THE MOLECULAR STATUS OF THE LARGE POLYPEPTIDES OF ERYTHROCYTE MEMBRANES

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1. Introduction

The 'solubilisation' of membrane proteins by sodium dodecyl sulphate (SDS) and subsequent fractionation of the protein–detergent complexes by polyacrylamide gel electrophoresis or gel exclusion chromatography in the presence of SDS is now by far the most widely used technique in the analysis of membrane proteins. It is claimed that this method is preferable to others as the results are not complicated by protein aggregation as proteins are always dissociated into their constituent polypeptides, which may then be separated on the basis of their differing molecular weights. On the grounds of the observations we describe here, and other reports in the literature, we submit that this claim is not completely valid and that results obtained by this method need to be interpreted with caution.

Several authors [1–3] have observed that the proteins extracted from erythrocyte ghosts by dilute solutions of ethylenediamine tetraacetic acid (EDTA) are seen by SDS gel electrophoresis to consist predominantly of proteins of low mobility and hence of high molecular weight. Most workers [2, 3] record a molecular weight in the region of 200 000 for these proteins and also note a smaller amount of protein of high mobility (mol. wt about 40 000). We find that an analagous fractionation of this same mixture of proteins can be achieved by polyacrylamide electrophoresis in a buffer of 'Tris'/glycine/EDTA. There is protein of low and high mobility, and the protein of low mobility in 'Tris'/glycine is also slow moving in SDS and that of high mobility in 'Tris'/glycine also fast moving in SDS. The two types of protein when re-electrophoresed run true in both buffers. However, if the fast moving protein from the 'Tris'/glycine system is passed down a Sephadex G-200 column and then examined by SDS electrophoresis it is found to contain slow moving (200 000) bands which were not present before the chromatography and must have been generated on the column from the '40 000' protein. As '200 000' complexes which are stable in SDS can be formed from smaller components the possibility that the '200 000' proteins in the original EDTA extract are of this nature and not large polypeptides must be countenanced.

2. Experimental

2.1. *EDTA extraction*

Ghost cells were prepared from ox (*Bos taurus*) blood as previously described [4] by a modification of the procedure of Dodge et al. [5]. Four vol of 0.5 mM EDTA, adjusted to pH 7.5 with NaOH were added and the suspension left to dialyse against this EDTA solution at 4°C for 16 hr. The insoluble material was removed by a centrifugation of 1 hr at 65 000 *g* [6].

2.2. *Polyacrylamide gel electrophoresis*

Analytical polyacrylamide gel electrophoresis was carried out using either a 'Tris'/glycine/EDTA buffer system [6] or an SDS buffer system [7].

Preparative gel electrophoresis was carried out by a slab method using the 'Tris'/glycine/EDTA buffer system [6]. 130 ml of the acrylamide solution were polymerised in a mould 20 × 20 × 0.4 cm. The slab was pre-run for 6 hr at 300 V, and then 5 ml of pro-

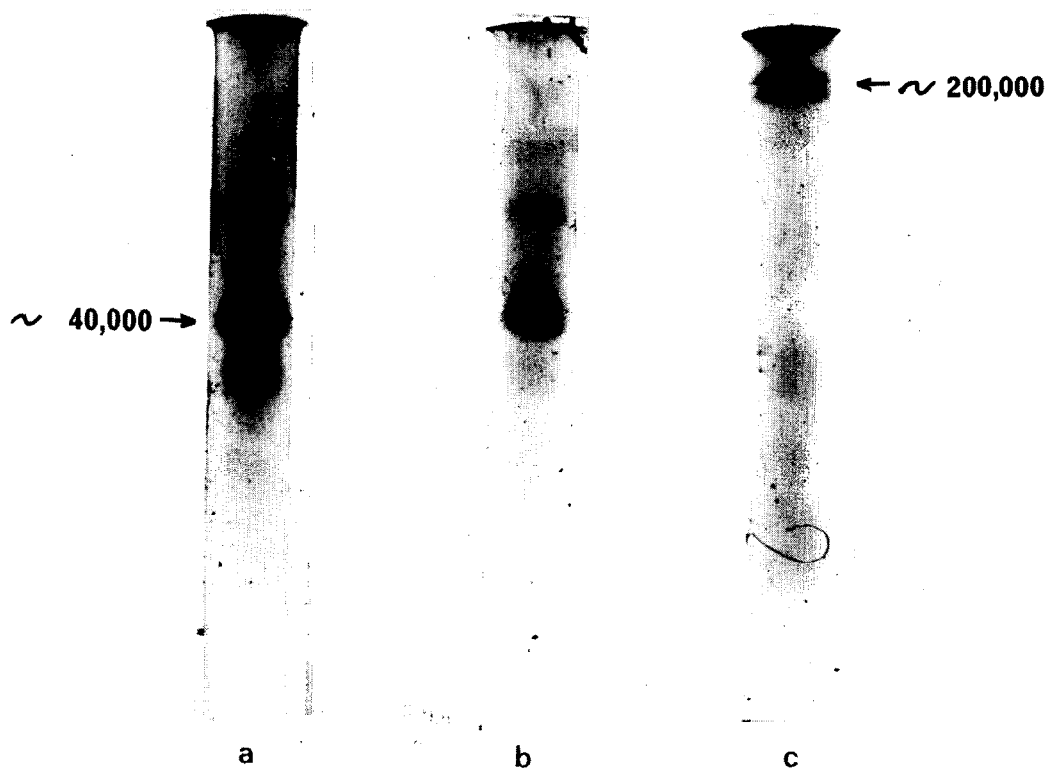

Fig. 1. The effect of Sephadex G-200 chromatography in dilute phosphate buffer on the apparent molecular weights of membrane proteins as determined by the SDS electrophoresis method: a) The material applied to the Sephadex column; b) The excluded components; c) The retarded components.

tein solution (5 mg/ml) were applied and electrophoresed for 18 hr at 6 V cm^{-1}.

After electrophoresis the slab was cut up into 0.5 cm slices and each slice homogenised in 2.5 mM phosphate + 0.5 mM EDTA buffer (pH 8.0, 8.0 ml). The homogenates were spun, re-extracted twice with the same buffer and the supernatants pooled.

2.3. *Column chromatography*

Column chromatography was carried out on Sephadex G-200 using 2.5 mM PO_4, 0.5 mM EDTA, pH 8.0 buffer as the eluant.

3. Results

When the unfractionated EDTA extract was subjected to electrophoresis by the 'Tris'/glycine system, three major groups of bands of low mobility (a, b and c) were observed together with bands of higher mobility. Using preparative gel slab electrophoresis in a 'Tris'/glycine buffer system it was possible to fractionate the EDTA extract. The bands with a low mobility on 'Tris'/glycine gels (i.e. a, b and c) all consisted of protein in the '200 000' region of SDS gels. The bands with a higher mobility in the 'Tris'/glycine system consisted of protein bands of low apparent molecular weight by the SDS method, including the major component at '40 000'.

When slab fractions consisting of high molecular weight protein by the SDS gel system were chromatographed on a Sephadex G-200 column, as would be expected all of the protein was eluted in the void volume (V_0). This material when re-run on SDS gels gave a pattern indistinguishable from that of the original sample which had been applied to the column, i.e. it consisted of protein migrating in the 200 000 mol. wt region. However, when the slab fraction of low molec-

ular weight was subjected to G-200 column chromatography it was fractionated into protein which eluted in the V_0 and a component which was retarded by the column. By SDS gel electrophoresis the protein in the V_0 appeared similar to the starting material i.e. '40 000', but surprisingly the material which was retarded by the column was located in the 200 000 mol. wt. region of the SDS gels (fig. 1). Protein of this size was absent from the sample which had been applied to the column. It should be pointed out that as the chromatography is carried out in the absence of detergent the molecular weights do not necessarily apply on the column as these relate to proteins after detergent treatment. Thus the exclusion of '40 000' proteins is not necessarily anomalous, for in dilute buffer the proteins may have a different size and/or Stokes' radius. All of the original EDTA extract is excluded by G-200 unless it is treated with detergents.

Both the excluded and retarded components were concentrated and recycled through the G-200 column. The excluded material again fractionated into two components, one in the V_0 and one which was retarded. The retarded material was once again found to comprise of 200 000 mol. wt material by SDS gels. The retarded component from the first G-200 cycle was excluded when recycled as would normally be expected of high molecular weight protein and it contained only protein of low mobility on SDS gels.

4. Discussion

Our central observation is that during passage down a G-200 column, proteins, which by SDS electrophoresis are recognised as having a mol. wt. of 40 000 are converted to proteins with a mol. wt. of 200 000 by the same method. The aggregation presumably involves some interaction between protein and the large surface area of the Sephadex. An interaction would be expected to produce the observed anomalous retardation of the large complexes.

It is not certain whether the '200 000' complex generated *in vitro* is identical with the material of this size in the original membrane extract but this possibility is suggested by two pieces of information. Firstly, we have found by N-terminal analysis using dansylation [8] of EDTA-extract fractions that the same N-terminal amino acids are present both in the material from the 200 000 mol. wt. region of the gels and in the protein from the '40 000' region. The N-terminal acids are tyrosine, lysine, glycine, threonine, serine, aspartic and glutamic acids. Secondly, the *in vitro* generation of '200 000' protein is correlated with the formation of the fibrous structures that may be seen by the electron microscope in the unfractionated EDTA extract. These fibres are absent in the '40 000' fraction. (D. Starling, unpublished observations).

The molecular basis for the aggregation is unknown One may consider a covalent cross-linking of the monomers or their non-covalent association. It seems improbable that the chromatography could bring about formation of covalent bonds but the recent report [9] of covalent cross-links between membrane polypeptides, including those extracted by EDTA, means that this alternative explanation must be considered. If covalent cross-links are being generated during the Sephadex treatment it could well be that we are observing the formation of a protein superstructure that could be highly significant in the architecture of the membrane.

The alternative explanation based on non-covalent association would provide another example of a membrane protein complex which cannot be dissociated by SDS and gives further basis for the doubts regarding the general validity of this technique for the analysis of membrane proteins. Although the technique seems valid for the majority of soluble proteins [10] a number of anomalous results have been reported, e.g. the effect of maleylation [11], of high charge density [12, 13] and of the presence of sugar residues [14]. In the case of membrane proteins Lenard [7] found that in addition to SDS, EDTA was also essential for all the erythrocyte ghost-protein to enter the gels, and under certain conditions calcium ions can cause formation of stable aggregates [15]. Furthermore, Fairbanks et al. [2], although they were unable to further dissociate SDS–protein complexes using dissociating agents, found that some agents actually caused an aggregation of '90 000' peptide which could not be reversed by SDS. Such irreversible aggregations have also been reported by other workers [16]. We have shown that membrane proteins can form multimolecular complexes which persist in the presence of SDS. In the particular case of the '200 000' proteins, studies using 6 M guanidine hydrochloride [17] have been cited as additional evidence, contrary to our

claim, that they are single polypeptides rather than multi-molecular complexes, and we must argue that the complex is capable of withstanding the effects of both SDS and guanidine hydrochloride. However there is no a priori reason why such complexes should not exist and agarose beads, although paradoxically they are used to study 'undissociated molecules', are a familiar example of non-covalent structures that are not disrupted by SDS or guanidine. The inability of guanidine hydrochloride to destroy all membrane protein interactions has also been previously reported [18,19].

We concluded that certain '200 000' protein complexes of '40 000' subunits can exist which SDS is unable to dissociate. It is improbable that the complex is formed by covalent linkage and its existence may reflect the unreliability of the SDS technique.

It is permissable to conclude that a protein is in an undissociated state in the presence of SDS only when there is unequivocal corraborative evidence from other techniques, and we consider that more caution should be exercised in the interpretation of results using SDS systems when applied to membrane proteins, and other intractable proteins. Consequently the molecular status of the reputedly very large 'polypeptides' of membranes needs careful re-examination.

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