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Heterogeneity in the human erythrocyte Band 3 anion-transporter revealed by Triton X-114 phase partitioning

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Triton X-114 phase partitioning used in conjunction with countercurrent distribution was utilized to examine the phasing properties of the human erythrocyte Band 3 anion-transport protein. Phase partitioning and countercurrent distribution of Band 3 protein followed by electrophoresis and immunoblotting revealed that Band 3 protein possesses biphasic properties with approx. 65% of the Band 3 97000-M_r species being localized in the detergent phase and 35% isolated in the aqueous phase. The bidirectional phasing of the anion-transporter does not appear to be a result of glycosylation or phosphorylation, since treatment of alkali-washed ghosts with glycosidases or phosphatase respectively did not significantly alter the phasing profiles. Chymotrypsin treatment of erythrocytes followed by the purification of the 60000-M_r fragment, and exposure of this fragment to phase separation and countercurrent distribution also revealed biphasic partitioning with 70% of the species being isolated in the aqueous phase and 30% in the detergent phase. These data demonstrate that the human erythrocyte Band 3 anion-transport protein is heterogeneous by Triton X-114 phase partitioning and that this heterogeneity is preserved in the 60000-M_r chymotryptic fragment of Band 3 protein.

INTRODUCTION

Triton X-114 is a non-ionic detergent of the Triton series with an average of 7–8 oxyethylene groups per molecule. Solutions of Triton X-114 are capable of separating into two phases at temperatures in excess of 20 °C (Goldfarb & Sepulveda, 1969). This temperature, known as the cloud point, apparently causes aggregation of the detergent micelles and, at higher temperatures, results in the formation of a detergent-rich phase and a detergent-depleted phase (Mulley, 1967; Staples & Tiddy, 1978). The same phenomenon also occurs with the more common detergent Triton X-100, but requires elevated temperatures (> 60 °C) (Maclay, 1956). Bordier (1981) has developed the procedure of phase separation in which protein samples in solutions of Triton X-114 are brought above the cloud point, resulting in the formation of a large aqueous phase and a small detergent phase separated from each other by a sucrose barrier. Hydrophilic proteins are generally found in the aqueous phase after the partitioning procedure, whereas hydrophobic proteins are usually localized in the detergent phase. Because of the mild conditions utilized in this method, Triton X-114 phase separation has been used for the purification and characterization of various proteins (Alcaraz *et al.*, 1984; Bouvier *et al.*, 1985; Maher & Singer, 1985; Matsas *et al.*, 1985; Escuyer *et al.*, 1986; Holm *et al.*, 1986; Pryde & Phillips, 1986).

It has been suggested that the phase-partitioning properties of proteins were affected by the relative hydrophobicity of the protein surfaces and were not merely a result of whether the protein could bind detergent (Alcaraz *et al.*, 1984). Phasing studies performed with membrane proteins that possess a channel for transport functions have demonstrated peculiar partitioning effects, that is species of these proteins were found in both the aqueous (detergent-poor) and the detergent-enriched phase. The most unusual case was for the acetylcholine receptor, which partitioned almost exclusively into the aqueous phase (Maher & Singer, 1985).

The human erythrocyte Band 3 protein is the major glycosylated integral membrane protein of erythrocytes (Fairbanks *et al.*, 1971; Tanner & Boxer, 1972). The membrane domain of Band 3 protein (M_r 52000) possesses anion-transport capabilities (Cabantchik & Rothstein, 1974; Ho & Guidotti, 1975; Passow *et al.*, 1975), in particular the exchange of Cl^- and HCO_3^- anions, whereas the cytoplasmic domain of Band 3 protein (M_r 43000) associates with constituents of the cytoskeleton (Bennett & Stenbuck, 1980; Hargreaves *et al.*, 1980; Pasternack *et al.*, 1985). The amino acid sequence of mouse Band 3 protein, inferred from the cDNA sequence, is consistent with the presence of a 52000-M_r hydrophobic membrane domain that contains as many as 12 membrane-spanning segments (Kopito & Lodish, 1985). Direct studies using chemical modification and proteolysis have provided evidence for at least eight membrane crossings (Jennings *et al.*, 1986). The predicted mouse Band 3 protein sequence was also found to share a high degree of amino acid identity with peptide fragments of the human anion-transporter (Mawby & Findlay, 1982; Brock *et al.*, 1983; Brock & Tanner, 1986). In the present study the phase-partitioning properties have been investigated for the Band 3 anion-transporter, which appears to undergo bidirectional phase partitioning. Our results demonstrate that Triton X-114 phase separation coupled with the method of countercurrent distribution can be used as a simple method to isolate unique species of proteins that exhibit heterogeneity due to biphasic partitioning.

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EXPERIMENTAL

Materials

Outdated human erythrocytes were obtained from the DeGowin Blood Center, The University of Iowa Hospitals and Clinics. Triton X-114, trypsin, α-chymotrypsin, alkaline phosphatase, bovine serum albumin, bacteriorhodopsin, cytochrome *c* and cytochrome *c* oxidase were all purchased from Sigma Chemical Co. Endo-1,4-β-galactosidase and endo-β-*N*-acetylglucosaminidase F were obtained from Miles Laboratories and New England Nuclear respectively. Prestained protein standards were from Bethesda Research Laboratories. Protein assay reagent was from Pierce Chemical Co. and peroxidase-conjugated goat $F(ab')_2$ anti-(mouse IgG) antibody was purchased from Cappel Laboratories.

Preparation of erythroycte ghosts

Erythrocyte ghosts were prepared according to the procedure of Dodge *et al.* (1963) with slight modification (Carter-Su *et al.*, 1982). Briefly, cells were washed three times with 150 mM-NaCl/5 mM-Na_2HPO_4, pH 7.4, and pelleted by centrifugation at 1500 *g* for 5 min. Then the cells were slowly added to 20 vol. of 0.1 mM-EDTA/5 mM-Na_2HPO_4, pH 8.0, and stirred at 4 °C for 15 min. The mixture was centrifuged at 30000 *g* for 15 min to pellet the ghosts. The supernatant was aspirated off and the bottles were tilted so that the button could likewise be removed. The ghosts were repeatedly washed with the hypo-osmotic buffer until the supernatant after centrifugation was clear. The ghosts were then assayed for total protein (Bradford, 1976) and diluted to a concentration of 4 mg/ml with 5 mM-Na_2HPO_4, pH 8.0.

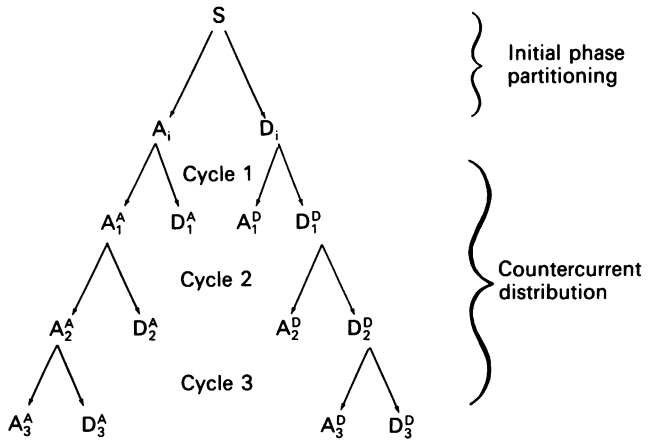
Alkali-washed ghosts were prepared by mixing the ghosts (4 mg/ml) with 5 vol. of 15.4 mM-NaOH/2 mM-EDTA/0.2 mM-dithiothreitol (Gorga & Lienhard, 1981). After this mixture had been stirred for 15 min at 4 °C, alkali-extracted ghosts were sedimented by centrifugation at 50000 *g* for 15 min. The pellets were washed and centrifuged twice with 5 mM-Hepes, pH 7.4, and assayed for protein as before. Base-washed ghosts were resuspended at 4 mg/ml and stored at −70 °C until use.

Triton X-114 precondensation

The method of precondensation (Bordier, 1981), used to remove hydrophilic contaminants from Triton X-114 (Maclay, 1956), was utilized to purify the detergent before undertaking the phase-separation experiments. Following precondensation, 17.5% (w/v) Triton X-114 was detected in the detergent phase, and 0.05% (w/v) was determined in the last aqueous phase. The detergent phase isolated from the precondensation procedure was used as the stock solution of Triton X-114 for the experiments described.

Phase partitioning and countercurrent distribution procedure

The phase-separation experiments were carried out in a similar way to the method previously described by Bordier (1981). Bovine serum albumin and cytochrome *c* were used as hydrophilic standards and appeared exclusively in the aqueous phase following the partitioning procedure (results not shown). Similarly, bacteriorhodopsin and cytochrome *c* oxidase were utilized as hydrophobic standards and partitioned entirely into the detergent phase. For the phasing studies with Band 3 protein, protein samples (0.5–2 mg/ml) were solubilized with 2% Triton X-114 for 15 min at 4 °C followed by centrifugation at 31000 *g* for 15 min. The supernatant was isolated and used for the phasing experiments.



Scheme 1. Schematic representation of phase partitioning and the countercurrent distribution procedure

S represents a sample of the original material (supernatant following solubilization with Triton X-114), which, after undergoing phase separation (Bordier, 1981), results in the formation of the initial aqueous (A_i) and initial detergent (D_i) phase. The initial aqueous and detergent phases can then be isolated by pipetting them into separate tubes so that the method of countercurrent distribution can be employed. The countercurrent distribution procedure involves the addition of fresh detergent and aqueous solution respectively to A_i and D_i followed by phase separation to give isolated phases resulting from the initial aqueous (superscript A) or the initial detergent (superscript D) phase. This method is repeated for two more cycles, after which portions proportional to the total volume of all the isolated phases are run on gels.

The phase partitioning of the solubilized supernatant resulted in the formation of the initial aqueous and detergent phases (Scheme 1). These phases can be isolated by pipetting them into separate tubes. At this point, a type of partition chromatography known as countercurrent distribution can be employed (Craig & Craig, 1950). The rationale for the usage of countercurrent distribution studies in relation to phase separation was to ensure that the bidirectional phasing properties demonstrated by Band 3 protein did not result merely from equilibrium distribution of the protein (because of its partition coefficient) between the two phases. The procedure used in the countercurrent distribution studies is actually identical with the initial phase separation except that an isolated phase (either aqueous or detergent) from a prior phase partitioning is exposed to another separation (Scheme 1). The volume of the individual isolated phases is then measured and fresh detergent added to aqueous samples, and likewise buffer is added to detergent samples so that all samples are adjusted to 2% Triton X-114 before undergoing another round (cycle) of partitioning.

Gel electrophoresis and Western blotting

SDS/polyacrylamide-gel electrophoresis (5–12% linear-gradient gels) run in the Laemmli (1970) system

was employed for all Band 3 protein phasing studies. Samples proportional to the volume of the isolated phases were mixed with dithiothreitol and Laemmli sample buffer [10% (v/v) glycerol/0.05% Bromophenol Blue/1.0% (w/v) SDS/50 mM-Tris, pH 6.9] and boiled before electrophoresis. The prestained M_r markers used were: myosin (M_r 200000), phosphorylase *b* (M_r 97000), bovine serum albumin (M_r 66000), ovalbumin (M_r 45000), chymotrypsinogen (M_r 25000) and cytochrome *c* (M_r 12000). After the gels were run, they were transferred to nitrocellulose sheets and subjected to an immunoblotting procedure (Towbin *et al.*, 1979) utilizing Tween 20 (0.05%, v/v) as the blocking agent (Batteiger *et al.*, 1982). The monoclonal antibodies II E 1 and IV F 12, directed against distinct regions of the Band 3 molecule, were used as primary antibodies in the blotting procedure (Jennings *et al.*, 1986). Peroxidase-conjugated goat $F(ab')_2$ anti-(mouse IgG) antibody was the secondary antibody, and the protein bands were developed with a mixture of H_2O_2 in Tris buffer, pH 7.4, and methanolic 4-chloro-1-naphthol. Following development, the blots were dried and photographed. Densitometric scanning of the blots was utilized as a method for quantifying of the experimental results.

RESULTS

The results of exposing alkali-extracted solubilized ghosts to phase separation and countercurrent distribution followed by specific observation of Band 3 species by immunoblotting with the monoclonal antibody IV F 12 are shown in Fig. 1. Densitometric scanning revealed approx. 65% of Band 3 97000-M_r species appearing in the detergent phase and 35% localized in the aqueous phase (compare D_3^D with A_3^A). Also illustrated in Fig. 1, the ratios from initial phase partitioning (compare A_i with D_i) do not reflect the ratios after countercurrent distribution. After each successive washing step, the amount of carry-over into the other phase decreased until distinct aqueous- and detergent-preferring forms were obtained. Therefore the phasing of Band 3 protein is partially dependent upon the partition coefficients of the various Band 3 species, but, if carried out far enough, two defined subpopulations of Band 3 97000-M_r species can be identified. One subpopulation contains the aqueous-preferring forms whereas the other comprises the detergent-localized species. We have also consistently observed that, similarly to other hydrophobic proteins (e.g. glucose-transport protein), Band 3 anion-transporters in high-detergent solutions tend to aggregate irreversibly, and, since the self-association of Band 3 protein appeared to vary from experiment to experiment, we have chosen to quantify only the results from the Band 3 97000-M_r species observed in the immunoblots.

To investigate what effects, if any, the heterogeneous glycosylation of Band 3 protein (Findlay, 1974) may play upon its bidirectional phase partitioning, we incubated base-washed ghosts with the glycosidic enzymes endo-1,4-β-galactosidase (Fukuda *et al.*, 1979) and endo-β-*N*-acetylglucosaminidase F followed by phase separation and countercurrent distribution (Fig. 2). Exposure of erythrocyte ghosts to these glycosidases resulted in a marked sharpening and increased mobility of Band 3 protein on electrophoresis in SDS/polyacrylamide gels (compare S with S+). Similarly to the distribution of Band 3 protein observed in Fig. 1, the deglycosylated form of Band 3 protein (S+) resulted in the appearance of approx. 60% of the Band 3 97000-M_r species into the detergent phase after countercurrent distribution (compare D_3^D with A_3^D). The heterogeneity in the carbohydrate moieties of Band 3 protein, therefore, does not significantly affect the distribution of Band 3 subpopulations revealed by Triton X-114 phase partitioning.

Since Band 3 protein is known to be phosphorylated by a tyrosine-specific protein kinase associated with erythrocyte membranes (Dekowski *et al.*, 1983), base-washed ghosts were treated with alkaline phosphatase followed by phase separation and countercurrent distribution. Immunoblots from alkaline-phosphatase-treated ghosts showed no change in the profile as compared with untreated samples (results not shown). This result is consistent with the observation by Holm *et al.* (1986) that the phasing profiles of the phosphorylated and unphosphorylated forms of hormone-sensitive lipase were not significantly different when exposed to Triton X-114 phase separation. Phase partitioning of 4,4′-diisothiocyano-2,2′-stilbenedisulphonate-labelled ghosts also resulted in a profile that was not significantly

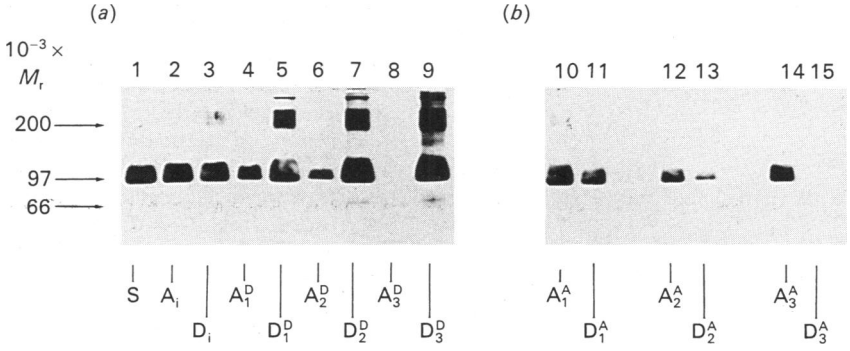


Fig. 1. Triton X-114 phase partitioning and countercurrent distribution of the intact Band 3 molecule

Alkali-washed ghosts (2 mg/ml) were solubilized with 2% Triton X-114 and centrifuged. Portions of all isolated samples from phase partitioning and countercurrent distribution were subjected to electrophoresis on SDS/polyacrylamide gels, transferred to nitrocellulose and immunoblotted with the monoclonal antibody IV F 12. Samples are labelled utilizing the designations illustrated in Scheme 1.

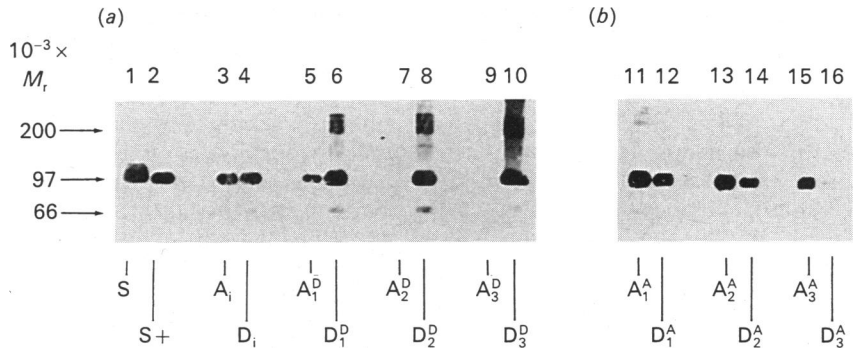


Fig. 2. Phase separation and countercurrent distribution on deglycosylated Band 3 protein

Base-washed erythrocyte ghosts (2 mg/ml) were incubated in the absence (S) or with the combination of endo-1,4-β-galactosidase (55 munits/ml) and endo-β-*N*-acetylglucosaminidase F (250 munits/ml) for 20 h at 37 °C (S+). This mixture was solubilized with 2% Triton X-114, centrifuged and exposed to the phasing procedure illustrated in Scheme 1. The monoclonal antibody IV F 12 was used in the immunoblotting procedure.

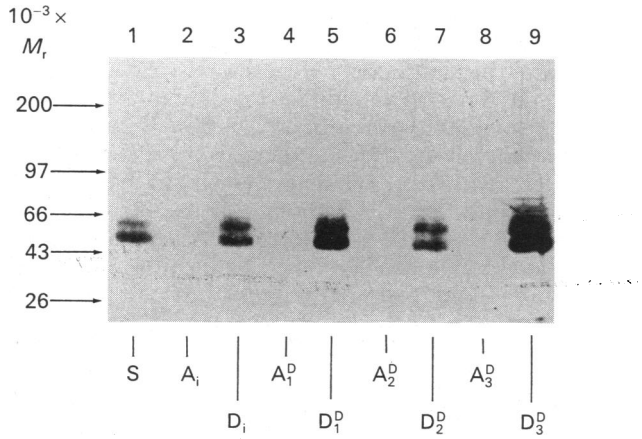


Fig. 3. Triton X-114 phase separation and countercurrent distribution of the anion-transporter membrane-spanning region

The membrane-spanning region (M_r 52000) of Band 3 protein was prepared by trypsin digestion of unsealed ghosts (Jennings *et al.*, 1986). Alkali-washed ghosts (0.12 mg/ml) were mixed with trypsin (50 μg/ml) in 150 mM-NaCl/5 mM-Na_2HPO_4, pH 7.4, and incubated for 45 min at 37 °C. L-Tosyl-lysylchloromethane (70 μM) was then added and the mixture centrifuged at 48000 *g* for 15 min. The pellet was washed with 250 mM-sucrose/2 mM-EDTA/10 mM-Tris/HCl, pH 7.4, three times, resuspended to 2 mg/ml in the same buffer and solubilized with 2% Triton X-114. After the phasing experiment and SDS/polyacrylamide-gel electrophoresis, blotting was performed with the monoclonal antibody IV F 12.

different from that for alkali-washed ghosts (results not shown).

To determine if the biphasic partitioning of Band 3 protein could be preserved in a subdomain of the molecule, proteolytic fragments of Band 3 protein were prepared and exposed to the phase partitioning procedure. Trypsin digestion of unsealed ghosts (Jennings *et al.*, 1986) followed by Triton X-114 phasing studies performed on the solubilized fraction revealed that the transmembrane region (M_r 52000) of Band 3 protein was localized entirely into the detergent phase (Fig. 3). Since the transmembrane region must contain the amphipathic helices responsible for anion-transport functionality, these amphipathic domains apparently do not disrupt the incorporation of this fragment into the detergent micelle, as was suggested by Maher & Singer (1985) for the acetylcholine receptor.

Exposure of erythrocytes to α-chymotrypsin digestion results in the formation of a 60000-M_r fragment of Band 3 protein, and this can be purified by gel-filtration chromatography (Jennings & Adams, 1981). When the purified 60000-M_r peptide is subjected to Triton X-114 phase separation coupled with countercurrent distribution, bidirectional phasing is observed with approx. 30% isolation into the detergent phase and 70% of the species being localized into the aqueous phase (Fig. 4). This apparent reversal in the ratio of aqueous to detergent forms compared with intact Band 3 protein is most probably due to the fact that the entire hydrophilic domain (M_r 43000) of Band 3 protein is present in the 60000-M_r fragment, however, only a small portion (M_r 17000) of the transmembrane sequence remains in this 60000-M_r fragment.

It has been observed that acidic pH causes an increase in hydrophobicity in the colicin E3 molecule as determined by Triton X-114 phase partitioning (Escuyer *et al.*, 1986). We attempted to manipulate the phasing profiles for Band 3 97000-M_r species by incubating the isolated aqueous and detergent phases, following countercurrent distribution, at various pH values encompassing the physiological range. The results obtained throughout the pH range from 6.5 to 8.5 revealed that the aqueous form(s) of Band 3 protein remained totally in the aqueous phase (Fig. 5). Likewise, the detergent species also remained in the detergent phase throughout this pH range. The same result was observed when alkali-washed ghosts were subjected to various pH values before phase separation (results not shown).

DISCUSSION

Maher & Singer (1985) have previously observed that transport proteins, including the Band 3 anion-transporter, displayed unusual phase partitioning properties because an appreciable amount of these integral membrane proteins was localized into the aqueous phase. Bordier (1981) was able to quantify phasing results of proteins from human erythrocyte membranes by labelling

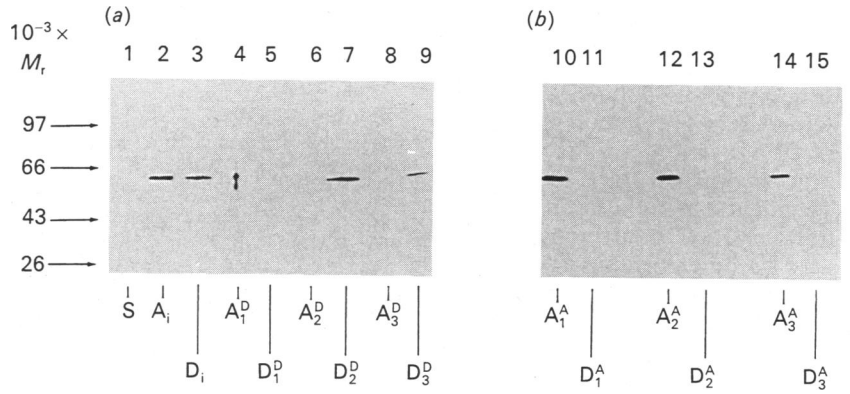


Fig. 4. Phasing of the purified M_r 60000 fragment of Band 3

α-Chymotrypsin digestion of erythrocytes and purification of the 60000-M_r peptide fragment was performed according to the method of Jennings & Adams (1981). Samples of the purified 60000-M_r peptide were adjusted to 2% Triton X-114 and subjected to phase partitioning and countercurrent distribution. The monoclonal antibody II E 1 was used in this immunoblotting procedure.

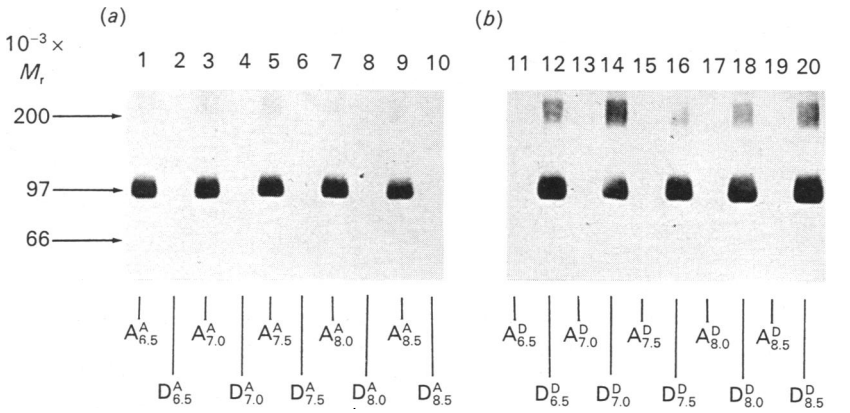


Fig. 5. pH-dependence of the isolated aqueous and detergent Band 3 species

Triton X-114 phase partitioning and countercurrent distribution of alkali-washed ghosts were performed as described in Fig. 1. The final aqueous (A_3^A) and detergent (D_3^D) phases were isolated and made equivalent in Triton X-114 concentration. Samples were then exposed to phase separation at various pH values in 165 mM-NaCl/100 mM-Tris. Samples were adjusted to the appropriate pH (6.5–8.5) and incubated at 37 °C for 10 min followed by neutralization of the pH at room temperature. Both the aqueous and the detergent fractions were individually phased and portions of the phases were run on gels. Immunoblotting was performed with the monoclonal antibody IV F 12.

the glycoproteins with tritium and following the label through the phasing experiment. By this method, 74% of Band 3 protein was recovered in the detergent phase. This value is in good agreement with our value of 65% in the detergent phase for the Band 3 97000-M_r species. Our results most probably underestimate the amount isolated in the detergent phase because aggregation of Band 3 protein was more prevalent in this phase. The observation that Band 3 protein appears to be heterogeneous has also been independently determined by the method of isoelectric focusing. Three distinct forms of the monomeric anion-transporter with pI values ranging from 5.25 to 5.70 were observed for the purified Band 3 protein (Ideguchi *et al.*, 1982). However, covalent modification by glycosylation and/or phosphorylation of the anion-transporter cannot account for the bidirectional phasing properties, since treatment of Band 3 protein with glycosidases or alkaline phosphatase did not cause a marked change in the partitioning profiles.

Equilibrium effects on partitioning were obviated by employing the countercurrent distribution procedure (Craig & Craig, 1950), which involves washing and rephasing the isolated aqueous and detergent phases a number of times (three cycles) in order to observe the relative ratios of the protein species localized in the two phases. This could be utilized as an effective technique for isolating different solution-preferring forms of an apparently homogeneous protein in order to determine the possible cause for biphasic partitioning, and to determine differences, if any, in functional activity of the protein species in the two phases.

The concentration of Triton X-114 in the partitioning process has been shown to be dependent on ionic strength and temperature (Alcaraz *et al.*, 1984; Bordier, 1981), and the phasing profiles of proteins probably depend on a multitude of factors. We have routinely observed a Triton X-114 concentration of 8–10% in the detergent phase and 0.04–0.05% in the aqueous phase following the phase separation procedure under our conditions. The critical micellar concentration of Triton X-114 is approx. 0.01% (Bordier, 1981). Other workers utilizing the Triton X-114 phase separation method have

observed the presence of hydrophobic proteins in the aqueous phase and have suggested that the low concentration of Triton X-114 present in the aqueous phase may be sufficient to accommodate a portion of the integral membrane protein molecules (Holm *et al.*, 1986; Pryde & Phillips, 1986). However, our results with the 52000-M_r transmembrane region of Band 3 protein demonstrated that this fragment partitioned entirely into the detergent phase with no extraction into the aqueous phase through three cycles of countercurrent distribution. This further supports the conclusion that intact Band 3 protein possesses distinct aqueous- and detergent-preferring species.

The fact that the purified 60000-M_r chymotryptic fragment of Band 3 protein exhibits biphasic partitioning suggests that the heterogeneity of Band 3 protein is preserved to a certain extent in this fragment. This observation is not surprising, since isoelectric focusing of the 60000-M_r fragment resulted in the formation of three bands in the pI range 4.75–5.30 (Ideguchi *et al.*, 1982). The shift in the ratio for the 60000-M_r peptide in the predicted manner suggests that phasing may be dependent on the relative hydrophilic and hydrophobic surfaces of the protein. Triton X-114 phase partitioning of the IgE receptor has demonstrated that the receptor and its individual subunits partitioned preferentially into the detergent phase. However, IgE bound to the receptor caused the IgE–α-subunit complex as well as the IgE–IgE receptor complex to partition mainly into the aqueous phase (Alcaraz *et al.*, 1984). Phasing studies of an integral membrane protein (p63) of *Leishmania* promastigotes demonstrated both a hydrophobic and a hydrophilic form of this protein that appeared to differ only by the presence of covalently bound myristoyl residues in the hydrophobic form (Bouvier *et al.*, 1985). Therefore, the presence of a small hydrocarbon moiety on a large protein can cause a dramatic alteration in its phase-partitioning profile.

Although the mechanism responsible for the biphasic partitioning of Band 3 protein has not been elucidated to date, the heterogeneity observed by isoelectric focusing suggests that a change in the overall charge balance may account for the different forms. Also, it is conceivable that the phasing procedure itself may elicit the heterogeneity observed in Band 3 protein. Clearly, further work needs to be accomplished in order to determine the cause(s) of heterogeneity in Band 3 protein and other transport proteins by Triton X-114 phase separation. The heterogeneity that these transport proteins display may be inherent to the properties that these proteins possess. The procedure of Triton X-114 phase partitioning used in conjunction with countercurrent distribution is a simple procedure utilizing mild conditions that could be employed to isolate and characterize differences in protein structure and perhaps function.

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