Volume expansion of little skate (Raja erinacea) erythrocytes increases the affinity of ankyrin binding without altering in the number of binding sites. Potassium iodide-stripped inside-out vesicles (KI-IOV) were used to assess ankyrin binding under volume-expanded conditions. Under isoosmotic conditions, ankyrin binds nearly exclusively to a single class of sites ($B_{\text{max}}$, $52 \pm 12 \mu g/mg; K_d$, $150 \pm 28 \text{ nM}$). KI-IOV from volume-expanded cells (either with one-half osmolarity medium or with inclusion of the permeant solute ethylene glycol) demonstrate two ankyrin-binding populations. A high affinity population occurs transiently under volume-expanded conditions. This population has a $B_{\text{max}}$ of $18 \pm 7 \mu g/mg$ and a $K_d$ of $25 \pm 9 \text{ nM}$. Total binding of high and low affinity sites is $57 \pm 17 \mu g/mg$. This change in ankyrin affinity is reversible on volume regulatory decrease. A major target protein in the KI-IOV was identified as the skate homolog of the mammalian red cell anion exchanger band 3. Inclusion of the purified cytoplasmic domain of band 3 competes away more than 80% of the ankyrin binding. To determine whether increased ankyrin affinity is due to band 3 tetramer formation that occurs in volume expansion, cells were treated with pyridoxal 5-phosphate or 4,4'-dinitrostilbene-2,2'-disulfonic acid, two agents that increase tetramer formation under isoosmotic conditions. Both treatments altered the binding affinity with a shift toward higher affinity binding without significant alteration in the number of binding sites.

On volume expansion, skate erythrocytes exhibit a stimulated efflux of solutes, primarily the $\beta$-amino acid taurine, to accomplish a volume decrease (Goldstein et al., 1996). A number of biochemical events parallel the changes in solute efflux although a precise mechanism of regulation is not understood. One protein that we have identified as a volume-regulated protein is the skate homolog of the mammalian anion exchanger protein band 3 (Musch et al., 1994a, 1994b). After volume expansion, a tetrameric state of band 3 is the predominant form in the membrane rather than the dimer that is normally found (Musch et al., 1994b). Band 3 normally exists in the membrane as a dimer (Jennings, 1985; Casey and Reithmeier, 1991; Wang et al., 1994). The tetrameric form of band 3 binds the cytoskeletal protein ankyrin with a greater affinity than that of the dimer (Casey and Reithmeier, 1991).

Ankyrin is a major link between the cytoskeleton and band 3. Ankyrin is a 210-kDa protein that binds to a number of proteins (Bork, 1993). Ankyrin binds to the $\beta$ chain of spectrin (Bennett and Gilligan, 1993; Peters and Lux, 1993) as well as to a variety of membrane proteins including band 3 (Hargreaves et al., 1980; Bennett and Stenbuck, 1980; Alper et al., 1988; Jennings, 1985; Low, 1986; Davis and Bennett, 1990a), Na,K-ATPase (Davis and Bennett, 1990b), and a neuronal voltage-dependent sodium channel (Srinivasan et al., 1988). The interaction with band 3 has been determined to reside in an 89-kDa fragment that contains 24 ankyrin repeats. Each repeat is 33 amino acids, and 4 subdomains of 6 repeats each are formed that are responsible for the interaction with the various membrane proteins (Michaely and Bennett, 1993; Peters and Lux, 1993). In the human erythrocyte, the major membrane protein that ankyrin interacts with is the anion exchanger band 3. Ankyrin binds to the cytoplasmic N-terminal domain of band 3 (which can be isolated as a 41-kDa soluble chymotryptic fragment (Willardson et al., 1989). Because of the unique interaction of ankyrin with both spectrin and band 3, a linkage is formed that may modulate the activity of band 3. Changes in ankyrin by mutation have demonstrated that this linkage is important for maintenance in structure that can be altered in such conditions as hemolytic spherocytosis, ovalocytosis, and anemia (J arolim et al., 1991, 1992; Sarabia et al., 1993).

In the present study, we have determined that a physiological stimulus that causes volume expansion also causes an altered interaction between band 3 and ankyrin. Ankyrin binds more avidly to vesicles derived from volume-expanded erythrocytes. This interaction occurs coincident with the volume-expanded conditions and reverses on volume recovery. High affinity ankyrin binding can be displaced by the purified cytoplasmic domain of band 3 which suggests that a majority of ankyrin binding is to band 3. Other interventions that promote tetramer formation, pyridoxal 5-phosphate and DNDS, also induce high affinity ankyrin binding.

**EXPERIMENTAL PROCEDURES**

Preparation of KI-IOV, Purification of Ankyrin, and the Cytoplasmic Domain of Band 3—Potassium iodide-stripped inside-out vesicles were prepared from erythrocytes incubated at 10% hematocrit in isoosmotic elasmobranch incubation medium (940 mosm/liter or 940 EIM, composition in mM/liter: 300 NaCl, 5.2 KCl, 2.7 MgSO4, 5 CaCl2, 15 HEPES, pH 7.5, and 370 urea); hypotonic EIM (460 mosm/liter or 460 EIM, where urea was reduced to 250 mM and NaCl to 100 mM); ethylene glycol EIM (where 200 mM ethylene glycol replaced 100 mM NaCl); or 940 EIM with 2 mM PLP or 0.5 mM DNDS. Following incubation, ghosts were isolated by dilution in 10 volumes of lysis buffer (120 mM Tris pH 7.4, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, with 10 $\mu$g/ml DNDS and 0.5 $\mu$g/ml PLP) and recentrifuged at 9000 $\times$ g for 10 min.

**Notes:**

1. The abbreviations used are: DNDS, 4,4'-dinitrostilbene-2,2'-disulfonic acid; KI-IOV, potassium iodide-stripped inside-out vesicles; EIM, elasmobranch incubation medium; PLP, pyridoxal 5-phosphate; MES, 4-morpholinolinesulfonic acid.

2. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

3. To whom correspondence should be addressed: Dept. of Physiology, Box G, B-311, Brown University, Providence, RI 02912. Tel.: 401-863-3341; Fax: 401-863-1222; E-mail: Leon_Goldstein@brown.edu.

4. The abbreviations used are: DNDS, 4,4'-dinitrostilbene-2,2'-disulfonic acid; KI-IOV, potassium iodide-stripped inside-out vesicles; EIM, elasmobranch incubation medium; PLP, pyridoxal 5-phosphate; MES, 4-morpholinolinesulfonic acid.
Volume Expansion Alters Ankyrin Binding

To determine whether volume expansion alters ankyrin binding, KI-IOV were made at 10 min from cells exposed to medium of one-half osmolarity or incubated in ethylene glycol medium. As shown in Fig. 1, control inside-out vesicles demonstrated ankyrin binding with an affinity similar to that measured in human KI-IOV (Thevinin and Low, 1990). In some cases of control KI-IOV, an additional population is noted that corresponds to a higher affinity binding. This population in cases of control KI-IOV, an additional population is noted that is not significantly, under volume-expanded conditions (Table I). These two populations of ankyrin binding have affinities for the KI-IOV of 155 ± 38 nM for the low and 25 ± 9 nM for the high affinity sites (Table I). The low affinity K_d site was not altered significantly in control versus volume-expanded cells.

The change in ankyrin affinity binding was determined to be a reversible effect. KI-IOV were made from cells at varying times after volume expansion during the regulatory volume decrease. Swelling is maximal at 5–10 min with a gradual return toward basal volumes by 60 min in hypotonic medium. The time course of changes in ankyrin binding is presented in Fig. 2. The data are presented as the number of binding sites, rather than as percentages, as the total binding increases only slightly in the volume-expanded condition. The change in ankyrin binding is similar to that of changes in volume.

To demonstrate that band 3 is involved in this binding, the cytoplasmic domain of band 3 was purified from human red cells and included in the reaction. Ten μg of this fragment was included in the binding assay using KI-IOV from control and hypotonically treated cells at 10 min. As demonstrated in Fig. 3, a majority of the binding of ankyrin is to band 3 since it can be inhibited away by inclusion of the cytoplasmic domain of band 3. Binding to the low as well as the high affinity sites is inhibited although not eliminated by cytoplasmic domain of band 3, suggesting that other proteins may be present in the

![Image 317x251 to 562x461](http://www.jbc.org/Downloaded from http://www.jbc.org/)

Table I

| Condition     | Low affinity sites |                      | High affinity sites |                      | Total sites |
|---------------|--------------------|----------------------|---------------------|----------------------|-------------|
|               | Capacity (μg/mg protein) | K_d (nM) | Capacity (μg/mg protein) | K_d (nM) | Capacity (μg/mg protein) | K_d (nM) |
| Control       | 52 ± 12            | 150 ± 28             | ND                  | ND                  | 52 ± 12     |
| Hypotonic     | 39 ± 11            | 155 ± 38             | 18 ± 7              | 25 ± 9              | 57 ± 16     |
| Ethylene glycol| 41 ± 14            | 162 ± 35             | 15 ± 10             | 32 ± 11             | 56 ± 17     |

* ND, not detectable.
membranes that bind ankyrin. The affinity of these remaining sites was calculated to be 185 ± 65 nM.

To determine if the binding of ankyrin to skate band 3 resembled that in the human, the pH dependence was measured. Thevinin and Low (1990) showed that incubation of human erythrocyte KI-IOV at low pH increases the affinity of ankyrin binding over periods of incubation >1 h. In addition, significantly greater numbers of ankyrin binding sites were measured at low pH. Skate erythrocyte KI-IOV were pelleted and resuspended in ankyrin-binding buffer, pH 6.3, using 5 mM MES to buffer the solution. Scatchard analysis was performed with KI-IOV, and binding assays were allowed to proceed for 60 as well as 480 min. As in KI-IOV from human red cells, ankyrin binding was greater at the lower pH (Fig. 4). Even at 60 min, there was a small percentage of ankyrin bound to the high affinity sites. By 480 min, much of the binding had changed to the high affinity sites. However, only a small increase in the number of binding sites was observed.

To determine if other interventions that modulate the oligomeric state of band 3 alter ankyrin binding, the anion exchanger inhibitors PLP (2 mM) and DNDS (0.5 mM) were used. Under isoosmotic conditions, both agents induced the formation of tetramers of band 3 (Salhany et al., 1990; Musch et al., 1994a). As shown in Fig. 5, both agents induced a high affinity ankyrin-binding population, suggesting that modulation of the oligomeric state in situ can modulate the affinity for ankyrin.

FIG. 2. Time course of effect of hypotonic stress on ankyrin binding. The zero point represents data from cells in 940 EIM. KI-IOV were used at 100 μg/ml final concentration and 125I-ankyrin at 1.25–250 μg/ml, and binding incubation was for 60 min. Binding sites were calculated from Scatchard analysis. Values are means ± S.E. from three experiments.

FIG. 3. Effect of inclusion of cytoplasmic domain of band 3 on ankyrin binding. 10 μg of the 41-kDa fragment of band 3 was included in binding assays with KI-IOV (final concentration of 100 μg/ml) taken at 10 min exposure to hypotonicity or isoosmotic EIM. 125I-Ankyrin was used at 1.25–250 μg/ml, and incubations were for 60 min. Results shown are from one experiment repeated with similar results on two occasions.

FIG. 4. Effect of reduced pH on ankyrin binding. KI-IOV were made from cells under isoosmotic conditions. Following isolation, vesicles were resuspended in ankyrin-binding buffer, pH 6.3, and binding reactions were allowed to proceed for 60 or 480 min. KI-IOV were used at 100 μg/ml and 125I-ankyrin at 1.25–250 μg/ml. Results shown are from one experiment repeated with similar results on two occasions.

Volume expansion alters ankyrin binding

DISCUSSION

Volume expansion of skate red blood cells causes a number of dramatic biochemical events, many of which may be related. We have previously demonstrated that skate band 3 undergoes an allosteric change after volume expansion and forms a tetramer rather than the dimer that is normally present (Musch et al., 1994b). The present studies demonstrate that a physiological stimulus that promotes tetramer formation increases the affinity of ankyrin binding to band 3 in the membrane. Volume expansion as well as two pharmacologic agents that have been shown to promote tetramer formation (PLP and DNDS) have induced an increase in the affinity of ankyrin binding to KI-IOV. This altered binding is reversible on volume recovery of the cell, and the binding is specific for band 3 since a purified cytoplasmic portion of this protein can inhibit the binding.

The interaction of ankyrin with membrane proteins is a complex process that is regulated by a number of factors. The dimer of band 3 is capable of binding ankyrin as demonstrated by the ability of the isolated cytoplasmic domain of band 3, which exists only as a dimer, to bind ankyrin (Bennett and Stenbuck, 1980; Hargreaves et al., 1980). Still, a large number of observations suggest that multiple forms of the interaction of band 3 and ankyrin exist and that many of these are of differ-

Downloaded from http://www.jbc.org/ by guest on April 26, 2019
ent affinity. A preferred form of interaction may be between the tetramer of band 3 and ankyrin. Positive cooperativity is observed in the interaction of band 3 and ankyrin, suggesting that ankyrin must be able to bind to a higher oligomer of band 3 (Bennett and Stenbuck, 1980). In sedimentation analyses, the major form of band 3 to co-sediment with ankyrin is the tetramer (Mulzer et al., 1990). Electron micrographs have shown that ankyrin-dependent aggregation of band 3 results in a complex of a size that is consistent with two band 3 dimers (Pinder et al., 1995).

Interaction between the ankyrin repeats could be important in the increased affinity of the ankyrin for the band 3 tetramer. The 89-kDa fragment of ankyrin containing all ankyrin repeats binds band 3 with higher affinity than fragments that contain only repeats 1–12 or 13–24. As presented in a model by Michaely and Bennett (1995), one site may interact with one band 3 dimer and the other with a different band 3 dimer. Thus the ankyrin would serve as a bridge between two band 3 dimers, resulting in a tetramer of band 3 with one bridging ankyrin.

Other factors may modulate the interaction of ankyrin with band 3 and could alter the affinity of the dimeric or tetrameric form for ankyrin. Potentially, other areas of ankyrin or unidentified accessory proteins (such as glycoporin as suggested by Thevinin and Low (1990)) may modulate the binding to band 3 so that the tetramer would bind with greater affinity.

The oligomeric state of band 3 correlates closely with the activation of taurine efflux under volume-expanded conditions (Brill et al., 1992). Therefore, band 3 appears to play a role in this volume-activated transport. During hypotonic stress, band 3 forms tetramers that bind ankyrin more avidly. This would lead to a stronger association with the cytoskeleton, transmitting forces from the cytoskeleton to band 3 through its strengthened interaction with ankyrin. This could result in the opening of an osmolyte channel, or in near the band 3 tetramer, that transports taurine (as well as polyols and trimethylamines) (Goldstein et al., 1996).

Support for the involvement of band 3 as a mediator of volume-activated osmolyte permeability has recently been derived using doned trout band 3. The transport activity of band 3 is normally as an electroneutral anion exchanger. However, band 3 from erythrocytes of trout can act as a membrane channel (Fievet et al., 1995; Goldstein et al., 1996). This channel activity is increased by volume expansion and could mediate taurine fluxes. In these studies, it is unknown whether the exchanger itself is responsible for increased permeability or if band 3 acts as a participant with other cellular elements to form the channel.

Cytoskeletal elements have been demonstrated to be pivotal modulators of channel activity. Ankyrin has been demonstrated to bind to a neuronal voltage-dependent sodium channel (Srinivasan et al., 1988). The channel-ankyrin interaction is hypothesized to be responsible for the cellular distribution of the channel. However, no experiments addressed whether channel activity was regulated by ankyrin. Another prominent member of the cytoskeleton that has been shown to modulate channel activity is actin. Actin regulation of both sodium and chloride channels has been established and may modulate second messenger regulation of the channel activity (Prat et al., 1995; Cantillió et al., 1991; Schwebert et al., 1994). Whether ankyrin itself or an additional cytoskeletal protein may modulate the volume-expanded permeability of red blood cells is not known.

In addition, how the interaction between ankyrin and band 3 (Soong et al., 1987) is regulated is not yet known. Phosphorylation of ankyrin has been demonstrated to alter its interaction with band 3 (Cianci et al., 1988; Costa et al., 1990) and hypothesized to influence the stoichiometry as well as affinity of the interaction. However, no apparent change in the phosphorylation state of ankyrin occurs after volume expansion in skate erythrocytes (Musch et al., 1994a), and the occurrence of such phosphorylation events regulated under physiological processes in other species is unknown.

**REFERENCES**

Alper, S. L., Kapito, R. R., Libresco, S. M., and Lodish, H. F. (1988). J. Biol. Chem, **263**, 17092–17099.

Bennett, V. (1983) Methods Enzymol. **96**, 313–324.

Bennett, V. and Gilligan, D. (1993) Annu. Rev. Cell Biol. **9**, 27–66.

Bennett, V. and Stenbuck, P. J. (1980) J. Biol. Chem. **255**, 6424–6432.

Bork, P. (1993) *Proteins* **17**, 363–374.

Brill, S. R., Musch, M. W., and Goldstein, L. (1992) J. Exp. Zool. **264**, 19–25.

Cantillió, H. F., Stow, J. L., Prat, A. G., and Ausiello, D. A. (1991) *Am. J. Physiol.* **261**, C882–C888.

Casey, J. R., and Rethmier, R. A. F. (1991) J. Biol. Chem., **266**, 15726–15737.

Cianci, C. D., Giorgi, M., and Morrow, J. S. (1988) J. Cell. Biochem. **37**, 219–233.

Costa, F., Agre, P., Watkins, P., Winkelmann, J., Tang, T., John, K., Lux, S., and Forget, B. (1990) *N. Engl. J. Med.* **323**, 1046–1050.

Davis, L. H., and Bennett, V. (1990a) J. Biol. Chem. **265**, 10589–10596.

Davis, J. Q., and Bennett, V. (1990b) J. Biol. Chem. **265**, 17252–17256.

Fievet, B., Gabillet, N., Borgese, F., and Motais, P. (1995) *Biochimie* **77**, 5158–5169.

Goldstein, L., Davis-Amiral, E. M., and Musch, W. M. (1996) Kidney Int. **49**, 1690–1694.

Hairgrove, W. R., Giehl, K. N., Verkleij, A., and Branton, D. (1980) J. Biol. Chem. **255**, 11965–11972.

Jarolim, P., Paléj, J., Amato, D., Hassan, K., Sapak, P., Nurse, G., Rubin, H., Zhai, R., Sahr, K., and Liu, S. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 11202–11206.

Jarolim, P., Rubin, H., Liu, S., Cho, M., Brabec, V., Derick, L., Yi, Y., Saad, S., Alper, S., Brugnara, C., Golan, D., and Paléj, J. (1992) *Clin. Invest.** **93**, 121–130.

Jennings, M. L. (1985) Annu. Rev. Physiol. **47**, 513–533.

Low, P. S. (1986) *Biochim. Biophys. Acta* **864**, 145–167.

Michaely, P., and Bennett, V. (1993) J. Biol. Chem. **268**, 27703–27709.

Michaely, P., and Bennett, V. (1995) J. Biol. Chem. **270**, 22050–22057.

Mulzer, K., Kampmann, L., Petrasch, P., and Schubert, D. (1990) *Colloid Polym. Sci.** **268**, 60–64.

Musch, M. W., Leffingwell, T. R., and Goldstein, L. (1994a) *Am. J. Physiol.* **266**, R65–R74.

Musch, M. W., Davis, E. M., and Goldstein, L. (1994b) J. Biol. Chem. **269**, 19683–19686.

Peters, L., and Lux, S. (1993) *Semin. Hematol.* **30**, 85–118.

Pinder, J., Pokrun, S., Maggs, A., Brain, I., and Gratzer, W. (1995) *Blood* **85**, 2951–2961.

Prat, A. G., Xiao, X. F., Ausiello, D. A., and Cantillió, H. F. (1995) *Am. J. Physiol.*
Salihany, J. M., Sloan, R. L., and Cordes, K. A. (1990) J. Biol. Chem. 265, 17688–17693
Sarabia, V. E., Casey, J. R., and Reithmeier, R. A. F. (1993) J. Biol. Chem. 268, 10676–10680
Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660–672
Schwiebert, E. M., Mills, J. W., and Stanton, B. A. (1994) J. Biol. Chem. 269, 7081–7089
Soong, C.-J., Lu, P.-W., and Tao, M. (1987) Arch. Biochem. Biophys. 254, 509–517
Srinivasan, Y., Elmer, L., Davis, J., Bennett, V., and Angelides, K. (1988) Nature 333, 177–180
Thevinin, B. J.-M., and Low, P. S. (1990) J. Biol. Chem. 265, 16166–16172
Wang, D. N., Sarabia, V. E., Reithmeier, R. A., and Kuhlbrandt, W. (1994) EMBO J. 13, 3230–3235
Willardson, B. M., Thevinin, B. J.-M., Harrison, M. L., Kuster, W. M., Benson, M. D., and Low, P. S. (1989) J. Biol. Chem. 264, 15893–15899
High Affinity Binding of Ankyrin Induced by Volume Expansion in Skate Erythrocytes
Mark W. Musch and Leon Goldstein

J. Biol. Chem. 1996, 271:21221-21225.
doi: 10.1074/jbc.271.35.21221

Access the most updated version of this article at http://www.jbc.org/content/271/35/21221

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 16 of which can be accessed free at http://www.jbc.org/content/271/35/21221.full.html#ref-list-1