ASSOCIATION OF HIGH-MOLECULAR WEIGHT PROTEINS IN THE RED CELL MEMBRANE

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Received 26 June 1972

1. Introduction

Red cell membranes contain two species of polypeptide chains of very high molecular weight [1], which make up about a quarter or more of the total protein. They belong to the class of so-called "tektins" [2], which are extracted into media of low ionic strength, and they contain no carbohydrate. In polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, the two species can be separated [3,1,4], and their molecular weights have been variously estimated as 200,000 to 240,000, and 220,000 to 255,000. It seems likely that the "spectrin" component of the membrane, studied by Marchesi and his co-workers [5], can be identified with this same fraction, even though their estimates of molecular weights, based on different methods, are much lower. Marchesi et al. have shown that when the ionic strength is raised, their preparations form filamentous structures. In the absence of denaturants, the two high molecular weight chains do not separate electrophoretically or in gel filtration or velocity sedimentation, and only a single precipitin line is obtained in immunodiffusion experiments [6]. Trayer et al. [4] have suggested that spectrin may be simply a mixture of proteins adventitiously extracted from the membrane at low ionic strength.

We present here the results of cross-linking experiments on intact ghosts, in which we show that there is a correspondence between the two high-molecular weight chains, from which it can be inferred that they are uniquely associated with one another in a pairwise manner. Our evidence is that they are arranged in a highly aggregated system, with few contacts, if any, between like chains.

* Recipient of a Fellowship from the Deutsche Forschungsgemeinschaft.

2. Methods

Ghosts from normal human red cells were prepared by standard procedures [7]. The cross-linking reagent was dimethyl suberimidate [8], which was synthesized from suberonitrile, as described by Davies and Stark [8]. The ghosts at 0.1 mg/ml were suspended in 0.02 M Tris buffer pH 8.5, containing 3×10^{-5} M $CaCl_2$, and the reagent was dissolved in the same buffer and immediately added to the ghosts. The reaction was allowed to proceed for 3 hr at an optimal reagent concentration of 0.2 mg/ml. The pH was then brought to neutral with dilute acetic acid and the ghosts were washed by suspension in the Tris-$CaCl_2$ buffer. They were then solubilised in the gel electrophoresis buffer, made 3% in sodium dodecyl sulphate and 1% in 2-mercaptoethanol, with heating for 3 min at 60°, and applied to 2.5 or 3.5% polyacrylamide gels, containing 5% methylenebisacrylamide. Electrophoresis in the system of Weber and Osborn [9] was performed at 3–4 V/cm. The gels were stained with Coomassie Brilliant Blue, and destained by agitation in a mixture of water, acetic acid and methanol (35:3:2). The ammonolysis reaction for cleavage of the cross-links was performed directly on the separated components in slices of polyacrylamide, cut from stained or unstained gels. The unstained samples were shaken for about 1.5 hr in a mixture of concentrated ammonium

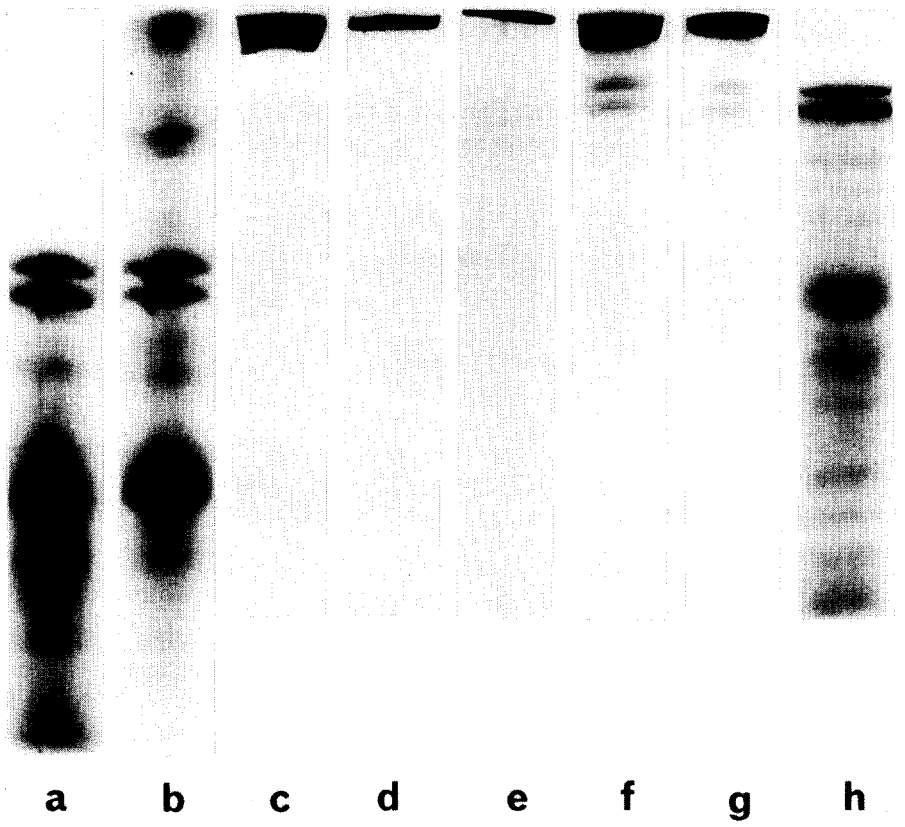

Fig.1. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate of high-molecular weight red cell membrane proteins, before and after cross-linking and ammonolysis. (a) Untreated red cell membrane proteins; note that the gel above the two high-molecular weight components ("tetkins" or "spectrin") is clear; (b) Ghosts solubilized after exposure to 0.2 mg/ml dimethyl suberimidate, showing appearance of new components in the dimer position and higher; (c) Re-electrophoresis of "dimer" seen in (b); (d) Re-electrophoresis of next higher-molecular weight zone generated by cross-linking; (e) Re-electrophoresis of high-molecular weight zone from the top of the gel, generated by cross-linking; (f) Electrophoresis of "dimer" component after partial ammonolysis (see text), showing re-appearance of original bands; (g) Electrophoresis of higher molecular weight (middle) component after partial ammonolysis; (h) Untreated red cell membrane proteins, as (a) but at higher polyacrylamide concentration. The same pattern as in (f) is also produced when the three major components of the dimer are individually subjected to ammonolysis. Polyacrylamide concentrations 2.5% in (a) and (b), and 3.5% in (c) to (h).

hydroxyde (35%), acetic acid, 2-mercaptoethanol and 10% sodium dodecyl sulphate in water, in the volume ratio 75:5:1:10. Stained samples were treated in the same way after prior solubilisation of the precipitated protein by heating for 3 min at 60° in 0.1 M phosphate buffer, 3% sodium dodecyl sulphate, 1% 2-mercaptoethanol pH 7.2. The gel slices were finally re-equilibrated with the same buffer for 1 hr, with several changes, applied directly to the tops of cylindical polyacrylamide gels, and subjected to electrophoresis and staining as before.

3. Results

Gel electrophoresis of the solubilised membrane proteins in the presence of sodium dodecyl sulphate gives results similar to those obtained by other workers [1,3,4]. At the low gel concentrations used, the two chains were easily resolved, and it is also apparent (fig.1) that there are no components of higher molecular weight. Increasing exposure to dimethyl suberimidate causes the progressive appearance of zones in the high-molecular weight region, with diminution

in concentration of the original "spectrin" components (as well as changes in other proteins). When the dimethyl suberimidate concentration exceeds about 1 mg/ml, it is no longer possible to achieve clean separations of the ghost proteins. At low concentrations one sees only a group of three zones close together, in the region of the gel in which dimers of 400–500,000 molecular weight would be expected to migrate. At higher reagent concentrations further, apparently heterogeneous, components appear, as well as still higher molecular weight material, which barely enters the gel (fig. 1). That the first group of zones in fact arises from the spectrin components is demonstrated by ammonolysis: this gives rise, as also shown in fig.1, only to the two original components in equal intensity, and no other protein bands. The same results are obtained whether or not the protein has gone through the staining process. The second, higher molecular weight band, when subjected to ammonolysis, breaks down to give the original chains and some of the dimer, and the yet larger material from the top of the gel similarly generates all the lower molecular weight components. In no case can any proteins migrating more rapidly than the spectrin bands be detected. When the ghost protein after cross-linking was examined in 2.0% polyacrylamide gels, at least four oligomeric species could be resolved between the dimer zones and the top of the gel.

The possibility must next be considered that the three components of the dimer have different compositions: if the two spectrin components are denoted by A and B, oligomers containing both might be expected to give rise to the three species , AA, AB and BB. We therefore performed the ammonolysis experiments on the three dimer zones, individually cut out of the gel after staining. All three zones gave rise after ammonolysis to both A and B, in apparently equal proportions. It thus appears that the three zones differ only in the mode of cross-linking and that no significant number of cross-links between like chains can be formed.

4. Discussion

Our results seem to leave no room for doubt that the two high-molecular weight polypeptide chains of the red cell membrane are associated with one another, as subunits of an aggregate. By contrast with the situation in oligomeric proteins in solution [8], cross-linking by intermolecular reaction is impossible, at least in the absence of free translational diffusion of proteins in the membrane. It thus follows that the higher cross-linked forms also arise from inter-chain contacts, and are of the kind $(AB)_n$. The existence of three dimeric forms is curious and suggests that there may be only three regions on each chain where lysines occur sufficiently closely to lysines in the neighbouring chain to permit cross-linking. A cross-link joining the A and B chains near their middles will cause a reduction in the hydrodynamic radius of the unfolded dimeric chain, compared with a cross-link joining the chains near their ends. It is interesting that Clarke [10] has found that the two chains in the extracted protein in aqueous solution can be similarly cross-linked, and also apparently give rise to a triplet. Clarke indeed concluded that the chains were in an oligomeric form, possibly a dimer, in these conditions. One may surmise that the geometry of the association in the membrane is preserved in solution, at least as between pairs of chains. The further association of the hetero-dimer unit, which our results demonstrate, must be of a heterologous kind, and may be related to the filament-forming tendency of the whole spectrin [5,6].

The absence of homodimer species in our experiments suggests that there are no, or spatially limited, AA or BB contacts in the aggregate, which must on the basis of our results contain at least twelve chains and very probably a much larger number, the higher species not being resolved in the gel. The simplest model would involve some kind of sandwich structure of the type ...ABABAB... though another possibility would be AB dimers in which the chains, which appear to be highly disymmetric [10], lie parallel, or at least extensively in contact, the dimers themselves being associated by way of sites of small contact area. It seems likely that the aggregates have a structural role in maintaining the state of the intact cell.

The ammonolysis reaction cannot in our hands be driven to completion, for under more extreme conditions peptide hydrolysis ensues, with the formation of smears in the polyacrylamide gels. Presumably those neighbours joined by the smallest number of cross-links are most readily cleaved. Since the completion of this work, Steck [11] has reported that a num-

ber of the proteins in red cell ghosts can be cross-linked to one another by other reagents than are used here. The high-molecular weight chains are involved in these reactions. Steck's results are not equivalent to ours, but do not seem to be in any sense incompatible with our conclusions.

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