Characterization of the stilbenedisulphonate binding site on band 3 Memphis variant II (Pro-854 → Leu)

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Band 3 Memphis variant II is a mutant anion-exchange protein associated with the Diego a⁺ blood group antigen. There are two mutations in this transporter: Lys-56 → Glu within the cytoplasmic domain, and Pro-854 → Leu within the membrane-bound domain. The Pro-854 mutation, which is thought to give rise to the antigenicity, is located within the C-terminal subdomain of the membrane-bound domain. Yet, there is an apparent enhancement in the rate of covalent binding of H₂DIDS (4,4'-diisothiocyanatodihydro-2,2'-stilbenedisulphonate) to 'lysine A' (Lys-539) in the N-terminal subdomain, suggesting widespread conformational changes. In this report, we have used various kinetic assays which differentiate between conformational changes in the two subdomains, to characterize the stilbenedisulphonate site on band 3 Memphis variant II. We have found

a significantly higher H₂DIDS (a C-terminal-sensitive inhibitor) affinity for band 3 Memphis variant II, due to a lower H₂DIDS 'off' rate constant, but no difference was found between mutant and control when DBDS (4,4'-dibenzamido-2,2'-stilbenedisulphonate) (a C-terminal-insensitive inhibitor) 'off' rates were measured. Furthermore, there were no differences in the rates of covalent binding to lysine A, for either DIDS (4,4'-diisothiocyanato-2,2'-stilbenedisulphonate) or H₂DIDS. However, the rate of covalent intrasubunit cross-linking of Lys-539 and Lys-851 by H₂DIDS was abnormally low for band 3 Memphis variant II. These results suggest that the Pro-854 → Leu mutation causes a localized conformational change in the C-terminal subdomain of band 3.

INTRODUCTION

Band 3 Memphis was discovered on the basis of its abnormal migration on SDS/PAGE [1]. This transporter was shown to have a point mutation at position 56 where glutamate is substituted for lysine [2,3]. Hsu and Morrison [4] showed that there are at least two variants of band 3 Memphis which can be distinguished by differences in H₂DIDS (4,4'-diisothiocyanatodihydro-2,2'-stilbenedisulphonate) covalent binding to the membrane-bound domain of band 3. Band 3 Memphis variant I had normal H₂DIDS binding, whereas band 3 Memphis variant II had an apparently increased rate of H₂DIDS covalent binding. Spring et al. [5] then discovered that band 3 Memphis variant II was associated with the Diego a⁺ blood group antigen, and Bruce et al. [6] showed that this antigen was associated with a second point mutation at position 854 (Pro → Leu) in the membrane-bound domain of band 3.

An important question raised by the work of Bruce et al. [6] concerns the specific location of the Diego a⁺ antigenic epitope within the membrane-bound domain of band 3 Memphis variant II. The authors noted that Pro-854 was located in what is believed to be a small extracellular loop [7] between putative transmembrane helices 13 and 14 within the C-terminal subdomain of the membrane-bound domain of band 3. Yet, the characteristic functional change associated with this mutation was an apparently higher rate of H₂DIDS covalent binding to 'lysine A' (Lys-539) which is located within the N-terminal subdomain [7]. The genetic work would suggest that the antigenic

epitope should be located in the C-terminal subdomain which contains the Pro-854 → Leu mutation. On the other hand, the functional studies suggest that the antigenic epitope may arise from a widespread conformational change which is transmitted to Lys-539 within the N-terminal subdomain [6].

To discriminate between these two possibilities (localized versus widespread conformational changes), it is necessary to have assays of the structure of band 3 which differentiate between conformational changes in the two well-defined subdomains (N-terminal and C-terminal) of the membrane-bound domain of band 3 [8,9]. We have developed a series of high-resolution methods to study the kinetics of both reversible and covalent binding of stilbenedisulphonates to band 3 [10–15]. Our work with band 3 HT (Pro-868 → Leu) [13] has demonstrated that the H₂DIDS 'off' rate constant is uniquely sensitive to conformational changes within the C-terminal subdomain of band 3, while the DBDS (4,4'-dibenzamido-2,2'-stilbenedisulphonate) 'off' rate constant is not. Similarly, H₂DIDS covalent binding to lysine A was slowed by about 10-fold, while covalent binding of DIDS (4,4'-diisothiocyanato-2,2'-stilbenedisulphonate) was unaffected by the Pro-868 → Leu mutation. Such differential sensitivity is believed to arise from the ability of H₂DIDS to assume a configuration within the stilbenedisulphonate binding pocket which is distinct from the configurations assumed by the more rigid DBDS or DIDS molecules [13].

In the present study, we have applied these and two new methods to the study of band 3 Memphis variant II in an attempt to see if the mutation at residue 854 produces a localized or

Abbreviations used: H₂DIDS, 4,4'-diisothiocyanatodihydro-2,2'-stilbenedisulphonate; DIDS, 4,4'-diisothiocyanato-2,2'-stilbenedisulphonate; DBDS, 4,4'-dibenzamido-2,2'-stilbenedisulphonate; PBS, 5 mM sodium phosphate, pH 8.0, plus 150 mM NaCl; 5P8, 5 mM sodium phosphate, pH 8.0; IOV, inside-out vesicle; C₁₂E₈, poly(oxyethylene-8-lauryl ether).

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widespread conformational change in the integral domain of the transporter.

MATERIALS AND METHODS

Materials

DIDS was purchased from Sigma (St. Louis, MO, U.S.A.) and its concentration was determined spectrophotometrically using an absorption coefficient of $36000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 340 nm [16]. H_2DIDS was purchased from Molecular Probes and its concentration was also determined spectrophotometrically using an absorption coefficient of $39000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 283 nm [17]. The sources of all other materials used in this study have been described before [10,11,14]. Fresh underweight units of whole blood were donated by the Omaha Chapter of the American Red Cross. A unit of homozygous Diego $\text{Di}(\text{a}^+\text{b}^-)$ blood was generously provided by Dr. Yasuto Okubo of the Osaka Red Cross Blood Center and sent to Omaha on ice by overnight delivery.

Preparations

Control and Diego (a^+b^-) blood samples were washed in PBS (5 mM sodium phosphate, pH 8.0, plus 150 mM NaCl) and unsealed ghosts were prepared as described previously [18]. Membranes were stripped of residual haemoglobin and glyceraldehyde-3-phosphate dehydrogenase by washing twice in PBS and then three times in 5P8 (5 mM sodium phosphate, pH 8.0). Inside-out vesicles (IOVs) were prepared as described in [19]. The amount of band 3 present in ghosts and IOVs was determined by fluorescence titration with DIDS [14,20]. IOVs were titrated in the presence and absence of poly(oxyethylene-8-lauryl ether) (C_{12}E_8) in order to expose all of the band 3 to the inhibitor.

Stilbenedisulphonate release kinetics

H_2DIDS release rates were measured by premixing H_2DIDS with ghosts and then immediately (< 1 min) mixing that sample with excess DIDS. This reaction was monitored by observing the change in protein fluorescence (excitation 280 nm, with fluorescence emission measured through a 315 nm short-wavelength-cutoff filter) subsequent to conversion from H_2DIDS /band 3 into DIDS/band 3 reversible complexes [10]. DBDS release rates were also measured by rapidly mixing saturated DBDS/band 3 complexes with excess DIDS. Here, the change in fluorescence between free and bound DBDS was monitored by exciting the sample at 335 nm and observing fluorescence through a 445 nm short-wavelength-cutoff filter. The experimental details and theory of these replacement reactions have been described in two previous reports [10,11].

Kinetics of H_2DIDS reversible binding

The kinetics of H_2DIDS binding to unsealed ghosts was measured as described previously [15], by observing protein fluorescence quenching of band 3 subsequent to reversible binding of H_2DIDS [10]. The time course of the reaction is biphasic, with the fast-phase rate constant showing classical second-order kinetics, and the slow-phase rate constant showing saturation behaviour [15]. These two phases were analysed according to a two-step [21] uncoupled [15] binding mechanism:

where S is stilbenedisulphonate, B is the band 3 subunit, and SB

and SB^* are two types of bound complexes. We have determined that

$$K_1 = k_{-1}/k_1 \quad (2)$$

and

$$K_2 = k_{-2}/k_2 \quad (3)$$

and that

$$K_d = (K_1 \cdot K_2)/(1 + K_2) \quad (4)$$

The H_2DIDS dependence of the fast-phase rate constant was analysed by fitting data using the equation

$$k_{\text{fast}} = k_1[\text{S}] + k_{-1} \quad (5)$$

For the slow phase the following equation was used

$$k_{\text{slow}} = \{k_2[\text{S}]/(K_1 + [\text{S}])\} + k_{-2} \quad (6)$$

The values of k_{-2} were taken from the replacement reaction, since the values for H_2DIDS are too small to determine accurately in forward-flow kinetic binding studies. Justification for this approach was presented elsewhere by comparing the larger k_{-2} values for DBDS using both forward-flow binding and replacement reactions [12].

Kinetics of DIDS covalent binding to band 3

DIDS covalent binding was monitored by observing the increase in fluorescence after forming a 1:1 DIDS/band 3 reversible complex (excitation 360 nm, emission 450 nm), as described previously [14]. The reactions were performed using unsealed ghosts in 10 mM sodium phosphate, pH 7.4, plus 150 mM NaCl at 25 °C.

Kinetics of H_2DIDS covalent binding

In general, the H_2DIDS covalent binding reaction was studied using erythrocyte ghosts which were solubilized in 5 mM sodium phosphate, 150 mM NaCl, pH 7.4, plus 0.5% C_{12}E_8 at 25 °C. The sample was then centrifuged at 8800 *g* to remove particulates, and equilibrated at 37 °C. Aliquots (1 ml) of solubilized preparation were placed in both sample and reference cuvettes of an Hitachi 100-60 spectrophotometer. The reaction with H_2DIDS was initiated by adding a small aliquot of buffer to the reference cuvette and the same volume of an H_2DIDS stock to the sample cuvette. A more detailed description of the method used in measuring the rate of H_2DIDS covalent binding is given in the Results section below.

Kinetics of H_2DIDS intrasubunit cross-linking

Control and Diego (a^+b^-) erythrocytes were washed in PBS and treated with chymotrypsin exactly as described by Schopfer and Salhany [14]. Cells were washed in PBS and mixed with H_2DIDS to give a final volume of 200 ml and a concentration of $12 \mu\text{M}$ H_2DIDS and 5% haematocrit. The samples were incubated at 37 °C. One aliquot was incubated without H_2DIDS . At various times, 20 ml of the erythrocyte suspension were removed and mixed with sodium azide to give a final azide concentration of 100 mM. We have shown in quantitative kinetic experiments that this azide concentration reacts with the isothiocyano groups of reversibly or monovalently bound H_2DIDS molecules in less than 1 min [14]. After samples were collected at all time points, membranes were isolated as described above. These membranes were stripped of peripheral proteins and solubilized and electrophoresed as described [14]. Two prominent bands were observed, one is the 60 kDa fragment, and the other is the 90 kDa fragment [22]. We quantitatively analysed these two bands by scanning the

Coomassie Blue-stained gels using an on-line gel scanning system described in a previous report [23].

RESULTS

H₂DIDS release and binding kinetics

Figure 1(A) shows the time courses for typical H₂DIDS/DIDS replacement reactions for wild-type band 3 and band 3 Memphis

Figure 1 Time courses of stilbenedisulphonate replacement reactions for wild-type band 3 (○) and for band 3 Memphis variant II (●)

(A) The H₂DIDS/DIDS replacement reaction for wild-type band 3 and for band 3 Memphis variant II unsealed ghosts were performed as described in the Materials and methods section of the text, by mixing ghosts containing 2.2 μ M band 3 and 6 μ M H₂DIDS, added just prior to (< 1 min) initiating the reaction, with 13 μ M DIDS. The temperature was 25 °C, and the buffer was 5 mM sodium phosphate, pH 7.4, plus 150 mM NaCl. Fluorescence was monitored through a 315 nm short-wavelength-cutoff filter, exciting the sample at 280 nm. ΔF is defined as the difference between the final fluorescence and the fluorescence at any time. The term ΔF_{total} is defined as the difference between the final fluorescence and the fluorescence at time $t = 0$. 1000 data points were collected and the time courses were fitted to a single exponential function: $\Delta F/\Delta F_{\text{total}} = \exp(-kt)$. Ghosts containing wild-type band 3 (○) gave a value of $k = 0.26 \pm 0.02 \text{ s}^{-1}$. Diego (a⁺b⁻) ghosts containing band 3 Memphis variant II gave a value of $k = 0.13 \pm 0.01 \text{ s}^{-1}$. (B) The DBDS/DIDS replacement reactions for wild-type band 3 and for band 3 Memphis variant II were performed by premixing unsealed ghosts with DBDS to yield concentrations of 2 μ M band 3 and 6 μ M DBDS in 150 mM NaCl, 5 mM sodium phosphate, pH 7.4. Temperature = 25 °C. The sample was then mixed in the stopped-flow with 13 μ M DIDS prepared in the same buffer. Fluorescence was monitored by exciting at 335 nm, and monitoring the emission through a 415 nm short-wavelength-cutoff filter. Control ghosts containing wild-type band 3 gave a single exponential time course with a rate constant of $1.05 \pm 0.01 \text{ s}^{-1}$. Diego (a⁺b⁻) ghosts containing band 3 Memphis variant II showed indistinguishable kinetics with a rate constant of $1.01 \pm 0.02 \text{ s}^{-1}$.

variant II. Both time courses were monophasic and exponential, which is consistent with the fact that each sample contains a homogeneous population of band 3 subunits. The rate constant for band 3 Memphis variant II was about 2-fold lower ($0.13 \pm 0.01 \text{ s}^{-1}$) than the rate constant for wild-type band 3 ($0.26 \pm 0.02 \text{ s}^{-1}$), all measured at 25 °C. Such results were obtained in three out of three independent preparations from the same units of blood. Furthermore, it is important to note that the wild-type rate constant of 0.26 s^{-1} is in excellent agreement with values reported in three previous independent studies using unsealed ghosts where values of $0.24 \pm 0.01 \text{ s}^{-1}$ [13] and $0.31 \pm 0.05 \text{ s}^{-1}$ [24] were obtained for measurements at 25 °C, and where a value of $0.23 \pm 0.02 \text{ s}^{-1}$ was obtained at 23 °C [11]. The value of the H₂DIDS 'off' rate constant for solubilized band 3 [10] is considerably higher than the value found for membrane-bound band 3 (see [12] for further details). The observed rate constant is taken as being equivalent to the value of k_{-2} in eqn. (1) [12].

H₂DIDS binding kinetics showed typical biphasic time courses for both wild-type band 3 and band 3 Memphis variant II (results not shown, but see Figure 2 of the paper by Salhany [15] for an example of the data). The various kinetic constants were determined by studying the reaction at several concentrations of H₂DIDS as described previously [15]. There was no difference in the kinetics between wild-type band 3 and band 3 Memphis variant II, and the kinetic constants of eqn. (1) were the same as reported previously [15]: $k_1 = 2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_{-1} = 0.5 \text{ s}^{-1}$; $k_2 = 1.8 \text{ s}^{-1}$. With these constants and the values of k_{-2} reported above, we calculated the overall K_d using eqns. (2)–(4). The K_d for wild-type band 3 was $32 \times 10^{-9} \text{ M}$, while the K_d for band 3 Memphis variant II was $17 \times 10^{-9} \text{ M}$.

DBDS release kinetics

In studies with band 3 HT (Pro-868 → Leu), we have shown that H₂DIDS is uniquely sensitive to conformational changes in the C-terminal subdomain, while the DBDS release rate was insensitive to the mutation in that subdomain. The results in Figure 1(B) illustrate that the same principle of differential sensitivity between H₂DIDS and the other stilbenedisulphonates also holds for band 3 Memphis variant II. No significant difference was observed for the kinetics of DBDS release.

Kinetics of covalent binding of DIDS

The DIDS adduct formation rate was followed by observing the increase in DIDS fluorescence after forming the DIDS/band 3 reversible complex (see Figure 1 of [14]). The reactions were all monophasic and exponential, and there was no significant difference in the observed rate constant between wild-type band 3 ($0.28 \pm 0.044 \text{ min}^{-1}$, $n = 4$), and band 3 Memphis variant II ($0.35 \pm 0.043 \text{ min}^{-1}$, $n = 6$), suggesting that the conformation of the protein around helix 5 is the same in each case. Although the kinetic constants were not significantly different, we did notice that the quantum yield for the fluorescence change associated with adduct formation was smaller in band 3 Memphis variant II than in wild-type band 3 ($1.21 = \text{wild type/mutant}$). This difference was not associated with a shift in either the excitation or the emission spectra.

Absorbance assay for H₂DIDS/band 3 covalent adduct formation

In order to determine if the conformation of Lys-539 had changed in the Memphis variant II, we chose to determine the rate of H₂DIDS covalent binding. In previous studies, it was possible to obtain quantitative values for this rate from a gel

Figure 2 Difference spectral and kinetic changes associated with the formation of the monovalent $H_2DIDS/Lys-539$ covalent adduct on band 3

(A) Time-dependent changes in the absorbance spectrum of H_2DIDS upon reaction with band 3 at 30 °C. The sample and reference cuvettes were both loaded with 2.5 ml of IOVs solubilized in 5 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl containing 0.5% $C_{12}E_8$. Absorbance at 280 nm was 0.8. The band 3 concentration was 2.2 μM in each cuvette. The reaction was initiated by adding 70 μl of buffer to the reference and 70 μl of 78.5 μM H_2DIDS to the sample cuvettes, giving 2.2 μM H_2DIDS in the reaction mixture. The solid line in (A) is the spectrum seen immediately after mixing H_2DIDS with solubilized IOVs. The dashed line is the spectrum of the same sample after 90 min has elapsed. (B) Time course for the reaction of H_2DIDS with band 3 at 37 °C. An aliquot (3 ml) of unsealed ghosts containing 3.4 μM band 3, was solubilized in 10 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl containing 0.5% $C_{12}E_8$. The solution was clarified by centrifugation at 8800 g for 15 min and then equilibrated at 37 °C for 10 min. Aliquots (1 ml) of the solubilized preparation were placed in both sample and reference cuvettes. The reaction was initiated by adding 18.8 μl of buffer to the reference and 18.8 μl of 160 μM H_2DIDS to the sample cuvettes, giving 3.0 μM H_2DIDS in the reaction mixture. The progress of the reaction was followed at 245 nm.

electrophoresis assay [25], or by using the $H_2DIDS/DIDS$ replacement reaction [13]. For our purposes, however, it was necessary to develop a method which could measure that rate directly. In the course of the present study, we have discovered that there is a significant change in the absorbance spectrum of H_2DIDS upon formation of the covalent adduct with band 3. This is illustrated in Figure 2(A), where 2.2 μM band 3 from solubilized IOVs was reacted with 2.2 μM H_2DIDS . Solubilized IOVs were used since the background absorbance was too high for accurate measurement when unsolubilized IOVs were used. Since the non-covalent dissociation constant for H_2DIDS binding to band 3 is about 0.1 μM under these specific solution conditions [10], all of the H_2DIDS should be expected to bind to band 3 leaving no excess H_2DIDS for non-specific reaction with other components.

Immediately after mixing H_2DIDS with the solubilized IOVs, the spectrum showed two closely spaced peaks, at 274 and

287 nm, which are characteristic of H_2DIDS (Figure 2A, solid line) [26]. Subsequent spectra, at later times, revealed an isosbestic perturbation of the starting spectrum into one which had a single, broad peak around 275 nm (Figure 2A, broken line). Isosbestic points were at 294, 285 and 274 nm. A substantial increase in absorbance was seen at both 245 and 310 nm.

We have used the absorbance change at 245 nm to follow the kinetics of H_2DIDS covalent binding to band 3. We have applied this assay to solubilized erythrocyte ghosts, solubilized IOVs and isolated band 3 protein, obtaining equivalent rates of reaction for each preparation. The most demanding substrate has been erythrocyte ghosts, since the turbidity of the solubilized material resulted in an unacceptably large background absorbance at 245 nm. To resolve this problem, we centrifuged the solubilized ghost preparation, pelleting the insoluble cytoskeleton. An example of one protocol used with ghosts is given in the legend to Figure 2(B). If an excess of H_2DIDS was mixed with the band 3 preparation, a slower secondary reaction occurred. This secondary reaction was even present when isolated band 3 was used (results not shown). We believe that this slower reaction represents non-specific covalent binding of H_2DIDS to band 3 lysines. Our assay was therefore restricted to conditions where H_2DIDS concentration did not exceed the concentration of band 3. Since variable amounts of band 3 were precipitated along with the cytoskeleton pellet during the centrifugation/clarification step, the amount of H_2DIDS which yielded a first-order time course had to be empirically determined. Once the reaction conditions were established, a reproducible, first-order absorbance change of about 0.03 could be obtained with good signal-to-noise (see Figure 2B). Background absorbance at 245 nm varied from 0.4 to 0.8 depending on the preparation, which was suppressed by utilizing matching protein solutions in both the sample and reference cuvettes.

Comparison of the H_2DIDS reaction kinetics for wild-type band 3 and band 3 Memphis variant II were made using erythrocyte ghosts at pH 7.4 and 37 °C. Solubilization and centrifugation was performed as described in the legend to Figure 2(B). The ratio of starting band 3 concentration to final H_2DIDS concentration was varied from 1.0 to 1.9 using 3–4 μM band 3 in each reaction. All time courses were first-order. The average value of the observed rate constant for wild-type band 3, from four such experiments, was $0.23 \pm 0.04 \text{ min}^{-1}$. This value is in reasonably good agreement with the rate constant determined by Kampmann et al. [25] for the rate of H_2DIDS covalent adduct formation at 37 °C using erythrocytes at pH 7.4 (0.1 min^{-1}). Band 3 Memphis variant II gave essentially the same rate constant as wild-type band 3 ($0.29 \pm 0.01 \text{ min}^{-1}$, $n = 5$).

Kinetics of H_2DIDS intrasubunit cross-linking

To measure the kinetics of H_2DIDS intrasubunit cross-linking, it is necessary to have a rapid method to stop the reaction. Here, we used the newly discovered finding that azide reacts rapidly with the isothiocyano groups of either reversibly or monovalently bound H_2DIDS molecules in complex with band 3 [14]. The exact procedure is described in the Materials and methods section and the results are shown in Figure 3. The gels associated with these experiments are not shown since a related gel was published previously (see Figure 7 of [14]). It is apparent that the rate of appearance of the cross-linked 90 kDa gel band (Figure 3A) correlates with the rate of disappearance of the 60 kDa band on the gel (Figure 3B), as is now well established [22,25]. A semi-logarithmic plot of the disappearance of the 60 kDa gel band is shown as an insert to Figure 3(B). We find, in agreement with Kampmann et al. [25], that the kinetics are

Figure 3 Time course of H_2DIDS intrasubunit cross-linking of chymotrypsin-treated wild-type band 3 (○) and band 3 Memphis variant II (●)

Experimental details are given in the Materials and methods section of the text. The temperature was 37 °C. (A) Increase in cross-linked 90 kDa fragment with time, and (B) corresponding decrease in 60 kDa uncross-linked fragment. y -axes represent the percentage of each fragment per total 90 kDa plus 60 kDa staining intensity. The insert shows a semi-logarithmic plot of the disappearance of the 60 kDa fragment versus time. Wild-type band 3 gave an apparent rate constant of 0.006 min^{-1} , while band 3 Memphis variant II gave an apparent rate constant of 0.003 min^{-1} .

monophasic and exponential within the experimental uncertainties of this assay. We obtained a rate constant for the control of 0.006 min^{-1} for this cross-linking reaction, which was conducted at pH 8 and 37 °C using intact erythrocytes. This value is in excellent agreement with the same rate constant reported by Kampmann et al. [25] (0.0063 min^{-1}) under essentially the same experimental conditions, but using acid pH instead of azide to quench the cross-linking reaction. The cross-linking reaction for band 3 Memphis variant II was significantly slower (0.003 min^{-1}), for otherwise identical experimental conditions. Such significant differences were obtained in two out of two independent gel electrophoresis studies using the same units of control and Diego (a^+b^-) blood.

DISCUSSION

The results of this study suggest that the conformation of band 3 in the region of Lys-851 is abnormal in band 3 Memphis variant II, while the conformation of Lys-539 (lysine A) remains unperturbed by the point mutation at position 854 (Pro → Leu). Strong support for the conclusion that the conformation of lysine A is not altered comes from our direct measurements of the apparent rate constants for monovalent H_2DIDS and $DIDS$

covalent adduct formation, which showed no difference between wild-type and mutant band 3. The value of the rate constant for monovalent H_2DIDS adduct formation was in reasonable agreement with the value determined by Kampmann et al. [25] measured at 37 °C, pH 7.4. Such agreement supports our contention that the spectral change in Figure 2(A) is associated with formation of the H_2DIDS /Lys-539 covalent bond.

If the rate of H_2DIDS monovalent adduct formation is not altered, how can one explain that two laboratories have shown the presence of more tritiated H_2DIDS covalently bound to band 3 in gel electrophoresis assays using band 3 Memphis variant II? The apparent answer to this question comes from our measurements of the kinetics of H_2DIDS reversible binding and release, where we found that the 'off' rate constant was lowered significantly for band 3 Memphis variant II (Figure 1A). The lower 'off' rate results in a higher H_2DIDS affinity, since the 'on' rate constants were not altered by the mutation. We believe that because of the extraordinarily high affinity of H_2DIDS for band 3 in intact membranes [15], washing procedures to remove unreacted H_2DIDS may have been incomplete. Since band 3 Memphis variant II has a higher H_2DIDS affinity than wild-type band 3, such incomplete removal would be exaggerated for this mutant, and this difference could result in the appearance of more covalently bound H_2DIDS in the gel electrophoresis assay [4,6].

If the conformation of lysine A has not changed with the mutation of Pro-854 → Leu, then the observation of altered H_2DIDS reversible binding may be attributed to a localized conformational change in the vicinity of the mutation at residue 854, and also to the unique ability of H_2DIDS to sense conformational changes in the C-terminal subdomain of band 3. This view is supported by the results in Figure 1(B), where we see that the DBDS 'off' rate constant was insensitive to the conformational change which was sensed by H_2DIDS . A similar differential sensitivity was observed between H_2DIDS and DBDS in their reactions with band 3 HT, which also has a point mutation in the C-terminal subdomain (Pro-868 → Leu) [13]. Furthermore, the results in Figure 3 offer strong support for the localized nature of the conformational change in band 3 Memphis variant II, by illustrating that the rate of H_2DIDS intramonomeric cross-linking from Lys-539 to Lys-851 was lowered significantly. Since Lys-851 is only three residues away from the site of the mutation at residue 854, we suggest that the conformational change in band 3 Memphis variant II may be localized to a putative small extracellular loop between transmembrane helices 13 and 14 [7,27].

Our suggestion that the conformational change in band 3 Memphis variant II is localized to the vicinity of band 3 containing the Pro-854 → Leu mutation implies that the epitope for the Diego a^+ antigen is somehow revealed by such a localized conformational change. This may occur through an intramonomeric conformational change, or through subunit interaction effects. It is worth noting that recent evidence suggests that conformational changes in the C-terminal subdomain are also sensitive to subunit interaction effects [24]. Thus, conversion of Pro-854 into Leu could perturb a region of subunit contact within the band 3 dimer, and thereby expose an antigenic epitope at the interface between subunits. Independently of the exact mechanism for antigenicity in band 3, it seems clear from our studies that the conformational change produced in band 3 Memphis variant II is not transmitted to lysine A, but rather is localized to the C-terminal subdomain of the transporter.

We thank Dr. David Anstee for arranging delivery of the Diego (a^+b^-) blood sample and Dr. Y. Okubo for generously providing a unit of that blood. We thank Karen

Cordes for preparing the illustrations. This work was supported by the Medical Research Service of the Veterans Administration.

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Received 10 January 1996/18 March 1996; accepted 21 March 1996