Cysteine-directed cross-linking localizes regions of the human erythrocyte anion-exchange protein (AE1) relative to the dimeric interface

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The human erythrocyte anion-exchanger isoform 1 (AE1) is a dimeric membrane protein that exchanges chloride for bicarbonate across the erythrocyte plasma membrane. Crystallographic studies suggest that the transmembrane anion channel lies at the interface between the two monomers, whereas kinetic analysis provides evidence that each monomer contains an anion channel. We have studied the structure–function relationship of residues at the dimeric interface of AE1 by cysteine-directed cross-linking. Single cysteine mutations were introduced in 16 positions of putative loop regions throughout AE1. The ability of these residues to be chemically cross-linked to their partner within the dimeric protein complex was assessed by mobility of the protein on immunoblots. Introduced cysteine residues in extracellular loops (ECs) 1–4 and intracellular loop 1 formed

disulphide cross-linked dimers. Treatment with homobifunctional maleimide cross-linkers of different lengths (6, 10 and 16 Å; 1 Å ≡ 0.1 nm) also cross-linked AE1 with introduced cysteines in EC5 and close to the start of transmembrane segment (TM) 1. On the basis of these data, tentative positional constraints of TMs 1–4 and 6 relative to the dimeric interface are proposed. Neither disulphide- nor maleimide-mediated cross-linking perturbed AE1 transport function, suggesting that loop–loop contacts across the dimeric interface are not primarily responsible for allosteric interactions between monomers within the functional dimeric protein complex.

Key words: Band 3, helical packing, membrane.

INTRODUCTION

The anion-exchanger (AE) family of proteins mediates electro-neutral exchange of chloride for bicarbonate in most mammalian membranes. Human erythrocyte AE isoform 1 (AE1) is the best-characterized member [1,2]. Although the kinetics of anion exchange have been studied extensively for AE1 [3,4], relating these kinetic data to a mechanistic model for protein function requires structural information describing the anion-translocation channel. Considerable data are available concerning the topology of the AE1 membrane domain. The topology of the first eight transmembrane segments (TMs; these are numbered according to the folding model presented in Figure 1) is well defined [5–7], whereas controversial topology models containing non-helical membrane-spanning segments have been proposed for the C-terminal region of the TM domain [6,8]. Mutagenesis studies indicate that TM8 of AE1 may form part of the anion-translocation channel [9]. Histidine residues 703 [intracellular loop (IC) preceding TM9], 734 [extracellular loop (EC) 5, preceding TM10], 819 (region with undetermined topology) and 834 (TM12) may also be required for anion translocation [10,11]. Complementation studies suggest further that TMs 6 and 7 are not required for anion transport [12].

Although it is important to determine the topology of polytopic membrane proteins and to identify key residues required for function, an understanding of the structure–function relationship requires insight into the packing organization of TMs. Two-

dimensional crystallography revealed an AE1 dimer 120 Å long, where a central depression at the dimeric interface has been interpreted as a possible site of anion translocation [13] (1 Å = 0.1 nm). The resolution of the structure (20 Å) precludes assignment of the positions of TMs. Conversely, kinetic studies indicate that each monomer within the dimeric protein complex contains an anion-translocation channel [14]. It is also possible that a single channel at the surface of the protein bifurcates within the protein structure to form separate channels within each monomer. Whether or not the anion channel is at the dimeric interface, a large body of evidence indicates that monomer–monomer interactions influence both anion transport and inhibition by ligands [15–18]. Identification of regions of the protein at the dimeric interface is therefore critical to the understanding of how monomer–monomer interactions affect the anion-transport process.

In the present study, we performed a protein-wide analysis of AE1 loop regions to identify which loops are close to the dimeric interface. Specifically, we introduced single cysteines into putative loop regions of AE1C[−], a functional cysteineless form of AE1 [19]. The proximity of loop regions to the dimeric interface was revealed by cysteine-directed cross-linking, and the cross-linking event was correlated with anion-exchange function. On the basis of the cross-linking data we have provided structural constraints for the positions of AE1 TMs 1–4 and 6 relative to the dimeric interface. We also demonstrate that cross-linking these loop regions does not affect anion-exchange function.

Abbreviations used: AE, anion exchanger; AE1, AE isoform 1; AE1C[−], cysteineless form of AE1; BCECF-AM, 2',7'-bis-(2-carboxyethyl)-(5 and 6)-carboxyfluorescein acetoxymethyl ester; biotin maleimide, 3-(N-maleimidylpropionyl)biocytin; BMH, bismaleimido-hexane; C₁₂E₈, octaethylene glycol monododecyl ether; CuP, Cu(II)-(1,10-phenanthroline)₃; DTT, dithiothreitol; EC, extracellular loop; IC, intracellular loop; o-PDM, N,N'-o-phenylenedimaleimide; p-PDM, N,N'-p-phenylenedimaleimide; TM, transmembrane segment.

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Figure 1 Topology model of the membrane domain of the human erythrocyte AE1

Cysteines were introduced into putative loop regions at the black circles. The topology model is based on a wide range of information, including proteolysis data and introduced cysteine-accessibility data [5,6,8,24,37,38].

EXPERIMENTAL

Materials

ECL chemiluminescence reagent, horseradish peroxidase conjugated to sheep anti-mouse IgG and Hyperfilm were from Amersham Pharmacia Biotech. *Pwo* polymerase and restriction endonucleases were from Roche Molecular Biochemicals. TSK G4000SW_{XL} HPLC size-exclusion column was from TosoHaas. Octaethylene glycol monodecyl ether ($C_{12}E_8$) was from Nikkol Chemical. *N,N'*-*o*-Phenylenedimaleimide (*o*-PDM), *N,N'*-*p*-phenylenedimaleimide (*p*-PDM) and 1,10-phenanthroline were from Sigma. Bismaleimidoethane (BMH) was from Pierce. PVDF was from Millipore. 2',7'-Bis-(2-carboxyethyl)-(5 and 6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and biotin maleimide [3-(*N*-maleimidylpropionyl)biocytin] were from Molecular Probes. The monoclonal antibody IVF-12 was a kind gift from Dr M. Jennings (Department of Physiology and Biophysics, University of Arkansas, Little Rock, AR, U.S.A.).

Construction of mutant anion exchangers

A human AE1 cDNA construct in which all five endogenous cysteine codons were mutated to serine codons was previously constructed in the eukaryotic expression vector pRBG4 [20] and called AE1C⁻. Individual cysteine codons were introduced into AE1C⁻ using a PCR megaprimer mutagenesis strategy and verified by sequencing [21].

Protein expression and chemical labelling

Anion exchangers were expressed by transient transfection of HEK-293 cells by calcium phosphate precipitation, as described previously [5]. Post-transfection (48 h) cells were harvested by centrifugation (228 *g*, 5 min), washed once with 140 mM NaCl/10 mM Tris, pH 7.4, lysed by Dounce homogenization on ice in

8% (w/v) sucrose/100 μ M PMSF/10 mM Tris, pH 7.4, and cell debris removed by low-speed centrifugation (4000 *g*, 5 min, 4 °C). Membranes were collected by centrifugation (35000 *g*, 5 min, 4 °C) and resuspended in 150 mM NaCl/20 mM NaH₂PO₄, pH 7.0.

Disulphide cross-linking was performed at 20 °C for 15 min by addition of 100 μ M Cu(II)-(1,10-phenanthroline)₃ (CuP) to membranes (5 mg/ml total protein) and reactions quenched by addition of EDTA to a final concentration of 10 mM. Maleimide-mediated cross-linking was achieved by incubation of membrane suspensions (5 mg/ml total protein, 20 °C, 15 min) with 100 μ M of *p*-PDM, *o*-PDM or BMH. Maleimide reagents were added from 10 mM stock solutions in DMSO. Cross-linking was terminated by addition of cysteine to a final concentration of 10 mM. Both disulphide cross-linked and maleimide cross-linked membrane samples were sedimented (30000 *g*, 2 min, 4 °C), then resuspended in 150 mM NaCl/20 mM NaH₂PO₄, pH 7.0, containing 10 mM *N*-ethylmaleimide. Membranes were solubilized by $C_{12}E_8$ (1%, w/v) and insoluble material removed by centrifugation (90000 *g*, 20 min, 4 °C). Solubilized proteins were mixed with SDS/polyacrylamide-gel loading buffer with and without 1% (v/v) 2-mercaptoethanol, and samples (5 μ g of total protein) subjected to electrophoresis in SDS/polyacrylamide gels (6%) [22]. Proteins were transferred on to PVDF membrane and AE1 was detected using the monoclonal antibody IVF-12, as described elsewhere [5]. Bands on immunoblots were quantified by densitometry using NIH Image 1.60 software. The 'cross-linking efficiency' was defined as $[1 - (1 - a)/(1 - b)]$, where *a* and *b* are the fractions of AE1 located at the dimer molecular mass under non-reducing and reducing conditions respectively. Cross-linking efficiency determined in this way corrects for the presence of small amounts of SDS-resistant AE1, migrating at the same position as the cross-linked dimer. The cross-linking efficiency from experiments with the homobifunctional cross-linkers was obtained similarly, except that *a* and *b* were the percentages

of AE1 located at the dimer molecular mass in the presence of maleimide cross-linker and DMSO respectively. Statistical analyses of mean values for cross-linking were performed using the Student's *t* test and a value of *P* < 0.05 considered significant.

The accessibility of introduced cysteines to chemical modification was assessed by their ability to react with biotin maleimide. Reaction with biotin maleimide was as described elsewhere [5], except that labelling was performed on isolated cell membranes under conditions identical with that used for cross-linking with maleimide cross-linking reagents. Biotin incorporation was detected by probing immunoblots with biotinylated streptavidin-conjugated horseradish peroxidase. The amount of protein present was determined by stripping immunoblots and reprobing with the monoclonal anti-AE1 antibody, IVF-12 [5]. Each biotinylation signal was normalized to the amount of protein for that mutant [8], i.e. (biotinylation_{mutant}/protein_{mutant}) and (biotinylation_{Y555C}/protein_{Y555C}). The relative biotinylation, defined as the normalized biotinylation_{mutant}/normalized biotinylation_{Y555C}, enabled comparison of the ability of each mutant to be labelled with biotin maleimide.

Size-exclusion HPLC

Determination of AE1 oligomeric state was achieved by size-exclusion HPLC, as described previously [19,23]. Fractions collected were analysed for AE1 content by dot-blot analysis using the monoclonal anti-AE1 antibody (IVF-12, 1:3500 dilution).

Functional analysis

HEK-293 cells grown on polylysine-coated glass coverslips (7 mm × 11 mm) were transfected as described above. To investigate the effect of CuP cross-linking on AE1 function, 48 h post-transfection cells were washed (150 mM NaCl/20 mM NaH₂PO₄, pH 7.0) and cross-linked by addition of CuP (100 μM, 15 min, 20 °C). The coverslip, with adherent cells, was then washed (serum-free Dulbecco's modified Eagle's medium), loaded with BCECF-AM (4 ml of serum-free Dulbecco's modified Eagle's medium/2 μM BCECF-AM, 15 min, 37 °C) and mounted in a fluorescence cuvette with perfusion capabilities [5]. The cuvette was perfused alternately with Ringer's buffer (5 mM glucose, 5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM MgSO₄, 2.5 mM NaH₂PO₄, 25 mM NaHCO₃ and 10 mM Hepes, pH 7.4) containing either chloride (140 mM NaCl, 'chloride buffer') or gluconate (140 mM sodium gluconate, 'chloride-free buffer') [5]. Both buffers were bubbled continuously with air containing 5% CO₂, and pH maintained by addition of NaOH (1 M). BCECF fluorescence, monitored in a Photon Technologies International RCR/Delta Scan spectrofluorometer (excitation wavelengths 440 and 502 nm and emission wavelength 520 nm), measured intracellular pH changes. Initial rates of pH change (both alkalization and acidification), determined by linear regression analysis using Felix software (Photon Technologies International), provided a measure of AE1 activity. Dithiothreitol (DTT) was then added to a final concentration of 1 mM and AE1 activity re-measured. The effect of oxidative cross-linking upon AE1 transport was calculated as: fraction transport activity after treatment = average initial rate of pH change before DTT/average initial rate of pH change after DTT.

The effect on anion translocation of treatment of AE1 mutants with o-PDM was investigated by measuring the initial rate of anion exchange, incubating with 100 μM o-PDM (15 min, 20 °C) and re-examining the ability of AE1 to mediate anion exchange. Analysis of data following cross-linking with o-PDM was similar

to that used to quantify the effect of CuP cross-linking on AE1 function. The effect of o-PDM upon AE1 transport was calculated as: fraction transport activity after treatment = average initial rate of pH change after o-PDM/average initial rate of pH change before o-PDM. The calculation for cells treated with DMSO alone was performed in a similar manner.

RESULTS

Accessibility of introduced cysteines to chemical modification

Figure 1 presents a topology model for AE1, on the basis of hydropathy, proteolysis, glycosylation and chemical-labelling analyses [24]. To identify the positions of sites relative to the dimeric interface, we introduced Cys residues into a functional, cysteineless form of AE1, AE1C⁻ [19]. Introduced Cys mutants for the cross-linking studies were selected on the basis of the following criteria: (i) place residues into extramembraneous loops and (ii) test for cross-linking only residues that can be labelled by thiol reagents. The first requirement was applied in order to scan the entire AE1 molecule. The second requirement was met by testing for cross-linking only those sites that were accessible to biotin maleimide, since the inability to form cross-links at a site is uninformative if the reason for the failure is that the site is buried or otherwise inaccessible to chemical reaction. The threshold for accessibility to biotin maleimide was set to a minimum of 0.25 times the level of the Y555C mutant. Sites meeting these criteria were identified on the basis of previously published topology studies of AE1 [5,8] and an analysis of the C-terminal region of AE1 (Q. Zhu and J. R. Casey, unpublished

Figure 2 Accessibility of introduced cysteines to chemical modification by biotin maleimide

Cells transfected with AE1 cDNA were labelled with biotin maleimide, and solubilized samples were immunoprecipitated with anti-AE1 antibody. Samples were resolved by SDS/PAGE and transblotted to PVDF membranes. The amount of biotinylation was determined by probing with streptavidin-horseradish peroxidase and development with ECL. The amount of AE1 in each sample was determined by stripping the blot and reprobing with the monoclonal anti-AE1 antibody IVF-12. The amount of biotin incorporation and AE1 in each sample was established by scanning and densitometry. The Figure quantifies biotinylation relative to the Y555C mutant ± S.D. (*n* = 5–8).

Figure 3 Intra-dimeric cross-linking of AE1 loop regions

Membranes were prepared from HEK-293 cells expressing AE1 introduced cysteine mutants. (A) Disulphide cross-linking from the monomer position (M) to the dimer position (D) was catalysed by addition of CuP. Samples were subjected to SDS/PAGE (6% gel) under either reducing (+) or non-reducing (–) conditions. (B) Membranes prepared from HEK-293 cells expressing AE1 introduced cysteine mutants were treated with DMSO solvent carrier (lanes 1), o-PDM (lanes 2), p-PDM (lanes 3) or BMH (lanes 4).

work). In the latter study each residue from the W831–Q884 region was individually mutated to cysteine and assessed for its ability to be labelled by the thiol reagent biotin maleimide [5,8]. This analysis showed that introduced Cys residues in the C-terminal region were poorly accessible to chemical reaction. However, the intracellular C-terminal tail region (N880–Q885) was accessible, consistent with the prior determination that this region forms an extramembraneous intracellular region [25]. Among the other unreported mutants, only A855C fulfilled the two criteria for cross-linking studies.

Figure 1 shows the positions of introduced cysteine mutants, used for cross-linking studies. Each of these mutants was characterized in anion-exchange assays and shown to be functional (results not shown and [5,8]). Therefore these mutants have a native conformation. Since only introduced Cys residues that displayed chemical reactivity towards thiol reagents were candidates for cross-linking, we measured the ability to label each introduced Cys mutant with biotin maleimide. Figure 2 shows reactivity towards biotin maleimide for each introduced Cys mutant, normalized to the value for the Y555C mutant. Although the efficacy of cysteine modification by biotin maleimide was different at each position, all introduced Cys mutants assessed in cross-linking assays were labelled by the probe, implying that these residues are located in extramembraneous loops [5]. Furthermore, prior incubation of the mutants with the maleimide cross-linking reagents used in this study blocked subsequent labelling with biotin maleimide (results not shown), implying that all introduced cysteine mutants examined react with these cross-linking reagents. Therefore, in subsequent cross-linking studies, absence of cross-linked product is not due to the

Figure 4 Cross-linking efficiency of AE1 introduced cysteine mutants

Membranes prepared from HEK-293 cells expressing AE1 introduced cysteine mutants were treated with either (A) CuP or (B) maleimide cross-linking reagents. (A) Disulphide-bond formation was quantified by densitometry and expressed as the mean percentage cross-linking efficiency $\pm$ S.D. ($n = 5$). (B) Maleimide cross-linking was achieved by addition of o-PDM (black bars), p-PDM (white bars) or BMH (grey bars) to membranes. The efficiency of these reagents to covalently cross-link AE1 monomers was quantified by densitometry and expressed as the mean percentage cross-linking efficiency $\pm$ S.D. ($n = 5$).

inability of the introduced cysteines to react with maleimides. AE1C[–] is slightly reactive towards biotin maleimide (0.08 ± 0.06 times as reactive as Y555C), probably because of deprotonated lysine side chains.

Cross-linking analyses

The ability to form inter-monomeric cross-links within AE1 dimers was assessed for each introduced Cys mutant. Figure 3(A) presents representative immunoblots for two introduced Cys mutants following incubation with CuP, which catalyses disulphide-bond formation [26]. Following cross-linking, samples

Figure 5 Determination of the oligomeric state of Y555C cross-linked with BMH

Membranes expressing Y555C were solubilized with 1% (v/v) $C_{12}E_8$ and applied to a TSK 4000SW_{XL} size-exclusion column eluted with 0.1% (v/v) $C_{12}E_8$, 150 mM NaCl and 20 mM NaH_2PO_4 , pH 7.0. The elution positions of $C_{12}E_8$ -solubilized Y555C (50 μ g of total protein) both before (□) and after (○) cross-linking with BMH were determined by immunoblotting. The column was calibrated with standard proteins, as described in [23]. The positions of AE1 dimers and tetramers are both indicated by arrowheads. Inset: an aliquot of the same $C_{12}E_8$ -solubilized Y555C before (lane 1) and after (lane 2) cross-linking was also analysed by SDS/PAGE and immunoblotting with anti-AE1 antibody IVF-12, confirming that cross-linking had occurred.

were resolved by SDS/PAGE under either non-reducing or reducing conditions and analysed by immunoblotting. The Figure illustrates the behaviour of T431C, which is almost completely cross-linked to dimer by CuP. By contrast D821C shows little cross-linking. Similarly, AE1C⁻ does not demonstrate cross-linking following treatment with CuP, consistent with the protein's lack of Cys residues. Figure 3(B) shows immunoblots obtained from cross-linking these representative mutants with o-PDM (lanes 2; a homobifunctional cross-linker with a 6 Å rigid spacer), p-PDM (lanes 3; a homobifunctional cross-linker with a 10 Å rigid spacer) and BMH (lanes 4; a homobifunctional cross-linker with a 16 Å flexible, alkane-chain spacer). Samples treated with solvent carrier (DMSO) acted as controls (lanes 1). Figure 3(B) shows that neither D821C nor AE1C⁻ was affected by the maleimide cross-linking compounds. However, maleimide cross-linkers were substantially effective in cross-linking T431C to a dimer.

Figure 4 quantifies cross-linking of introduced Cys mutants by CuP and maleimide cross-linking reagents. Data represent the fraction of AE1 shifted from monomer to dimer by the cross-linking reagent. For some of the mutants, notably A456C, Y486C and S731C, some SDS-resistant dimers of AE1 were observed even in the absence of cross-linking reagent. To correct for these cross-linking independent dimers, cross-linking efficiency was calculated by comparing the percentage of AE1 found at the position of the dimer relative to total AE1 under both reducing and non-reducing conditions. Statistical analysis of the results showed that T431C, G456C, Y486C, Y555C, G565C, R656C and A751C formed disulphide-cross-linked product ($P < 0.05$, when compared with AE1C⁻). Of these mutants, G456C and R656C were unable to form cross-linked product with maleimides. A402C, which was unable to form disulphide cross-linked product ($P > 0.05$, when compared with AE1C⁻),

was cross-linked by all the maleimide compounds. Additional experiments, performed with T431C, Y486C, Y555C, G565C and A751C for shorter times at lower temperatures to reduce thermal motions, were unable to distinguish the rates of cross-linking of the different-length cross-linkers (results not shown).

To be confident that the observed cross-linking events were intra-dimeric, as opposed to inter-dimeric, through collisions between two neighbouring AE1 dimers, we analysed the cross-linked products by size-exclusion HPLC [23,27]. Since the oligomeric state of AE1C⁻ expressed in HEK-293 cells is dimeric [19], intra-dimeric cross-linking will not affect the oligomeric state of the protein, whereas inter-dimeric cross-linking will produce an AE1 tetramer. Figure 5 shows size-exclusion HPLC analysis of mutant Y555C both before and after cross-linking with BMH. Before cross-linking, the protein eluted with a Stokes radius of 78 Å, previously ascribed to dimeric AE1 [23]. No tetramers or higher-molecular-mass aggregates of AE1 were detected. The elution profile before cross-linking was essentially identical with that after cross-linking, indicating that the cross-linking event was intra-dimeric. Similar results were obtained by cross-linking Y555C with CuP (results not shown). Mutants T431C and G565C were also chosen for analysis, since these are most efficiently cross-linked by both CuP and BMH (Figure 4). Both these mutants eluted at a position consistent with a dimer before and after cross-linking with either CuP or BMH, again implying that the cross-linking event is intra-dimeric (results not shown).

Functional consequences of cross-linking

Formation of inter-monomeric cross-links could inhibit transport if the cross-links spanned the catalytic site of the protein, or if large conformational rearrangements in the cross-linked region were part of the transport cycle. Anion-exchange activity of introduced Cys mutants was assessed following cross-linking in whole cells. The basis for the assay is that transfected HEK-293 cells express AE1 in their plasma membrane. Assays monitor changes of intracellular pH in cells loaded with the pH-sensitive fluorescent dye BCECF-AM [5]. To measure chloride/bicarbonate exchange activity, cells were alternately perfused with chloride-containing or chloride-free Ringer's buffer. AE1 mediates the movement of bicarbonate in the opposite direction to chloride, leading to changes in cellular pH. Prior to initiation of the assay, cells expressing the Y555C mutant were incubated with CuP (Figure 6). The effect of oxidative cross-linking was quantified by determining the initial anion-transport rates before and after reduction for 15 min with 1 mM DTT. Figure 6 shows that CuP treatment had no effect on transport activity of Y555C. The effect of maleimide cross-linking on anion-exchange function was investigated by measuring the initial rate of anion exchange, incubating with maleimide and re-measuring anion-translocation rates. The cross-linking efficiency of the three maleimides used was similar with all mutants examined. We therefore chose to study only the effect of o-PDM on anion-exchange function, because it has the shortest spacer arm (6 Å) and would therefore be expected to most strongly perturb AE1 structure/dynamics/function. We also examined the effect of treatment with DMSO, the solvent carrier for o-PDM. This transport assay is sensitive to small changes in activity, since assays performed in the presence of the anion-exchange inhibitor dinitrostilbene-2,2'-disulphonate (DNDS) showed that transport was inhibited by 7% at 0.2 μ M DNDS (the lowest level studied). Using this assay the K_i for inhibition was accurately measured as 2 μ M, which compares well with the 4 μ M value found for AE1 in erythrocytes [28].

Figure 6 Anion-exchange assay to determine the effect of CuP on AE1 function

Cells expressing mutant Y555C were grown on glass coverslips, incubated with CuP, loaded with BCECF, suspended in a fluorescence cuvette and intracellular pH monitored following alternate perfusion with chloride-containing (black bars) and chloride-free (white bars) Ringer's buffer. The y-axis shows the ratio of emission recorded at 520 nm with excitation wavelengths 502 nm and 440 nm. The ratio is directly proportional to intracellular pH. The ability of AE1 to mediate anion exchange was quantified by measurement of initial rates of alkalization and acidification, induced by changing the direction of the chloride gradient. Following incubation with chloride-free 1 mM DTT in Ringer's buffer, anion-exchange function was re-examined.

Figure 7 Effect of cross-linking on AE1 function

Anion-exchange assays were performed on HEK-293 cells transfected with mutant AE1 cDNA. Transport rates were determined from the initial rates of alkalization and acidification, as measured for coverslips of cells suspended in a fluorescence cuvette (see Figure 6). Cells were treated in one of two ways in the fluorometer cuvette. Cells were pre-treated with CuP (black bars), then alternately perfused with chloride-containing and chloride-free Ringer's buffer. Cells were then incubated for 15 min with 1 mM DTT in chloride-free Ringer's buffer, followed by alternating chloride-containing and chloride-free Ringer's buffers. Alternatively, transfected cells were subjected to the same alternating cycles of perfusion with chloride-containing and chloride-free Ringer's buffer. They were then incubated for 15 min with chloride-free Ringer's buffer containing DMSO solvent carrier (1%, v/v; white bars) or Ringer's medium containing DMSO (1%, v/v) and 100 μ M o-PDM (grey bars). The fraction of transport activity after treatment quantifies the relative anion-exchange rate following treatment with DMSO or o-PDM in DMSO. In the case of CuP-treated samples, it quantifies the rate of transport prior to DTT treatment compared with that following DTT treatment. Data are means $\pm$ S.D. ($n = 4$).

Figure 7 summarizes the effect of CuP- and o-PDM-mediated cross-linking on AE1 transport function. Treatment with CuP did not have a significant effect on the transport function of any mutants examined ($P > 0.05$, compared with AE1C⁻). Treatment with o-PDM increased the initial rate of anion transport for mutants AE1C⁻, T431C, Y555C, G565C and A751C, whereas A402C was unaffected. However, DMSO, the solvent carrier of o-PDM, similarly increased the transport function of AE1C⁻, T431C, Y555C, G565C and A751C; A402C was again unaffected. No significant o-PDM-mediated changes in transport function were observed for any of the mutants investigated ($P > 0.05$, by comparing the initial rates after addition of o-PDM in DMSO and after addition of just DMSO). The reason for the effects seen upon addition of 1% (v/v) DMSO is unclear, but indicates that the accelerated initial rates of anion transport following o-PDM were not due to cross-linking. Parallel experiments, where cross-linking was performed in whole cells prior to making membranes and immunoblotting, confirmed that cross-linking by both CuP and o-PDM was effective under the conditions of the functional assay for the mutants investigated, as was disulphide-bond reduction by DTT (results not shown). We conclude that intradimeric cross-linking of introduced cysteine mutants with CuP or o-PDM did not impair transport function.

DISCUSSION

Identification of regions that contribute to the dimeric interface of AE1 provides the basis for understanding the communication between monomer subunits within the functional dimeric protein complex. Subunit interactions have been shown to play a role in both anion transport and inhibition of this transport by ligands [15–18]. Regions of the protein contributing to the dimeric interface have not previously been identified, although co-immunoprecipitation studies seem to exclude TMs 6–8, 13 and 14 [29]. The strategy chosen in the present study was to introduce single cysteines into putative loop regions of the protein. The ability of cysteines in one monomer to be cross-linked to the corresponding cysteine in the other monomer maps the loop region of the protein relative to the dimeric interface. This approach allows a protein-wide scanning of loops located near to the dimeric interface and aids the design of future studies aimed at cross-linking across the dimeric interface within the plane of the membrane.

The results of this study show, for the first time, that EC1, IC1, EC2, EC3, EC4 and EC5 form disulphide cross-linked product (Figures 3 and 4). Using cross-linking reagents with different-length spacers, we also identified a cross-linked product from a cysteine introduced immediately preceding TM1 (Figures 3 and 4). Size-exclusion chromatography confirmed that only intradimeric cross-linked dimers were formed (Figure 5). No effect of inter-monomeric cross-linking upon anion-transport activity was observed, which suggests that constraining loop regions by cross-linking does not perturb, either directly or allosterically, the anion-transport process (Figures 6 and 7).

Can the results presented here provide insight into the TM arrangement of AE1 helices within the dimeric unit? Both the location of the introduced cysteines to the TM of interest and the dynamic nature of proteins must be considered when attempting to utilize data obtained from this investigation to provide constraints on the packing of TMs. In particular, interpretation of cross-linking data is complicated by the distance that loop regions may extend from the end of a TM. Assuming that the TMs are α -helical with a diameter of 10 Å [30], disulphide cross-linking of a residue in a loop region that is located maximally 0–10 Å from the TM of interest indicates that this TM

is located at the dimeric interface. Similarly, disulphide cross-linking of a residue located 10–20 Å from the TM of interest indicates that this TM either is located at the dimeric interface or is removed from the dimeric interface by one TM. Therefore estimation of the maximum distance of a cross-linked residue from the end of a TM allows constraints upon the position of this TM relative to the dimeric interface to be inferred. For example, T431C is located 5 and 3 residues from the ends of TM1 and TM2 respectively, according to the model presented in Figure 1. In a fully extended conformation T431C could thus be 17.5 and 10.5 Å from the ends of the TMs 1 and 2 respectively, since each residue in extended conformation spans 3.5 Å [31]. To these distances must be added 3.5 Å for half the distance between α -carbons in a disulphide bond [32]. Thus, disulphide cross-linking of T431C may occur maximally at a distance of 21 and 14 Å from the ends of TMs 1 and 2 respectively. This constrains TMs 1 and 2 to no more than two TMs and one TM away from the dimeric interface, respectively. It should be noted that this analysis relies upon the validity of the folding model presented in Figure 1 to obtain maximal distances of introduced cysteines to TMs of interest.

Similar arguments applied to G456C, Y486C and Y565C further localize TM2 to the dimeric interface, constrain TMs 3 and 6 to no more than one TM span from the dimeric interface, and localize TM4 to no more than two TM spans from the dimeric interface. Furthermore, by combining our results with co-immunoprecipitation studies indicating that TM6 is not located at the dimeric interface [29], we may deduce that TM6 is next to TMs that are at the dimeric interface.

Although disulphide-cross-linked products were obtained for Y555C, R656C and A751C, the distance of these residues from the predicted ends of TMs precludes deduction of useful structural constraints. Similarly, no structural information was obtained from the data obtained using the homobifunctional cross-linkers, which increase the allowed distance between α -carbons of two cross-linked cysteines and so reduce the resolution of inferred structural data.

Together, maleimide-mediated and disulphide cross-linking provide information on protein dynamics. Specifically, we found that T431C, Y486C, Y555C, G565C and A751C, which were zero-distance cross-linked, were also cross-linked by all the maleimide compounds, indicating that regions of the protein surrounding these sites are flexible enough to accommodate cross-linkers with spacer lengths up to 16 Å. A402C was unable to be zero-distance cross-linked, but maleimide-mediated cross-linking was observed for all of the homobifunctional reagents used (Figure 4). This suggests that the region of the protein surrounding this residue is flexible and that the distance between the two A402C residues is greater than the maximal distance of 7.5 Å required for disulphide-bond formation. Alternatively, the geometric requirements for successful disulphide-bond formation cannot be satisfied. G456C was unable to be cross-linked by maleimides even though zero-distance cross-linked product was obtained, suggesting an inflexible region of the protein surrounding this site, which is consistent with location of this residue in a small loop between TMs 2 and 3. R656C formed a disulphide cross-linked product, although no maleimide-mediated cross-linked product was observed. Previously, residues 656–663 in EC4 were suggested to provide an extramembraneous 'funnel' guiding substrate to the anion channel [9]. Flexibility in this funnel region would presumably be undesirable by slowing both the rates of anion binding and release from AE, with concomitant reduction in the optimal rate of AE function. Region 656–663 may lie beneath the surface of the membrane, forming part of a TM with open structure, as has been suggested

from proteolytic studies [33]. This interpretation would be consistent with the observed restricted dynamics. The lack of cross-linked product formed by reaction of R656C with the homobifunctional maleimides may also be a consequence of the geometric requirement for maleimide cross-linking, and EC4 may be as flexible as the other loops [34].

Experiments to examine the effect of inter-monomeric cross-linking were designed to test the possibility that the anion-translocation channel is found at the dimeric interface, as was proposed from analysis of the two-dimensional crystal of AE1 [13]. Formation of cross-links at the transport site may impair transport by either restriction of motion or blockage of the channel if the cross-links spanned the centre of the channel. We found that cross-linking loop regions EC1, EC3, EC4 and EC5, as well as the region of the protein immediately before TM1, had no effect upon the ability of AE1 to transport anions. It has also previously been demonstrated that cross-linking EC3 with bis(sulphosuccinimidyl)suberate did not perturb anion-exchange function of AE1 [35]. Although our findings cannot discriminate between an anion channel located at the dimeric interface and one located within each monomer, we are able to refine an allosteric model for anion exchange [15,16]. Previous work indicated the importance of monomer–monomer contacts in anion transport, since perturbation of these contacts following stilbene disulphonate binding may provide an allosteric mechanism for inhibition by these ligands [17,36]. The present findings suggest that interaction between TMs rather than loop regions at the dimeric interface is important for the allosteric model of anion transport, since constraining the conformation/dynamics of extramembraneous regions of the protein located near the dimeric interface by cross-linking did not alter AE1 transport function.

In conclusion, cysteine-directed cross-linking analysis of loop regions of AE1 has allowed a protein-wide scan to determine regions of the protein close to the dimeric interface. From the data obtained, we tentatively locate TM2 at the dimeric interface. TMs 3 and 6 are maximally one TM removed and TMs 1 and 4 are maximally two TMs removed from the dimeric interface. It should be stressed that these deductions require knowledge of the position of introduced cysteine residues, relative to the ends of TMs. Thus our interpretation is only as good as the topology model (Figure 1). In addition, it should be noted that non- α -helical membrane-spanning structure located close to the dimeric interface would add considerable uncertainty to our deductions on the location of TMs relative to this interface. Although functional analysis of the cross-linked products was unable to localize the anion channel to the dimeric interface, the data suggest that allosteric coupling between monomers during the anion-transport cycle occurs primarily through TM–TM rather than loop–loop interactions. These findings aid the design of future cross-linking studies within the membrane plane aimed at examining the role of monomeric subunit interactions in the anion-transport process.

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REFERENCES

- 1 Passow, H. (1986) Molecular aspects of Band 3-mediated anion transport across the red blood cell membrane. *Rev. Physiol. Biochem. Pharmacol.* **103**, 61–223
- 2 Reithmeier, R. A. F. (1993) The erythrocyte anion transporter (Band 3). *Curr. Opin. Struct. Biol.* **3**, 515–523

- 3 Knauf, P. A., Gasbjerg, P. K. and Brahm, J. (1996) The asymmetry of chloride transport at 38 °C in human red blood cell membranes. *J. Gen. Physiol.* **108**, 577–589
- 4 Knauf, P. A. and Brahm, J. (1989) Functional asymmetry of the anion-exchange protein, capnophorin: effects on substrate and inhibitor binding. *Methods Enzymol.* **173**, 432–453
- 5 Tang, X. B., Fujinaga, J., Kopito, R. and Casey, J. R. (1998) Topology of the region surrounding Glu681 of human AE1 protein, the erythrocyte anion exchanger. *J. Biol. Chem.* **273**, 22545–22553
- 6 Popov, M., Tam, L. Y., Li, J. and Reithmeier, R. A. F. (1997) Mapping the ends of transmembrane segments in a polytopic membrane protein. Scanning N-glycosylation mutagenesis of extracytosolic loops in the anion exchanger, band 3. *J. Biol. Chem.* **272**, 18325–18332
- 7 Reithmeier, R. A. F., Chan, S. L. and Popov, M. (1996) Structure of the erythrocyte band 3 anion exchanger. In *Transport Processes in Eukaryotic and Prokaryotic Organisms*, vol. 2 (Konings, W. N., Kaback, H. R. and Lolkema, J. S., eds.), pp. 281–309, Elsevier Science, New York
- 8 Fujinaga, J., Tang, X.-B. and Casey, J. R. (1999) Topology of the membrane domain of human anion exchange protein, AE1. *J. Biol. Chem.* **274**, 6626–6633
- 9 Tang, X.-B., Kovacs, M., Sterling, D. A. and Casey, J. R. (1999) Identification of residues lining the translocation pore of human AE1, plasma membrane anion exchange protein. *J. Biol. Chem.* **274**, 3557–3564
- 10 Müller-Berger, S., Karbach, D., König, J., Lepke, S., Wood, P. G., Appelhans, H. and Passow, H. (1995) Inhibition of mouse erythroid band 3-mediated chloride transport by site-directed mutagenesis of histidine residues and its reversal by second site mutation of Lys 558, the locus of covalent H2DIDS binding. *Biochemistry* **34**, 9315–9324
- 11 Müller-Berger, S., Karbach, D., Kang, D., Aranibar, N., Wood, P. G., Ruterjans, H. and Passow, H. (1995) Roles of histidine 752 and glutamate 699 in the pH dependence of mouse band 3 protein-mediated anion transport. *Biochemistry* **34**, 9325–9332
- 12 Groves, J. D., Wang, L. and Tanner, M. J. A. (1998) Complementation studies with co-expressed fragments of human red cell band 3 (AE1): the assembly of the anion-transport domain in *Xenopus* oocytes and a cell-free translation system. *Biochem. J.* **332**, 161–171
- 13 Wang, D. N. (1994) Band 3 protein: structure, flexibility and function. *FEBS Lett.* **346**, 26–31
- 14 Jennings, M. L., Whitlock, J. and Shinde, A. (1998) Pre-steady state transport by erythrocyte band 3 protein: uphill countertransport induced by the impermeant inhibitor H2DIDS. *Biochem. Cell. Biol.* **76**, 807–813
- 15 Schopfer, L. M. and Salhany, J. M. (1995) Characterization of the stilbenedisulfonate binding site on band 3. *Biochemistry* **34**, 8320–8329
- 16 Salhany, J. M., Schopfer, L. M., Kay, M. M., Gamble, D. N. and Lawrence, C. (1995) Differential sensitivity of stilbenedisulfonates in their reaction with band 3 HT (Pro-868 → Leu). *Proc. Natl. Acad. Sci. U.S.A.* **92**, 11844–11848
- 17 Taylor, A. M., Grobner, G., Williamson, P. T. and Watts, A. (1999) Binding properties of the stilbene disulfonate sites on human erythrocyte AE1: kinetic, thermodynamic, and solid state deuterium NMR analyses. *Biochemistry* **38**, 11172–11179
- 18 Verkman, A. S., Dix, J. A. and Solomom, A. K. (1983) Anion transport inhibitor binding to Band 3 in red blood cell membranes. *J. Gen. Physiol.* **81**, 421–449
- 19 Casey, J. R., Ding, Y. and Kopito, R. R. (1995) The role of cysteine residues in the erythrocyte plasma membrane anion exchange protein, AE1. *J. Biol. Chem.* **270**, 8521–8527
- 20 Lee, B. S., Gunn, R. B. and Kopito, R. R. (1991) Functional differences among nonerythroid anion exchangers expressed in a transfected human cell line. *J. Biol. Chem.* **266**, 11448–11454
- 21 Sarkar, G. and Sommer, S. S. (1990) The megaprimer method of site-directed mutagenesis. *Biotechniques* **8**, 404–407
- 22 Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 23 Casey, J. R. and Reithmeier, R. A. F. (1991) Analysis of the oligomeric state of Band 3, the anion transport protein of the human erythrocyte membrane, by size exclusion high performance liquid chromatography: oligomeric stability and origin of heterogeneity. *J. Biol. Chem.* **266**, 15726–15737
- 24 Casey, J. R. and Reithmeier, R. A. F. (1998) Anion exchangers in the red cell and beyond. *Biochem. Cell. Biol.* **76**, 709–713
- 25 Lieberman, D. M. and Reithmeier, R. A. F. (1988) Localization of the carboxyl terminus of Band 3 to the cytoplasmic side of the erythrocyte membrane using antibodies raised against a synthetic peptide. *J. Biol. Chem.* **263**, 10022–10028
- 26 Careaga, C. L. and Falke, J. J. (1992) Thermal motions of surface α -helices in the D-galactose chemosensory receptor. *J. Mol. Biol.* **226**, 1219–1235
- 27 Falke, J. J. and Koshland, Jr, D. E. (1987) Global flexibility in a sensory receptor: a site-directed cross-linking approach. *Science* **237**, 1596–1600
- 28 Cabantchik, Z. I. and Greger, R. (1992) Chemical probes for anion transporters of mammalian membranes. *Am. J. Physiol.* **262**, C803–C827
- 29 Groves, J. D. and Tanner, M. J. (1999) Structural model for the organization of the transmembrane spans of the human red-cell anion exchanger (band 3; AE1). *Biochem. J.* **344**, 699–711
- 30 Chothia, C., Levitt, M. and Richardson, D. (1981) Helix to helix packing in proteins. *J. Mol. Biol.* **145**, 215–250
- 31 Creighton, T. E. (1983) *Proteins: Structures and Molecular Properties*, W. H. Freeman and Co., New York
- 32 Katz, B. A. and Kossiakoff, A. (1986) The crystallographically determined structures of atypical strained disulfides engineered into subtilisin. *J. Biol. Chem.* **261**, 15480–15485
- 33 Hamasaki, N., Okubo, K., Kuma, H., Kang, D. and Yae, Y. (1997) Proteolytic cleavage sites of band 3 protein in alkali-treated membranes: fidelity of hydropathy prediction for band 3 protein. *J. Biochem. (Tokyo)* **122**, 577–585
- 34 Pakula, A. A. and Simon, M. I. (1992) Determination of transmembrane protein structure by disulfide cross-linking: the *Escherichia coli* Tar receptor. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4144–4148
- 35 Jennings, M. L. and Nicknisch, S. J. (1985) Localization of a site of intermolecular cross-linking in human red blood cell Band 3 protein. *J. Biol. Chem.* **260**, 5472–5479
- 36 Salhany, J. M., Sloan, R. L., Cordes, K. A. and Schopfer, L. M. (1994) Kinetic evidence for ternary complex formation and allosteric interactions in chloride and stilbenedisulfonate binding to band 3. *Biochemistry* **33**, 11909–11916
- 37 Mori, A., Okubo, K., Kang, D. and Hamasaki, N. (1995) A structural study of the carboxyl terminal region of the human erythrocyte band 3 protein. *J. Biochem. (Tokyo)* **118**, 1192–1198
- 38 Okubo, K., Kang, D., Hamasaki, N. and Jennings, M. L. (1994) Red blood cell band 3. Lysine 539 and lysine 851 react with the same H₂DIDS (4,4'-diisothiocyanodihydrostilbene-2,2'-disulfonic acid) molecule. *J. Biol. Chem.* **269**, 1918–1926

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