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Cytoskeleton-membrane connections in the human erythrocyte membrane: band 4.1 binds to *tetrameric* band 3 protein

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Abstract

Band 4.1 provides, besides ankyrin, the main linkage between the erythrocyte membrane and its cytoskeleton. Its predominant binding sites in the membrane are located on the glycophorins. However, the cytoplasmic domain of band 3 can also bind band 4.1. We have studied which of the different band 3 oligomers observed (monomers, dimers, tetramers) can act as band 4.1 binding sites, by equilibrium sedimentation experiments on mixtures of purified band 3 and dye-labelled band 4.1 in solutions of a nonionic detergent. At low molar ratios of band 4.1 and band 3, the sedimentation equilibrium distributions obtained could all be perfectly fitted assuming that only two dye-labelled particles were present: uncomplexed band 4.1 and a complex formed between one band 4.1 molecule and one band 3 tetramer. The presence of small amounts of complexes containing band 3 monomers or dimers could not be completely ruled out but is unlikely. On the other hand, stabilized band 3 dimers effectively bound band 4.1. At higher molar band 4.1/band 3 ratio, the band 3 tetramer apparently could bind up to at least four band 4.1 molecules. The band 4.1/band 3 tetramer complex was found to be unstable. The results described, together with those reported previously, point at a prominent role of tetrameric band 3 in ligand binding.

Keywords: Band 3 protein; Band 4.1 protein; Heterologous association; Complex stoichiometry; Erythrocyte membrane; Analytical ultracentrifugation

1. Introduction

The erythrocyte cytoskeleton is linked to the integral proteins of the plasma membrane by two proteins: ankyrin (band 2.1) and band 4.1 [1,2]. Ankyrin binds to the anion exchanger, band 3 (AE 1), the red cell's most abundant intrinsic membrane protein [1–3]. The predominant binding partners of band 4.1 are the glycophorins (in particular glycophorins C/D) [1–7]. However, the cytoplasmic domain of band 3 can also bind band 4.1 [1–7]. The preference of band 4.1 for binding either to the glycophorins or to band 3 seems to be determined by the state of phosphorylation of a membrane phospholipid of low abundancy, phosphatidylinositol [8]. In addition, the association

Abbreviations: $C_{12}E_9$, nonaethyleneglycol lauryl ether; DTT, dithiothreitol; Rh-band 4.1, band 4.1 labelled by treatment with carboxytetramethylrhodamine succinimide ester

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of band 4.1 and band 3 is strongly inhibited by phosphorylation of band 4.1 by protein kinase C [9].

Band 3 is heterogeneous with respect to its state of self-association: dimers and tetramers of the protein were detected by a variety of techniques [10], and the existence of monomers (in an association equilibrium with dimers and tetramers) was deduced from studies on detergent-solubilized band 3 [11,12]. Thus, the question arises which of the different band 3 oligomers is favoured as a binding site for band 4.1. Recent studies showed that ankyrin, hemoglobin and the glycolytic enzyme aldolase bind predominantly if not exclusively to the band 3 tetramer [13–16]. With respect to band 4.1, no information was available up to now.

To address the problem described, we analyzed the mixed complexes formed by band 4.1 and band 3 in solutions of a nonionic detergent, nonaethyleneglycol lauryl ether ($C_{12}E_9$). In this detergent, band 3 is native with respect to both its anion transport function and its function as a binding site for proteins of the cytoskeleton and cytosol [16,17]. Applying analytical ultracentrifugation for studying mixed associations between proteins [18,19], we show in this paper that tetrameric band 3 also represents the sole or at least by far the predominant binding site for band 4.1. The methodological approach applied has been recently reviewed [20].

2. Materials and methods

Band 3 protein was isolated from human erythrocyte ghosts using the nonionic detergent $C_{12}E_9$ ('Thesit'; Boehringer Mannheim, Germany), according to a modified version [11] of a method of Yu and Steck [21]. All solutions were thoroughly degassed unless otherwise stated. Stable dimers of band 3 were derived from the purified protein by two different reactions: (a) by inducing the formation of disulfide bridges, using $CuSO_4$/o-phenanthroline as catalyst [22]; (b) by the action of oxidizing degradation products in the detergent during an incubation period of approx. 45 h, in non-degassed buffer and in the absence of reducing agents, which yields noncovalent dimers [11,12,15]. In both cases, nondimeric protein was separated by gel filtration on Sephacryl S-300 [15]. Protein concentration was determined photometrically [23]. The cytoplasmic domain of band 3 and ankyrin were isolated from spectrin-depleted inside-out vesicles of human erythrocytes according to Ref. [24]. The isolation of the erythrocyte cytoskeletal protein band 4.1 essentially followed the method of Ohanian and Gratzer [25]. The method applies the dissociation of purified membrane skeletons at neutral pH (in 2 M Tris-HCl; W.B. Gratzer, personal communication), followed by gel filtration and anion exchange chromatography. In order to prevent aggregation of the protein, the solutions used during the final isolation steps contained 0.02% $C_{12}E_9$. After isolation, the protein was concentrated 3- to 5-fold by dialysis against buffered solutions of 25% (w/v) polyethylene glycol 20 000 (Merck, Darmstadt, Germany).

All protein samples showed one single band, without any visible contaminants, after SDS polyacrylamide gel electrophoresis followed by staining with Coomassie blue [26]. In some band 3 preparations, however, a faint band corresponding to band 4.2 protein could be detected after silver staining of the gels. The glycophorins, which are virtually not stained by Coomassie blue [26], are absent from the band 3 samples prepared by us, as shown in previous studies [11,21] (most of the material stainable by periodic acid/Schiff reagent is already separated in the preextraction step [11]).

The physical method for studying heterologous protein–protein associations applied in this paper requires labelling of the ligand protein with a dye, in a way that the binding properties of the labelled protein remain unaffected. Labelling of band 4.1 was done by using the dye carboxytetramethylrhodamine succinimide ester (Sigma, Deisenhofen, Germany), as described by Pagliaro and Taylor [27]. The concentration of the labelled protein was determined photometrically [27,28]. The ability of labelled band 4.1 to bind to the cytoplasmic domain of band 3 was confirmed by using the binding assay described previously [29]. In brief, the cytoplasmic domain of band 3 (or a reference protein) was adsorbed to nitrocellulose dots. After blocking of unoccupied protein binding sites with low fat milk, the dots were incubated with labelled or unlabelled band 4.1 protein, followed by extensive washing. The bound band 4.1 was visualized by the immunoperoxidase method, using an antibody specific for human protein 4.1, appropriate

peroxidase-labelled secondary antibodies, and chloronaphtol as chromogen.

Analytical ultracentrifugation was performed in a Beckman Optima XL-A ultracentrifuge, using an An-60 Ti rotor. The proteins and protein mixtures were studied in a variety of buffer systems, in most cases containing 5–10 mM Tris-HCl (pH 7.5–8.5), 10–100 mM NaCl, 0.2–0.4% $C_{12}E_9$, 0.2 mM dithiothreitol (DTT) and 0–1 mM EDTA. The concentrations of band 3 and band 4.1 were independently adjusted to values between 80–350 μg/ml (band 3) and 50–310 μg/ml (band 4.1). Most experiments were of the sedimentation equilibrium type and used 6-channel centerpieces. Rotor speed was between 8000 and 15 000 rpm, and rotor temperature 4°C. In order to selectively monitor the behaviour of the labelled band 4.1 and its complexes with band 3, the absorbance-versus-radius distributions A(r) were monitored at 557 nm, an absorption maximum of rhodamine. Stable A(r)-profiles were obtained during 24–38 h after starting the runs. The behaviour of unlabelled controls, in particular those of band 3 samples, was monitored at 280 nm. The evaluation of the measurements used the strategy described in detail previously [20] and computer programs developed by P. Schuck [30]. The latter also allow an estimation of the statistical accuracy of the results obtained [30,31]. The general equation used for fitting was the following:

$$A(r) = \sum A_i(r) = \sum A_i(r_o)\exp\left[M_{ieff}\omega^2\left(r^2 - r_o^2\right)/2RT\right] \quad (1)$$

with $A_i(r)$, $A_i(r_o)$: absorbance of macromolecular species 'i' at any radial position r or at the fixed position r_o, respectively; $M_{ieff} = M_i(1\text{-}\bar{v}_i\,\rho_o)$: effective molar mass of particle 'i', $\bar{v}_i$ denoting the particle's partial specific volume and ρ_o the solvent density; ω: angular velocity of the rotor. Particle 'i' may itself be composed of k components: $M_{ieff} = \sum M_{ikeff}$ [18,19]. A few control calculations were performed in order to rule out non-ideal sedimentation behaviour of the samples [15].

In addition to sedimentation equilibrium experiments, we also conducted sedimentation velocity runs at a rotor speed of 40 000 rpm. Most of them used synthetic boundary double sector Epon centerpieces of the capillary type.

The partial specific volume $\bar{v}$ of band 4.1 was calculated according to Durchschlag [32], using the basic data originating either from Cohn and Edsall or Zamyatnin (refined data) (see Table 1 in Ref. [32]). Values of $\bar{v} = 0.739$ ml/g or 0.719 ml/g, respectively, were obtained. $M_{eff} = M(1\text{-}\bar{v}\rho_o)$ for band 4.1, as used in the evaluations, was determined experimentally; see below. For the band 3 protomer, the evaluations applied a value of 32 200 for $M(1\text{-}\bar{v}\rho_o)$. It was calculated [32] based on the following data: $M_r = 101\,800$ for the polypeptide part of the glycoprotein [2,33], a carbohydrate content of 10% (w/w) [34], a value of $\bar{v} = 0.74$ ml/g for the glycoprotein, as determined by experiment [23] (which is close to the calculated $\bar{v}$ [35]), a value of 0.64 g of bound $C_{12}E_9$ per g of glycoprotein [11], $\bar{v} = 0.944$ ml/g for $C_{12}E_9$ [36], and a (mean) solvent density $\rho_o = 1.008$ g/ml. This value for $M(1\text{-}\bar{v}\rho_o)$ is by 10% higher than the value determined by us in earlier experiments [18]. Its maximum uncertainty is estimated to be $\pm$7%. The conclusions drawn below (and in earlier papers [13–16]) are unaffected by the stated difference in $M(1\text{-}\bar{v}\rho_o)$ (which is most probably due to a certain degree of heterogeneity in the 'dimeric' band 3 samples studied in [18]).

3. Results

3.1. Association behaviour and binding properties of rhodamine-labelled band 4.1

In sedimentation equilibrium runs, unmodified and rhodamine-labelled band 4.1 were found to be identical with respect to the molar mass: for both protein samples, an effective molar mass $M_{eff} = M(1\text{-}\bar{v}\rho_o)$ of (18 600 $\pm$ 600) g/mol (S.D., $n = 6$) was obtained. Depending on the figure used for $\bar{v}$ (see above), this leads (together with a value for ρ_o of 1.008 ml/g) to a molecular mass of 72 900 or 67 600 kDa, respectively. When compared to the molecular mass of 66 300 kDa calculated from the cDNA sequence [37], our data show that both proteins were monomeric.

Rhodamine-labelling of band 4.1 did not affect its binding affinity towards the cytoplasmic domain of band 3, as demonstrated by a dot blot assay [29]: both the labelled and unlabelled proteins bound specifically and at apparently identical amounts to the im-

mobilized band 3 fragment, as judged by virtually identical immunosignals. No binding of band 4.1 (labelled or unlabelled) was seen when the band 3 fragment was replaced by ankyrin [38] or bovine serum albumin. The observed lack of influence of labelling of band 4.1 via succinimide ester-linked rhodamine on the band 3/band 4.1 association is in line with results from a previous study [29]. In this study, succinimide ester-mediated labelling of band 4.1 with biotin was successfully applied to identify the sequence motifs involved in the binding between the two proteins. In addition, the sequence identified as the major band 3 binding motif on band 4.1, LEEDY, and its vicinity of 8 amino acids, do not contain lysine residues, to which succinimide esters couple preferentially [29].

3.2. Determination of the predominant binding site for band 4.1

Sedimentation equilibrium experiments on mixtures of band 3 and rhodamine-labelled band 4.1, at low band 4.1/band 3 ratio, were used to identify that band 3 oligomer which serves as the predominant binding site for the ligand protein band 4.1. The A(r)-profiles obtained were analyzed by fitting to these data the sum of two individual Boltzmann distributions (Eq. (1)): one representing unbound band 4.1, and the other one representing a complex of fixed effective molar mass $M_{eff} = \Sigma M_k\ (1\text{-}\bar{\nu}_k\ \rho_o)$. This mass was systematically varied between that of the smallest and that of the largest complex conceivable. The parameters to be optimized were the absorbancies, at a fixed radial position r_o, of both components. The result of primary interest, namely the dependency of the sum of the squared residuals, σ, on M_{eff} is shown in Fig. 1. It is obvious that $\sigma(M_{eff})$ has a rather well-defined minimum for a complex mass which agrees with that of one band 4.1 molecule plus the band 3 tetramer. On the other hand, complexes containing, beside one band 4.1 molecule, either one band 3 monomer or dimer, led to σ-values which are larger than the former one by one order of magnitude. Thus, the band 3 tetramer, but not the monomer or dimer, seems to represent the predominant binding site for band 4.1. This conclusion is

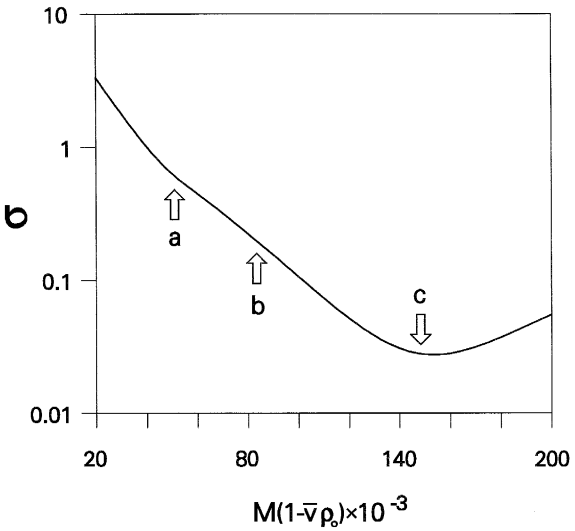

Fig. 1. Sedimentation equilibrium analysis of a band 3/band 4.1 mixture: Dependency of the sum of the squared residuals of the fit, σ, on the effective mass, M_{eff}, of an assumed complex. The arrows indicate the effective molar mass corresponding to a complex consisting of one band 4.1 molecule and either a band 3 monomer (a), dimer (b) or tetramer (c). The concentration of band 3 was 340 μg/ml, that of band 4.1 107 μg/ml. Buffer: 10 mM Tris-HCl (pH 8.0), 80 mM NaCl, 1 mM EDTA, 0.2 mM DTT, 0.2% $C_{12}E_9$. Rotor speed: 10000 rpm.

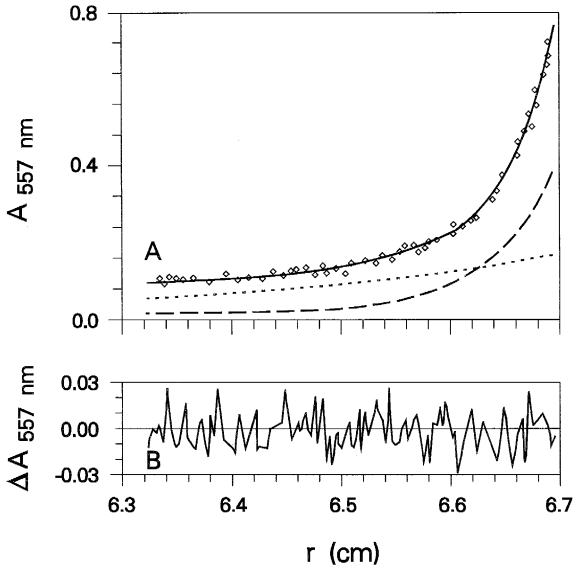

Fig. 2. An extension of the analysis of Fig. 1: (A) Experimental A(r)-sedimentation equilibrium data (◇); curve fitted to them under the assumption that the only dye-labelled protein species present were uncomplexed band 4.1 and a complex of one band 4.1 molecule and the band 3 tetramer (————); calculated contributions of unbound band 4.1 (· · ·) and complex (— — —). (B) Residuals of the fit to the experimental data.

supported by Fig. 2, in which for the band 4.1/band 3 tetramer complex a comparison is made of the experimental and the fitted A(r)-data (together with the calculated local contributions of the two types of particles). The local residuals of the fit, as plotted in Fig. 2b, are not only within the uncertainty of the experimental data but are virtually statistically distributed along the r-axis and thus demonstrate the excellent quality of the fit. The results described were independent of the initial concentrations of both proteins, of pH and ionic strength.

The lack of influence of ionic strength and of protein concentration on the quality of the fits supports our assumption that, under the conditions used in this study, the samples show ideal sedimentation behaviour [18,20]. This conclusion is reinforced by the results of control calculations which allowed for non-ideal sedimentation of the complex: the fits deteriorated or at least could not be improved by introducing a non-zero value for the second virial coefficient [15].

It should be noted that with pure band 3, under the conditions of the experiments described above, the distribution of the different band 3 oligomers (monomer/dimer/tetramer) was found to be approx. 30:50:20% (w/w), when averaged over the cell volume. Thus, the band 3 tetramer represented the least abundant component among the band 3 oligomers present.

3.3. Contributions of other band 3 oligomers to band 4.1 binding

The possible contribution of band 3 monomers or dimers as binding sites for band 4.1 can be evaluated by simple calculations: Terms which represent fixed amounts of complexes containing band 3 monomers or dimers are added to the set of the two Boltzmann distributions applied above, and their influence on the sum of the squared residuals of the fits is monitored [20]. Typical results, using the same data set as in Figs. 1 and 2, are shown in Fig. 3. It is obvious from this figure that the consideration of terms representing band 4.1 bound to band 3 monomers or dimers does not improve the quality of the fits. Instead, the corresponding complexes, when assumed to represent more than a few percent of the complexed band 4.1, will deteriorate its quality. However, Fig. 3 also

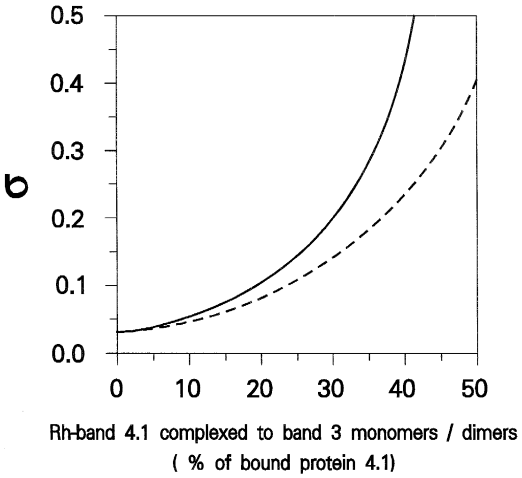

Fig. 3. A search for additional complexes: Dependency of σ on the addition of fixed amounts of complexes consisting of one band 4.1 molecule plus either a band 3 monomer (————) or dimer (— — —).

shows that the presence of small amounts of complexes containing band 3 monomers and dimers cannot be ruled out definitely.

The above data were collected 24–38 h after starting the ultracentrifuge run (32–46 h after solubilizing band 3). When data collected at longer running times were analyzed (40–55 h), the minima of the curves for the band 3 dimer/band 4.1 complex were found to be slightly shifted to non-zero complex concentrations. The observed shift increased with time. This suggests that, with increasing time, stable band 3 dimers were formed [11,12,15]) which, as shown below, also represent binding sites for band 4.1.

3.4. Number of band 4.1 molecules bound by the band 3 tetramer

The experiments described above, performed at molar ratios between band 4.1 and band 3 below 0.5, could be perfectly fitted by assuming the presence of only one complex of one band 4.1 molecule and one band 3 tetramer, with an effective molar mass, M_{eff}, of 147 000. At higher relative abundance of band 4.1, fits of that type were of inferior quality. However, fully satisfactory fits could be retrieved by using higher figures for the complex mass, the optimum figure depending on the band 4.1/band 3 ratio. The highest optimum complex mass which could be de-

tected reproducibly corresponded to that of one band 3 tetramer plus four band 4.1 molecules and thus to a complex of 1:1 stoichiometry of the two proteins (Fig. 4). This limiting stoichiometry is in agreement with the results obtained by others [4]. In one experiment, however, a molar ratio between band 4.1 and band 3 of approx. 6 was observed, which would be in line with the presence of two band 4.1 binding sites per band 3 protomer [29]. It is clear that the optimum molar complex mass determined from the fits corresponds to a mean value, which is averaged over the particle population. This notion is also favoured by the finding that the sum of the squared residuals of the fits could be slightly reduced by assuming that the samples contained two different complexes of different band 4.1 content instead of one.

As described above, all experimental A(r)-distributions collected at low molar band 4.1/band 3 ratio could be best fitted assuming the presence of only complexes of stoichiometry 1:4. This indicates that the binding of band 4.1 to band 3 does not show positive cooperativity, which again is in agreement with the data in the literature [4] [1].

3.5. Binding of band 4.1 to stable band 3 dimers

The heterogeneous band 3 samples used in the above experiments were converted into stable dimers essentially in two ways: (1) By chemical cross-linking via S-S-bridges induced by the catalytic action of $CuSO_4$/o-phenanthroline [22], and (2) by oxygen-catalyzed chemical reactions in the polyether detergent applied (which finally lead to the formation of peroxides); in this case, no covalent bonds are formed [11,12,15]. These stable band 3 dimers, when incubated with band 4.1, also readily formed heterologous complexes. For one of those samples, containing band 3 modified by chemical reactions in the detergent, and two different band 4.1 concentrations, the dependency of the sum of the squared residuals of the fits on the molar mass of the complex is shown in Fig. 5. As in the case of unmodified band 3, complexes were formed, the molar mass of which increased with increasing supply of band 4.1. For the lower band 4.1 concentration, the effective molar complex mass corresponding to the best fit was virtually identical to that of the complex of one band 4.1 molecule and one band 3 dimer. The complex obtained with the higher 4.1 concentration contained, besides the band 3 dimer, two 4.1 molecules; it represented the largest complex found with dimeric band 3. The same results were obtained when stable band 3 dimers resulting from cross-linking via S-S-bridges were used (data not shown). With incompletely 'dimerized' samples, the A(r)-data could be fitted best by assuming that complexes both of 1:2 and of 1:4-stoichiometry were present. It is thus clear that 'stabilized' band 3 dimers (which we assume to be without biological relevance) also represent binding sites for band 4.1.

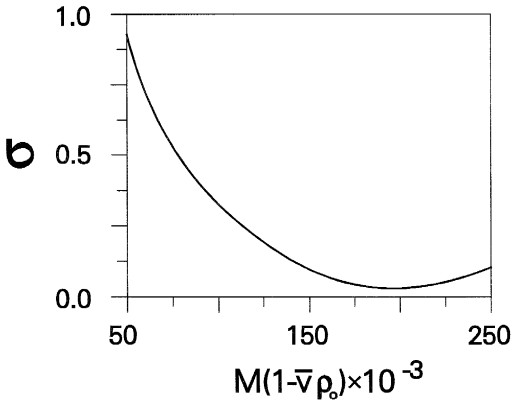

Fig. 4. Detection of larger complexes: Dependency of σ on M_{eff} of the assumed complex at high molar ratios of band 4.1 and band 3. The minimum of the curve corresponds to the effective molar mass of a complex consisting of four band 4.1 molecules plus a band 3 tetramer. The concentration of band 3 was 180 μg/ml and that of band 4.1 310 μg/ml. Buffer: 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5 mM EDTA, 0.2 mM DTT, 0.2% $C_{12}E_9$. Rotor speed: 8500 rpm.

3.6. Stability of the band 4.1 / band 3 complexes

The sedimentation equilibrium experiments described above do not discriminate between stable

[1] The ease by which the complex of the band 3 tetramer and one band 4.1 molecule could be detected, despite the availability of 3 (or possibly 7) additional binding sites for band 4.1, may even indicate negative cooperativity of ligand binding to the band 3 tetramer. The same argument holds for the binding of hemoglobin [14] and aldolase [15]. A shift of the association equilibrium of band 3 towards the tetramer following ligand binding could, however, also explain the experimental findings (at least in part).

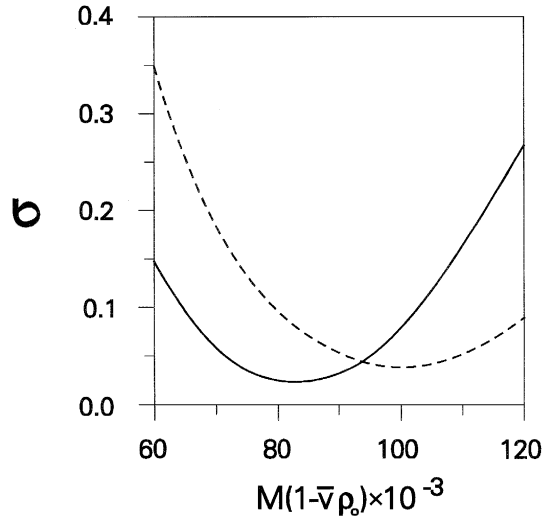

Fig. 5. Binding of band 4.1 to stable band 3 dimers: Dependency of σ on M_{eff} of the assumed complex in samples containing noncovalent stable band 3 dimers, at low and high molar ratios of band 4.1 and band 3. The minima of the curves correspond to effective molar masses of complexes consisting of one band 3 dimer and either one (————) or two (— — —) band 4.1 molecules. The concentration of band 3 was 210 μg/ml and that of band 4.1 110 μg/ml (————) and 270 μg/ml (— — —), respectively. Buffer: 10 mM Tris-HCl (pH 8.0), 20 mM NaCl, 0.5 mM EDTA, 0.2% $C_{12}E_9$. Rotor speed: 10 000 rpm.

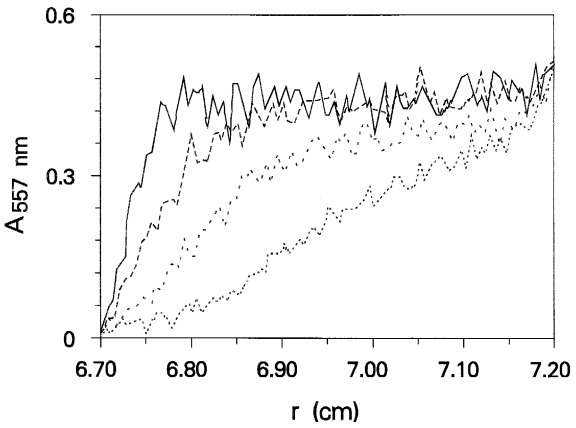

Fig. 6. Nonequilibrium centrifugation of band 4.1/band 3 mixtures: Sedimentation velocity profiles A(r,t). The concentration of band 3 was 270 μg/ml and that of band 4.1 120 μg/ml. Buffer: 10 mM Tris-HCl (pH 8.0), 40 mM NaCl, 0.5 mM EDTA, 0.2 mM DTT, 0.2% $C_{12}E_9$. Rotor speed: 40 000 rpm. The scans were recorded 10, 20, 30, and 60 min (left to right) after reaching the selected rotor speed.

aggregates and aggregates which are part of an association equilibrium. One way to discriminate between the two possibilities is to study the system by sedimentation velocity runs. A typical experiment on a mixture of rhodamine-labelled band 4.1 and unmodified band 3 is shown in Fig. 6. In this figure, consecutive A(r)-diagrams monitored at 557 nm are plotted for different running times. The figure shows, instead of two boundaries of $s_{20,w} = 3.0–3.5$ S (for unliganded band 4.1 ([4,25] and own observations)) and > 12 S (for the band 4.1/band 3 complex [23]), as expected in the case of a stable complex, a single but broad boundary of $s_{20,w} = 4.9$ S. This is indicative of an association equilibrium between the two proteins, the relaxation of which is intermediate on the time-scale of the technique [39]. Thus, the band 4.1/band 3 complex is not stable but part of an association equilibrium.

4. Discussion

Band 3 is a multifunctional protein. Its most prominent function is to transport anions across the erythrocyte membrane [40,41]. Another function is of equal importance, namely anchoring the membrane skeleton to the lipid bilayer. The main linker used for that purpose is ankyrin (band 2.1); however, there is good evidence that band 4.1 represents another linker [1–7]. In addition, band 3 is able to bind other proteins of the membrane skeleton and the cytoplasm, including three glycolytic enzymes and hemoglobin. For reviews of the other functions of band 3, see, e.g., Refs. [41,42].

The influence of the oligomeric state of band 3 on its diverse functions certainly represents an interesting problem. In this respect, anion transport has been the most intensely studied function of band 3, in particular with respect to the ‘functional unit’ of the transport (the smallest oligomer that can perform the function) [10]. Our group has presented evidence that already monomeric band 3 protein can transport anions [43], though other authors favour the view that dimeric band 3 is required for transport function (e.g., Refs. [41,44]). We have also studied the functional units of the binding of ankyrin [13,18], hemoglobin [14] and the glycolytic enzyme aldolase [15]. Surprisingly, it turned out that only the band 3

tetramer is able to serve as a binding site for these proteins. Band 3 dimers were found to bind these proteins only after being converted, from an unstable form (in an association equilibrium) into noncovalently or covalently linked stable aggregates. Using the same technique as in these previous studies, sedimentation equilibrium analysis of the solubilized proteins in solutions of a nonionic detergent, we now extended our studies to the band 3/band 4.1 association. The results obtained are completely analogous to those found earlier: it is the band 3 tetramer, but not the monomer or dimer, which represents the by far predominant or even the sole binding site for band 4.1. Possible reasons for the preference to the tetramer, as well as the problem of the biological relevance of our in vitro-studies, have been discussed earlier [15,16]. Again, however, stabilized band 3 dimers turned out to be capable of binding band 4.1 [2].

A particularly interesting aspect of the band 3/band 4.1 association is the capability of the band 3 tetramer to bind up to 4 (or even up to 8) band 4.1 molecules. This behaviour is not limited to band 4.1: It was observed already with hemoglobin [14] and aldolase [15] (but, up to now, not with ankyrin). Just this similarity, however, opens the possibility that the band 3 tetramer may represent a base on which a heterogeneous complex may self-assemble, allowing for positive or negative cooperativity and mutual control (for a review, see Ref. [46]). Sedimentation equilibrium analysis of the detergent-solubilized aggregates may yield some insight into this problem, as well as into that of competition for binding sites on the tetrameric base (e.g., between band 4.1 and ankyrin [5] or the glycolytic enzymes and hemoglobin [46]).

[2] With respect to the noncovalent stable band 3 dimers prepared by us, our findings again demonstrate that, in solutions of a polyether detergent, band 3 can easily be converted into a state with binding properties different from the initial one [11,12,15]. In our opinion, this is a noteworthy argument in the persisting debate on the state(s) of association of detergent-solubilized band 3 protein ([11,12] versus, e.g., Refs. [35,45]). The same argument holds with respect to the recent findings of Pinder et al. [35] on the band 3/ankyrin association, as compared to those of Mulzer et al. [13,18].

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