Near-UV Circular Dichroism of Band 3. Evidence for Intradomain Conformational Changes and Interdomain Interactions†

Michael M. Batenjany‡ and Hiroshi Mizukami*

Division of Regulatory Biology and Biophysics, Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202

James M. Salhany

Veteran's Administration Medical Center and Departments of Internal Medicine and of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska 68105

Received July 13, 1992; Revised Manuscript Received October 20, 1992

ABSTRACT: Near-UV circular dichroism (CD) was used to identify differences in the tertiary structure of human erythrocyte band 3, the chloride/bicarbonate exchange protein, consequent to covalent binding of anion transport inhibitors to the intramonomeric stilbenedisulfonate (ISD) site. Isolated intact band 3 and its membrane domain (B3MD) were compared. Spectral differences were observed which involved *intradomain* effects, in that they were seen both with intact band 3 and with B3MD, or *interdomain* effects, in that they were observed only for B3MD, but were inhibited when the cytoplasmic domain was attached. The intradomain effect involved a significant loss in optical activity in the Phe/Tyr region of the spectrum below 280 nm. It was seen only when the ISD site had stilbenedisulfonates bound covalently at pH 7.4. Raising the pH to 9.6 after adduct formation "normalized" this spectral change irreversibly. The interdomain effect was identified in the Trp spectral region at 292 nm. There was a significant increase in optical activity at 292 nm when bulky covalent ligands such as DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonate) were bound to B3MD, but not when the same ligands were bound to intact band 3. These latter results offer evidence that certain aspects of the conformational response of the integral domain are inhibited by the presence of an attached cytoplasmic domain. The potential significance of interdomain interactions to band 3 function is discussed briefly.

Band 3 is an integral membrane protein which facilitates the exchange of chloride for bicarbonate across the erythrocyte membrane and also provides binding sites for certain cytoskeletal and cytosolic proteins (Passow, 1986; Salhany, 1990). This glycoprotein (M_r 95 000) exists as a mixture of dimers and tetramers in situ (Salhany et al., 1990; Casey & Reithmeier, 1991) and is composed of two domains. The N-terminal *cytoplasmic domain* (M_4 40 000) extends into the cell and provides the protein binding sites (Low, 1986). The C-terminal *integral domain* (M_r 55 000) spans the bilayer and can transport anions without the aid of an attached cytoplasmic domain (Lepke & Passow, 1976; Grinstein et al., 1978; Lepke et al., 1992). It is the integral domain of the protein which contains binding sites for a large class of inhibitors of anion exchange (Passow, 1986; Salhany, 1990; Cabantchik & Greger, 1992).

The predominant view of band 3 structure/function has been that a transport site exists within the integral domain which can bind one anion at a time and alternate conformation between inside-facing and outside-facing states in a manner which is independent of the functioning of neighboring subunits within the various oligomeric forms which exist in situ (Jennings, 1976, 1989; Gunn & Frohlich, 1979; Macara & Cantley, 1983; Lindenthal & Schubert 1991). Furthermore, the structure and conformation of the integral domain have been considered to be totally independent of the presence of an attached cytoplasmic domain (Appell & Low, 1982; Low, 1986; Jennings et al., 1988). In contrast, an opposing view has emerged suggesting (a) that the intrinsic mechanism of anion exchange involves at least two anion binding sites per monomer, with formation of a ternary complex occurring during the transport cycle (Salhany & Rauenbuehler, 1983; Salhany et al., 1987); (b) that there can be significant allosteric interactions between the subunits of band 3 (Salhany & Swanson, 1978; Salhany & Gaines, 1981; Salhany, 1990, 1992; Salhany et al., 1990, 1991); and (c) that significant conformational interactions can occur between the integral and the cytoplasmic domains of band 3 (Salhany et al., 1980).

Considerable evidence has been published recently supporting a "two-site" allosteric interpretation for band 3 structure–function. Several laboratories now seem to agree that band 3 transport kinetics are not consistent with a single-site ping-pong mechanism (Benaroch & Gunn, 1992; Restrepo et al., 1989, 1991). Furthermore, new transient-state and steady-state kinetic evidence (Salhany & Cordes, 1992), as well as in situ protein cross-linking studies (Salhany et al., 1990; Salhany, 1992), offers further support for the existence of allosteric interactions between active monomers, possibly within a tetrameric structural unit. Finally, several papers can be cited which indicate that the two domains of the band 3 monomer are conformationally (Salhany et al., 1980; Hsu & Morrison, 1983; Macara et al., 1983; Sami et al., 1992) and functionally (Ducis et al., 1988; Kopito et al., 1989; Yamaguchi & Kimoto, 1992; Zang & Solomon, 1992) interactive in situ.

In the present study, we sought to obtain direct structural evidence for interactions between the two domains of band 3. To accomplish this goal, we studied the near-UV CD spectrum of both isolated band 3 and its integral domain, in the absence and presence of various covalently attached inhibitors of anion

† This work was supported by NIH Grant HL 16008 (to H.M.) and by the Department of Veterans Affairs Medical Research Funds (to J.M.S.).

* To whom correspondence should be addressed.

‡ Present address: Laboratory of Chemical Physics, NIH/NIDDK, Building 2, Room B1-03, Bethesda, MD 20892.

0006-2960/93/0432-663$04.00/0

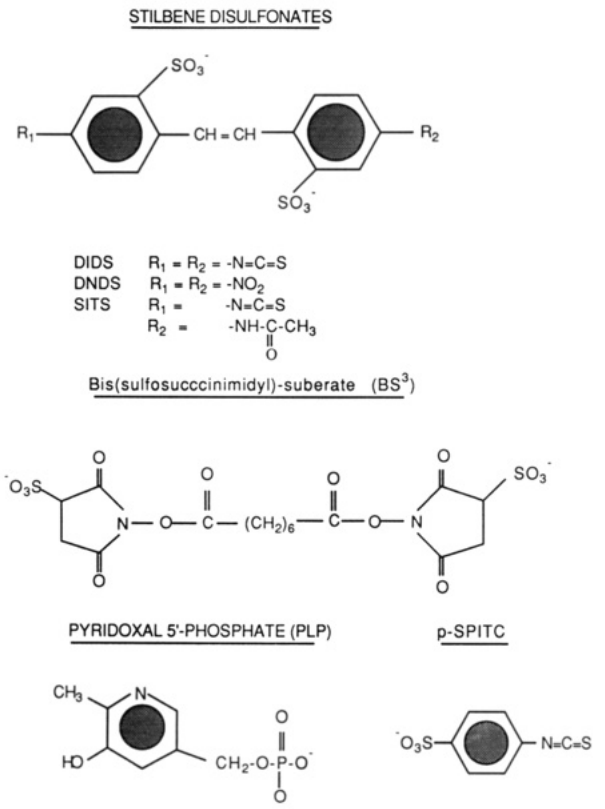

FIGURE 1: Structures of the various inhibitors of band 3 anion exchange used in this study. These inhibitors are all apparently competitive inhibitors of band 3 anion exchange (Passow, 1986; Salhany, 1990).

exchange. The inhibitors used in this study are shown in Figure 1. All of these inhibitors bind to lysine residues contained within or very near the intramonomeric stilbenedisulfonate binding site (Salhany et al., 1987; Passow, 1986; Salhany, 1990). The reversible component of inhibitor binding to this site shows apparent competitive inhibition of anion exchange toward substrate anions (Passow, 1986). Stilbenedisulfonates bind to the integral domain of band 3 with high affinity and with a 1:1 monomer stoichiometry (Jennings & Passow, 1979). The stoichiometries of bis(sulfosuccinimidyl) suberate (BS^3)[1] (Salhany et al., 1991) and p-SPITC (Drickmeier, 1976) binding are also 1:1 with the monomer under properly chosen conditions. On the other hand, PLP binds to two lysines within the stilbene binding pocket, each of which is equally capable of inhibiting anion exchange (Salhany et al., 1987; Wood et al., 1992).

The inhibitors shown in Figure 1 may be expected to cause some perturbation of the conformation of the amino acid side chains when they bind to the stilbenedisulfonate site. Near-UV CD should be one of the most sensitive methods available to detect such conformational perturbations (Cantor & Schimmel, 1980). In the near-UV region, aromatic side chains would be the predominant chromophore, and changes in conformation consequent to inhibitor binding should be readily detectable by measuring the CD spectrum between 250 and 320 nm, where the stilbenedisulfonate molecule has an absorbance minimum (Rao et al., 1979). Near-UV CD contrasts with the more widely used far-UV CD spectrum, in that the former detects changes in tertiary structure, while the latter is a measure of protein secondary structure. Our near-UV CD results provide direct spectroscopic evidence that the conformational response of integral domain tryptophans to the binding of bulky covalent inhibitors depends on the presence or absence of an attached cytoplasmic domain. Other inhibitor-induced spectral changes are identified and discussed.

[1] Abbreviations: DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; BS^3, bis(sulfosuccinimidyl) suberate; p-SPITC, 4-sulfophenyl isothiocyanate; PLP, pyridoxal 5′-phosphate; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 5P8, 5 mM sodium phosphate, pH 8; B3MD, band 3 membrane domain.

MATERIALS AND METHODS

Materials. DIDS, PLP, PMSF, DTT, BSA, and Sepharose CL-4B and DEAE-Sepharose CL-6B were all purchased from Sigma (St. Louis, MO). SITS was purchased from Sigma and Calbiochem (San Diego, CA). Different lots were used to eliminate the possibility of a DIDS-contaminated SITS reagent. The BCA protein assay kit and BS^3 were purchased from Pierce (Rockford, IL). p-SPITC was purchased from Aldrich (Milwaukee, WI). Lubrol poly(X) was purchased from Sigma and purified by the method of Chang and Bock (1980) or obtained in purified protein grade from Calbiochem. The AE-Sepharose was prepared by the method of Casey et al. (1989). All other chemicals were of analytical grade.

Preparation of Intact Red Blood Cells. The preparation was carried out at 4 °C. Recently outdated, packed, human red blood cells were purchased from the Michigan Chapter of the American Red Cross. The red blood cells were washed in 2 volumes of cold 106 mM sodium phosphate buffer at pH 7.5 and centrifuged for 20 min at 3000*g*, and the supernatant and any buffy coat were aspirated. The cells were washed 3 more times and resuspended in an equal volume of the same buffer.

Incubation of Intact Red Cells with Various Inhibitors. Red blood cell suspensions were treated at 50% hematocrit, unless otherwise stated. All inhibitor solutions were prepared in 106 mM sodium phosphate, pH 7.5, within 10 min of use, and added to separate aliquots of the red blood cells suspensions to give final concentrations of 50 μM or 5 mM BS^3, 50 μM DIDS, 1 mM SITS, or 7.5 mM p-SPITC. These suspensions were mixed, covered with aluminum foil, and incubated at 37 °C for 1 h, as was an aliquot of the red blood cell suspension without inhibitors, as a control. At the end of the incubations, unbound inhibitor was removed by washing the red blood cells twice with an equal volume of 106 mM sodium phosphate, pH 7.5, at 4 °C containing 0.5% (w/v) BSA and then twice with 2 volumes of cold 106 mM sodium phosphate buffer, pH 7.5, without BSA. The untreated red cell suspension was washed in the same manner. In the case of PLP treatment, the red blood cell suspension was at 10% hematocrit, and freshly prepared PLP in 106 mM sodium phosphate buffer, pH 7.5, was added to the red blood cell suspension to give a final concentration of 50 mM, enough to saturate available sites in the band 3 protein (Salhany et al., 1987). The PLP suspension was mixed, covered with foil, and shaken gently at room temperature for 20 min and then on ice for another 15 min. In order to convert PLP/band 3 Schiff base linkages into stable products, solid $NaBH_4$ was added to the cold suspension to give a final concentration of 55 mM and shaken gently on ice, for another 15 min. This suspension was centrifuged at 3000*g* for 20 min at 4 °C, and the supernatant was aspirated. The red blood cells were washed twice with an equal volume of cold 106 mM sodium phosphate buffer, pH 7.5, containing 0.5% (w/v) BSA and twice with the same buffer without BSA.

DIDS, SITS, or p-SPITC Treatment of Intact Red Blood Cells at pH 7.4 and 9.6. The cold 106 mM sodium phosphate buffer, pH 7.5, gave a room temperature pH value of 7.4. The

band 3 or band 3 membrane domain (B3MD) isolated from the treated red blood cells was used to obtain the CD profiles designated as the near-UV CD spectra at pH 7.4. In order to obtain the profiles designated as the near-UV CD spectra at pH 9.6, aliquots of the various treated cells were washed in 2 volumes of 150 mM $NaHCO_3$ with a room temperature pH of 9.6, then resuspended in 4 volumes of 150 mM $NaHCO_3$, at pH 9.6, and incubated for 1 h at 37 °C. After this incubation, the cells were centrifuged at 4 °C for 20 min at 3000*g*, and the supernatant was aspirated. These cells were then washed twice in 3–4 volumes of ice-cold 106 mM sodium phosphate buffer, pH 7.5.

Purification and Isolation of Band 3 and the Band 3 Membrane Domain (B3MD). Extractions and purifications were performed at 4 °C, unless otherwise stated. The red blood cell suspensions were hypotonically lysed in 15 volumes of cold 5P8, containing 0.1 mM PMSF, and subsequently washed in the same solution, until white ghosts were obtained. Alkali stripping of the membranes was avoided in order to maintain the pH of the samples at 7.4 and to avoid denaturation of the cytoplasmic domain in band 3 (Low, 1986). The hypotonically lysed ghosts were stripped of cytoskeletal and peripheral proteins by incubating then at 37 °C for 1 h, under the following conditions: (a) twice with 10 volumes of a low ionic strength buffer containing 2 mM EDTA, 0.2 mM DTT, and 0.1 mM PMSF, at pH 8.0 (cold); (b) once with 10 volumes of a high ionic strength buffer containing 1 M KI in 7.5 mM sodium phosphate, 1 mM DTT, and 0.1 mM PMSF, at pH 7.5 (room temperature) (Casey et al., 1989). Residual KI, from the high ionic strength incubations, was removed by washing twice in 10 volumes of 5P8 containing 0.1 mM PMSF. These ghosts were first resuspended using a 20-gauge needle and 3 cm^3 syringe into an equal volume of 5P8; then 5 volumes of 1% (w/v) Lubrol poly(X) in 5P8 containing 0.1 PMSF were added. The ghosts were solubilized in this solution, by gentle mixing over several hours at 4 °C. The solubilized ghosts were centrifuged for 1 h at 100000*g*, and the supernatant was collected for the isolation of band 3 or B3MD.

The B3MD was prepared by using a modification of the method of Jennings and Passow (1979). Briefly, the ghosts were washed in 106 mM sodium phosphate, pH 7.5, resuspended in 20 volumes of the same buffer, and incubated with 100 μg/mL trypsin at 37 °C for 1 h. The proteolysis was stopped by placing the mixture on ice and adding PMSF to 2 mM. The sample was centrifuged at 19000*g* for 30 min and the supernatant aspirated to waste. The trypsinized ghosts were washed twice in ice-cold 5P8 containing 0.1 mM PMSF, then stripped, and solubilized as described for the intact band 3 preparation.

Increasing step gradient anion-exchange column chromatography was performed, using AE-Sepharose CL-4B for band 3 and DEAE-Sepharose CL-6B for B3MD (Casey et al., 1989). The isolated proteins were concentrated in an Amicon concentrator using a PM-10 membrane and were dialyzed overnight against 200 volumes of 0.1% Lubrol poly(X) in 5P8. Protein concentrations were usually determined using the BCA protein assay and sometimes estimated from their absorbencies at 280 nm. We used the specific absorptivity values of 1.55 L g^{-1} cm^{-1} for band 3 and 2.44 L g^{-1} cm^{-1} for B3MD, which we derived by correlating multiple BCA assays with absorbance readings at 280 nm of the same protein samples in 0.1% Lubrol poly(X) in 5P8. The BCA standard curve was obtained using various concentrations of BSA in 0.1% Lubrol poly(X) in 5P8.

Near-UV CD Studies. Circular dichroism spectroscopy was performed on samples not more than 10 days old at a protein concentration between 1 and 2 mg/mL under solution conditions given in the legends to the figures. A model J-600 spectropolarimeter (Jasco, Tokyo, Japan) was used which was calibrated with the camphorsulfonic acid standard [ϵ = 2.36 M^{-1} cm^{-1}, 290.5 nm, 1-cm cell (Yang et al., 1986)]. All samples were run in the same 1-cm quartz cuvette (Hellma, Jamaica, NY) for near-UV CD studies (250–320 nm), at room temperature. Spectra were collected in 0.2-nm increments at a scan speed of 2 nm/min, 1-nm band width, and 10–20 mdeg sensitivity and using an 8-s time constant. They were base-line-corrected for buffer contributions and adjusted to zero CD at 320 nm. Preliminary studies using matching inhibitor concentrations demonstrated that no spectral correction was needed between 250 and 320 nm. Molar ellipticities were obtained using mean residue molecular masses of 111.7 and 111.2 g mol^{-1} for band 3 and B3MD, respectively. The spectra were smoothed by Fourier transform filtering using the Passage II, version 2.10 program (Passage Software, Inc., Fort Collins, CO) written for Macintosh (Apple Computer, Inc., Cupertino, CA).

RESULTS

Conformational Response of the Integral Domain to Inhibitor Binding. All inhibitors of band 3 anion exchange which can make a covalent adduct with the protein are found to reside in the integral domain (Salhany, 1990). Figure 2 shows CD spectra of the integral domain of band 3 with various covalent inhibitors added within intact cells. There are two regions of the spectrum which we wish to focus on in our description. The first is the 292-nm peak which is assigned to Trp. The second region is the one below 280 nm, which is assigned to Phe/Tyr. It is apparent that formation of DIDS or SITS covalent adducts significantly alters the CD spectrum as compared to the unlabeled form of B3MD under pH 7.4 cellular reaction conditions. It should be noted that both of these inhibitors form covalent adducts with band 3 under the cellular reaction conditions used here [see Table 4 of Cabantchik and Greger (1992)].

There was a significant loss in optical activity in the Phe/Tyr region below 280 nm, and a large apparent increase in the Trp CD at 292 nm consequent to covalent binding of DIDS or SITS (Figure 2). Raising the pH of the cells *after adduct formation* reversed or "normalized" the Phe/Tyr region, but the intense optical activity in the Trp region was retained for both stilbenedisulfonate adducts. Keep in mind that the pH values indicated in the figures are cellular incubation values present after covalent adduct formation. Exposure of the adduct to pH 9.6 buffer was followed by returning the pH to 7.4, and all isolation procedures were at pH 7.4 or 8 (see Materials and Methods). CD spectra were collected in 0.1% Lubrol poly(X) in 5 mM sodium phosphate, pH 7.2. Thus, returning the pH 9.6 treated cells to near-neutral pH prior to isolation of band 3 did not restore the original spectrum in the Phe/Tyr region for the DIDS adduct. Furthermore, raising the pH to 10 after isolation of DIDS-labeled band 3 also caused an irreversible "normalization" of the spectrum in the Phe/Tyr region (data not shown). In contrast, inhibitor-free band 3 showed no significant spectral changes over the same pH range (data not shown).

CD spectra of band 3 adducts formed by using the aliphatic homobifunctional cross-linking reagent BS^3 (Staros, 1982; Staros & Anjaneyulu, 1989), or formed using the monofunctional reagent p-SPITC (Drickamer, 1976), differed from the spectra observed when stilbenedisulfonates were bound (Figure 2). The former inhibitors bind to the intramonomeric stilbenedisulfonate site in a mutually competitive fashion with

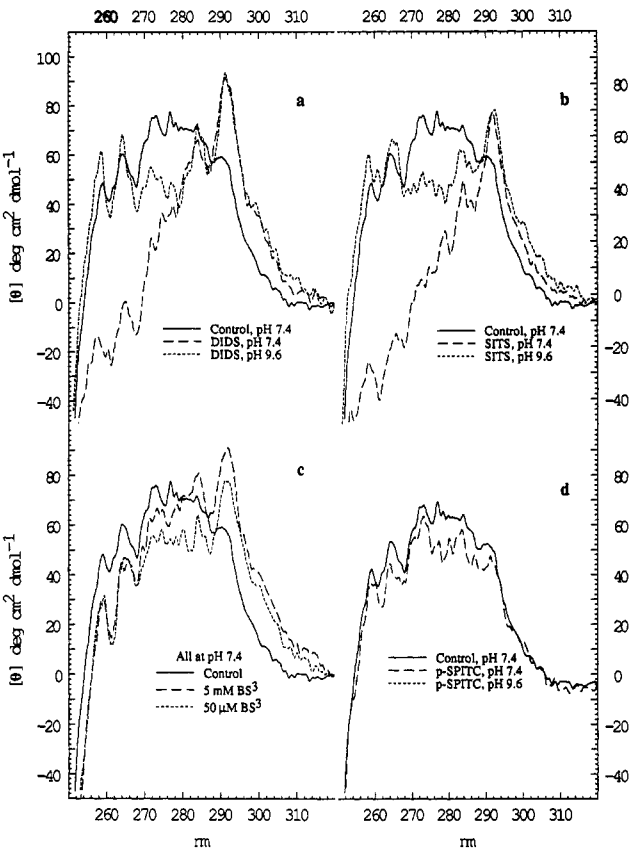

FIGURE 2: Near-UV CD of the integral domain of band 3 (B3MD) in the absence and presence of covalently bound inhibitors of anion exchange. Intact red blood cells were incubated at 50% hematocrit in the presence or absence of 50 μm DIDS, 1 mM SITS, 5 mM or 50 μM BS^3, or 7.5 mM p-SPITC in 106 mM sodium phosphate buffer, pH 7.4 (room temperature), for 1 h at 37 °C. A portion of the DIDS- and SITS-treated cells was removed and washed and incubated further in 4 volumes of 150 mM sodium bicarbonate buffer, pH 9.6 (room temperature), for 1 h at 37 °C. Afterward, B3MD was formed by tryptic cleavage of band 3 in leaky red cell ghosts and isolated into 0.1% Lubrol poly(X) in 5 mM sodium phosphate buffer, pH 8.0 (cold). Near-UV CD spectra were colelcted at room temperature in 0.1% Lubrol poly(X) in 5 mM sodium phosphate, pH 7.2. The pH values listed in the figure are cellular incubation pH values. Except for the pH 9.6 cellular washing step, all steps involving isolation of the protein were performed at pH values of 7.4 or 8.0. Molar concentrations refer to mean residue moles per liter. Details of cell treatments as well as CD parameters and corrections are described under Materials and Methods.

SITS or DIDS (Salhany, 1990). While BS^3 caused an intense increase in the 292-nm Trp region, no change was seen in the Phe/Tyr region of the spectrum. p-SPTIC had no significant effect on the near-UV CD spectrum of B3MD (Figure 2).

Conformational Response of Intact Band 3 to Inhibitor Binding. The response of the CD spectrum of B3MD in the Phe/Tyr region to covalent binding of DIDS or SITS was also observed using intact band 3 (Figure 3). These samples had the pH adjusted to the levels of indicated while band 3 was still membrane-bound in intact cells. Raising the pH to 9.6 after cellular incubation with inhibitors reversed or "normalized" the spectrum in the Phe/Tyr region (Figure 3).

The response of the Trp region (292 nm) of the CD spectrum of intact band 3 to covalent adduct formation (Figure 3) differed considerably when compared to B3MD (Figure 2). Neither the DIDS nor the SITS covalent adduct caused a significant increase in optical activity at 292 nm (Figure 3). Furthermore, BS^3 adduct formation had no effect on the CD spectrum of intact band 3 either in the Phe/Tyr or in the Trp region (Table I). This lack of effect of BS^3 on the CD spectrum in the Phe/Tyr region was the same as that seen using B3MD

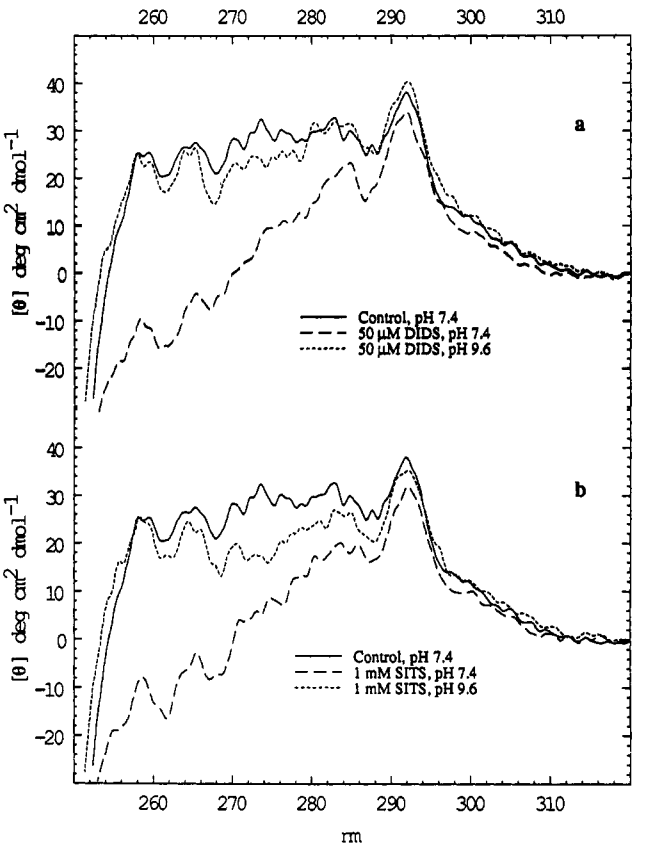

FIGURE 3: Near-UV CD of the intact band 3 in the absence and presence of covalent inhibitors of anion exchange. Intact red blood cells were incubated at 50% hematocrit in the absence or presence of 50 μM DIDS or 1 mM SITS in 106 mM sodium phosphate buffer, pH 7.4 (room temperature), for 1 h at 37 °C. A portion of the cells was removed and washed and then incubated further in 4 volumes of 150 mM sodium bicarbonate buffer, pH 9.6 (room temperature), for 1 h at 37 °C. Band 3 was subsequently isolated into 0.1% Lubrol poly(X) in 5 mM sodium phosphate buffer, pH 8.0 (cold). Near-UV CD spectra were collected at room temperature in 0.1% Lubrol poly-(X) in 5 mM sodium phosphate, pH 7.2. The pH values listed in the figure and the conditions for isolation are as in Figure 2. Molar concentrations refer to mean residue moles per liter. Details of cell treatments as well as CD parameters and correction are described under Materials and Methods.

Table I: Effect of Inhibitors on the Near-UV CD Spectrum of Band 3[a]

	intact band 3		B3MD	
inhibitor type	Trp region	Phe/Tyr region	Trp region	Phe/Tyr region
bulky covalent inhibitors				
50 μM DIDS	–	+	+	+
1 mM SITS	–	+	+	+
5 mM BS^3	–	–	+	–
50 uM BS^3	–	–	+	–
other inhibitors				
7.5 mM p-SPITC	nd	nd	–	–
50 mM PLP	–	–	nd	nd

[a] Calculations were based on differences in inhibitor minus control values. Plus sign indicates >20 deg cm^2 dmol^{-1}; minus sign indicates <20 deg cm^2 dmol^{-1}. nd indicates not determined.

(Figure 2). However, the lack of effect of BS^3 in the Trp region at 292 nm for intact band 3 differed significantly from the large increase in optical activity at 292 nm seen using B3MD (Figure 2). Covalent modification of intact band 3 with PLP had no effect on the near-UV CD spectrum (Table I). The effects of the various inhibitors on the near-UV CD spectrum of B3MD and intact band 3 are summarized in Table I.

DISCUSSION

We have studied the tertiary structure of isolated band 3 in detergent solution using near-UV CD to search for conformational differences in the protein related to the binding of transport inhibitors to the intramonomeric stilbenedisulfonate site. The inhibitors selected for study included the important class of apparently competitive inhibitors of anion exchange known as the stilbenedisulfonates (SITS and DIDS) (Salhany, 1990). The results show that covalent binding of stilbenedisulfonates produces significant spectral perturbations within the integral domain of the porter. However, covalent inhibitor binding along is an insufficient explanation for the observed differences. Several smaller inhibitors which bind covalently, presumably to the same lysine (Salhany, 1990), do not produce significant spectral changes. The various results are summarized in Table I, and they suggest that ligand bulk *and* covalency are both necessary characteristics to produce the observed changes.

Bulky covalent ligands such as DIDS, SITS, and BS^3 all affect the Trp region of the spectrum within the isolated integral domain, while only DIDS and SITS seem to produce differences in the Phe/Tyr region. Using near-UV CD, the results may be interpreted most simply in terms of local conformational changes of amino acid side chains at the stilbenedisulfonate binding site (Cantor & Schimmel, 1980). However, without knowledge of the detailed three-dimensional structure of band 3, it is not possible to offer a unique molecular interpretation for these differences. Nevertheless, both DIDS and SITS are known to bind covalently to lysine A, which has been identified as being either Lys-539 (Bartel et al., 1989) or Lys-542 (Garcia & Lodish, 1989) of the human protein sequence (Tanner et al., 1988; Lux et al., 1989). The specific lysine residues involved in the BS^3 reaction at the stilbene site have not been identified, but are expected to at least include lysine A (Salhany et al., 1991). It is worth noting that four Phe residues (524F, 526F, 532F, and 537F) and one Tyr (534Y) all lie on a segment of the peptide containing the two reactive lysines. This segment is thought to form a transmembrane helix (helix 5) (Tanner et al., 1988), which makes up part of the hydrophobic pocket containing the stilbenedisulfonate site (Passow, 1986). In addition, it is known that several Trp residues exist in the integral domain of band 3 (Kleinfield et al., 1980) and that covalent binding of DIDS quenches the fluorescence of these residues (Macara et al., 1983). Thus, it is likely that there are Trp residues which are also close to the intramonomeric stilbenedisulfonate binding site. These structural considerations imply that spatial proximity of Phe/Tyr and Trp side chains to a covalently bound stilbenedisulfonate molecule is a reasonable explanation for the changes in the CD spectrum of the protein seen consequent to covalent binding of stilbenedisulfonates.

The pH "normalization" effect observed in the Phe/Tyr region of the CD spectrum (Figures 2 and 3) occurs when the pH of the stilbenedisulfonate–band 3 adduct is raised to 9.6. Returning the pH to lower values does not restore the original spectrum below 280 nm. Since SITS and DIDS are attached to the protein covalently, this "normalization" effect cannot be attributed to ligand dissociation. Furthermore, although DIDS can make an intramonomeric cross-link when the pH of the adduct is raised to 9.6 (Jennings & Passow, 1979), SITS cannot form such a cross-link because it lacks a second isothiocyano group (Figure 1). In addition, BS^3 makes an intramonomeric cross-link at a site mutually exclusive with stilbenedisulfonate binding (Jennings & Nicknish, 1985; Salhany et al., 1991), yet it does not cause a perturbation of the CD spectrum in the Phe/Tyr region (Figure 2). Thus, intramonomeric cross-linking per se cannot explain the spectral change observed below 280 nm for the stilbenedisulfonate–band 3 adducts, nor can such cross-linking explain the apparent "normalization" effect. Rather, we suggest that covalent attachment of DIDS or SITS at pH 7.4 in situ *traps* band 3 in a state with conformationally perturbed Phe and Tyr side chains. Raising the pH to 9.6 is then proposed to release the conformational trap by somehow allowing the affected side chains to relax to a conformation more nearly like that of inhibitor-free protein. Once this conformational trap is released, it cannot be reset by lowering the pH. The absence of a similar spectral change after BS^3 intramonomeric cross-linking (50 μM BS^3; Salhany et al., 1991) may be due to the lack of a ring structure in this molecule and/or to the conformational flexibility of the aliphatic chain during the initial cross-linking reaction. BS^3 is expected to react most rapidly and monofunctionally with lysine A, and through a "tethering" mechanism find a second lysine to complete the cross-link (Staros & Anjaneyulu, 1989). Such a tethering effect may allow an alternate cross-linking pathway to be followed by BS^3 which cannot take place when using the stilbenedisulfonate molecule.

One of the interesting and potentially important features of the spectral differences we have identified is the apparent influence of the cytoplasmic domain on the conformational response of the integral domain to covalent binding of DIDS, SITS, and BS^3. This difference is seen exclusively in the Trp spectral region at 292 nm and does not depend on the pH used to posttreat the cells (Figure 2). When the cytoplasmic domain is absent, there is a large increase in the Trp molar ellipticity of B3MD for the DIDS, SITS, and BS^3 adducts compared to control (Figure 2 and Table I). In contrast, no significant differences were seen for intact band 3 in the Trp region of the spectrum (Figure 3 and Table I). Thus, attachment of the cytoplasmic domain inhibits the conformational response of the integral domain tryptophans to inhibitor binding. This result suggests that some type of interaction exists between the two domains within the isolated intact protein which prevents conformational changes of the integral domain tryptophans from occurring. The strength of these interactions in energetic terms need not be large in order to explain the observed difference in response to ligand binding.

The results of this paper offer direct spectroscopic evidence for interdomain interactions within isolated band 3, in support of the original work of Salhany et al. (1980) and the other data in the literature (see the introduction). Although it seems clear that the integral domain can transport anions without an attached cytoplasmic domain (Grinstein et al., 1978; Lepke et al., 1992), it is not clear whether the band 3 turnover number in inside-out vesicle preparations is comparable to the in situ turnover number where cytoskeletal and cytosolic proteins are bound to the band 3 tetramer (Mulzer et al., 1990; Schuck & Schubert, 1991). It is interesting to note that removal of nine amino acids constituting an hydrophobic subdomain at the junction between the putative first transmembrane helix and the cytoplasmic domain causes complete inhibition of anion exchange and also causes total inhibition of covalent binding of DIDS (Schofield et al., 1992a,b). Since the deleted amino acids are located far from the DIDS binding site, the results with this mutant seem to support our findings suggesting that long-range conformational interactions can occur on band 3.

The role of interdomain interactions in band 3 function is not established at present. However, evidence suggests certain possibilities for cytoplasmic modulation of the anion exchange activity: (a) by oxygen-linked hemoglobin binding to the

cytoplasmic domain (Chetrite & Cassoly, 1985; Ducis et al., 1988); (b) by phosphorylation of the cytoplasmic domain (Bursaux et al., 1984; Yannoukakos et al., 1991); (c) by calcium binding to the cytoplasmic domain (Passing & Schubert, 1983; Salhany & Cordes, 1991; Low, 1978; Gunn et al., 1979; Joshi & Gupta, 1990). There is some evidence that trout red cells use oxygen-linked hemoglobin binding to band 3 as part of the mechanism to regulate cell volume (Borgese et al., 1991; Motais et al., 1991).

In summary, our results define significant differences in the tertiary structure of band 3 related to the binding of certain important inhibitors of anion exchange. In addition, spectral evidence was presented suggesting the presence of interdomain interactions. The significance of such interactions to band 3 function remains to be established.

ACKNOWLEDGMENT

We thank Roger Romanick, Veronica Riha, and Karen Cordes for manuscript preparation and Drs. Patrick Kelly and Howard Petty for discussions and critiques.

REFERENCES

Appell, K. C., & Low, P. S. (1982) *Biochemistry 21*, 2151–2157.
Bartel, D., Hans, H., & Passow, H. (1989) *Biochim. Biophys. Acta 985*, 355–358.
Benaroch, R. G., & Gunn, R. (1992) *Biophys. J. 61*, A522.
Borgese, F., Motais, R., & Garcia-Romeu, F. (1991) *Biochim. Biophys. Acta 1066*, 252–256.
Bursaux, E., Hilly, M., Bluze, A., & Poyart, C. (1984) *Biochim. Biophys. Acta 777*, 253–260.
Cabantchik, Z. I., & Greger, R. (1992) *Am. J. Physiol. 262*, C803–827.
Cantor, C. R., & Schimmel, P. R. 91980) *Biophysical Chemistry, Part* II, pp 409–433, W. H. Freeman & Co., San Francisco.
Casey, J. R., & Reithmeier, R. A. F. (1991) *J. Biol. Chem. 266*, 15726–15737.
Casey, J., Lieberman, D., & Reithmeier, R. (1989) *Methods Enzymol. 173*, 494–512.
Chang, H., & Bock, E. (1980) *Anal. Biochem. 104*, 112–117.
Chetrite, G., & Cassloy, R. (1985) *J. Mol. Biol. 185*, 639–644.
Drickamer, K. (1976) *J. Biol. Chem. 251*, 5115–5123.
Ducis, I., Kandrach, A., & Racker, E. (1988) *J. Biol. Chem. 263*, 8544–8550.
Garcia, A., & Lodish, H. (1989) *J. Biol. Chem. 264*, 19607–19613.
Grinstein, S., Ship. S., & Rothstein, A. (1978) *Biochim. Biophys. Acta 507*, 294–304.
Gunn, R. B., & Frohlich, O. (1979) *J. Gen. Physiol. 74*, 351–374.
Gunn, R. B., Frohlich, O., Macintyre, J. D., & Low, P. S. (1979) *Biophys. J. 25*, 106a.
Hsu, L., & Morrison, M. (1983) *Arch. Biochem. Biophys. 227*, 31–38.
Jennings, M. L. (1976) *J. Membr. Biol. 28*, 187–205.
Jennings, M. L. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 397–430.
Jennings, M. L., & Passow, H. (1979) *Biochim. Biophys. Acta 554*, 498–519.
Jennings, M. L., & Nicknish, J. S. (1985) *J. Biol. chem. 260*, 5472–5479.
Jennings, M. L., Anderson, M. P., & McCormick, S. J. (1988) in *Cell Physiology of Blood* (Gunn, R. B., & Parker, J. C., Eds.) pp 164–180, Rockefeller University Press, New York.
Joshi, R., & Gupta, C. M. (1990) *J. Membr. Biol. 117*, 233–242.
Kleinfeld, A. M., Lukacovic, M., Matayoshi, E. D., & Solomon, A. K. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1714.
Kopito, R. R., Lee, B. S., Simmons, D. M., Lindsey, A. E., Morgans, C. W., & Schneider, K. (1989) *Cell 59*, 927–937.
Lepke, S., & Passow, H. (1976) *Biochim. Biophys. Acta 455*, 353–370.
Lepke, S., Becker, A., & Passow, H. (1992) *Biochim. Biophys. Acta 1106*, 13–16.
Lindenthal, S., & Schubert, D. (1992) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6540–6544.
Low, P. S. (1978) *Biochim. Biophys. Acta 514*, 264–273.
Low, P. S. (1986) *Biochim. Biophys. Acta 864*, 145–167.
Lux, S. E., John, K. M., Kopito, R. R., & Lodish, H. F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9089–9093.
Macara, I. G., & Cantley, L. C. (1983) *Cell Membr., Methods Rev. 1*, 41–87.
Macara, I. G., Kuo, S., & Cantley, L. C. (1983) *J. Biol. Chem. 258*, 1785–1792.
Motais, R., Guizouarn, H., & Garcia-Romeu, F. (1991) *Biochim. Biophys. Acta 1075*, 169–180.
Mulzer, K., Kampmann, L., Petrasch, P., & Schubert, D. (1990) *Colloid Polym. Sci. 268*, 60–64.
Passing, R., & Schubert, D. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364*, 873–878.
Passow, H. (1986) *Rev. Physiol. Biochem. Pharmacol. 103*, 61–203.
Rao, A., Martin, P., Reithmeier, R. A. F., & Cantley, L. C. (1979) *Biochemistry 18*, 4505–4516.
Restrepo, D., Kozody, D. J., Spinellu, L. J., & Knauf, P. A. (1989) *Am. J. Physiol. 257*, C520–C527.
Restrepo, D., Cronise, B. L., Snyder, R. B., Spinelli, L. J., & Knauf, P. A. (1991) *Am. J. Physiol. 260*, C535–C544.
Salhany, J. M. (1990) *Erythrocyte Band 3 Protein*, CRC Press, Boca Raton, FL.
Salhany, J. M. (1992) *Prog. Cell Res. 2*, 191–206.
Salhany, J. M., & Swanson, J. C. (1978) *Biochemistry 17*, 3354–3362.
Salhany, J. M., & Gaines, E. D. (1981) *J. Biol. Chem. 256*, 11080–11085.
Salhany, J. M., & Rauenbuehler, P. B. (1983) *J. Biol. Chem. 258*, 245–249.
Salhany, J. M., & Cordes, K. A. (1991) *Biochem. Biophys. Res. Commun. 174*, 975–982.
Salhany, J. M., & Cordes, K. A. (1992) *Biochemistry 31*, 7301–7310.
Salhany, J. M., Cordes, K. A., & Gaines, E. D. (1980) *Biochemistry 19*, 1447–1454.
Salhany, J. M., Rauenbuehler, P. B., & Sloan, R. L. (1987) *J. Biol. Chem. 262*, 15965–15973.
Salhany, J. M., Sloan, R. L., & Cordes, K. A. (1990) *J. Biol. Chem. 265*, 17688–17693.
Salhany, J. M., Sloan, R. L., & Cordes, K. A. (1991) *Biochemistry 30*, 4097–4104.
Sami, M., Malik, S., & Watts, A. (1992) *Biochim. Biophys. Acta 1105*, 148–154.
Schofield, A. E., Reardon, D. M., & Tanner, M. J. A. (1992a) *Nature 355*, 836–838.
Schofield, A. E., Tanner, M. J. A., Pinder, J. C., Clough, B., Bayley, P. M., Nash, G. B., Dluzewski, A. R., Reardon, D. M., Cox, T. M., Wilson, R. J. M., & Gratzer, W. B. (1992b) *J. Mol. Biol. 223*, 949–958.
Schuck, P., & Schubert, D. (1991) *FEBS Lett. 293*, 81–84.
Staros, J. V. (1982) *Biochemistry 21*, 3950–3955.
Staros, J. V., & Anjaneyulu, P. S. R. (1989) *Methods Enzymol. 172*, 609–628.
Tanner, M., Martin, P., & High, S. (1988) *Biochem. J. 256*, 703–712.
Wood, P. G., Muller, H., Sovak, M., & Passow, H. (1992) *J. Membr. Biol. 127*, 139–148.
Yamaguchi, T., & Kimoto, E. (1992) *Biochemistry 31*, 1968–1973.
Yang, J., Wu, C., & Martinez, H. (1986) *Methods Enzymol. 130*, 208–269.
Yannoukakos, D., Meyer, H. E., Vasseur, C., Draincourt, C., Wajcman, H., & Bursaux, E. (1991) *Biochim. Biophys. Acta 1066*, 70–76.
Zhang, Z., & Solomon, A. K. (1992) *Biochim. Biophys. Acta 1106*, 31–39.