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Ultracentrifugation studies on the transmembrane domain of the human erythrocyte anion transporter Band 3 in the detergent $C_{12}E_8$

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Abstract The dilute solution behaviour of the transmembrane domain (TMD) of the human erythrocyte anion exchanger Band 3 was studied by analytical ultracentrifugation. Sedimentation velocity and equilibrium studies of the TMD solubilized with the detergent $C_{12}E_8$ demonstrate that the protein is a stable dimer in the concentration range 0.1 to 1 mg/ml. There is no evidence of a dissociation at low concentrations or of an association at higher concentrations. Hydrodynamic calculations applying a prolate ellipsoid of revolution and assuming a hydration of $w = 0.35$ result in an asymmetrical particle with an axial ratio (a/b) of ~3.5.

Key words Band 3 · Membrane protein · Analytical ultracentrifugation · Hydrodynamics

Introduction

Band 3, the erythrocyte anion exchange protein, consists of two major domains, a soluble, cytoplasmic domain of ~42 kDa and a transmembrane domain (TMD) of ~55 kDa (Reithmeier 1993). TMD retains its anion exchange function after removal of the cytoplasmic domain with trypsin, and may be purified in non-ionic detergents (Reithmeier 1979). Two-dimensional crystals have been grown from TMD in $C_{12}E_8$ and the structure solved to a resolution of 20 Å (Wang et al. 1993; Wang et al. 1994). TMD shows an elongated dimeric structure with a large stain-filled pore, assumed to be the anion transport channel.

Although the transmembrane domain of Band 3 lacks the soluble cytoplasmic domain, it is more suitable for crystallization for a number of reasons. Firstly, the hinge between TMD and the cytoplasmic domain is very flexible (Wang 1994) which would hinder packing of the intact protein into a crystal lattice. Furthermore, the cytoplasmic domain is highly elongated and has another flexible hinge region in the middle (Low 1986). TMD is also more stable than the intact protein (Sami et al. 1992).

Intact Band 3 exhibits complex behaviour in non-ionic detergent solution and has been reported to be dimeric (Clarke 1975), a dimer-tetramer mixture (non-interconverting) (Casey et al. 1991), a monomer-dimer-tetramer equilibrium (Pappert and Schubert 1983), and a dimer-hexamer mixture (Wong 1993). Recently, the conditions under which some of these oligomeric states exist have been determined (Taylor et al. 1998), and it appears that the native functional unit is the Band 3 dimer.

We have previously investigated the oligomeric state of the Band 3 cytoplasmic domain and concluded that it is dimeric, although it dissociates to monomers at low concentrations (Cölfen et al. 1997). Recently, the self-association of TMD to form denatured heterogeneous aggregates has also been studied (Vince et al. 1997; Salhany et al. 1997). Here we examine the oligomeric state of TMD in $C_{12}E_8$ by analytical ultracentrifugation, and conclude that the protein forms stable dimers in solution.

Materials and methods

Material. Outdated human red blood cells were purchased from the Tisch Hospital Blood Bank, New York. $C_{12}E_8$

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(octaethylene glycol n-lauryl monoether) was purchased from Nikko Chemical Co. (Tokyo). All other materials were from Sigma Chemicals and were of analytical grade. Band 3 transmembrane domain (TMD) was prepared in 0.1% $C_{12}E_8$ according to the method described in Casey et al. (1989).

Solvent densities and viscosities. Solvent densities have been determined at (20.00±0.01)°C using an Anton-Paar model DMA 60 & DMA 602 precision density meter, calibrated with CsCl solutions. Solvent viscosities at (20.00±0.01)°C were determined using an automatic (Schott-Geräte, Darmstadt, FRG) viscometer. For each density and viscosity value, 10 consistent readings were obtained to permit an estimation of experimental error to be made. The density was found to be ρ=(1.001258±0.000001) g/ml, the viscosity η=(1.023±0.003) cP.

Partial specific volume $\bar{v}$. The $\bar{v}$ of the pure TMD of Band 3 was calculated from the amino acid composition (Tanner et al. 1988) to be 0.7642 ml/g (Perkins 1986). From the 0.945 g of bound detergent $C_{12}E_8$ per g TMD (Casey et al. 1991), the $\bar{v}$ of the TMD-$C_{12}E_8$ complex was calculated to be 0.866 ml/g assuming volume additivity.

Analytical ultracentrifugation. A Beckman (Palo Alto, USA) Optima XL-I analytical ultracentrifuge equipped with automatic scanning absorption optics and on-line Rayleigh interference optics was used for all investigations at a temperature of (20.0±0.1)°C. Standard 12 mm optical path length double sector cells were used with buffer as reference solvent in every case.

Sedimentation velocity analysis. The sedimentation velocity experiments were carried out at speeds of 45 000 and 50 000 RPM using a wavelength of 280 nm as well as the Rayleigh interferometer. The sedimentation coefficients were measured in two ways. In the first, conventional determination via the boundary movement was used. As a second approach, the sedimentation velocity profiles were fitted to approximate solutions of the Lamm differential equation (in this case the Fujita (1973) function was used together with the Fujita-MacCosham relation) to give the sedimentation and approximate diffusion coefficient, and thus a molar mass using the well known Svedberg equation (Svedberg and Pedersen 1940). For this purpose, we used the program SVEDBERG (Philo 1994, 1997).

The sedimentation coefficients $s_{20,b}$ measured in buffer, *b*, at a temperature of (20.0±0.1)°C were corrected to standard conditions of solvent viscosity, η and density, ρ, (water at 20.0±0.1°C) using the correction formula (Tanford 1961).

$$s_{20,w} = \left\{\frac{(1-\bar{v}\rho)_{20,w}}{(1-\bar{v}\rho)_{T,b}}\right\}\left\{\frac{\eta_{T,b}}{\eta_{20,w}}\right\} s_{T,b} \qquad (1)$$

Sedimentation equilibrium analysis. Sedimentation equilibrium measurements were performed at a rotor speed of 10 000 RPM. Scanning wavelengths of 280 & 260 nm were used. Molecular weights were analysed using the QUICKBASIC routine MSTARA (Cölfen and Harding 1997). This routine, amongst other features evaluates (i) the apparent (i.e. for a given loading concentration) weight average molecular weight, $M_{w,app.}$ using the M* procedure of Creeth and Harding (1982) and (ii) point weight average apparent molar masses, $M_{w,app.}$ (r) as a function of radial displacement r.

As a second independent evaluation, the radial equilibrium absorption profiles were fitted to the model of an ideal 1 or 2 component system using the routines IDEAL1 & IDEAL2 from Beckman instruments (McRorie and Voelker 1993).

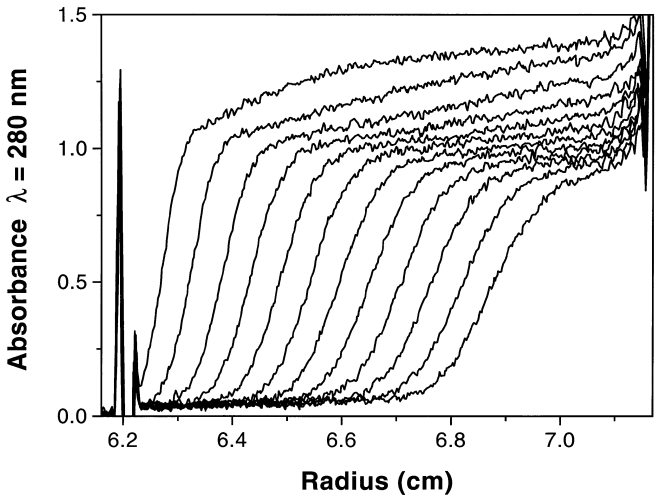

Fig. 1 Sedimentation velocity profiles for 0.6 mg/ml TMD of Band 3 in $C_{12}E_8$ at 50 000 rpm, 20.0°C and 280 nm, Scan interval 12 min. (Not all scans shown for figure clarity)

Results

Sedimentation velocity

From the sedimentation velocity profiles it can be seen that the Band 3 TMD preparation contains a significant amount (~30% of the total weight concentration) of a polydisperse and high molecular weight aggregate sedimenting considerably faster ($s_{20,w}$~14.5 S) than the (macromolecular) TMD component. The macromolecular component however appears to be sedimenting uniformly as a single stable species.

Attempts were made to fit the velocity profiles (Fig. 1) as a two component system with the SVEDBERG routine, but the fits were of poor quality probably because of the ill-defined nature of the aggregates. Thus, only scans were used for the fits to a one component system where the aggregates had sedimented completely: this still left 13–24 scans for analysis. Fits performed in this way were of good quality and yielded small and statistically distributed residuals. The weight average molar mass estimated from

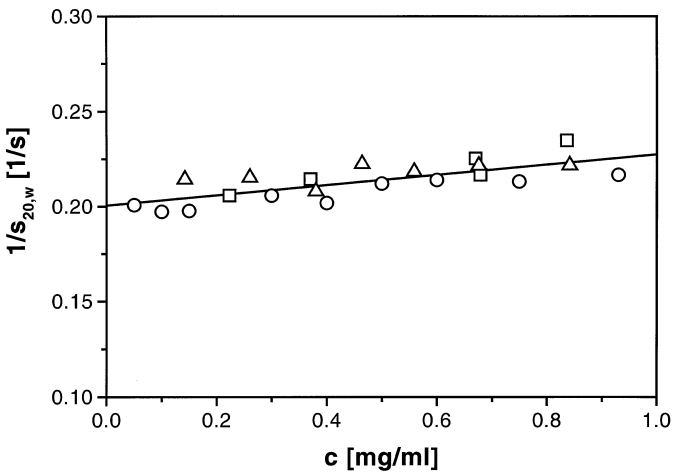

Fig. 2 Concentration dependence of $1/s_{20,w}$ for TMD of Band 3 in $C_{12}E_8$. The concentrations were corrected for radial dilution. See materials and methods for experimental details. □ Absorption optics 280 nm from fitting; ○ Absorption optics 280 nm from boundary movement; △ Interference optics from boundary movement

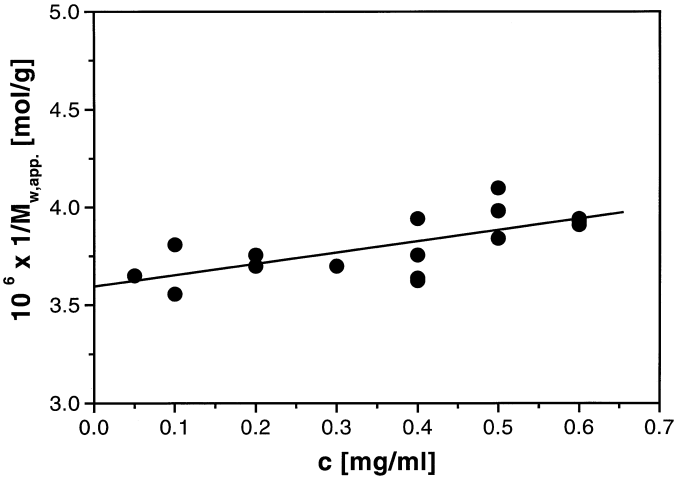

Fig. 3 Concentration dependence of the reciprocal apparent molar mass ($M_{w,app}$) for TMD of Band 3 in $C_{12}E_8$, determined by sedimentation equilibrium at 20 °C

the derived sedimentation and translational diffusion (*D*) coefficients via the Svedberg equation is (224,000 ± 17,000 g/mol). This value is in reasonable agreement with the theoretically expected molar mass of 239,000 g/mol for the dimeric TMD-$C_{12}E_8$ complex (see sedimentation equilibrium) although estimations of the molar mass from sedimentation velocity data are only approximate due to the possible high errors in *D*. In the fitting procedure applied, monodispersity is assumed and thus *D* is overestimated if the sample exhibits slight polydispersity, which in turn leads to an underestimate of M.

In Fig. 2, the sedimentation coefficients derived from the fitting procedure are plotted together with those from the conventional evaluation of absorption and interference records, giving good agreement.

Evaluation of the concentration dependence of the inverse sedimentation coefficients yields an $s^0_{20,w}$ of (4.99 ± 0.07) S. The concentration dependence shows no evidence of dissociative behaviour at low concentrations.

Sedimentation equilibrium

The sedimentation equilibrium experiments yield a weight average molar mass of (278,000 ± 4,900) g/mol for the TMD-$C_{12}E_8$ complex from the concentration dependence of $1/M_{w,app}$ (Fig. 3).

There is no indication of a dissociation at low concentration or of an association at higher concentrations. The calculated molar mass of the TMD-dimer from the amino acid composition (Tanner et al. 1988) is 122,800 g/mol. Taking into account the amount of bound detergent (0.945 g detergent/g TMD) (Casey et al. 1991) this gives a theoretical molar mass of 238,846 g/mol for the detergent complex of the dimer. The molar mass derived from sedimentation equilibrium data is some 40,000 g/mol higher than the crude estimate from sedimentation velocity but both may clearly be assigned as dimeric. The difference may be attributable to the small amount of high molar mass contaminants which was detected in sedimentation velocity experiments, but not used in the analysis of s (see Fig. 1): these aggregates would however make a contribution to the weighted averaged M_w from sedimentation equilibrium.

Fitting the sedimentation equilibrium concentration profiles to different models showed that the ideal 1 component system describes the experimental data best. The results from these fits agree well with the results from the independent M* evaluation and are therefore included in Fig. 3. All fits were of good quality as indicated by the small and statistically distributed residuals.

Hydrodynamic calculations

From the molar mass and $s^0_{20,w}$, one can calculate the frictional ratio f/f_0 (see e.g. Tanford, 1961) by using:

$$\left(\frac{f}{f_0}\right)=\left[\frac{M\,(1-\bar{\nu}\,\rho_{20,w})}{N_A\,(6\,\pi\,\eta_{20,w}\,s^0_{20,w})}\right]\left(\frac{4\,\pi\,N_A}{3\,\bar{\nu}\,M}\right)^{1/3} \qquad (2)$$

f is the frictional coefficient of the macromolecule and f_0 the corresponding coefficient for a sphere of the same mass and anhydrous volume as the macromolecule. N_A is Avogardo's number and the subscript "20,*w*" for viscosity and density refers to pure water at 20.0 °C. From the frictional ratio, the Perrin shape function P can be calculated (Perrin 1936; Squire and Himmel 1979; Harding 1995):

$$P=\left(\frac{f}{f_0}\right)\left(\frac{w}{\bar{\nu}\,\rho_0}+1\right)^{-1/3} \qquad (3)$$

with *w* the so called "hydration" (Tanford 1961).

Using the molar mass of a dimer complex of 240,000 g/mol and an $s^0_{20,w}$ of (4.99 ± 0.07) S, one obtains f/f_0 = (1.29 ± 0.02). Taking the apparent hydration *w* (italic) as ~0.35 (Tanford 1961; Zhou 1995) then P = (1.15 ± 0.05). Using the

routine ELLIPS1 for a prolate ellipsoid (Harding and Cölfen 1995; Harding et al. 1997), this gives an axial ratio (a/b)~ (3.5±0.8). This axial ratio can only be an *estimate* as the exact hydration is not known. If the hydration is less than 0.35, then the asymmetry would be much higher. Nonetheless, this axial ratio is in reasonable agreement with that found for the 20 Å resolution structure, estimated from two-dimensional crystals by electron microscopy and image reconstruction to be 110×40 Å (a/b = 2.8) and for a second conformation 120×35 Å (a/b = 3.4) (Wang et al. 1993). However, in a more recent paper, the dimensions for a 3D structure were given as 110×60×80 Å which would correspond to (a/b)~1.6 (Wang et al. 1994).

Discussion

The results from the sedimentation equilibrium experiments clearly indicate that TMD of Band 3 is a dimer, with no evidence of dissociation to monomers at lower cencentrations (at least down to 0.1 mg/ml). The dissociation constant must therefore be less than 10^{-6} M. This is in contrast to the behaviour observed for the cytoplasmic domain of Band 3 (Cölfen et al. 1997) with a dissociation at low concentration with a K_d of ~3 μM. Our finding of a strongly bound dimer for the Band 3 TMD in $C_{12}E_8$ is consistent with recent observations of Vince et al. (1997) and Salhany et al. (1997) who have used chromatographic based approaches. The different K_d's of the cytoplasmic and transmembrane domain of Band 3 would appear to confirm that it is the transmembrane domain, and not the cytoplasmic domain, which dictates the strong binding of the intact Band 3 into dimers.

Sedimentation velocity experiments (Fig. 1) indicate also a proportion (~30% of the extinction at 280 nm) of high molecular weight material ($s_{20,w}$~14.5 S). The presence of this material may explain why the M_w from sedimentation equilibrium was found to be 40,000 g/mol higher than the value of ~240,000 g/mol calculated from the amino acid sequence and the amount of bound detergent.

It is possible to obtain a further estimate for $M_{w,app.}$ by analysing the sedimentation profiles of Fig. 1 in terms of the Lamm equation, according to the procedure described by Philo (1997): this procedure, in addition to providing a check on the sedimentation coefficient obtained by conventional boundary analysis, also yields an estimate for the apparent translational diffusion coefficient and hence from the Svedberg equation (Svedberg and Pedersen 1940) an estimate for the molar mass. The molar mass for the TMD found by this method is lower (M_w = 224,000±17,000 g/mol) than that from sedimentation equilibrium and shows a much higher standard deviation but also confirms the TMD dimer.

Although the concentration range studied here is still relatively low compared with that required for other biophysical studies (NMR & crystallization trials), we have confirmed a tight dimer formation with an asymmetric structure of axial ratio consistent with the results from electron microscopy (Wang et al. 1993, 1994). This gives a possible basis for further investigations of the quaternary structure.

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