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THE STATE OF ASSOCIATION OF BAND 3 PROTEIN OF THE HUMAN ERYTHROCYTE MEMBRANE IN SOLUTIONS OF NONIONIC DETERGENTS

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Band 3 protein, the anion transport protein of the human erythrocyte membrane, was solubilized and purified in aqueous solutions of two nonionic detergents: Ammonyx-LO (dimethyl laurylamine oxide) and $C_{12}E_9$ (nonaethylene glycol lauryl ether). The state of association of the purified protein was studied by analytical ultracentrifugation. Band 3 protein solubilized and studied in solutions of Ammonyx-LO was found to be in a monomer/dimer/tetramer association equilibrium. Band 3 protein freshly prepared in $C_{12}E_9$ showed the same behaviour; however, during aging the protein was converted into stable noncovalent dimers. The conversion was retarded by the presence of β-mercaptoethanol or by treatment of the samples with iodoacetamide; it seems to be due to oxidation of the protein by degradation products of the detergent. It is concluded that a monomer/dimer/tetramer association equilibrium is the native state of association of band 3 protein solubilized by nonionic detergents. Since nonionic detergents are assumed not to interfere with protein-protein interactions among membrane proteins, the results strongly support the claim that, in the erythrocyte membrane, band 3 is in a monomer/dimer/tetramer association equilibrium (Dorst, H.-J. and Schubert, D. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 1605–1618).

Introduction

There seems to be general agreement that the associations among integral membrane proteins will not, or at least not grossly, be disturbed if the proteins are solubilized by means of nonionic detergents [1–3]. Analysis of the state of association of integral membrane proteins in solutions of nonionic detergents by analytical ultracentrifugation, which is based on firm physicochemical principles [2,4], has therefore become a main source of information on protein association in biological membranes [2–4]. In the present paper, this concept has been applied to the study of the self-association of band 3, the main integral protein of the human erythrocyte membrane and the membrane's anion transport protein [5,6].

The self-association of band 3 protein both in the erythrocyte membrane and after solubilization has already been intensely studied. Earlier data, mainly from cross-linking experiments on intact membranes [5,7,8] and from band centrifugation of band 3 solubilized by the nonionic detergent Triton X-100 [9,10], seemed to indicate that the protein is a stable, noncovalent dimer under both conditions. The view of band 3 protein being dimeric has been widely accepted (despite a contrary claim that the protein is a stable tetramer [11]), and it has even been used as a structural

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basis of models of anion transport and other membrane transport processes [3,12]. However, during the last few years, the dimer model of band 3 protein has been challenged [13–19]. It was concluded from electronmicroscopic observations on freeze-fractured erythrocyte membranes that band 3 protein may be partly [13] or even predominantly [14,15] tetrameric. In ultracentrifugation and gel electrophoresis studies on detergent-solubilized band 3 protein, it was observed that the protein exists as a mixture of stable dimers and stable tetramers [16,17], and other authors have detected, besides dimers, monomers of band 3 [18]. In addition, band 3 solubilized by means of acetic acid and afterwards transferred into aqueous solutions was found to be in a monomer/dimer/tetramer association equilibrium, and it was suggested that the protein would show the same behaviour in the erythrocyte membrane [19]. The present study tries to clarify this conflicting situation by reinvestigating the state of association of band 3 solubilized by nonionic detergents. Two different detergents were used: $C_{12}E_9$ (nonaethylene glycol lauryl ether), a detergent of the Brij series which is a polyoxyethylene derivative like Triton X-100, but is transparent in the near-ultraviolet [1,2], and Ammonyx-LO (dimethyl laurylamine oxide), which lacks polyoxyethylene groups. Some of our results have been briefly reported [20–22].

Materials and Methods

Materials. Ammonyx-LO was purchased from Onyx Chemical Co. (Jersey City, NJ, U.S.A.) and $C_{12}E_9$ from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Brij 58, β-mercaptoethanol and the reagents for gel electrophoresis were obtained from Serva (Heidelberg, F.R.G.). Cellex D was from Bio-Rad (Richmond, CA, U.S.A.) and Sephadex G-25 from Pharmacia (Uppsala, Sweden). ^2H_2O (99.7%) was a kind gift of the Karlsruhe Nuclear Research Center. All other reagents were from Merck (Darmstadt, F.R.G.), and were of analytical grade (if available).

Isolation of band 3 protein. 20 ml of human erythrocyte membranes (prepared according to [23]) were mixed with 50 ml buffer A (50 MM NaCl/10 mM Tris-HCl (pH 8.0)) containing 1% Brij 58, incubated (20 min, 0°C) and centrifuged for 20 min at 40000 × *g* (this solubilizes most of the glycophorin and some proteolytic activity). To solubilize band 3 protein, the pellet was resuspended in 50 ml buffer A containing 0.3% Ammonyx-LO or, alternatively, 1% $C_{12}E_9$, incubated (20 min, 0°C) and centrifuged for 60 min at 40000 × *g* [24]. If stability of band 3 against proteolytic degradation and irreversible aggregation for more than several hours was desired, the solutions added also contained 0.05% deoxycholate. The supernatant was applied to a column of Cellex D (1.5 × 12 cm) equilibrated with buffer A plus 0.2% Ammonyx-LO (0.4% $C_{12}E_9$) [25]. For washing the column, the concentration of NaCl was increased to 130 mM (100 ml), and band 3 protein was eluted by further increasing NaCl concentration to 250 mM [25]. Immediately afterwards, the ionic strength in the sample was reduced by gel filtration on a Sephadex G-25 column (2.6 × 30 cm) run in buffer A plus 0.2% Ammonyx-LO (0.4% $C_{12}E_9$). All centrifugations and column runs were done at 3–5°C, and all detergent solutions were prepared immediately prior to use.

Analytical ultracentrifugation. A Spinco Model E ultracentrifuge (Beckman Instruments (Palo Alto, CA, U.S.A.)) equipped with ultraviolet scanning system, monochromator and multiplexer was used. For technical details and the evaluation of the data see Ref. 19. The measurements were performed at 5°C. Protein concentration was between 20 and 500 μg/ml for solutions of Ammonyx-LO and between 20 and 1000 μg/ml for solutions of $C_{12}E_9$. When necessary, the protein was concentrated by ultrafiltration using the cell model 12 and filters XM 50 from Amicon (Lexington, MA, U.S.A.).

Other procedures. The determination of phosphorus and sialic acid content and SDS gel electrophoresis were done as in Ref. 26. The determination of the amount of oxidizing impurities in the detergents used and the purification of $C_{12}E_9$ were performed as described by Chang and Bock [27]. Protein concentration was determined from the ultraviolet absorbance of the samples [19]. Solution densities were measured by a Paar densitometer DMA 02 (Anton Paar (Graz, Austria)).

Results

Purification of band 3 protein

The procedure used by us is essentially a modified version of that of Yu and Steck [25]. Several of the modifications introduced by us have, in similar form, already been applied by others [24,28,29], though not in combination. As in the original method, the isolated band 3 protein had a purity of 95% (Fig. 1), at a yield of approximately 20% [25]. The average content of phosphorus and of sialic acid of the preparations was 2–3 mol of both components per mol protein; however, in some preparations it was below 1 mol per mol protein. The main improvement gained by the modifications was an increase in the stability of the isolated band 3 protein against proteolytic degradation and against the (irreversible) formation of high molecular weight aggregates: partial degradation and aggregation became apparent only after 3–4 days in our preparations, but already after several hours if the original method was used.

State of aggregation of band 3 protein prepared and studied in solutions of Ammonyx-LO

Solubilized band 3 protein shows an association equilibrium. In sedimentation velocity runs in the analytical ultracentrifuge, band 3 protein as isolated by us showed a single broad boundary, the sedimentation coefficient of which increased strongly and reversibly with increasing protein concentration, c (Fig. 2). This is evidence for the occurrence of an association equilibrium between different band 3 oligomers, the relaxation of which towards equilibrium is intermediate or rapid on the time scale of the experiment [30,31]. The smallest $s_{20,w}$ value observed (for $c < 70$ μg/ml) was (3.5 ± 0.3) S; the highest values were approximately 11 S ($c = 500$ μg/ml). Virtually identical results were obtained: (1) when deoxycholate was omitted from the preparation procedure of the protein; (2) when, for the ultracentrifugation experiments, the concentration of the detergent was varied between 0.1 and 0.5%; or (3) when the NaCl concentration in the samples was lowered to 10 mM or increased to 150 mM.

Since the yield of our isolation procedure for band 3 protein is only 20%, there remained the possibility that the data described may be representative for a minor subpopulation of band 3 only. We have therefore performed sedimentation velocity runs on the crude band 3 extracts which contained approx. 80% of the band 3 protein originally present. Though these samples also contained significant amounts of lipids, band 4.5 and glycophorin and traces of bands 1, 2 and 4.2, band 3 undoubtedly was the dominating component in their near ultraviolet absorption spectra and thus in the sedimentation diagrams. The results obtained were virtually identical to those described for the purified protein, both with respect to the

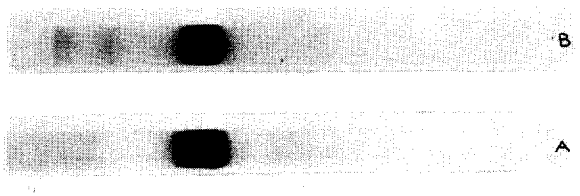

Fig. 1. SDS gel electrophoresis pattern of band 3 protein samples isolated by the use of Ammonyx-LO (A) and $C_{12}E_9$ (B) after staining by Coomassie brilliant blue. 50 μg protein applied per gel.

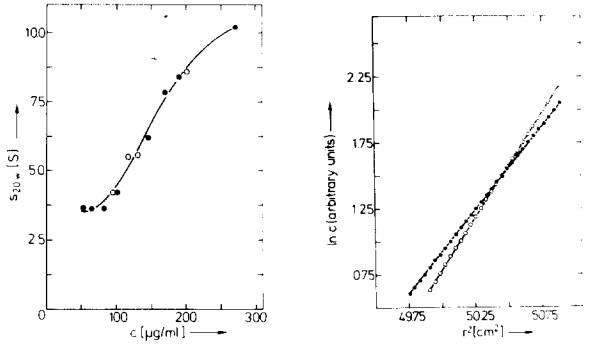

Fig. 2. Dependency of $s_{20,w}$ on protein concentration, c, for band 3 protein isolated by the use of Ammonyx-LO and diluted from a stock solution ($c_0 = 270$ μg/ml) (●) or concentrated up to 3-fold after dilution to $c = 65$ μg/ml (○).

Fig. 3. Plots of ln c versus r^2 from sedimentation equilibrium runs at low protein concentration ($c_0 = 45$ μg/ml) for protein isolated in Ammonyx-LO. The solutions contained either ^1H_2O (○) or $^1H_2O/^2H_2O$ (1:1, v/v) (●). Rotor speed was 15000 rpm.

sedimentation coefficients and to the shape of the boundaries. The occurrence of an association equilibrium in the samples of purified band 3 protein, as isolated by us, is thus a characteristic property of the whole band 3 population, or at least of its major part.

Identification of the smallest component of the association equilibrium. Plots of ln c versus r^2 obtained from sedimentation equilibrium runs of samples with $s_{20,w} = 3.5$ S showed straight lines (Fig. 3). This demonstrates that the particle population studied is homogeneous. The slope of the straight lines yielded $M_c(1 - \bar{v}_c\rho) = 29\,700 \pm 500$ if ^1H_2O was used as a solvent (buffer density $\rho =$ 1.0022 g/ml), whereas corresponding runs in $^1H_2O/^2H_2O$ (1 : 1 v/v, $\rho = 1.0513$ g/ml) yielded $M_c(1 - \bar{v}_c\rho) = 24\,000 \pm 500$, where M_c is the particle molecular weight (protein plus bound detergent) and $\bar{v}_c$ the partial specific volume of the particles [2]. From this, one obtains $M_c = 140\,000 \pm 23\,000$ and $\bar{v}_c = (0.79 \pm 0.03)$ ml/g. Since the molecular weight of band 3 protein is approx. 97 000 [5,19], it follows that the protein particle with an $s_{20,w}$ value of 3.5 S described above, and thus the smallest component of the association equilibrium of band 3, is the monomer of the protein which has bound, per g, approx. 0.4 g of Ammonyx-LO.

Identification of the higher oligomers contributing to the association equilibrium. Equilibrium sedimentation runs performed at initial protein concentration above 100 μg/ml (in ^1H_2O) yielded curvilinear ln $c(r^2)$ plots, which shows that the samples contained a heterogeneous population of band 3 particles. Apparent weight average molecular weights, M_w, derived from runs at different rotor speeds and/or different initial protein concentrations, when plotted versus c, led to overlapping curves, which again demonstrates the existence of an association equilibrium of band 3 [30]. The M_w-values obtained were between 180 000 and > 500 000 (including bound detergent), and thus indicated the presence of monomers of band 3 as well as of protein aggregates consisting of at least three subunits. The particles contributing to the $c(r)$ distributions were identified by mathematical analysis of the curves in terms of a superposition of distributions of the different oligomers [19,32,33], assuming the absence of solution nonidealities * and taking as a basis the data for the monomer-detergent complex given above. Excellent fits of the experimental curves were obtained for a monomer/dimer/tetramer model of self-association, as shown in Fig. 4a. The relative contributions of the different band 3 oligomers to the calculated curve are shown in Fig. 4b. Inclusion of trimers into the model neither changed the calculated $c(r)$-curves nor the calculated contributions $c_i(r)$ of the different oligomers significantly, the contribution of the trimer term being virtually zero. On the other hand, omission of the dimer or tetramer term from the calculations in all cases led to much less satisfactory or completely unsatisfactory fits of the experimental data. Thus, the band 3 oligomers present in significant amounts and taking part in the association equilibrium are, besides monomers, dimers and tetramers. Of course, our data do not rule out that at much higher values of c, still higher oligomers of band 3 (hexamers, octamers) may take part in the association equilibrium, as was shown for band 3 solubilized by acetic acid [19], nor do they rule out the presence of a small percentage of trimer (see also Ref. 19).

State of aggregation of band 3 in solutions of $C_{12}E_9$

Freshly prepared band 3 is in an association equilibrium. During the first few hours after purification of the protein, the results from sedimentation velocity runs of the samples prepared and studied in solutions of $C_{12}E_9$ were very similar to those described above: the boundaries were broad, and the $s_{20,w}$ values increased strongly and reversibly with increasing protein concentration, and were between 3.8 and 7–8 S (Fig. 5). Thus, in these

* Of the different types of nonideality, both excluded volume effects and Donnan effects can be neglected, due to the low protein concentrations and the relatively high salt concentrations used in our study (this is also demonstrated by the straight lines in Fig. 7, which would be concave downward if these effects were operative [34]). A dependency of the partial specific volume of the protein-detergent complexes on pressure or on the state of association of the protein is ruled out by the overlap of the $M_w(c)$ curves obtained at different rotor speeds or initial protein concentrations (see above) [33,35]. The latter also shows that the different band 3 oligomers contain approximately the same amount of bound detergent per g protein.

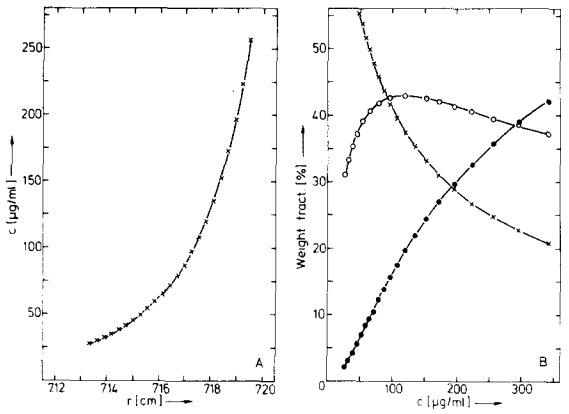

Fig. 4. (A) $c(r)$ profile (×) from a sedimentation equilibrium run ($c_0 = 170$ µg/ml, 15200 rpm) on band 3 protein prepared by Ammonyx-LO, and least-squares fit to the experimental data according to a monomer/dimer/tetramer model of self-association (———). (B) Relative contributions of monomer (×), dimer (○) and tetramer (●) to the calculated $c(r)$ distribution. The data can be characterized by the equilibrium constants $K_{12} = 27$ l/g and $K_{14} = 5 \cdot 10^3$ l^3/g^3.

samples band 3 protein is in an association equilibrium, as described above for band 3 in solutions of Ammonyx-LO. Identical results were obtained if 10 mmol/l β-mercaptoethanol were added to the isolated protein (plus 1 mM EDTA to suppress the development of excess ultraviolet absorbance [36] * or if, during protein preparation, the pre-extraction step and the solubilization of the protein were done in the presence of 5 mM iodoacetamide (Fig. 5). However, with the latter modifications, the sedimentation behaviour described was unchanged within 40 h after purification of the protein, thus allowing analysis by sedimentation equilibrium runs. $M_w(c)$ values derived, for protein treated with iodoacetamide, from data for two cells with different initial protein concentrations, c_0, are shown in Fig. 6A. The overlap between the two curves again demonstrates that the particles studied are in an association equilibrium [30]. The $c(r)$ distribution for the cell of higher c_0 of Fig. 6A is shown in Fig. 6B, together with a fit to the experimental data by use of a monomer/dimer/tetramer model of self-association. Fig. 6C shows the calculated distributions $c_i(c)$ for the different oligomers. It is evident from Fig. 6B that a good agreement exists between the experimental and the theoretical $c(r)$ distribution. It is also evident that, at a given protein concentration, the relative amount of tetramer calculated is distinctly smaller than shown above for band 3 isolated by the use of Ammonyx-LO (Fig. 4B). In some experiments, the contribution of tetramer was so small that the curves could also be satisfactorily fitted assuming a monomer/dimer association equilibrium (data not shown).

The occurrence of stable noncovalent dimers of band 3 in aged samples. The association equilibrium described above for band 3 freshly prepared by $C_{12}E_9$ was not stable: after the time intervals given above, the broad boundaries originally observed in sedimentation velocity runs became superimposed by a sharp boundary of $s_{20,w} = (6.8 \pm 0.2)$ S (independent of protein concentration), and after sufficient time all protein present sedimented in this boundary. Analogously, the $\ln c(r^2)$ plots obtained from sedimentation equilibrium runs progressively became less curvilinear and finally were straight lines, indicating homogeneity of particle weight. The state of association of the band 3 particles formed was determined by combining data from equilibrium sedimentation runs in ^1H_2O and a $^1H_2O/^2H_2O$ mixture containing 51.5% ^2H_2O and by this, since its density of 1.0564 g/ml just equals that of the detergent, cancelling the contributions of bound detergent to apparent particle weight [2,4]. From the data shown in Fig. 7 we obtained $M_c = 326000 \pm 15000$, $\bar{v}_c = (0.82 \pm 0.02)$ ml/g and, using a value of 0.74 ml/g for the partial specific volume of the protein [19], $M_p = 199000 \pm 6000$ for the molecular weight of the protein part of the band 3-detergent complex [2,4]. Thus, the stable particle replacing the association equilibrium of band 3 is a dimer which has bound (0.64 ± 0.15) g detergent per g protein. Since the protein migrated as a monomer during SDS gel electrophoresis both after and without treatment of the samples with 2% β-mercaptoethanol, the dimer is stabilized by noncovalent interactions only.

As noted above, the conversion of particles in an association equilibrium into stable dimers could

* Virtually identical results were also found in crude band 3 extracts solubilized by $C_{12}E_9$ in the presence of up to 300 mM β-mercaptoethanol (plus 1 mM EDTA).

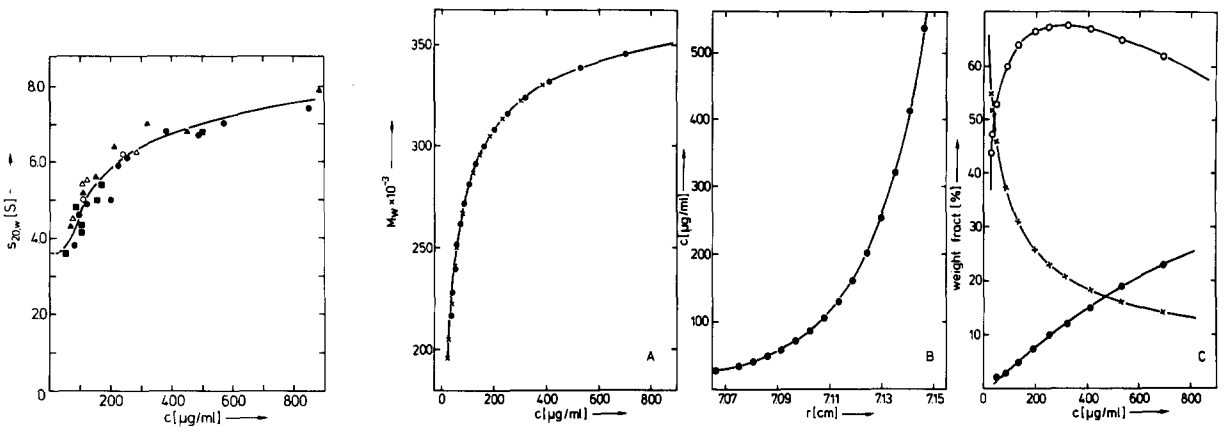

Fig. 5. Dependency of $s_{20,w}$ on protein concentration c for band 3 protein freshly isolated in $C_{12}E_9$. ▲, △, Unmodified protein diluted from a stock solution of $c_0 = 880$ µg/ml (▲) or concentrated after dilution to $c = 75$ µg/ml (△); ■, unmodified protein diluted from $c_0 = 500$ µg/ml, in the presence of 10 mM β-mercaptoethanol; ●, ○, protein treated with 5 mM iodoacetamide and diluted from a stock solution of $c_0 = 850$ µg/ml (●) or concentrated after dilution to $c = 80$ µg/ml (○).

Fig. 6. Equilibrium sedimentation experiments on band 3 protein solubilized by $C_{12}E_9$ in the presence of 5 mM iodoacetamide. (A) M_w values derived from two cells with initial protein concentrations c_0 of 160 µg/ml (×) and 340 µg/ml (●). Rotor speed was 15000 rpm. (B) $c(r)$ Profile for the cell of $c_0 = 340$ µg/ml (●), and least-squares fit to the data by means of a monomer/dimer/tetramer model of self-association (———). (C) Relative contributions of monomer (×), dimer (○) and tetramer (●) to the calculated $c(r)$ distribution of part B. From the curves, equilibrium constants $K_{12} = 50$ l/g and $K_{14} = 1.8 \cdot 10^3$ l^3/g^3 can be derived. All calculations were based on the data for molecular weight and partial specific volume of the band 3 dimer-detergent complex (Fig. 7).

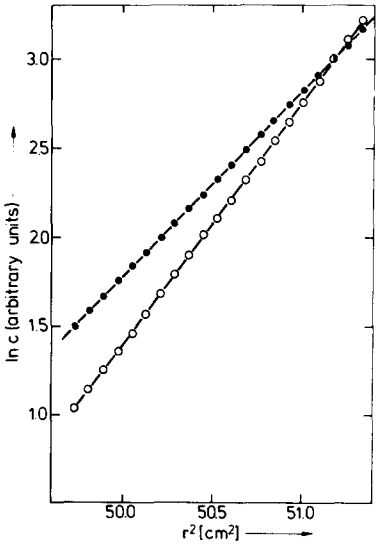

Fig. 7. Plots of ln c versus r^2 from sedimentation equilibrium runs using "aged" band 3 protein in solutions of $C_{12}E_9$. Solvent was either ^1H_2O (○) or $^1H_2O/^2H_2O$ (48.5:51.5, v/v) (●). Initial protein concentration: 60 µg/ml. Rotor speed: 12000 rpm.

be retarded, but not prevented, by the presence of β-mercaptoethanol or by treatment of the protein with iodoacetamide. On the other hand, little or no retardation of dimer formation was achieved when sulfhydryl oxidizing contaminants were eliminated from the detergent by the procedure of Chang and Bock [27].

Discussion

Self-association of band 3 protein in detergent solutions

The results described in this paper clearly demonstrate that band 3 protein solubilized and studied in solutions of the nonionic detergent Ammonyx-LO is in a monomer/dimer/tetramer association equilibrium, as is band 3 protein solubilized by acetic acid [19]. They also demonstrate that the protein freshly prepared in solutions of another nonionic detergent, $C_{12}E_9$, shows the same behaviour. On the other hand, after storage in solutions of this detergent for sufficient times, the protein is converted into a stable noncovalent di-

mer *. However, the circumstances of this conversion, in particular its retardation by the presence of β-mercaptoethanol, strongly suggest that the conversion is a secondary effect due to oxidation of the protein, and that therefore the native state also of $C_{12}E_9$-solubilized band 3 is a monomer/dimer/tetramer association equilibrium.

The occurrence of oxidizing impurities in polyoxyethylene-derived detergents (which include the Brij and Triton series) has already been noted by others (e.g., Refs. 27,37–39). Most of these impurities seem to arise from degradation of the ether moieties of the detergent's polyoxyethylene portion and include different types of peroxide [38,39] and, possibly, free radicals [39]. In protein solutions, the main target of these highly reactive impurities seems to be the SH-group of the cysteine side-chain; however, the side-chain of methionine is another preferred target [40]. It is interesting that iodoacetamide, which in our experiments strongly retarded dimer formation, is reactive towards both kinds of side-chain [40]. Thus, there seem to be several plausible ways to explain the formation of stable dimers of band 3 protein in solutions of $C_{12}E_9$ as an artifact due to chemical modification of the protein by impurities in the detergent **. In addition, these explanations would also be applicable to the stable dimers found after solubilization of band 3 by Triton X-100 [9,10]. We therefore think that not only the stable dimers of band 3 described in this paper are artifacts, but also those observed by others in Triton solutions. It may be worthwhile to rule out the possibility that similar artifacts were operative in those experiments (reviewed in Ref. 3) in which other membrane proteins were found to be dimeric after solubilization by Triton X-100.

Other workers have observed, in addition to the stable dimers, two additional states of association of band 3 protein solubilized by nonionic detergents: (1) Lukacovic et al. [18], studying band 3 in solutions containing 0.5% Triton X-100, have found by band centrifugation in a density gradient that the protein was present as a mixture of monomers and dimers. (2) Nakashima and colleagues [16,17], using human as well as bovine band 3 protein solubilized either by $C_{12}E_9$ or Triton X-100, have found that the protein was a mixture of stable dimers and stable tetramers [16,17]. The results of Lukacovic et al. certainly are consistent with our results, especially if it is considered that the existence of an association equilibrium most probably will remain undetected in standard band centrifugation experiments. The stable tetramers described by Nakashima et al. have been found in 'aged' samples of initial protein concentration of 1–2 mg/ml, which is 2–5-times higher than the initial protein concentration in our studies. This suggests that the 'stable' dimers observed by us and others may further aggregate irreversibly if stored at high protein concentration. In any case, there remains no serious discrepancy between our data and those described in the literature. However, the conclusions which have to be drawn from our data are entirely different from those drawn by others.

Self-association of band 3 in the erythrocyte membrane

Dorst and Schubert [19], combining their findings on the self-association of band 3 prepared in acetic acid with the data on band 3 self-association in the erythrocyte membrane reported by other workers, have suggested that, in the membrane, band 3 would be in a monomer/dimer/tetramer association equilibrium. It is clear that this suggestion is strongly supported by the results described in this paper (see Introduction). The arguments alleged by Dorst and Schubert in favour of their view will not be repeated here. We will, however, discuss two problems which have not been dealt with by these authors, namely, the possible influence on band 3 self-association of interactions of band 3 with glycophorin or with the membrane's cytoskeleton.

The possibility that, in the intact erythrocyte membrane, band 3 could be associated with glycophorin has been long discussed, though no definite

* Our observation that the relative amount of tetramers is always lower in band 3 samples freshly isolated by $C_{12}E_9$ than in those isolated by Ammonyx-LO could indicate that the first step in the conversion is a monomer/dimer association equilibrium.

** Our failure to prevent dimer formation by purifying the detergent could mean that degradation of the detergent molecules is a multistep process, and that the first degradation product is short-lived and is the most reactive one.

proof has been found. Recently, however, Nigg et al. [41] have deduced the existence of this association from observations on an immobilization of band 3 by antibody-induced crosslinking of glycophorin A. On the other hand, glycophorin can be extracted from the erythrocyte membrane by low concentrations of Triton X-100 [24,29] or by 1% Brij 58 (see above) without solubilization of band 3, and in solutions of Triton X-100 containing both band 3 and glycophorin no interaction between these two proteins has been detected [10]. The latter result is supported by our observation that band 3 shows the same sedimentation properties in crude band 3 extracts containing part or all of the sialoglycoproteins and after purification. According to the considerations described in the introduction, these findings would mean that in the erythrocyte membrane under physiological conditions, band 3 and glycophorin will not form a stable complex with each other and that transient associations would have to be very weak (weak transient interactions between the two proteins cannot be ruled out by this arguments, since in the erythrocyte membrane the concentration of both proteins will be much larger than after solubilization). This is supported by the recent finding that in the erythrocyte membrane no band 3-glycophorin complex is detectable by sensitive calorimetry [42]. An alternative interpretation of the observations of Nigg et al. [41] would thus be that, in their experiments, a band 3-glycophorin association is induced by the antibody used. Besides that, even if an association between the two integral membrane proteins existed in the unperturbed erythrocyte membrane, this would not necessarily abolish the association equilibrium of band 3.

In contrast to the band 3-glycophorin association, the existence of an association between band 3 and the cytoskeleton, which is mediated by band 2.1 and possibly involves also band 4.2, is firmly established [43,44]. Only 10–40% of band 3, however, takes part in this association, the remaining band 3 being unrestricted in its short-range mobility [43–45]. However, not only the latter fraction of band 3 could participate in the association equilibrium described above, but possibly also the former one, since associations of band 3 with the cytoskeleton probably only involve its highly polar cytoplasmic 40 kDa domain [45], whereas its self-association mainly involves hydrophobic regions of the protein molecules [19] and thus predominantly the transmembrane domains *. The same arguments will hold with respect to an influence of the interactions of band 3 with glyceraldehyde-3-phosphate dehydrogenase and aldolase [5,46,47] on band 3 self-association. In addition, the associations of these two enzymes with band 3 are also governed by association equilibria [5,46] and thus do not exist permanently. Therefore, there seems to be no reason to assume that the interactions discussed would prevent the majority or possibly all of the band 3 molecules from taking part in the association equilibria described for the solubilized protein, though they certainly may modify it.

Considering these arguments and those discussed by Dorst and Schubert [19], we conclude that a monomer/dimer/tetramer association equilibrium has indeed to be regarded as the most promising candidate for the state of association of membrane-bound band 3 protein, or at least of its major part.

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References

1 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79

2 Tanford, C. and Reynolds, J.A. (1976) Biochim. Biophys. Acta 457, 133–177

* Since, in a hydrocarbon environment, protein-protein associations cannot be stabilized by hydrophobic interactions, it seems that the actual driving force for the associations may be Van der Waals bonding.

3 Klingenberg, M. (1981) Nature 290, 449–454
4 Tanford, C. (1977) in Structure of Biological Membranes (Abrahamsson, S. and Pascher, I., eds.), pp. 497–508, Plenum Press, New York
5 Steck, T.L. (1978) J. Supramol. Struct. 8, 311–324
6 Cabantchik, Z.J., Knauf, P.A. and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239–302
7 Steck, T.L. (1972) J. Mol. Biol. 66, 295–305
8 Kiehm, D.J. and Ji, T.H. (1977) J. Biol. Chem. 252, 8524–8531
9 Clarke, S. (1975) J. Biol. Chem. 250, 5459–5469
10 Yu, J. and Steck, T.L. (1975) J. Biol. Chem. 250, 9176–9184
11 Wang, K. and Richards, F.M. (1974) J. Biol. Chem. 249, 8005–8018
12 Guidotti, G. (1976) Trends Biochem. Sci. 1, 11–13
13 Margaritis, L.H., Elgsaeter, A. and Branton, D. (1977) J. Cell Biol. 72, 47–56
14 Weinstein, R.S., Khodadad, J.K. and Steck, T.L. (1980) in Membrane Transport in Erythrocytes, Alfred Benzon Symposium 14 (Lassen, U.V., Ussing, H.H. and Wieth, J.O., eds.), pp. 35–50, Munksgaard, Copenhagen
15 Weinstein, R.S., Khodadad, J.K. and Steck, T.L. (1980) J. Cell Biol. 87, 209a
16 Nakashima, H. and Makino, S. (1980) J. Biochem. 88, 933–947
17 Nakashima, H., Nakagawa, Y. and Makino, S. (1981) Biochim. Biophys. Acta 643, 509–518
18 Lukacovic, M.F., Feinstein, M.B., Sha'afi, R.J. and Perrie, S. (1981) Biochemistry 20, 3145–3151
19 Dorst, H.-J. and Schubert, D. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 1605–1618
20 Pappert, G. and Schubert, D. (1981) Biochem. Soc. Trans. 9, 249 P
21 Pappert, G. and Schubert, D. (1982) in Protides of the Biological Fluids, Proceedings of the 29th Colloquium (1981) (Peeters, H., ed.), pp. 117–120, Pergamon Press, Oxford
22 Pappert, G. and Schubert, D. (1982) Hoppe-Seyler's Z. Physiol. Chem. 363, 904
23 Schubert, D., Poensgen, J. and Werner, G. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 1034–1042
24 Yu, J., Fishman, D.A. and Steck, T.L. (1973) J. Supramol. Struct. 1, 233–248
25 Yu, J. and Steck, T.L. (1975) J. Biol. Chem. 250, 9170–9175
26 Schubert, D. and Domning, B. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359, 507–515
27 Chang, H.W. and Bock, E. (1980) Anal. Biochem. 104, 112–117
28 England, B.J., Gunn, R.B. and Steck, T.L. (1980) Biochim. Biophys. Acta 623, 171–182
29 Wolosin, J.M. (1980) Biochem. J. 189, 35–44
30 Fujita, H. (1975) Foundations of Ultracentrifugal Analysis, pp. 236–278 and 377–429, John Wiley & Sons, New York
31 Cox, D.J. (1969) Arch. Biochem. Biophys. 129, 106–123
32 Osborne, J.C., Bronzert, T.J. and Brewer, H.B. (1977) J. Biol. Chem. 252, 5756–5760
33 Formisano, S., Brewer, H.B. and Osborne, J.C. (1978) J. Biol. Chem. 253, 354–360
34 Schachman, H.K. (1959) Ultracentrifugation in Biochemistry, pp. 201–214, Academic Press, New York
35 Howlett, G.J., Jeffrey, P.D. and Nichol, L.W. (1972) J. Phys. Chem. 76, 777–783
36 Wetlaufer, D.B. (1962) Adv. Protein Chem. 17, 303–390
37 Lever, M. (1977) Anal. Biochem. 83, 274–284
38 Ashani, Y. and Catravas, G.N. (1980) Anal. Biochem. 109, 55–62
39 Azaz, E., Segal, R. and Milo-Goldzweig, I. (1981) Biochim. Biophys. Acta 646, 444–449
40 Means, G.E. and Feeney, R.E. (1971) Chemical Modification of Proteins, pp. 162–165, Holden-Day, San Francisco
41 Nigg, E.A., Bron, C., Girardet, M. and Cherry, R. (1980) Biochemistry 19, 1887–1893
42 Davio, S.R. and Low, P.S. (1982) Biochemistry 21, 3585–3593
43 Bennett, V. and Stenbuck, P.J. (1979) Nature 280, 468–473
44 Sheetz, M.P. (1979) Biochim. Biophys. Acta 557, 122–134
45 Nigg, E.A. and Cherry, R. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4702–4706
46 Kliman, H.J. and Steck, T.L. (1980) J. Biol. Chem. 255, 6314–6321
47 Tsai, I., Murthy, S.N.P. and Steck, T.L. (1982) J. Biol. Chem. 257, 1438–1442