Effect of Band 3 Subunit Equilibrium on the Kinetics and Affinity of Ankyrin Binding to Erythrocyte Membrane Vesicles*

(Received for publication, September 24, 1997, and in revised form, March 25, 1998)

Heidi M. Van Dort†, Ryuichi Moriyama‡, and Philip S. Low‡‡

From the ‡Department of Chemistry, Purdue University, West Lafayette, Indiana 47907-1393

The membrane-spanning protein, band 3, anchors the spectrin-based membrane skeleton to the lipid bilayer via the bridging protein, ankyrin. To understand how band 3 subunit stoichiometry influences this membrane-skeletal junction, we have induced changes in the band 3 association equilibrium and assayed the kinetics and equilibrium properties of ankyrin binding. We observe that band 3 oligomers convert slowly to dimers and ultimately monomers following removal of ankyrin. Addition of excess ankyrin back to these membranes enriched in dissociated band 3 then shifts band 3 almost entirely to tetramers, confirming that the tetrameric form of band 3 constitutes the preferred oligomeric state of ankyrin binding. 4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) labeling of band 3, which is shown to shift most of the band 3 population to dimers, eliminates the majority of ankyrin-binding sites on the membrane and greatly reduces retention of band 3 in detergent-extracted membrane skeletons. Furthermore, DIDS-modified membranes lack all low affinity ankyrin-binding sites and roughly half of all high affinity sites. Since labeled membranes lack the rapid kinetic phase of ankyrin binding and exhibit only half of the normal amplitude of the slow kinetic phase, it can be concluded that the rapid phase of ankyrin association involves low affinity sites and the slow phase involves high affinity sites. A model accounting for these data and most previous data on ankyrin-band 3 interactions is provided.

Band 3 (MW ~101,000) is the predominant polypeptide of the human erythrocyte membrane, comprising ~25% of the total membrane protein. Band 3 contains two major structural domains, a membrane-spanning domain that may traverse the bilayer 14 times in α-helical segments (1–3), and an exposed cytoplasmic domain that exhibits an elongated segmented morphology (4, 5). The membrane-spanning domain (MW ~55,000) catalyzes anion transport across the phospholipid bilayer (6–8). It also serves as the major antigen responsible for immune-mediated removal of senescent and abnormal erythrocytes (9–16). The cytoplasmic domain participates in the mechanism of senescent/abnormal cell removal (9–15), binds and regulates glycoytic enzymes (17, 18), and provides the major link of the spectrin-based membrane skeleton to the bilayer (19, 20). This latter function is mediated by ankyrin, a protein that connects the β subunit of spectrin to the cytoplasmic domain of band 3 in a manner that is sensitive to pH (21), the concentration of diphosphoglycerate (22), and the association state of band 3.

Recent evidence suggests that the tetramer of band 3 may serve as the predominant ligand for ankyrin (21, 23–28), although a report that the dimer is responsible for ankyrin binding has also been published (29).

As a model of membrane-skeleton junctions, the ankyrin-band 3 interaction has been heavily investigated. The sites of ankyrin association on the cytoplasmic domain of band 3 have been localized to two distinct regions: one near a central proteolytically accessible hinge and a second more proximal to the anion transporter’s N terminus (30–34). Similarly, two sites of band 3 interaction with ankyrin have been identified and shown to separately reside in ankyrin repeats 7–12 and 13–24 (35). Perhaps related to this structural complexity is a similar complexity in the kinetics, affinity and magnitude of ankyrin binding to band 3. Thus, ankyrin association with the anion transporter in its membrane environment is characterized by populations of both low affinity (KD = 1.5 × 10⁻⁷ M) and high affinity (KD = 1.5 × 10⁻⁸ M) sites (21, 36). Furthermore, the low affinity sites gradually convert to high affinity sites during prolonged incubation, and a slow accrual of new sites is also observed as the binding reaction approaches completion (21). Although only ~100,000 ankyrin sites are detected in freshly prepared membranes (21, 36–38), during extended incubations at pH 6.35 with saturating ankyrin concentrations approximately 270,000 sites/cell can eventually be measured (21). Because removal of the cytoplasmic domain of band 3 eliminates virtually all ankyrin interactions with the red cell membrane (36, 37), it is assumed that band 3 somehow accounts for all of the kinetic and equilibrium phases of ankyrin association. It is the goal of this paper to place these unexplained kinetic and equilibrium binding data on a more firm physical foundation by identifying the sources of the fast and slow phases of ankyrin binding as well as the causes of the high and low affinity populations of sites.

EXPERIMENTAL PROCEDURES

Materials
Human blood was purchased from the Central Indiana Regional Blood Center and used within 2 weeks of its drawing date. DIDS1 and Bolton-Hunter reagent was obtained from ICN Pharmaceutical Co., and Sephacryl S-300 HR from Pharmacia. C₅₅₅₅ was purchased from Nikko Chemical Co., Triton X-100 was from Boehringer Mannheim, and α-octyl glucoside, octyl-β-D-thioglucopyranoside, and Zwittergent 3-14 were from Calbiochem. All other reagents were from major suppliers and of the highest purity available.

Methods
DIDS Labeling of Band 3—Washed red cells (50% hematocrit) were incubated with 50 μM DIDS for 1 h at 37 °C in 0.15 M NaCl, 5 mM

* This work was supported by National Institutes of Health Grants GM 24417 and T32 GM08296. 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.

† To whom correspondence and reprint requests should be addressed.

1 The abbreviations used are: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; IOV, inside-out erythrocyte membrane vesicles; KI-IOV, KI-stripped IOV; C₅₅₅₅, octadecylterephthalic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; α-octyl glucoside, α-octyl-β-D-glucopyranoside.
Sodium phosphate, pH 7.4. Cells were washed four times with 5 volumes of the same buffer containing 1% bovine serum albumin to remove unreacted DIOS and then three times without the serum albumin. Under these conditions, >95% of the DIOS resides on band 3 (39).

Ankyrin Purification and Binding Assay—Erythrocyte ankyrin and the IOVs for binding studies were prepared as described by Bennett (40) with minor modifications. Briefly, ankyrin was released with 0.5 M KCl from Triton X-100 extracted red cell membrane skeletons, precipitated with 32% ammonium sulfate, and purified by gel filtration chromatography on a Sephacryl S-300 HR column (2.8 × 120 cm) in 0.6 M NaBr, 0.1 mM EDTA, 20 mM sodium phosphate, 0.05% NaN₃, 10 mM dithiothreitol, and 0.1% Triton X-100 (41). The latter conducted in a buffer consisting of 5% sucrose, 50 mM sodium phosphate, 50 mM boric acid, 30 mM NaCl, 1 mM EDTA, 0.2 mM dithiothreitol adjusted to pH 6.5 or 7.8 as noted. An analysis of the band 3 dimer/tetramer ratio was not conducted on the specific SI-KI-IOV preparations used for the ankyrin binding studies, but an examination of a similar SI-KI-IOV preparation revealed a dimer/tetramer ratio of ~60:40. The more extensive KI stripping and 37 °C incubation procedures employed during preparation of band 3 for HPLC analyses yields a much lower fraction of band 3 tetramer (see “Discussion”).

DIDS Effects on Ankyrin-Band 3 Interactions—The 46-kDa fragment of ankyrin which contains the ankyrin-binding site for band 3 was expressed in E. coli BL21 (DE3) with the expression vector a kind gift of Vann Bennett. Protein expression and purification were performed as described by Davis and Bennett (41). Briefly, plasmid protein synthesis was induced in cultures grown to an A₅₆₀ of 0.5 with 1 mM isopropyl-β-D-thiogalactopyranoside for 2 h. The cells were harvested and washed with 10 mM sodium phosphate, 100 mM NaCl, pH 7.5, and then digested with lysosome (450 mg/ml) and DNase I (25 μg/ml) in 50 mM sodium phosphate, 1 mM sodium azide, 25% sucrose, 10 mM magnesium chloride, pH 8.7. The protein was solubilized with 10 mM sodium phosphate, 100 mM NaCl, 2 mM EDTA, 1 mM sodium azide, 1% dithiothreitol, 1% Triton X-100, and 1% deoxycholate, pH 7.5, then separated from unsolubilized material by spinning at 20,000 × g for 30 min. The ankyrin fragment was precipitated with 32% ammonium sulfate and purified on a Sephacryl S-300 gel filtration column in 10 mM sodium phosphate, 1 mM sodium bromide, 1 mM EDTA, 1 mM sodium azide, 1 mM dithiothreitol, pH 7.4. Peak fractions were collected and dialyzed against HPLC incubation buffer containing 5 mM sodium phosphate, pH 8, amended with 20 mM 2-mercaptoethanol, 80 μg/ml phenylmethanesulfonfluoride, 20 μg/ml leupeptin, and 20 μg/ml pepstatin A. Ankyrin fragment protein concentration was estimated by absorbance measurement at 280 nm using an extinction coefficient of 0.347 ml mg⁻¹ cm⁻¹ which we determined by the Edelhoch method (42).

Analysis of Band 3 Retention in Detergent-extracted Membrane Skeletons—Quantitation of intact band 3 in membrane skeletal fractions was difficult due to the diffuse nature of the anion transporter’s banding pattern and the desire to solubilize the detergent-solubilized band 3. Therefore, we have re-examined the issue by HPLC gel filtration chromatography using several different membrane preparations varying in their lengths of equilibration at 37 °C after removal of ankyrin and prior to detergent extraction time at 37 °C. Other laboratories have shown that when freshly prepared IOVs are stripped of peripheral proteins, solubilized in 1% C₅₋₆E₅, and separated by HPLC, the solubilized band 3 elutes as a mixture of predominately dimers with some tetramers and higher oligomers (25, 44). We have reproduced these observations...
tions, measuring a distribution of ~40% tetramer and 60% dimer, but we have also noted that the ratio between the dimeric and tetrameric forms of band 3 can be significantly affected by the membrane preparation procedure employed (data not shown). In order to keep band 3 native and to avoid unwanted aggregation/denaturation, the ghosts we employed for our HPLC analyses were not extensively washed prior to KI-IOV preparation. Furthermore, since we and others have found that NaOH stripping of peripheral proteins denatures the cytoplasmic domain of band 3 (37, 45–47), peripheral proteins were more gently (but thoroughly) removed by 1 M KI incubation. We additionally did not perform an anion exchange chromatography step before size analysis of band 3 by gel filtration chromatography, since anion exchange chromatography has been shown to remove essential lipids surrounding the solubilized band 3 and cause its aggregation (29, 48). In our hands, the band 3 was predominantly dimeric in both the unlabeled and DIDS-labeled preparations by the time the KI-IOV were solubilized in detergent solution (Fig. 1). Presumably, the KI stripping procedures at 37 °C allowed dissociation of most band 3 tetramers to dimers. Furthermore, as the KI-IOV were further incubated at 37 °C prior to solubilization, a gradual decrease in abundance of the dimeric band 3 was observed with a concomitant rise in a lower molecular weight species which we presume to be the monomer, but alternatively could be a hydrodynamically smaller dimer (Fig. 1A).

The influence of DIDS labeling on the band 3 subunit equilibrium was also manifested when the 46,500-dalton band 3-binding fragment of ankyrin (49) was added to a 4 molar excess of band 3 in the KI-IOV membranes to drive the band 3 subunit equilibrium back toward the tetramer, i.e. the preferred oligomeric state for ankyrin association (21, 27, 28). As seen in Fig. 2B (dotted line), unlabeled membranes allowed to incubate until band 3 dissociated to predominantly its monomeric state were shifted almost quantitatively to the tetrameric state upon 24 h incubation with ankyrin (solid line). This reassociation reaction occurred very slowly, with an approximate half-time of 12 h (data not shown). DIDS-labeled membranes, in contrast, re-equilibrated to approximately equal amounts of tetrameric and dimeric band 3 over the same time span (Fig. 2A, solid line). We did not extend this reassociation reaction in the DIDS-labeled membranes beyond 24 h, since studies described below argue that quantitative conversion to the tetramer would never have been achieved. These data thus suggest that DIDS stabilizes the dimeric state of band 3, resisting both an ankyrin-induced association to the tetramer and an unfacilitated slow dissociation to the monomer. The data further argue that the oligomeric form of unmodified band 3 preferred by ankyrin is the tetramer, since ankyrin shifts the subunit equilibrium to this association state.

Effect of DIDS on the Kinetics of 125I-Ankyrin Binding to KI-IOV—In view of the impact of DIDS on the band 3 subunit dissociation equilibrium, it was of interest to further explore the influence of DIDS labeling on ankyrin binding. Ankyrin has been shown previously to associate with band 3 in two kinetic

![Fig. 1. The shift in gel filtration HPLC profile of band 3 during incubation of unmodified and DIDS-labeled KI-IOV at 37 °C. KI-IOV prepared from control (A) and DIDS-labeled cells (B) were allowed to equilibrate at 37 °C in 5 mM sodium phosphate, pH 8, containing 20 mM 2-mercaptoethanol, 1 mM dithiothreitol, 80 μg/ml phenylmethanesulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml pepstatin. At the time points indicated, the membranes were solubilized in C12E8 and analyzed on a TSK-4000 SWXL column as described under Experimental Procedures. Shown on the x axis are the elution positions of the following standard proteins: T, thyroglobulin (Rg = 86 Å); F, ferritin (Rg = 63 Å); C, catalase (Rg = 52 Å); and A, aldolase (Rg = 46 Å). V0 indicates the elution position of the void volume which was determined using plasmid DNA (average Mw = 1 x 10^6). The tetramer, dimer, and monomer peaks of band 3 were found to elute at 6.5 ± 2, 9.5 ± 2, and 10.8 ± 3 ml, respectively (n = 14). Unlabeled minor peaks do not contain band 3, but are comprised instead of glycophorin and detergent, as revealed by SDS-PAGE.
DIDS Effects on Ankyrin-Band 3 Interactions

Fig. 2. Effect of ankyrin on the band 3 subunit equilibrium in preincubated control or DIDS-labeled KI-IOV. KI-IOV (2 mg/ml) were prepared from DIDS-labeled or unlabeled erythrocytes as described under “Experimental Procedures.” The KI-IOV were allowed to equilibrate at 37 °C for 150 h until the DIDS-labeled band 3 had dissociated to half-dimer and half-monomer (panel A, dashed line) and ~90% of the unmodified band 3 (panel B, dashed line) was shifted to the monomer. The 46.5-kDa ankyrin fragment (0.23 mg/ml) was then added, allowed to equilibrate for 24 h at 37 °C, and re-extracted with buffered 1 M KI to again remove the ankyrin fragment. The ankyrin-depleted membranes were then washed with 5 mM sodium phosphate, pH 8.0, solubilized in detergent (1% C12E8), and analyzed by gel filtration HPLC (solid lines), as described under “Experimental Procedures” and the legend to Fig. 1.

Fig. 3. Effect of DIDS on the kinetics of 125I-ankyrin binding to KI-IOV at pH 6.35. Red cells were either left unlabeled or DIDS-labeled as described under “Experimental Procedures.” KI-IOV were then immediately prepared for use in binding studies and kept on ice to prevent any further shift in the band 3 subunit equilibrium. 125I-Ankyrin (final 7 μg/ml) was then incubated at 24 °C with the DIDS-labeled (A) and unmodified (B) KI-IOV (final 35 μg/ml) in 50 mM NaH2PO4, 50 mM H3BO3, 20 mM NaCl, 1 mM EDTA, 0.2 mM dithiothreitol, 0.55 mg/ml bovine serum albumin, 5% sucrose, pH 6.35. At the times indicated, bound 125I-Ankyrin was separated from free 125I-ankyrin by pelleting the KI-IOV at 43,000 × g for 40 min through a 20% sucrose cushion, and the bound 125I-ankyrin was quantified by gamma-counting.

The difference between ankyrin bound to control and DIDS KI-IOV was then calculated by direct subtraction of the amount of ankyrin bound at each time point (C). This experiment was done in triplicate and also repeated on an independent set of membrane preparations. Since the error bars were generally less than twice the width of the data point symbols, they are not shown on the graph.

Fig. 4A. DIDS Effects on Ankyrin-Band 3 Interactions

Effect of DIDS Modification of Band 3 on Its Association with 125I-Ankyrin at True and Quasi-equilibrium—Previous studies have demonstrated that band 3 offers both high and low affinity binding sites to ankyrin (21, 36, 37) and that these sites are generated during the slow and fast phases of ankyrin association, respectively (21). Since DIDS reaction with the membrane-spanning domain of band 3 appears to diminish the fast phase of binding (Fig. 3), it would be predicted that DIDS might selectively remove the population of low affinity sites. As shown in the Scatchard analysis of Fig. 4A, DIDS not only eliminate all low affinity ankyrin sites, as anticipated, but it also reduces the quantity of high affinity sites to approximately half their normal number (Table 1).

Thevenin and Low (21) also observed that the low affinity sites seen during short incubation times with ankyrin were gradually converted to high affinity sites during the more extended slow phase of ankyrin binding. Since the data in Fig. 4A were obtained after more extended (8.5 h) incubation, we investigated whether low affinity sites might have been present earlier in the binding reaction, e.g. after only 1 h incubation. As displayed in Fig. 4B, under conditions where low affinity binding normally represents >95% of total binding (21), only the high affinity interaction is still detectable in DIDS-modified KI-IOV. We conclude that DIDS conjugation to band 3 eliminates low affinity sites at all time points. DIDS derivatization also initially reduces high affinity binding which nevertheless recovers partially during more extended incubation, i.e. similar to the slow accrual of high affinity sites seen with unmodified band 3 (21).

Effect of DIDS on Band 3 Retention in Detergent-extracted Membrane Skeletons—The DIDS-induced loss of all low affinity and substantial high affinity ankyrin-binding sites predicts that band 3 retention in membrane skeletons prepared directly from DIDS-labeled whole cells should be reduced. Because band 3 migrates as a highly diffuse polypeptide in SDS-PAGE gels of normal membrane skeletal preparations, its quantitation in the detergent-extracted membrane skeletons is difficult. This quantitation, however, can be facilitated by proteolytically releasing the carbohydrate-containing fragment from band 3 prior to detergent extraction to yield the sharply defined 65,000-dalton skeleton-anchored fragment of band 3 (39). Because this treatment has no known effect on anion transport, DIDS binding, or ankyrin association (39), it can be exploited to improve densitometric analysis of band 3 retention in the membrane skeletons. Fig. 5 shows an analysis of the retention of the 65,000-dalton band 3 fragment in n-octyl glucoside-insoluble membrane skeletons prepared from unmodified and DIDS-
FIG. 4. A, effect of DIDS on the equilibrium binding of 125I-ankyrin to KI-IOV at pH 7.8. Increasing amounts of 125I-ankyrin were incubated with KI-IOV (final 35 μg/ml) with (●) or without (●) DIDS modification (labeling was done on whole cells, and KI-IOVs were prepared and stored on ice until used) for 510 min at 24°C in 50 mM Na$_2$PO$_4$, 50 mM Na$_2$BO$_3$, 30 mM NaCl, 1 mM EDTA, 0.2 mM diithiothreitol, 0.25 mg/ml bovine serum albumin, 5% sucrose, pH 7.8. Ankyrin binding was quantitated, as described in the legend to Fig. 3 and under “Experimental Procedures.” The data are presented in a Scatchard plot. This experiment was conducted in triplicate and also repeated on a separate set of membrane preparations. Error bars were generally less than twice the width of the data point symbols, and similar results were obtained on an independent preparation of DIDS-labeled and control KI-IOVs. B, Scatchard plot of the binding of 125I-ankyrin to DIDS-labeled KI-IOV following a short (1 h) incubation at pH 7.8. Red cells were left unmodified or labeled with DIDS; then KI-IOVs were prepared before use. Various amounts of 125I-ankyrin were incubated with DIDS modified KI-IOV at 24°C for 1 h at pH 7.8 (●), and the binding was quantitated as described in the legend to Fig. 3. The line marked with solid triangles (●) is from the Scatchard plot of 125I-ankyrin binding to DIDS membranes following the 510-min incubation shown in Fig. 4A. The use of a Scatchard plot to present data at quasi-equilibrium has been justified elsewhere (20). This experiment was done in triplicate and repeated once on a separate KI-IOV preparation. Error bars generally fell within the width of the data point symbols, and similar results were obtained on an independent preparation of DIDS-labeled and control KI-IOVs.

treated whole cells. As anticipated, DIDS reduces retention of band 3 in the skeletal extract, exerting an increasingly greater impact as the time between DIDS labeling and detergent extraction proceeds. In contrast, the amount of band 3 retained in membrane skeletons from unmodified cells remains unchanged. By 1 h incubation, a reduction in band 3 content of ~55% was seen in four separate skeleton preparations from DIDS modified cells using n-octylglucoside as the extracting detergent (Fig. 5). While not all detergents yielded quantitatively the same result in this study, all detergents evaluated (which included Zwittergent 3-14, octyl-β-D-thioglucopyranoside, Triton X-100, and n-octylglucoside) revealed a consistent loss of band 3 from the membrane skeletons of DIDS-modified cells with no loss of band 3 from control skeletons (Table II). We interpret the differences in the amount of band 3 associated with membrane skeletons to simply reflect the differing solubilizing capacities of the several detergents. For example, octylglucoside-extracted cells retain roughly four times as much band 3 in their membrane skeletons as Triton X-100 extracted cells (50). Apparently, the band 3-ankyrin complexes most sensitive to DIDS modification constitute at least part of the population most readily extracted by Triton X-100.

**DISCUSSION**

We have provided evidence that DIDS binding to the membrane-spanning domain of band 3 significantly reduces 125I-ankyrin binding to the cytoplasmic domain. Not only were all low affinity (rapid phase) ankyrin-binding sites eliminated, but roughly half of all high affinity (slow phase) sites were also lost. Loss of sites was also observed in intact cells where the DIDS-mediated decrease in ankyrin binding resulted in elimination of more than half the normal linkages to the membrane skeleton (Fig. 5 and Table II). Presumably, the presence of high intracellular ankyrin concentrations prevented a more quantitative disappearance of binding sites in the whole erythrocyte.

While more than one mechanism can account for these observations, we hypothesize Scheme I to explain our current and previous data on ankyrin-band 3 interactions. As proposed by others (23–28) and ourselves (21), ankyrin appears to associate predominantly if not exclusively with the tetramer of band 3 in situ. Consistent with this observation is the fact that addition of ankyrin to KI-IOVs drives the band 3 subunit equilibrium nearly quantitatively to the tetramer (Fig. 2B). According to the above mechanism, the fast (low affinity) phase of ankyrin binding arises from its association with pre-existing band 3 tetramers, i.e., the receptive population at the time of ankyrin addition. By the same argument, the slow phase of ankyrin binding must derive from generation of new band 3 tetramers from band 3 dimers as ankyrin addition and complex formation shifts the subunit equilibrium to the right (Fig. 2). DIDS elimination of the fast phase of ankyrin binding may, therefore, result from depletion of pre-existing tetramers in the KI-IOV membranes, as noted by native gel electrophoresis (44). Retention of a slow phase of binding to DIDS-modified membranes (Fig. 3) would then simply represent the slow, but only partial recruitment of band 3 dimers back to tetramers, as seen in Fig. 2A. The structural basis for conversion of low to high affinity sites is the only unexplained feature of this model, but

**TABLE I**

| Incubation time | Low affinity sites | High affinity sites |
|-----------------|-------------------|---------------------|
|                 | Capacity | $K_D$ | Capacity | $K_D$ |
| min              | μg/mg    | μg/mg | min          | μg/mg |
| Control          | 510$^a$  | 148$^b$ | 150$^b$ | 118$^b$ | 17$^b$ |
| DIDS             | 510$^a$  | ND    | 60$^c$ | 52$^d$ | 16$^d$ |

$^a$ Taken from the data in Fig. 4A.

$^b$ The curved line Scatchard plots were assumed to result from the presence of two classes of independent sites characterized by binding capacities, $N_1$ and $N_2$, and dissociation constants, $K_D$ and $K_0$. Derivation of these four parameters was conducted by iteration using the equation,

$$B/F = \frac{N_1}{K_D + F} + \frac{N_2}{K_D + F}$$

where $B$ and $F$ represent the bound and free concentrations of ankyrin, respectively.

$^c$ ND, not detected.

$^d$ The monophasic Scatchard plots were analyzed for $K_D$ and $N$ according to the equation,

$$B/F = \frac{N}{K_D} - \frac{B}{K_D}$$

$^e$ Taken from Fig. 4B.
previous observations place significant constraints on any physical interpretation of this transition. For example, the two forms must be interconvertible and they must be generated in an ordered chronology, where low affinity sites are occupied before high affinity complexes can arise (21). Furthermore, the maximum number of high affinity complexes should approximate the potential number of band 3 tetramers in the membrane. While other explanations may be possible, the sequential interaction of two or more sites on band 3 with two or more sites on ankyrin represents an attractive mechanism to explain this interconversion. Thus, as noted in the Introduction, ankyrin is known to occupy two noncontiguous sites on band 3 (30–34), and recently two distinct domains of ankyrin have been shown to contribute to its high affinity association with band 3 (35). If completion of a single site interaction were required prior to isomerization to a multisite association, then sequential conversion of low affinity to high affinity sites would be expected. Furthermore, if most or all ankyrin-band 3 complexes eventually isomerize to their high affinity state (21), then the absence of low affinity sites in DIDS-labeled membranes would be required, since the rate-determining (kinetically visible) step in ankyrin binding would be the slow association of band 3 dimers to tetramers. Regardless of the interpretation, DIDS binding clearly shifts a subunit dissociation equilibrium in band 3 slowly toward the dimer and simultaneously eliminates all of the fast phase and most of the slow phase of ankyrin binding. It is our contention that these two perturbations are mechanistically linked.

We have attempted to analyze our HPLC, polyacrylamide gel electrophoresis, and ankyrin binding data as quantitatively as possible. While such quantitation can be useful in identifying conditions that shift the band 3 subunit equilibrium, caution must also be exercised to avoid overinterpretation of these data. Thus, the ratio of band 3 tetramers, dimers, and monomers in any sample has been shown to depend on: (i) the amount of ankyrin remaining in the sample (e.g. Fig. 2), (ii) the duration of KI-IOV incubation at 37 °C (Fig. 1), (iii) the temperature of the incubation (changes in subunit equilibrium are slow or nonexistent at 0 °C, Ref. 25), (iv) the extraction detergent employed (C12E8 and octyl glucoside are less disruptive of band 3 oligomers than Triton X-100 (50)), and (v) pH (25). Consequently, the oligomeric ratios observed in KI-IOV do not likely correspond to the ratios present in situ. Nevertheless, the qualitative shifts in this ratio induced by DIDS are undoubtedly real, since membrane skeletons extracted directly from whole cells display the impact of these shifts on band 3 retention.

Another laboratory has reported that DIDS derivatization causes little or no change in the size of the band 3 oligomer (25). In contrast, Salhany et al. (51), Tomida et al. (44), and ourselves observe that DIDS can substantially alter the subunit interactions of band 3. We suspect that this discrepancy does not arise from inaccuracies in the measurements, but rather from differences in the delay between DIDS labeling and band 3 analysis, or alternatively, in the temperature and method of preparation of peripheral protein-stripped membranes. In our

---

**TABLE II**

| Sample | Detergent                  | Time of 37 °C incubation before extraction | Percent band 3 versus actin concentration |
|--------|---------------------------|-------------------------------------------|------------------------------------------|
| Control | n-Octyl-glucoside          | 1 h                                       | 100                                      |
| DIDS   | n-Octyl-glucoside          | 1 h                                       | 45                                       |
| Control | Octyl-β-D-thioglucopyranoside | 1 h                               | 100                                      |
| DIDS   | Octyl-β-D-thioglucopyranoside | 1 h                               | 37                                       |
| Control | Zwittergent 3–14            | 1 h                                       | 100                                      |
| DIDS   | Zwittergent 3–14            | 1 h                                       | 48                                       |
| Control | Triton X-100               | 1 h                                       | 100                                      |
| DIDS   | Triton X-100               | 1 h                                       | 89                                       |
| Control | Triton X-100               | 24 h                                      | 100                                      |
| DIDS   | Triton X-100               | 24 h                                      | 45                                       |
hands, the subunit dissociation of band 3 was relatively slow, as was the reassociation induced by excess ankyrin (Fig. 2B). If different groups were to sample the band 3 population at different times after DIDS labeling, then the observed discrepancy would be expected. Additionally, we have observed that stripping of peripheral proteins from erythrocyte membranes with ice-cold 2 mM EDTA, pH 12, partially denatures band 3 and prevents the subunit dissociation events that we observe upon incubation of KI-stripped inside-out vesicles at 37 °C. 

The results of our studies provide a possible resolution to the controversy regarding the extent of interaction between the membrane-spanning and cytoplasmic domains of band 3. Evidence in favor of little or no contact between the two domains include: (i) the two domains unfold independently when examined in situ by differential scanning calorimetry (52, 53), (ii) the thermostabilities of the two domains can be independently regulated by changes in pH and ligand binding (52, 53), (iii) the two domains rotate in situ at different rates (54, 55), (iv) proteinases of different specificities can cleave band 3 between the two domains, yielding isolated membrane and cytoplasmic domains that display no affinity for each other (5), (v) the isolated membrane-spanning domain catalyzes anion transport similar to uncleaved band 3 (55–59), and (vi) the isolated cytoplasmic domain retains its binding sites for ankyrin, the glycolytic enzymes, band 4.1, band 4.2, hemoglobin, and, and possibly monomers (44, 51, 83–85), and since changes in this equilibrium can dramatically affect ankyrin binding, it would seem more likely that new bridges might preferentially re-establish with pre-existing band 3 tetramers (i.e. former ankyrin binding sites) rather than with dimers that must be slowly associated into receptive tetramers. Fortunately, band 3 oligomers dissociate only slowly, assuring that no significant loss of ankyrin-binding sites will occur during transient rearrangements of the membrane skeleton. However, because band 3 will eventually dissociate to its nonbinding states of association in the absence of ankyrin, it would be predicted that the number of available ankyrin sites in normal membrane preparations will never significantly exceed the number of ankyrin molecules in vivo. This expectation has, in fact, been confirmed by many researchers (21, 36–38).

Acknowledgment—We thank Dr. Vann Bennett for the generous gift of the host and expression plasmid for the 46.5-kDa ankyrin fragment.

REFERENCES
1. Lux, S. E., John, K. M., Kopito, R. R., and Lodish, H. F. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9089–9093
2. Alper, S. L. (1992) in Progress in Cell Research (Bamberg, E., and Passow, H., eds) Vol. 2, pp. 9–15 Elsevier Science Publishers, New York
3. Tanner, M. J. A. (1993) Semin. Hematol. 30, 34–57
4. Appell, K. C., and Low, P. S. (1993) J. Biol. Chem. 258, 11104–11111
5. Low, P. S. (1986) Biochim. Biophys. Acts 864, 145–167
6. Jennings, M. L. (1985) J. Biol. Chem. 260, 15714–15717
7. Cabantchik, Z. I., Kinant, P. A., and Rothstein, A. (1978) Biochim. Biophys. Acts 515, 293–302
8. Morgan, M., Hanke, P., Grygozyczek, R., Tintschel, A., Fasold, H., and Passow, H (1985) EMBO J. 4, 1927–1931
9. Kannan, R., Yuan, J., and Low, P. S. (1991) Biochem. J. 278, 57–62
10. Waugh, S. M., Willardson, B. M., Kannan, R., Labetka, R. J., and Low, P. S. (1986) J. Clin. Invest. 78, 1153–1160
11. Low, P. S., Waugh, S. M., Zincke, K., and Drenckhahn, D. (1985) Science 227, 531–533
12. Waugh, S. M., Walder, J. A., and Low, P. S. (1997) Biochemistry 26, 1777–1783
13. Yuan, J., Kannan, R., Shinar, E., Rachmilewitz, E. A., and Low, P. S. (1992) Blood 79, 3007–3013
14. Turrini, F., Mannu, F., Arase, P., Yuan, J., and Low, P. S. (1993) Blood 81, 3146–3152
15. Lutz, H. U., Bussolino, F., Flepp, R., Fasold, S., Kazatchkine, M. D., and Arase, P. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7368–7372
16. Ando, K., Kikutawa, K., and Beppu, M. (1997) Arch. Biochem. Biophys. 339, 250–257
17. Jenkins, J. D., Keedgy, P. F. J., and Steck, T. L. (1985) J. Biol. Chem. 260, 10426–10433
18. Low, P. S., Ratinathanu, P., and Harrison, M. L. (1993) J. Biol. Chem. 268, 14627–14631
19. Bennett, V., and Gillingham, D. (1993) Annu. Rev. Cell Biol. 9, 27–66
20. Bennett, V., and Stenbuck, P. J. (1979) Nature 280, 468–473
21. Thevenin, B. J.-M., and Low, P. S. (1990) J. Biol. Chem. 265, 16166–16172
22. Mokady, O., Lombardo, C. R., Workman, R. F., and Low, P. S. (1993) J. Biol. Chem. 268, 10990–10996
23. Mulzer, K., Pentrasch, P., Kampmann, L., and Schubert, D. (1989) Stud. Biophys. 143, 17–22
24. Tsuji, A., Kawasako, K., and Ohnishi, S. (1988) Biochemistry 27, 7447–7452
25. Casey, J. R., and Reithmeier, R. A. F. (1991) J. Biol. Chem. 266, 15726–15737
26. Salany, A. (1992) in Progress in Cell Research (Bamberg, E., and Passow, H., eds) Vol. 2, pp. 191–205, Elsevier Science Publishers, New York
27. Hanspal, M., Hanspal, J. S., Yi, S. J., Lui, S. C., and Patel, J. (1994) Blood 84, 305–311
28. Ding, Y., Casev, J. R., and Kopito, R. P. (1994) J. Biol. Chem. 269, 32011–32020
29. Ding, Y., Kobayashi, S., and Kopito, R. R. (1996) J. Biol. Chem. 271, 22494–22498
30. Michaelis, P., and Bennett, V. (1995) J. Biol. Chem. 270, 22050–22057
31. Bennett, V., and Steinbuch, P. J. (1980) J. Biol. Chem. 255, 15893–15899
32. Davis, L., Lux, S. E., and Bennett, V. (1989) J. Biol. Chem. 264, 9665–9672
33. Ding, Y., Casey, J. R., and Kopito, R. P. (1995) J. Biol. Chem. 269, 23862–23870
34. Davis, L., and Bennett, V. (1996) J. Biol. Chem. 265, 10589–10596
35. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 493–500
36. Bennett, V. (1983) Methods Enzymol. 119, 247–269
37. Hargreaves, R. W., Giedd, K., Verkleij, A., and Branton, D. (1980) J. Biol. Chem. 255, 11965–11972
38. Bennett, V., and Stenbuck, P. J. (1979) J. Biol. Chem. 254, 2533–2541
39. Jennings, M. L., and Passow, H. (1979) Biochim. Biophys. Acts 554, 498–519
40. Bennett, V. (1983) Methods Enzymol. 96, 313–324
41. Davis, L., and Bennett, V. (1985) J. Biol. Chem. 260, 15893–15899
42. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) Protein Sci. 4, 2411–2423
43. Bennett, V. (1983) Methods Enzymol. 119, 247–269
44. Tomida, M., Kondo, Y., Moriyama, R., Machida, H., and Makino, S. (1988) Biochim. Biophys. Acts 943, 493–500
45. Steck, T. L., and Yu, J. (1973) J. Supramol. Struct. 1, 220–232
46. Appell, K. C., and Low, P. S. (1982) Biochemistry 21, 2151–2157
47. Mori, A., Okubo, K., Dang, H., and Hamasaki, N. (1995) J. Biol. Chem. (Tokyo) 118, 1192–1198
48. Vince, J. W., Sarahia, V. E., and Reithmeier, R. A. F. (1997) Biochim. Biophys.
DIDS Effects on Ankyrin-Band 3 Interactions