Hydrodynamic Examination of the Dimeric Cytoplasmic Domain of the Human Erythrocyte Anion Transporter, Band 3

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ABSTRACT Solution studies of the cytoplasmic domain (molecular mass ≈40kDa) of band 3, the anion exchanger from human erythrocyte membranes, previously suggested a dimeric molecule on the basis of the relative techniques of calibrated gel filtration and calibrated preparative ultracentrifugation. This dimeric behavior is firmly established on an absolute basis by a combination of calibrated gel chromatography and absolute ultracentrifugation techniques. Sedimentation velocity in the analytical ultracentrifuge combined with calibrated gel chromatography give a molecular mass M of (77 ± 4) kDa, a value confirmed by low-speed sedimentation equilibrium. Velocity sedimentation in the analytical ultracentrifuge gave a single sedimenting species with an $s^{\circ}_{20,w}$ of (3.74 ± 0.07)S. Sedimentation equilibrium analysis was also used to establish the strength of the binding via the dissociation constant K_d, with a value from direct fitting of the concentration distribution curves of (2.8 ± 0.5) μM, confirmed by a value of ~3 μM obtained from fitting a plot of molecular weight $M_{w,app}$ versus cell loading concentration. Hydrodynamic calculations based on the classical translational frictional ratio showed that the protein was highly asymmetric, with an axial ratio of ~10:1, consistent with observations from electron microscopy.

INTRODUCTION

The band 3 protein is an integral membrane glycoprotein (molecular mass M_r = 95 kDa) of the erythrocyte membrane (Reithmeier, 1993). Its functions include the electroneutral exchange of anions across the membrane through an integral transmembrane domain (M_r = 53 kDa) (Passow, 1986) and the binding of cytoskeletal and cytosolic proteins by an extended cytoplasmic domain (M_r = 40 kDa). These domains may be separated by limited proteolysis of erythrocyte membranes and purified with their respective functions intact.

Whereas the intact band 3 and its transmembrane domain can be solubilized only by the use of detergents (Schubert et al., 1983; Casey et al., 1989), the cytoplasmic domain (CD) is readily soluble in aqueous solution, making it more amenable to solution characterization. In a previous study Appell and Low (1981) used a combination of calibrated gel chromatography with calibrated preparative ultracentrifugation (in a sucrose density, with the sedimentation rate compared with proteins of known sedimentation coefficient): A combination of the hydrodynamic or Stokes radius R_s with the apparent sedimentation coefficient led to the conclusion that the CD exists in solution in the dimeric form. Because of the relative nature of both techniques used (i.e., reliance on calibration standards), the firmness of this conclusion has remained open to some question: In the present study this dimeric form is firmly established by combining calibrated gel chromatography with the absolute sedimentation coefficient from the analytical ultracentrifuge and also by sedimentation equilibrium in the analytical ultracentrifuge, which gives (absolute) molecular weights directly. We further establish the strength of the interacting system by determining the dissociation constant from sedimentation equilibrium by using two approaches: 1) modeling the (weight average) apparent molecular weight data $M_{w,app}$ as a function of ultracentrifuge loading concentration c and 2) direct modeling of the concentration distribution data in the ultracentrifuge cell. We also use sedimentation equilibrium analysis to confirm the reversibility of the self-association process. We also comment on the extended conformation of the CD dimer, on the basis of classical translational frictional ratio calculations.

EXPERIMENTAL PROCEDURES

Materials

Recently outdated human blood was obtained from the Regional Transfusion Centre (Birmingham, UK). Gel permeation chromatography materials were obtained from Pharmacia (Milton Keynes, UK). All chemicals and reagents were of analytical grade.

The cytoplasmic CD of band 3 was purified and concentrated to 2 mg/ml by the use of an Amicon pressurized filter (with a cutoff of ~10,000 Da) (Appell and Low, 1981). The preparations were deemed >95% pure on the basis of Coomassie-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis. Protein concentrations were determined by a Coomassie-binding assay (Pierce Chemicals, Chester, UK). All measurements were made in a pH 8 Tris-HCl buffer containing 10 mM Tris-HCl, 10 mM NaCl, and 0.5 mM EDTA.

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Solvent densities and viscosities

Solvent densities were determined at 20°C by use of an Anton-Paar model DMA 02C precision density meter, calibrated with CsCl solutions (Kratky et al, 1973). Solvent viscosities at 20°C were determined with a Schott-Geräte AVS-310 automatic viscometer. For each density and viscosity value, 10 consistent readings were obtained to permit an estimation of experimental error to be made.

Gel filtration chromatography

Sepharose CL-4B (Pharmacia, Uppsala, Sweden) was agitated into suspension and carefully layered into a 300 mm × 5 mm column. The column was washed with 5 volumes of the Tris-HCl (pH 8) buffer at a rate of 0.25 ml/min to remove all traces of (storage) ethanol. All chromatographic runs were performed at 4°C, and the column was washed with 2–5 volumes of buffer before each protein preparation was injected.

Before sample loading, the head volume was allowed to run down to the top of the gel, allowing direct application of the sample onto the top of the column. After the sample had run into the gel, the head volume was replaced without disturbing the top of the gel. The column was immediately sealed and run at 0.1 ml/min. The eluate was monitored with an on-line UV monitor (Pharmacia) detecting at a wavelength of 280 nm. Peak volumes were standardized to a K_D value (Andrews, 1965)

$$K_D = (V_e - V_0)/(V_t - V_0), \quad (1)$$

where V_e is the elution volume of the center of a protein peak, V_0 is the void volume (determined by elution of blue dextran), and V_t is the total elution volume (determined by elution of β-mercaptoethanol).

Sedimentation velocity analysis in the analytical ultracentrifuge

A Beckman (Palo Alto, CA) Optima XL-A analytical ultracentrifuge equipped with automatic scanning absorption optics (at a wavelength of 280 nm) was used for all sedimentation velocity studies. Standard 12-mm optical path-length double sector cells with buffer were used as a reference solvent in every case. To check the presence or absence of high molar mass species we conducted sedimentation velocity experiments by initially monitoring for any moving boundaries at speeds of 3000, 5000, 10,000, and 15,000 revolutions/min before a final speed of 40,000 revolutions/min was selected. We corrected the sedimentation coefficients $s_{20,b}$ measured in the Tris-HCl (pH 8) buffer (represented by the subscript b) at a temperature of 20°C to standard conditions of solvent viscosity η and density ρ (water at 20°C), using the correction formula (Tanford, 1961)

$$s_{20,w} = \{(1 - \bar{\nu}\rho)_{20,w}/(1 - \bar{\nu}\rho)_{T,b}\}/\{\eta_{T,b}/\eta_{20,w}\} \cdot s_{T,b} \quad (2)$$

A value of 0.74 ml/g for the partial specific volume ν of the protein was used (Appell and Low, 1981). The infinite dilution sedimentation coefficient $s^o_{20,w}$ and the concentration-dependence coefficient k_s were obtained from the relation

$$s_{20,w} = s^o_{20,w}(1 - k_s c), \quad (3)$$

where c is the cell loading concentration (corrected for radial dilution).

Sedimentation equilibrium analysis in the analytical ultracentrifuge

Sedimentation equilibrium measurements were performed in the Optima XL-A ultracentrifuge at 20°C at a rotor speed of 10,000 rpm and with (12-mm) multichannel cells (three solution–solvent pairs) with 0.1 ml in the solution sectors and 0.12 ml in the reference solvent sectors. A scanning wavelength of 280 nm was used. We obtained the baseline absorbance (contribution from nonmacromolecular species) by overspeeding at the end of the run. Equilibrium solute distributions were analyzed with the Quickbasic routine MSTARA (Harding et al., 1992) now available on PC (Colfen and Harding, in preparation). This routine, among other features, evaluates 1) the apparent (i.e., for a given loading concentration) weight average molecular weight $M_{w,app}$ by use of the M^* procedure of Creeth and Harding (1982) and 2) point average apparent molecular weights $M_{w,app}(r)$ as a function of radial displacement r. As a check on the performance of the XL-A, the less convenient but more precise Beckman model E ultracentrifuge, equipped with a laser-based (5 mW, He–Ne) Rayleigh interferometer as detection optics, was used at a loading concentration of 0.93 mg/ml. The Rayleigh interference fringe patterns were captured off line by an Ultroscan XL laser densitometer (LKB Instruments, Bromma, Sweden) with appropriately modified software. Fringe data were captured into a PC and the variation in fringe shift with radial position evaluated with the Fourier-cosine series PASCAL algorithm ANALYSER (Rowe et al., 1992), and the meniscus concentration C_a was obtained by the intercept/slope method (Creeth and Harding, 1982). A modified form of MSTARA known as MSTARI was used to analyze the data (Harding et al., 1992; Colfen and Harding, in preparation).

Two methods were used to analyze the strength of the dimerization reaction of the protein. First the ASSOC4 routine of the XL-A software (Beckman Instruments, Palo Alto, CA), based on Eq. III27 of Kim et al. (1977), was used to represent directly a plot of the absorbance in the cell, $A_{280}(r)$, as a function of r at a loading concentration of 0.18 mg/ml, where nonideality effects can be safely assumed to be negligible. Second, a plot of $M_{w,app}$ versus loading concentration was used, fitted to Eq. III4 of Williams (1972): Because the highest loading concentration of protein used was 0.93 mg/ml, thermodynamic ideality can again be reasonably assumed. A value for the monomer molecular mass, M_1, of 40 kDa was used (Appell and Low, 1981). We monitored the reversibility of the association by overlaying plots of $M_{w,app}(r)$ versus $A_{280}(r)$ for different loading concentrations (Roark and Yphantis, 1969).

RESULTS AND DISCUSSION

Homogeneity and sedimentation coefficient

Sedimentation diagrams for the cytoplasmic domain, such as shown in Fig. 1 *A* for a loading concentration of 0.7 mg/ml, show only single symmetric sedimenting boundaries, strongly indicative of sample homogeneity, although the sloped plateau is indicative of a small amount of nonspecific aggregate and the visible low-s tail is indicative of a small amount of nonsedimenting material (also observed on sodium dodecyl sulfate polyacrylamide gel electrophoresis), probably caused by some proteolysis.

Fig. 1 *B* shows the corresponding dependence of $s_{20,w}$ on c. A linear fit to Eq. 3 yields a values for $s^o_{20,w}$ of (3.74 ± 0.07) S, which corresponds to the intact dimer. Our value is both consistent with the value obtained earlier by (preparative ultracentrifugation) for the apparent sedimentation coefficient of ~4.1 S obtained by Appell and Low (1981) and absolute because of its lack of dependence on calibration standards. No evidence for a dissociation of the dimer is evident over the protein concentration range used (~0.1–1.9 mg/ml) (apart from possibly the lowest concentration data point), which suggests a strong interaction of monomers with each other.

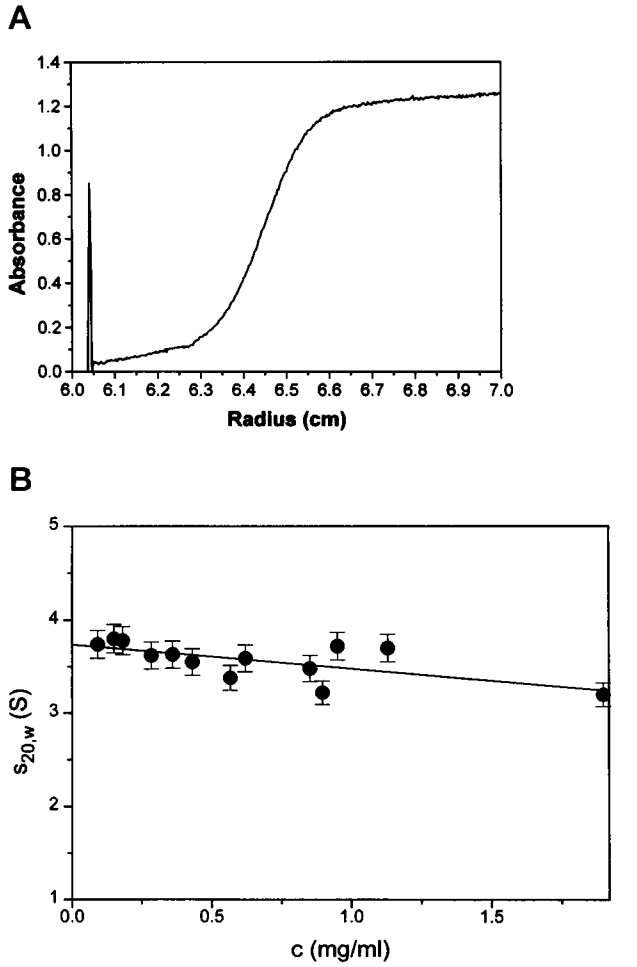

FIGURE 1 Sedimentation velocity of the CD of band 3. (*A*) Sedimentation diagram (absorption optical system) of the CD at 40,000 revolutions/min and 20°C. Protein loading concentration c = 0.7 mg/ml. (*B*) Dependence of measured $s_{20,w}$ on protein concentration c (corrected for radial dilution). Linear regression analysis gives $s^{\circ}_{20,w}$ = (3.74 ± 0.07) S.

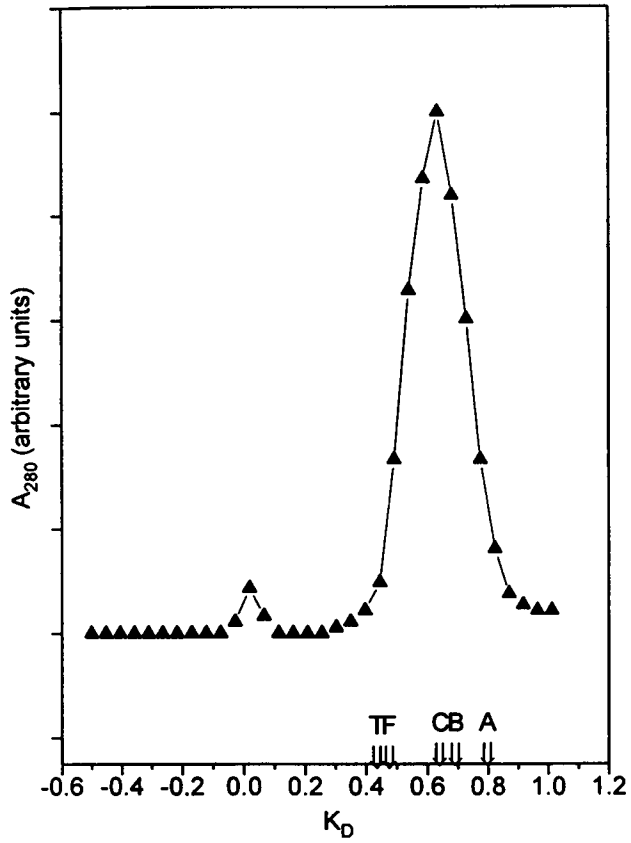

FIGURE 2 Gel filtration elution diagram of the CD of band 3 from a sepharose CL-4B column. Elution of standard proteins is indicated (*T*, thyroglobulin; *F*, apoferritin; *C*, catalase; *B*, bovine serum albumin; *A*, carbonic anhydrase). Loading protein concentration ~2 mg/ml.

Homogeneity and calibrated gel filtration chromatography

Elution profiles for the purified CD (Fig. 2) also show only a single macromolecular component, confirming the homogeneity observation of Fig. 1 *A* (and the presence of a small peak at K_D = 0 is also supportive of the observation from Fig. 1 *A* of a small amount of nonspecific aggregate). Inasmuch as column separation is more on the basis of molecular size than of molecular weight, the calibration procedure is based on the former: The arrows indicate the elution positions of the standard proteins of known Stokes radius, R_s: thyroglobulin (89 Å), apoferritin (69 Å), catalase (45.4 Å), bovine serum albumin (36.1 Å), and carbonic anhydrase (20.1 Å). Values of K_D were calculated from the elution position by Eq. 1 and were plotted against R_s for the standard proteins. Our R_s value of (46 ± 5) Å for the CD as calculated from a simple linear regression, although it is again consistent with a dimer form, is a little lower than that determined earlier by Appell and Low (1981) of 53 Å.

Calculation of M_r from R_s and $s^{\circ}_{20,w}$

The molecular mass of the band 3 cytoplasmic domain can be calculated from the modified form of the Svedberg equation (Svedberg and Pedersen, 1940):

$$M_r = \{6\pi\eta R_s s^{\circ}_{20,w}\}/(1 - \bar{\nu}\rho) \quad (4)$$

to give a value of (77 ± 10) kDa. Because the molecular mass of the CD monomer is 40 kDa, the dimer is confirmed. To obtain further quantitative information of the strength of the dimerization it is necessary to resort to the absolute molecular weight probe of sedimentation equilibrium in the analytical ultracentrifuge.

Sedimentation equilibrium: molecular weight and dissociation constant, K_d

The apparent weight average molecular weight (molar mass) $M_{w,app}$ dependence with ultracentrifuge cell loading concentration c is shown in Fig. 3 *A*. The value from the interference optical system of Beckman model E (at 0.93 mg/ml) appears to be consistent with the absorption optical data from the XL-A for the other loading concentrations. Graphical extrapolation to infinite dilution, ignoring the

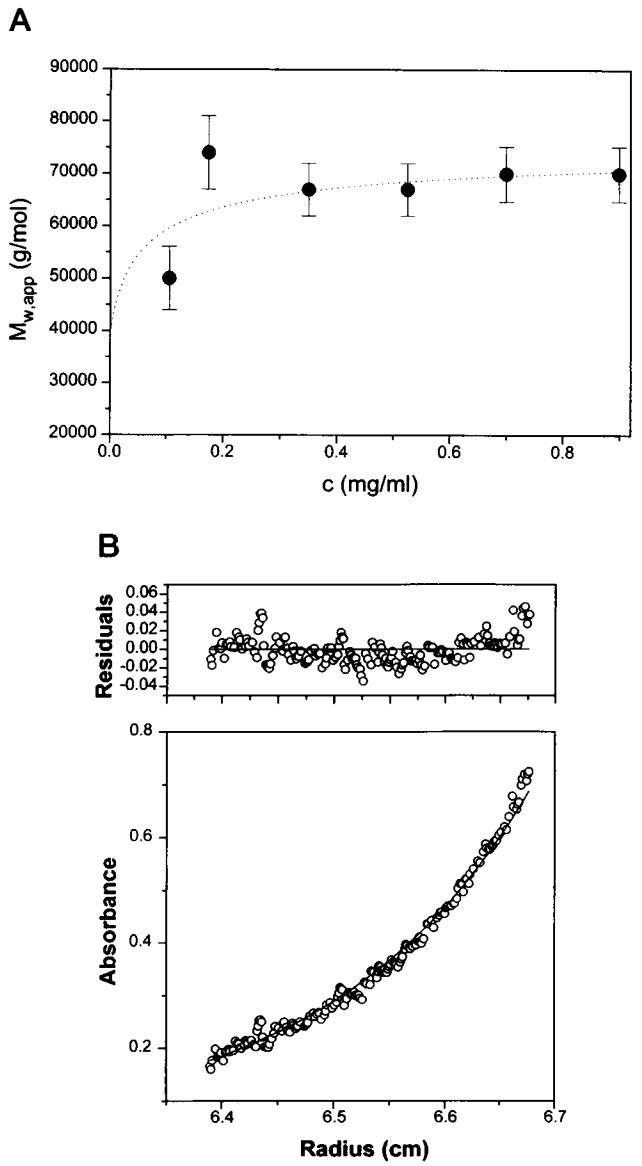

FIGURE 3 Sedimentation equilibrium distributions for the CD. (*A*) Plot of apparent weight average molecular weight $M_{w,app}$ versus cell loading concentration c. The dotted curve is a fit for an ideal reversible dimerization with dissociation constant K_d, ~3 μM. (*B*) Protein concentration (expressed in terms of UV absorbance at 280 nm in a 12-mm optical path-length cell) versus the radial displacement (cm) at a given position in the cell. Rotor speed 10,000 rpm; temperature 20°C; loading concentration c = 0.18 mg/ml. The line fitted is for an ideal reversible dimerization with dissociation constant K_d = (2.8 ± 0.5) μM.

first concentration point, is consistent with the expected value for cytoplasmic domain dimers (80,000 g/mol). A tentative fit to all the data for an ideal dimerizing system yields an estimate for the dimerization constant K_2 of ~(0.3 ± 0.2) μM^{-1}, equivalent to a dissociation constant K_d of ~(3 ± 2) μM. Direct fitting of the concentration distribution data (expressed as $A_{280}(r)$ versus r) for the 0.18-mg/ml loading concentration (Fig. 3 *B*), where nonideality effects can be completely disregarded, yields similar but more precise values of (0.36 ± 0.07) μM^{-1} for K_2 and (2.8 ± 0.5) μM for K_d, confirming a very strong interaction between monomers. Finally, Fig. 4 shows overlay plots of point average molecular weight data $M_{w,app}(r)$ as a function of $A_{280}(r)$ for four loading concentrations (0.18, 0.35, 0.58, and 0.7 mg/ml). Despite the noise (an unavoidable feature of point average molecular weight data calculated from absorption optical records unless heavy smoothing is used) the $M_{w,app}(r)$ data appear to follow the same general pattern, with no evidence of discontinuity, therefore confirming the reversible nature of the dimerization (Roark and Yphantis, 1969).

Gross solution conformation of the cytoplasmic domain

On the basis of the measured M_r and $s^\circ_{20,w}$ values for the cytoplasmic domain (dimer) it is possible to comment on its gross conformation by using the translational frictional ratio f/f_0 (where f is the frictional coefficient of the macromolecule and f_0 is the corresponding value for that of an anhydrous sphere of the same mass and volume), which relates to the sedimentation coefficient and molecular weight by (Tanford, 1961; Harding, 1995)

$$\left(\frac{f}{f_0}\right) = \left[\frac{M(1-\bar{\nu}\rho_0)}{N_A(6\pi\eta_0 s^\circ_{20,w})}\right]\left(\frac{4\pi N_A}{3\bar{\nu}M}\right)^{1/3}, \quad (5)$$

where N_A is Avogadro's number: a value for f/f_0 of (1.7 ± 0.2) is obtained. The frictional ratio can then be used to define the Perrin "frictional ratio due to shape" function P (Perrin, 1936; Squire and Himmel, 1979; Harding, 1995) by

$$P = \left(\frac{f}{f_0}\right)\left(\frac{w}{\bar{\nu}\rho_0} + 1\right)^{-1/3}, \quad (6)$$

where w is the so-called "hydration" (i.e., mass of solvent bound per unit mass of protein). If we take a "typical" value for w of ~0.35 (Tanford, 1961; Perkins, 1986) we can

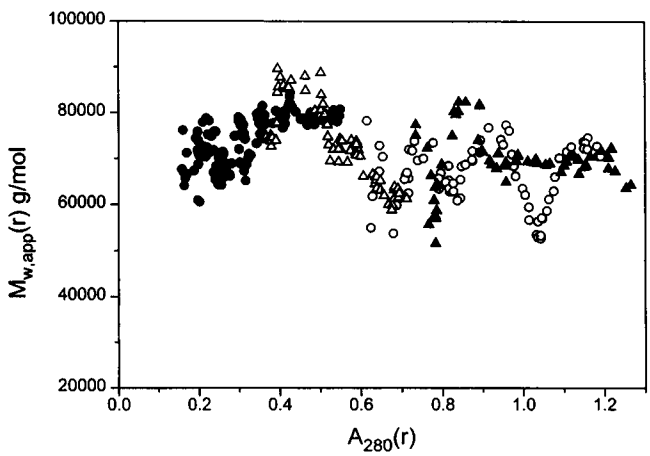

FIGURE 4 Sedimentation equilibrium point average $M_{w,app}(r)$ versus absorbance plots for different cell loading concentrations c: ●, c = 0.18 mg/ml; △, c = 0.35 mg/ml; ○, c = 0.58 mg/ml; ▲, c = 0.70 mg/ml

estimate P to be ~1.5; this corresponds, by simple inversion formulae (Harding and Colfen, 1995), to an asymmetric structure for the CD with an axial ratio of ~10 (equivalent prolate ellipsoid). This value is consistent both with the result of Appell and Low (1981) (obtained by use of an apparent sedimentation coefficient) and with the extended conformation of the domain as visualized by electron microscopy (Weinstein et al, 1978).

Implications for the quaternary structure of the intact band 3 protein

It is interesting to assess the implications of these findings for the CD on the overall quaternary structure of the integral band 3 protein (CD + transmembrane domain). There are currently two views as to the quaternary form of band 3 (in detergent solution): Schubert et al. (1992) believe that band 3 is a reversible equilibrium involving monomers, dimers, and tetramers. On the other hand, Casey and Reithmeier (1991) have proposed that band 3, like the CD, exists as a stable dimer. However, in contrast to the present finding, which suggest a K_d of 3 μM for the isolated CD, no dissociation of the band 3 dimers was observed down to 0.2 μM (Casey and Reithmeier, 1992): Inasmuch as there is no significant conformational change of the CD after cleavage from the transmembrane domain (Low, 1992), the most likely explanation is that the K_d of the transmembrane domain is significantly less than that of the CD, and hence it is the transmembrane domain that is chiefly responsible for holding the intact protein together. In a further study currently under way on both the intact protein and the isolated transmembrane domain, we hope to be able to address this question.

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