The nature of the stable noncovalent dimers of band 3 protein from erythrocyte membranes in solutions of Triton X-100

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Stable noncovalent dimers of band 3 protein from human erythrocyte membranes, in which state the protein is thought to exist after solubilization by the nonionic detergent Triton X-100, do not occur when purified batches of the detergent are used. Instead, the protein is in a monomer/dimer/tetramer association equilibrium. The stable dimers do appear, however, when the detergent has been 'aged'. They thus seem to be artifacts.

Erythrocyte membrane *Band 3 protein* *Stable noncovalent dimer* *Association equilibrium* *Triton X-100* *Peroxide*

1. INTRODUCTION

Band 3 protein, the anion transport protein of the human erythrocyte membrane and the membrane's main integral protein, can be easily solubilized and purified by the polyether-derived nonionic detergent Triton X-100 [1,2]. The state of association of the solubilized protein has been intensely studied and is regarded as being a stable noncovalent dimer [3–5] (though other authors have observed, besides stable dimers, stable tetramers [6]). On the other hand, our group has recently shown that the stable noncovalent dimers of band 3 which we observed after solubilization of band 3 by another polyether-derived nonionic detergent, nonaethylene glycol lauryl ether, are preparation artifacts caused by oxidizing impurities in the detergent, and we have suggested that the stable dimers (and stable tetramers) of band 3 in Triton X-100 could be of similar artifactual origin [7]. We have now studied the self-association of band 3 protein in solutions of Triton X-100 by analytical ultracentrifugation. We will show in this paper that, in the Triton solutions, stable dimers of band 3 do not occur if purified batches of the detergent are used.

2. MATERIALS AND METHODS

Triton X-100 ('peroxide-free', from which impurities were removed) was a gift of Boehringer Mannheim. For the sources of all other reagents used see [7].

2.1. *Isolation of band 3*

The protein was purified according to [7] but using Triton X-100 (0.5% (w/w) for solubilization and 0.4% during the column runs) instead of Ammonyx-LO or nonaethylene glycol lauryl ether. Before ultracentrifugation, the protein, in 50 mM NaCl plus 10 mM Tris–HCl (pH 8.0) plus 0.4% Triton X-100, was brought to 1.55% (w/w) sucrose and dialysed for 20 h against 19 vol. of D_2O containing the same additives.

2.2. *Analytical ultracentrifugation*

All experiments were of the high speed sedimentation equilibrium type [8]. They used absorption optics and were performed at 5°C. The basic techniques were those of [7,9,10]. The difficulties which arise from the strong UV absorbance of the detergent, from the sedimentation of the detergent micelles and from the contribution of protein-

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bound detergent to apparent particle weight were overcome by: (1) scanning the cells at a wavelength of 245 nm; (2) using single sector centrepieces of thickness 3 mm and filling the reference cell with water; and (3) using as a solvent the above-mentioned 95% D_2O/5% H_2O/buffer/sucrose mixture with a density (1.107 g/ml at 5°C) just equal to that of the detergent [11]. Details and controls on this approach will be described elsewhere.

2.3. *Determination of protein concentration*

Band 3 concentration c in the ultracentrifuge cell (or during isolation) was determined from the absorbance of the protein–detergent complex at 245 nm, using a value of $A^{1\%}_{1cm}$ (245 nm) = 8.8 ± 1.0. This value was calculated from (1) a value $A^{1\%}_{1cm}$ (280 nm) = 10.0 ± 0.5 for detergent-free band 3 protein [10], (2) an absorbance ratio $A^{1\%}_{1cm}$ (280 nm)/$A^{1\%}_{1cm}$ (245 nm) = 1.32 ± 0.09 measured for three preparations each of band 3 in Ammonyx-LO and nonaethylene glycol lauryl ether [7], (3) a binding capacity of band 3 of 0.77 g Triton X-100/g protein [3], and a value of $A^{1\%}_{1cm}$ (245 nm) = 1.65 for Triton X-100.

3. RESULTS

The isolation procedure for band 3 protein used in this paper yielded band 3 which is at least 95% pure and is virtually free of lipids, as the procedures in [7] which applied different detergents or the original method in [2]. When the protein was isolated by use of freshly prepared Triton solutions and then studied by sedimentation equilibrium runs in the analytical ultracentrifuge, curvilinear ln $c(r^2)$ plots were obtained, indicating heterogeneity of particle weight (fig.1a, curves A,B). The values for the apparent weight average molecular weight M_w of the protein derived from these plots [10] (using a value of 0.74 ml/g for the partial specific volume of the protein [10]) are shown in fig.1b. They are between about 130000 and 240000 and thus indicate the presence of monomers of band 3 as well as of particles larger than the dimer. In addition, the overlap of the curves A and B of fig.1b, which were derived from cells from the same run but with different initial protein concentrations, demonstrates that the different band 3 oligomers contributing to the M_w distribution are linked in an

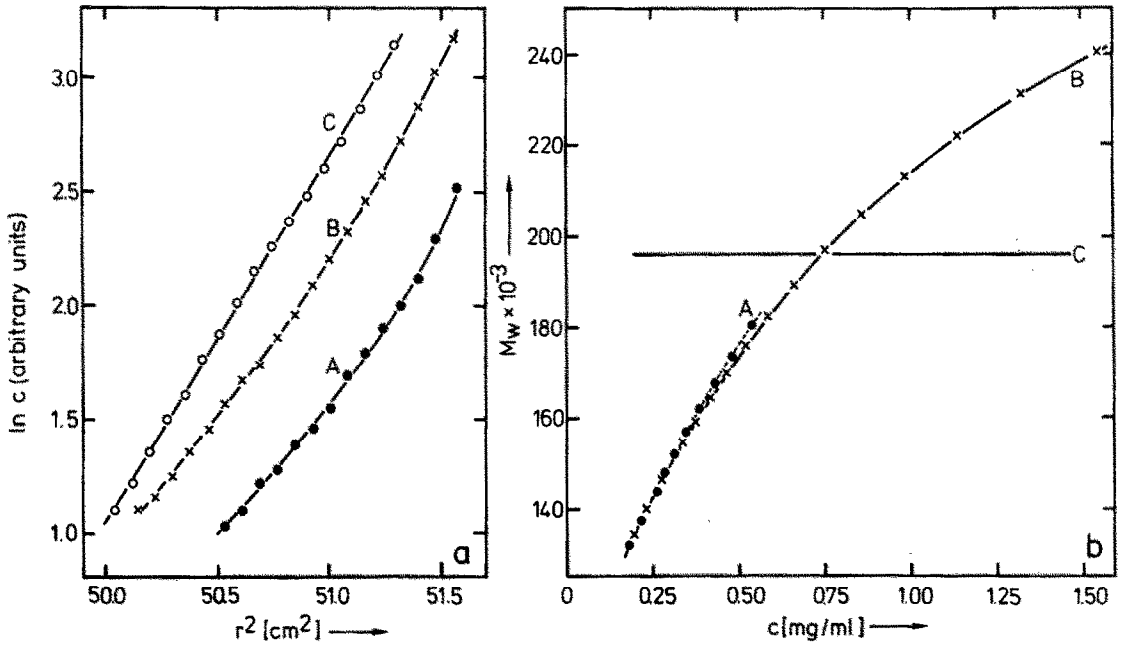

Fig.1. Equilibrium sedimentation of band 3 protein in Triton X-100: plots of ln c *vs* r^2 (a) and of M_w *vs* c (b). (A,B) Samples isolated with freshly prepared Triton solutions. Initial protein concentrations c_0: 0.19 mg/ml (A) and 0.38 mg/ml (B). (C) Sample of c_0 = 0.39 mg/ml isolated with aged Triton solutions. The data were collected 72 h after solubilization of the protein (48 h after starting the ultracentrifuge). Rotor speed was 14000 rpm.

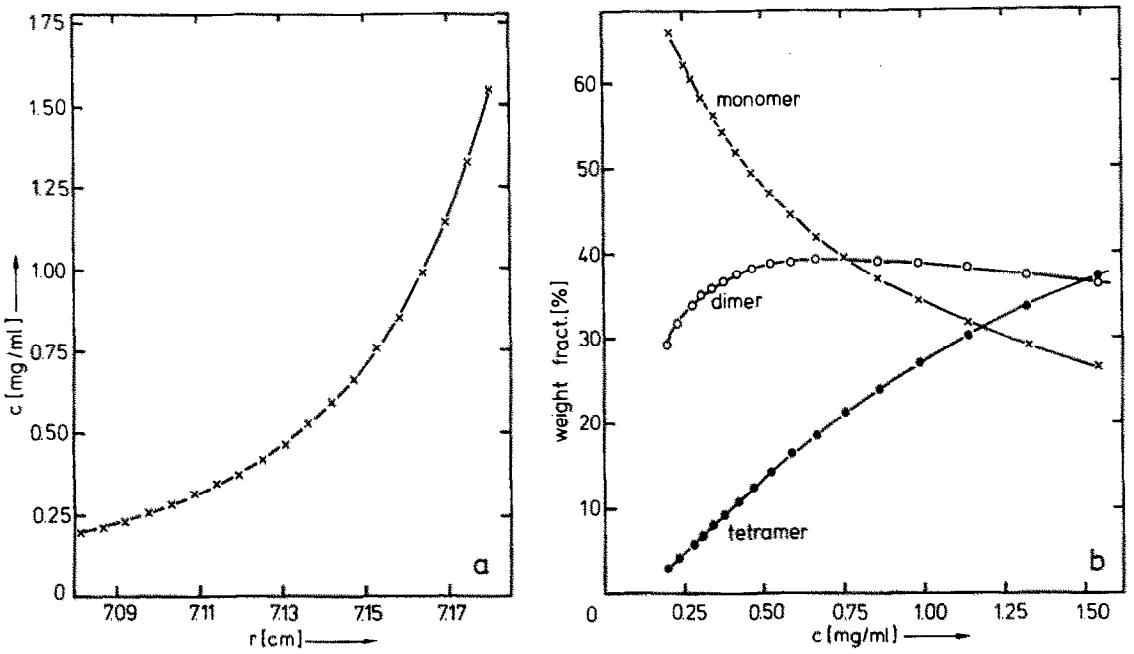

Fig.2. Mathematical analysis of a $c(r)$ distribution [7,10] obtained for band 3 isolated by the use of freshly prepared Triton solutions. The experimental data are those also analyzed in fig.1B. (a) Experimental $c(r)$ curve (×), and least-squares fit to the data based on a monomer/dimer/tetramer model of self-association (——). (b) Relative contributions of the different oligomers as a function of local protein concentration.

association equilibrium [12]. The $c(r)$ distributions can be satisfactorily fitted [10] assuming a monomer/dimer/tetramer model of reversible self-association (fig.2), as in the case of other band 3 preparations for which this model has been confirmed by more rigorous analyses [7,9,10].

After sedimentation equilibrium had been established, the ln $c(r^2)$ distributions described above were stable until, 4–5 days after solubilization of band 3, the $M_w(c)$ values decreased due to proteolytic degradation of the protein [7]. Different results were, however, obtained when the Triton solutions used for solubilization and purification of the protein were not prepared freshly but had been aged, before use, by storing them for several days at room temperature and in daylight, which is known to induce the formation of strongly oxidizing degradation products of the detergent [13,14]. In these cases, the ln $c(r^2)$ plots were curvilinear 2–3 days after solubilization of the protein but then became straight lines, indicating homogeneity of particle weight (line C of fig.1). The molecular weight of the particles was found to be 205000 ± 12000 (n = 4), corresponding to that of the band 3 dimer [7,10]. SDS gel electrophoresis in the absence of β-mercaptoethanol showed that the dimers were not stabilized by disulfide bonds.

4. DISCUSSION

As shown above, stable noncovalent dimers of band 3 protein, which are thought to be the state of association of the protein after solubilization by Triton X-100 [3–5], do not occur when purified, peroxide-free batches of the detergent are used. Instead, the protein is in a monomer/dimer/tetramer association equilibrium. Its association behavior thus agrees with that described for band 3 solubilized by acetic acid [10] and by Ammonyx-LO [7,9] or freshly prepared by nonaethylene glycol lauryl ether [7]. The stable dimers of band 3 do appear, however, when Triton solutions are applied in which the formation of oxidizing degradation products of the detergent had been induced [13,14]. It is therefore clear that these

dimers have to be regarded as preparation artifacts, as those observed for band 3 which had been stored, for prolonged times, in solutions of nonaethylene glycol lauryl ether [7]. For a discussion of these problems in relation to the state of association of band 3 protein in the erythrocyte membrane see [7,9,10].

Triton X-100 has been found to be a valuable tool in studies on membrane proteins [11,15–17]. Our results demonstrate that, in these studies, care should be taken to use detergent batches of sufficient purity.

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