized in 3 % sodium dodecylsulfate heated to 100 °C for 2 min in the presence of 0.25 M 1-mercaptoethanol and prestained with *O*-phthalaldehyde according to Weidekamm et al. [18], to each gel slab. The electrophoresis buffer is 0.04 M Tris, 0.02 M sodium acetate, 0.002 M EDTA(pH 7.4) containing 0.1 % sodium dodecylsulfate. Following electrophoresis, we visualize separated bands under ultraviolet light. We cut these out, homogenize the gels by forcing them through a syringe and elute overnight at 4 °C with 8 vol. of 5 mM phosphate buffer (pH 8.0) which does not contain sodium dodecylsulfate. We subject aliquots of the supernatant fluids to re-electrophoresis in sodium dodecylsulfate as controls.

For immunoelectrophoretic analyses, we concentrate the supernatant fluids over Amicon PM 10 membranes to estimated concentrations of 0.1–0.4 mg/ml, make these samples 1 % in Berol and stir for 30 min at 4 °C, whereby the proteins of Bands 1, 2, and 2.1 remain in soluble form. We perform crossed [19] and crossed-line [20] immunoelectrophoresis with purified, concentrated rabbit immunoglobulins against human erythrocyte membranes [21] in 1 % agarose (Batch 102 D, Litex, Glostrup, Denmark) containing 0.038 M Tris–0.10 M glycine buffer, (pH 8.9, 16 °C) and 1 % (w/v) Berol (Berol EMU-043, MoDoKemi, Stennungssund, Sweden) as in [21].

RESULTS

Fig. 1 shows the normal sodium dodecylsulfate–polyacrylamide-gel electrophoresis reference pattern of hypotonically lysed human erythrocyte membranes, the re-electrophoresed Bands 1, 2, and 2.1 obtained by preparative sodium dodecylsulfate–polyacrylamide-gel electrophoresis, and the pattern of crude "spectrin" which exclusively contains a mixture of the same Bands 1, 2, and 2.1. Sodium dodecylsulfate bands are numbered as in [10].

With antibodies against human erythrocyte membranes, crossed immunoelectrophoresis in 1 % Berol resolves each of the three sodium dodecylsulfate bands into 4–5 precipitation arcs (Fig. 2). The areas below the arcs are proportional to the volume applied. The same heterogeneity is also found in gels without incorporation of Berol, but the non-ionic detergent increases the sharpness of the precipitates and thereby the number of distinguishable components. Other studies [22] have shown that Berol does not cause artifacts in our electrophoresis system. Control experiments performed without antibodies, as well as with γ-globulins from non-immunized rabbits demonstrate that none of the observed precipitation arcs arise from non-specific aggregation of membrane proteins. The residual sodium dodecylsulfate present in the samples also does not create artifacts, because model experiments using crossed and line

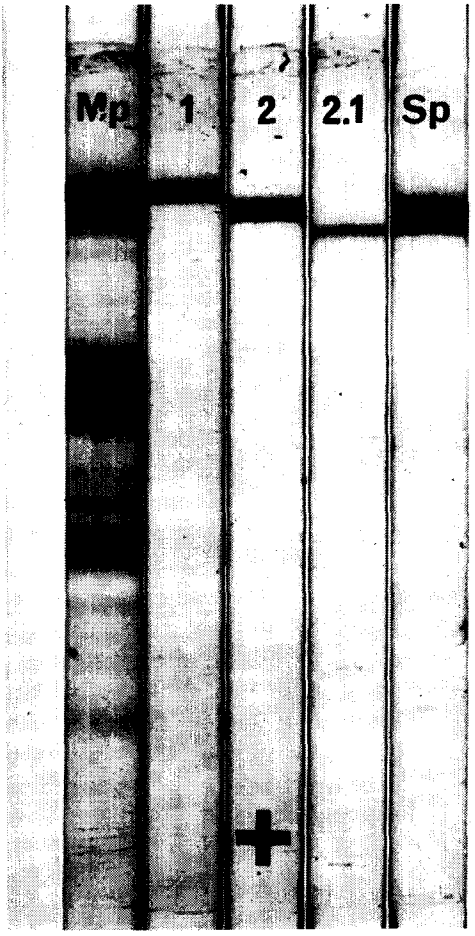

Fig. 1. Sodium dodecylsulfate–polyacrylamide-gel electrophoresis of human erythrocyte membrane proteins (Mp). Bands 1, 2 and 2.1 and crude spectrin (Sp) from preparative sodium dodecylsulfate–polyacrylamide-gel electrophoresis. Gel concentration 5 % with 2.5 % cross-linking. Buffer 0.04 M Tris, 0.02 M sodium acetate, 0.02 M EDTA and 1 % sodium dodecylsulfate (pH 7.4). Current 5 mA/gel. Duration of the run: 2 h. Staining: Coomassie Brilliant Blue.

immunoelectrophoresis with crude "spectrin" show that as much as 0.3–0.5 μmole of free sodium dodecylsulfate do not affect the precipitates when 1 % (w/v) Berol is incorporated in the samples and gels. We never exceed this limit in our experiments.

When sodium dodecylsulfate fractions are examined by Ouchterlony double diffusion against the same antibodies, only one precipitate can be visualized in each case. This agrees with earlier findings on crude "spectrin" [23] and demonstrates the higher resolving power of our immunoelectrophoretic system.

Crossed-line immunoelectrophoresis enables us to establish cross-reactions between the antigenic components of each sodium dodecylsulfate fraction [20]. We therefore perform crossed immunoelectrophoresis of a purified band but incorporate one of the other purified sodium dodecylsulfate bands in an intermediate gel strip

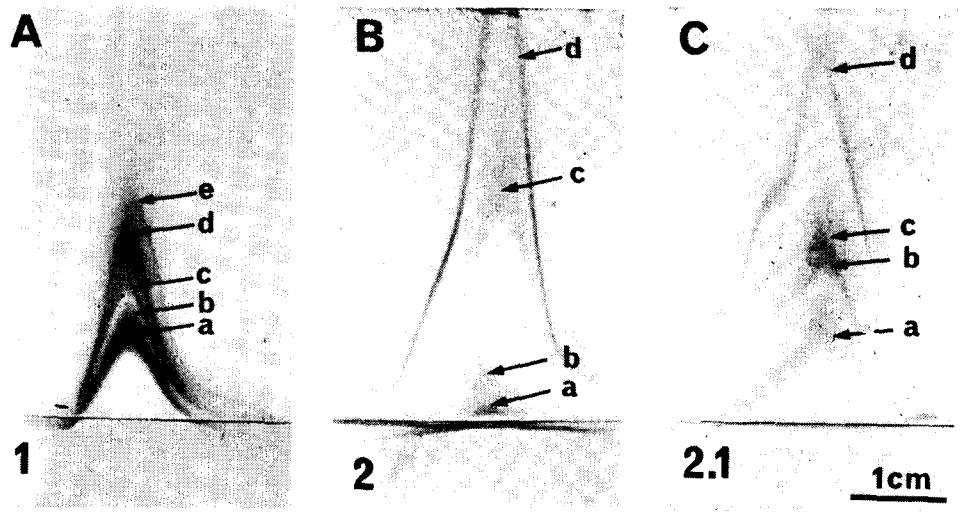

Fig. 2. Crossed immunoelectrophoresis of sodium dodecylsulfate Bands 1(A), 2(B) and 2.1(C). Arrows indicate the antigenic components. The electrophoresis was performed in presence of 1 % (w/v) Berol EMU-043 at pH 8.9 (16 °C) with 10 V · cm^{-1} for 35 min in the first dimension and 2 V · cm^{-1} for 18 h in the second dimension. Antibody content of the gels 0.12 mg · cm^{-2} (A,B) and 0.6 mg · cm^{-2} (C). Staining: Coomassie Brilliant Blue.

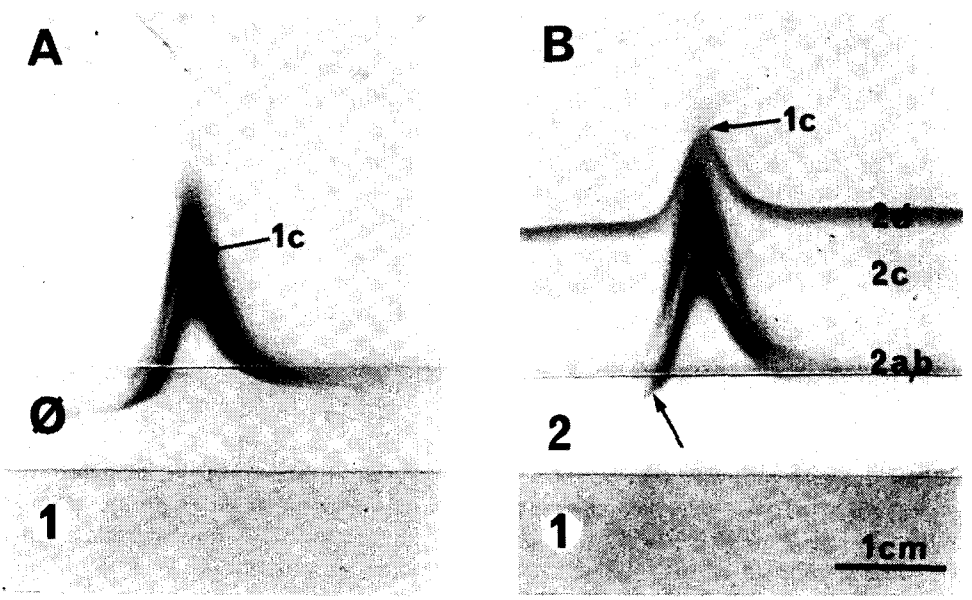

Fig. 3. Identification of common antigenic components. (A) Crossed immunoelectrophoresis of 10 μl of sodium dodecylsulfate Band 1 with blank intermediate gel (ϕ). (B) Crossed-line immunoelectrophoresis of 10 μl of band 1 with 50 μl of Band 2 in the intermediate gel. Antibody content of the gels of 0.12 mg · cm^{-2}. Conditions otherwise as for the experiment demonstrated in Fig. 2.

(see Fig. 3). Cross-reactions are established when (1) a precipitation arc is elevated in comparison with a control plate where a corresponding blank intermediate gel is used, or (2) when a precipitation arc is fused on to a line precipitate [24]. The results of crossed-line immunoelectrophoresis with Fractions 1 and 2 are shown in Fig. 3. Fraction 2 in the intermediate gel gives rise to lines which elevate all precipitates derived from Fraction 1. Therefore, all antigenic components of Band 1 are also present in Band 2. With this technique we also show that these antigenic components are present in Band 2.1. As expected, we find that when we incorporate crude "spectrin" into the intermediate gel the precipitation derived from this also elevates all the precipitation arcs derived from Band 1, 2, and 2.1.

Because the precipitation lines of each sodium dodecylsulfate bands represent antigenic components common to every fraction these experiments permit a semiquantitative comparison of the components. Fig. 2 shows that the relative concentrations of the antigenic components differ from band to band. Thus, the five antigenic components of Band 1 which we arbitrarily name 1a–1e from bottom (Fig. 2) are present in a ratio of approx. 1 : 1.7 : 2.1 : 3.3 : 4.4 (1a : 1b : 1c : 1d : 1e) as determined by planimetry of the area outlined by the respective arcs. In contrast, sodium dodecylsulfate Band 2 shows 4 distinct precipitation arcs named 2a–2d whose ratio is approx. 1 : 2.5 : 16 : 33. For Band 2.1 the ratio is 1 : 15 : 18 : 48. However, we cannot directly correlate the components of the bands, i.e. Component 1a need not correspond to Component 2a, etc. Such a correlation is possible in certain cases after crossed-line immunoelectrophoresis as shown in Fig. 3. Thus, in Fig. 3B we see that Band 2 incorporated in an intermediate gel gives rise to 4 precipitation lines, corresponding to the arcs 2a–2d seen in crossed immunoelectrophoresis of this band. When crossed-line immunoelectrophoresis is performed with Band 1 as a sample, we see elevation and clear fusion of one arc of Band 1 on to the major Component d of Band 2. A comparison between this plate and the control plate (Fig. 3A) shows that the uplifted arc corresponds to Component c of Band 1. Because of the small amount of Components 2a and 2b these lines are very faint and depressed, and it is not possible to correlate them exactly to the corresponding components of Band 1. Nevertheless, slight elevation of the Components 1a, 1b, 1d and 1e by components of Band 2 is always seen, and vice versa Components 2a–2d are always elevated by the components of Band 1 or 2 and 2.1, when they are incorporated as lines. In Fig. 3B (arrow) the descending part of Precipitate 1a crosses the lines of Components 2a and 2b because backward flow, owing to electroendosmosis, has brought antibodies in contact with the slowly moving part of Component 1a.

DISCUSSION

It is well established that one of the major proteins of the erythrocyte membrane, widely referred to as "spectrin" can be partly eluted by dialysis of membranes against buffers of low ionic strength [25]. Attempts to define "spectrin" on molecular terms were first hampered by the difficulty in avoiding aggregation and the lack of suitable methods for molecular analysis. However, by sodium dodecylsulfate–polyacrylamide-gel electrophoresis "spectrin" was defined molecularly as consisting of two major components, termed Band 1 and 2 [1]. On the basis of experiments employing this separation system one would conclude that each of the individual "spectrin" bands

indeed represents a unique molecular entity [26].

However, several recent investigations indicate that the molecular nature of "spectrin" might be more intricate than previously assumed. First, Maddy and Dunn [6] report that a low molecular weight erythrocyte membrane protein can aggregate to yield "spectrin"-like complexes. Second, N-terminal amino acid analysis of the individual "spectrin" Bands 1 and 2 [8] have shown these to possess multiple N-termini; surprisingly, the various N-termini of Band 1 were identical with those found for Band 2. Third, two-dimensional separation of EDTA-extractable erythrocyte membrane proteins [10] shows that the "spectrin" Bands 1 and 2 probably possess identical isoelectric points. Fourth, preparative isotachophoresis in 6 M urea yields two classes of components each with several immunochemically distinct constituents [27].

Our present data supplement these findings. Each "spectrin" band as defined by sodium dodecylsulfate–polyacrylamide-gel electrophoresis yields multiple precipitation lines in quantitative immunoelectrophoresis, revealing existence of several immunochemically distinct constituents. A surprising finding is the demonstration of cross-reactions between the "spectrin" bands. These data might be explained by the assumption that molecular heterogeneity exists in each band, or is induced by a factor in the antibody preparation; the cross-reactions reflect the existence of common antigenic determinants in the band. Such an assumption would still be compatible with the idea that sodium dodecylsulfate gives rise to monomers of "spectrin", and that the estimated molecular size of the components is correct as determined by detergent–gel electrophoresis.

Keeping the other published data [3–10, 28] in mind, however, one should also consider the possibility that multiple polypeptide "subunits" could give the "spectrin" pattern in sodium dodecylsulfate–polyacrylamide-gel electrophoresis. This would imply that "spectrin" in sodium dodecylsulfate does not represent monomers, but that the subunits are dissociable from each other during immunoelectrophoresis, due perhaps to equilibrium-shifting effects [28]. At this point, we cannot differentiate between these two possibilities, but further analyses aimed at determining the molecular weights of the individual antigenic components detectable by immunoelectrophoresis are now underway which should provide evidence in favour of one or the other hypothesis.

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