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DETECTION OF THE ASSOCIATED STATE OF MEMBRANE PROTEINS BY POLYACRYLAMIDE GRADIENT GEL ELECTROPHORESIS WITH NON-DENATURING DETERGENTS

APPLICATION TO BAND 3 PROTEIN FROM ERYTHROCYTE MEMBRANES

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Summary

Polyacrylamide gradient gel electrophoresis was carried out in micellar solutions of various detergents which differ in degree of potency to denature proteins. From the application of this method to band 3 protein from erythrocyte membranes, it was suggested that the procedure was useful in studying the molecular state of membrane proteins.

The electrophoretic behaviors of human and bovine band 3 protein did not show any species specificity in either a denature state and a state resembling the native state. As well as in nonionic detergent solutions, the dimeric and tetrameric structures of bovine band 3 protein were preserved in sodium deoxycholate solution, in which protein complexes maintained in nonionic detergent solutions are frequently dissociated. Even in dodecyltrimethylammonium bromide solution, which is a denaturant for water-soluble proteins, part of the band 3 protein was still present as the oligomer. The results suggest that the

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Abbreviations: $C_{12}E_9$, poly(oxyethylene glycol dodecyl ether) with an average of nine oxyethylene groups per molecule; SDS, sodium dodecyl sulfate; DTAB, dodecyltrimethylammonium bromide; TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

oligomeric form of band 3 protein is the stable structure and that the dimer and tetramer possibly coexist in membranes.

Introduction

Many membrane proteins are sparingly soluble in simple aqueous solutions and detergent solutions are the most suitable media for solubilization of the proteins [1]. However, detergents act on the proteins in various modes of interaction and, consequently, the molecular state of the proteins depends on the nature of detergent used [2].

Membrane proteins are frequently present as oligomers, i.e., composed of two or more identical or different polypeptide chains, which may relate to their biological function [3]. Such associated states are often retained in micellar solutions of appropriate mild detergents [2,3]. In this regard, in addition to an analysis of membrane proteins in denaturing detergents, a determination of the molecular state of membrane proteins in mild detergent solutions is of interest in relation to the function of the proteins. Furthermore, a reliable determination of whether an optically clear solution contains dispersed molecules or aggregates in the detergent solution used is important prior to the purification of a desired protein, especially in mild detergents. Conventional detection and resolving methods such as gel chromatography and analytical ultracentrifugation often do not afford easy solutions to these problems.

In a previous paper [4], we discussed preliminary the utility of polyacrylamide gradient gel electrophoresis in the presence of nonionic detergents for the study of the molecular state of membrane proteins in a state resembling the native state. In a further attempt to characterize the molecular state of membrane proteins, we extended the electrophoretic method to systems containing various detergents which differ in the extent to which they solubilize membrane proteins. This method was applied to band 3 protein, which is an anion transporter of erythrocyte membranes [5]. In the present paper, together with the results of the state of association of band 3 protein, the usefulness and the limitations of the electrophoretic method, especially in mild detergent solutions, are discussed.

Experimental Procedure

Materials

Polydisperse $C_{12}E_9$ was purchased from Nikko Chemicals, Triton X-100 was from Wako Chemicals, sodium deoxycholate was from Yoneyama Yakuhin Co., and SDS was from Nakarai Chemicals. These detergents were used without further purification. Dodecyltrimethylammonium chloride, which was obtained from Tokyo Kasei Kogyo Co., was converted to the bromide form, DTAB, by the addition of an excess of solid NaBr to a methanolic solution of the chloride salt. DTAB was recrystalized three times from methanol/ether.

Proteins used as standards of electrophoresis and gel chromatography were commercial preparations in routine use in this laboratory [4]. Sephacryl S-300 was obtained from Pharmacia. All other reagents were purchased from Wako Chemicals.

Methods

Human and bovine band 3 proteins purified in $C_{12}E_9$ solution, using ghosts stored at −20°C, were used in most experiments. The purification procedure has been reported in a previous paper [6]. In some cases, human band 3 protein was prepared from ghosts stored at 4°C in $C_{12}E_9$ and/or Triton X-100 solutions. Differences in storage temperature of ghosts and the use of different detergents as a solubilizing reagent, however, did not cause any change in the molecular associated state of human band 3 protein, as judged by polyacrylamide gradient gel electrophoresis with $C_{12}E_9$ or Triton X-100.

Displacement of $C_{12}E_9$ from the bovine band 3 protein-$C_{12}E_9$ complex by deoxycholate was performed by gel filtration. 50 mg of solid sodium deoxycholate were added to purified band 3 protein in 0.1% $C_{12}E_9$ (0.7 mg of protein per ml, approx. 4 ml), concentrated to 1 ml in a collodion bag under vacuum, and then the solution was applied to a Sephacryl S-300 column (1.4 × 84 cm) equilibrated with a 10 mM Tris-HCl/0.15 M NaCl/0.025% NaN_3 buffer, pH 8.5, containing 0.25% of sodium deoxycholate. This column was calibrated by using standard proteins with known Stokes' radii.

Polyacrylamide gradient gel electrophoresis was done on an Atto electrophoresis apparatus, SJ-1060 SD. Overall procedures were described in a previous paper [4]. The gel systems shown in Table I were used in the present experiments. In most experiments, band 3 protein purified in 0.1% $C_{12}E_9$ was electrophoresed without the removal of $C_{12}E_9$ and the presence of 0.1% $C_{12}E_9$ in sample solutions did not disturb the migration of the protein in the gels containing SDS, DTAB and deoxycholate, provided a large excess of the ionic detergents was added to the sample solutions before electrophoresis. To avoid heat denaturation [4], band 3 protein, without heat treatment, was electrophoresed and the unreduced material revealed the same electrophoretic pattern as that shown in the presence of 2-mercaptoethanol.

Gel in DTAB solution was photopolymerized in the presence of 0.0075 mg riboflavin per ml and 0.17% TEMED. Other gels were polymerized by the addition of ammonium persulfate and TEMED. 50-μl of sample solutions com-

TABLE I

GEL SYSTEMS USED IN THE PRESENT EXPERIMENTS

Detergent	Detergent concentration (%)	Buffer	Gel concentration with linear gradient (%)
$C_{12}E_9$ and Triton X-100	0.05 or 0.1	10 mM Tris/80 mM glycine, pH 8.3	3–15
Sodium deoxycholate	0.25	10 mM Tris/80 mM glycine, pH 8.3	3–20
DTAB	1.0	90 mM Tris/80 mM boric acid/2 mM EDTA, pH 8.3	3–20
SDS	0.2	40 mM Tris/20 mM sodium acetate/2 mM EDTA, pH 7.4	4–15

prised 10—20 μg of protein, usually 5% glycerol and the appropriate concentration of detergents (5% of SDS and DTAB, 1% of sodium deoxycholate and 0.1% of $C_{12}E_9$ and Triton X-100). The glycerol concentration was increased to 20% in the case of the DTAB-gel.

Electrophoresis in the presence of nonionic detergent and deoxycholate was performed in a cold room (4 ± 1°C). Electrophoresis in the presence of DTAB and SDS was run at room temperature. Electrophoresis was carried out at a constant voltage and the run was stopped when cytochrome *c* (DTAB-gel and SDS-gel) or Bromophenol blue ($C_{12}E_9$-gel, Triton X-100-gel and deoxycholate-gel) reached almost to the bottom of the gel.

Staining and destaining procedures for $C_{12}E_9$-gel, Triton X-100-gel and SDS-gel were described previously [4]. Before staining, deoxycholate-gel was soaked in an *n*-propanol/acetic acid/water (30 : 5 : 65%, v/v) mixture for 3 h at 40°C, washed with deionized water and soaked again in the same solution overnight at room temperature. The same procedure was also undertaken for the DTAB-gel because of a background otherwise observed after staining. Staining and destaining procedures were performed in the usual way.

Other measurements were made according to the procedures described previously [4].

Results

In this section, only electrophoretic results of band 3 protein are stated. The utility and the technical problems of the electrophoretic method will be described in Discussion.

Electrophoretic behavior of human and bovine band 3 protein in SDS and in nonionic detergent solutions

The details of the molecular weight determination of bovine band 3 protein have already been reported [4] and a value of 107 000 ± 5% was obtained by SDS-polyacrylamide gradient gel electrophoresis. In this experiment, the nearly equal value of 105 000 ± 5% was assigned for human band 3 by the same method (Fig. 1 a and c) and, furthermore, there was no appreciable difference between the mobilities of human and bovine band 3 protein in SDS-split gel electrophoresis (Fig. 1d). In $C_{12}E_9$ solution, it is apparent from the results of Fig. 2 that either human or bovine band 3 protein is composed of two major species, and that the migration distances and relative intensities of protein bands are essentially independent of the band 3 protein origins. As well as in the case of bovine band 3 protein [4], human band 3 protein was oxidatively cross-linked in $C_{12}E_9$ solution, as shown in Fig. 1b. These results indicate that the polypeptide molecular weights and the states of association of human and bovine band 3 protein are very similar to each other.

Judging from the results on associated state of the bovine band 3 protein in $C_{12}E_9$ solution [4], there is no doubt that the rapidly and slowly migrating components of human hand 3 protein in $C_{12}E_9$-polyacrylamide gradient gel electrophoresis correspond to the dimeric and tetrameric forms of the protein, respectively. Substitution of $C_{12}E_9$ with Triton X-100 did not affect the associated state of human band 3 protein.

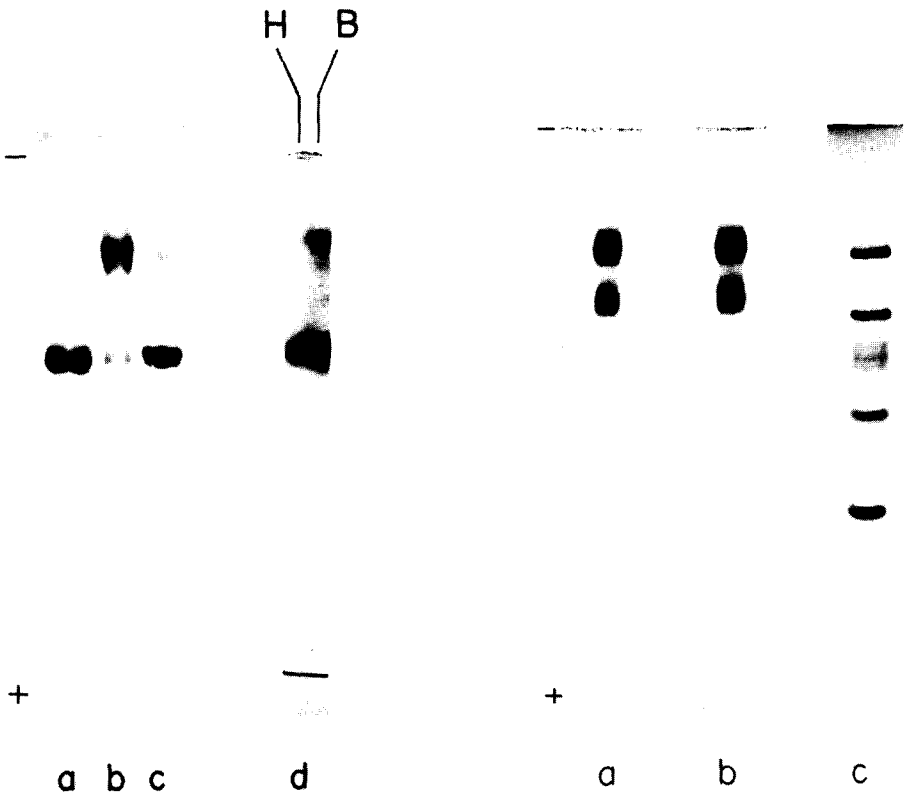

Fig. 1. SDS-polyacrylamide gradient gel electrophoresis (gels a—c) and split gel electrophoresis in 0.1% SDS-5% gel (gel d) of human and bovine band 3 protein purified in 0.1% $C_{12}E_9$. Gradient gel electrophoresis was run for 20 h at 70 V. Gel a, human band 3 protein; gel b, human band 3 protein treated with Cu^{2+}/*o*-phenanthroline in 0.1% $C_{12}E_9$; gel c, bovine band 3 protein; gel d, human 3 protein, left half denoted as H, and bovine band 3 protein, right half denoted as B. Except for gel b, samples were treated with 2-mercaptoethanol and about 20 μg of each sample were electrophoresed. Band 3 protein dimers were observable in the case of bovine band 3 protein. Migration was from top (—) to bottom (+).

Fig. 2. Polyacrylamide gradient gel electrophoresis in the presence of $C_{12}E_9$. Gel a, human band 3 protein; gel b, bovine band 3 protein; gel c, standard proteins (thyroglobulin, ferritin, catalase, lactate dehydrogenase and bovine serum albumin, from top to bottom, respectively). Electrophoresis was done in a 3—15% gradient gel for 16 h at 100 V at 4°C in 0.05% $C_{12}E_9$. Band 3 proteins were purified in 0.1% $C_{12}E_9$ and about 20 μg of each protein without reducing agent were loaded on the gel. Migration was from top (—) to bottom (+).

State of association of bovine band 3 protein in sodium deoxycholate and DTAB solutions

Bovine band 3 protein, which had been purified in $C_{12}E_9$ solution, was chromatographed on a Sephacryl S-300 column equilibrated with a buffer containing 0.25% of sodium deoxycholate (Fig. 3). Two protein components were eluted, without complete separation, at the positions of K_d = 0.10 and 0.18, respectively (K_d, partition coefficient). Electrophoretic profiles of the column fractions demonstrated that two molecular species migrated according to their molecular dimensions. The mobilities relative to standard proteins depended

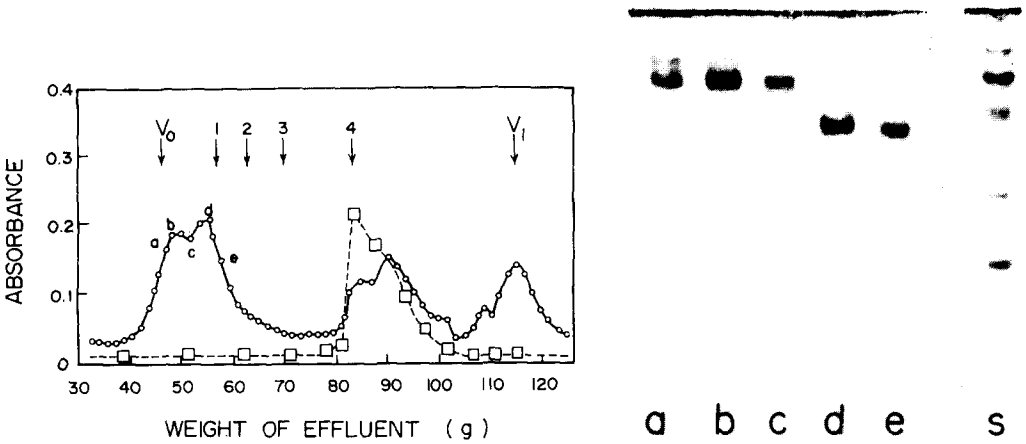

Fig. 3. Gel filtration of bovine band 3 protein on a Sephacryl S-300 column (1.4 × 80 cm) in 0.25% sodium deoxycholate. The preparation of the protein solution applied to the column is described in the text. Left panel: ○———○, absorbance at 280 nm; □- - - - - -□, absorbance at 320 nm of an ethylene dichloride extract of $C_{12}E_9$-ammonium cobalt thiocyanate complexes, indicating complete displacement of $C_{12}E_9$ with deoxycholate in the protein-detergent complex. Protein was eluted in the range of 45—60 g of eluant and the absorption peaks which appeared above 80 g of eluant corresponded to the elution positions of deoxycholate micelles and 2-mercaptoethanol. V_0 and V_i show the peak position of markers for the void and internal volume, respectively. Elution positions of standard proteins are indicated by arrows: 1, ferritin; 2, catalase; 3, bovine serum albumin; 4, myoglobin. Right panel: Fractions a—e were electrophoresed in the presence of 0.25% sodium deoxycholate for 17 h at 100 V. Gel s is the profile of standard proteins (see (Fig. 2). All samples were not reduced.

strongly on the V × h values applied to the gel, a property which was not observed in nonionic detergent-gel electrophoresis.

Only preliminary measurements were made in deoxycholate solution for an estimation of molecular weight, based on the procedure described previously [7]. The rapidly and slowly migrating components in the gel had sedimentation coefficients, $s_{20,w}$, and Stokes' radii of 8.7 ± 0.3 S and 6.5 ± 0.3 nm and 11.7 ± 0.9 S and 8.5 ± 0.5 nm, respectively, and the amount of deoxycholate bound to band 3 protein was 0.35 ± 0.15 g of deoxycholate per g of the protein. The molecular weight estimated using the values above and Eqn. 7 of Ref. 7 indicated that the rapidly migrating component is a dimer. The molecular weight of another molecular species ranged from 280 000 to 420 000, which corresponded to a trimer or tetramer of band 3 protein. We have not attempted further investigation of these results, since accurate determination of the molecular weight is not of interest for our purposes. However, it is clear that a band 3 protein monomer is not present in deoxycholate solution and thus it is reasonable to state that the two major components of band 3 protein in deoxycholate solution are the dimer and the tetramer.

In view of the results of several studies [8—11] concerning protein-cationic detergent interactions and gel electrophoresis in the presence of cationic detergents, it may be expected that many proteins are denatured in DTAB solution and, when reduced, they run in the gel as dissociated polypeptides bearing the charge of the detergent ion. This was confirmed for water-soluble proteins by the linear relationship of log(molecular weight) versus log(gel concentration

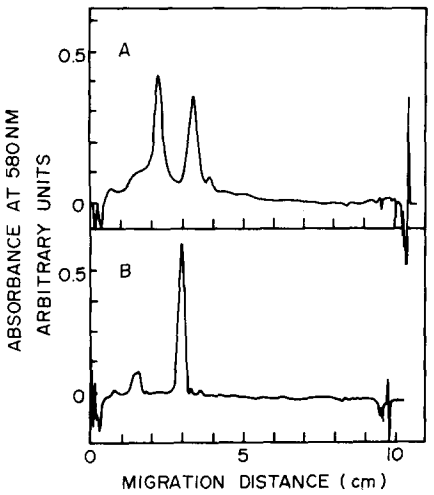

Fig. 4. Profiles of electrophoresis in the presence of DTAB and SDS of bovine band 3 protein. (A) DTAB-gel, (B) SDS-gel. Samples of approx. 20 μg which had been purified in 0.1% $C_{12}E_9$ were adjusted to 5% of the detergent and 1% of 2-mercaptoethanol and then electrophoresed in 1% DTAB (48 h, 25 V) and 0.2% SDS (20 h, 70 V). After staining with Coomassie blue and destaining, the gels were scanned with a Shimadzu CS-910 dual-wavelength TLC scanner.

reached by the protein). The molecular weight range covered was from 11 700 for cytochrome *c* to 134 000 for β-galactosidase and the mobilities were not affected by heat treatment of the proteins.

Bovine band 3 protein was soluble only in a high concentration of DTAB (above 1%) and under such conditions the electrophoretic pattern showed the existence of two major molecular species, as shown in Fig. 4A. This is completely different from the molecular state of band 3 protein in SDS solution, where the protein is present virtually in a single molecular state, the monomer (Fig. 4B). Because of the lack of other experiments to evaluate the molecular weight in DTAB solution, it may be inappropriate to state the details of the molecular state of bovine band 3 protein in detergent solution. However, providing that the molecular species observed in DTAB-gel electrophoresis migrate independently in the gel in the same manner as water-soluble proteins, the apparent molecular weights were assigned to be 88 000 ± 5% for the rapidly moving component and 215 000 ± 7% for the slowly migrating one. These values are close to those of the band 3 protein monomer and dimer, respectively.

Discussion

Polyacrylamide gradient gel electrophoresis in the presence of various detergents

For the purpose of a quick, reliable determination or detection of the molecular state of membrane proteins, polyacrylamide gradient gel electrophoresis was carried out in detergent solutions which differ in their action on proteins. It is known [12] that gradient gels are generally superior to homogeneous gels

in band resolution and reproducibility, and so we chose the following detergents; SDS, DTAB, sodium deoxycholate and $C_{12}E_9$ or Triton X-100. The first two detergents can be classified as denaturing detergents [2,8,9], however, the denaturing action of DTAB is less effective than that of SDS [8]. The others are non-denaturing detergents [2,13]. However, the weakening of the protein-protein interaction of oligomeric proteins is certainly greater in deoxycholate than in Triton X-100 and possibly in $C_{12}E_9$, as indicated in the cases of cytochrome *c* oxidase [14] and spike proteins of the Semliki Forest virus [15].

SDS-gel electrophoresis has proven very useful for resolving and characterizing protein components in the dissociated and denatured states. Cationic detergents have often been used for molecular weight determination by electrophoretic methods, in place of SDS [10,11]. Hence, no further discussion is needed concerning electrophoresis in the presence of SDS or DTAB. However, as indicated by Nozaki et al. [8] and shown in the present study, it should be stressed that DTAB and other cationic detergents are not suitable as detergents in procedures in which denaturation of proteins is essential and this weak denaturing action of cationic detergents is frequently advantageous, as seen in their successful use for reconstitutional studies of biological, active membrane proteins [16—18].

Deoxycholate is one of the detergents which are widely used in membrane protein research. However, the detergent is soluble only in alkaline solution and electrophoresis with deoxycholate is hampered by precipitation of the detergent in acidic solution which is needed for the fixation of proteins migrating in the gels. After several attempts, it was found that an *n*-propanol/acetic acid/water (30 : 5 : 65, v/v) mixture was suitable for removing the detergent from the gel without conspicuous shrinkage of the gel. The protein can be stained in the usual manner after the removal of deoxycholate.

Many membrane proteins are supposed to retain their native conformation in nonionic detergent solutions and the molecular species found in the detergent solution is probably present in the actual environment of the membrane [1, 2]. Thus, electrophoresis in the medium is very useful for detection of the molecular components. However, there were some problems in performing the electrophoresis. In a gel, inclusion of detergent at more than 0.1% reduced the migration rate of the proteins and, moreover, proteins did not run in the gel containing 1% of $C_{12}E_9$ or Triton X-100. Electrophoresis in dodecyldimethylamine oxide, which behaves as a nonionic detergent above neutral pH, did not give fine resolution of protein bands compared with that in $C_{12}E_9$ or Triton X-100. In addition, it is important to note that some globular proteins did not show well resolved protein bands. For example, aldolase exhibited unusually low electrophoretic mobility and a very diffuse band, while the protein was chromatographed on Sephadex gel in nonionic detergent solutions without showing any abnormality. These observations do not always imply that any nonionic detergent may be used in place of $C_{12}E_9$ or Triton X-100 and that any protein may be well resolved by this electrophoresis.

Nonionic detergents and deoxycholate can bind only to hydrophobic membrane proteins; binding does not occur at all with water-soluble proteins [13]. This means that deoxycholate causes the change of charges on membrane proteins by its binding and that the membrane protein-deoxycholate complexes

might migrate in electrophoresis more rapidly than water-soluble proteins possessing the same molecular size as the complexes. In the case of nonionic detergent-gel electrophoresis, on the other hand, the migration rates of membrane proteins relative to those of water-soluble proteins depend primarily upon the particle size of membrane protein-nonionic detergent complexes. Under the conditions that the complexes have an isoelectric point and a shape similar to those of standard water-soluble proteins, the Stokes' radii of the complexes can be determined by the electrophoretic method [4].

Thus, it is not possible to determine the molecular weight of a protein from electrophoresis with nonionic detergents or deoxycholate. When a protein shows different states of association in the media, however, this electrophoresis is useful especially for determination of the number and proportion of molecular components which cannot easily be resolved by conventional methods, though the data must be viewed with some caution for systems in association-dissociation equilibrium as indicated by Eisinger and Blumberg [19]. In addition, when applied to a two-dimensional system to detect proteins denatured by SDS in the second dimension after separation in the first dimension in the presence of mild detergents, this technique can provide valuable information regarding the multimeric structure.

Associated states of band 3 protein in various detergents

The present studies show that human band 3 protein is electrophoretically indistinguishable from bovine band 3 protein. By comparing with the results on the molecular characterization of bovine band 3 protein [4], it is apparent that human band 3 protein is present as a mixture of dimer and tetramer in nonionic detergents and probably in membranes. This contrasts with the reported observations [20,21] that human band 3 protein is present as a stable dimer in the nonionic detergent, Triton X-100. Possible sources for such a difference between our study and those of others have been discussed elsewhere [4].

The oligomeric structure of bovine band 3 protein retained in nonionic detergent solutions is preserved in deoxycholate solution. Even in DTAB solution, a denaturing medium for water-soluble proteins, it is suggested that the band 3 protein oligomers are not completely dissociated into the monomers and that part of the band 3 protein molecules still retains the native structure. This supports the results of optical studies of bovine band 3 protein [4] indicating that the band 3 protein oligomer is a stable molecular state. Furthermore, the result of DTAB-gel electrophoresis may account for the reason why functionally active band 3 protein was successfully reconstituted from DTAB-solubilized band 3 protein [18].

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