Carbonic Anhydrase II Binds to the Carboxyl Terminus of Human Band 3, the Erythrocyte Cl⁻/HCO₃⁻ Exchanger*

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In this study, we provide evidence that the 33-residue carboxyl-terminal (Ct) region of the human erythrocyte chloride/bicarbonate exchanger, band 3, binds carbonic anhydrase II (CAII). Immunofluorescence showed that tomato lectin-mediated clustering of band 3 in ghost membranes caused a similar clustering of CAII, indicating an in situ association. CAII cosolubilized and coimmunoprecipitated with band 3, suggesting that the two proteins form a complex. Band 3 (K_{d2} = 70 nM) or the membrane domain of band 3 (K_{d2} = 100 nM) bound saturably to immobilized CAII in a solid phase binding assay. The interaction with CAII was specifically blocked by an antibody to the Ct of band 3. Affinity blotting showed that a glutathione S-transferase (GST)-fusion protein (GST-Ct) containing the last 33 residues of human band 3 bound to CAII. The solid phase binding assay showed that binding of GST-Ct to immobilized CAII was saturable (K_{d2} = 20 nM). The binding rate was slow (t_{1/2} = 12 h) at physiological ionic strength and pH but was enhanced at low ionic strength or acidic pH. Intact band 3 (K_{t} = 15 nM), the membrane domain of band 3 (K_{t} = 100 nM), or antibodies to the Ct of band 3 were able to block GST-Ct binding to CAII, confirming the specificity of the interaction. Affinity chromatography showed that CAII bound to immobilized GST-Ct with a 1:1 stoichiometry. This work indicates that CAII, the bicarbonate supplier, is directly coupled to band 3, the chloride/bicarbonate exchanger in red blood cells.

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Human band 3 is a 911-amino acid glycoprotein (1, 2) present in the erythrocyte membrane at 1.2 × 10^6 copies per cell (3, 4). It is a member of the anion exchanger (AE)* gene family (5) and catalyzes the rapid, electroneutral exchange of bicarbonate for chloride (6, 7). Band 3 consists of two structural and functional domains: a 43-kDa amino-terminal cytoplasmic domain and a 55-kDa carboxyl-terminal membrane domain (8). The cytoplasmic domain is involved in protein-protein interactions with a number of structural proteins and metabolic enzymes (9). Several glycolytic enzymes bind electrostatically to the highly acidic extreme amino terminus (10–12), and this binding is regulated by tyrosine phosphorylation of band 3 (13). The membrane domain of band 3 spans the lipid bilayer 12 times (14) and is responsible for the anion transport function (15). The carboxyl-terminal (Ct) region of band 3 is negatively charged, 33-residue sequence that faces the cytosol (16, 17) and contains a tyrosine phosphorylation site (18). Evidence from antibody binding (16, 19) and protease accessibility studies (20, 21) suggest that the Ct sequence is involved in binding cytosolic proteins; however, these proteins have not been identified.

Along with band 3, carbonic anhydrase (CA) contributes to pulmonary gas exchange by catalyzing the hydration of CO₂ to produce bicarbonate (22). Two major isoforms of CA are found in human red cells; over 85% of erythrocyte carbonic anhydrase is CAI, whereas the remainder is predominantly the higher activity form, CAII (23). Interestingly, as the abundance of CAII in red cells is approximately 1 million (24), its ratio to band 3 is close to 1:1. Although CAI deficiency is not associated with any known medical disorder in humans (25), CAII deficiency leads to osteopetrosis with renal tubular acidosis and cerebral calcification (26). Both isoforms of CA have increased activity in the presence of erythrocyte membranes. Bovine CA, the homologue of human CAII, binds to erythrocyte membranes in vitro (27). Binding of a stilbene-disulfonate inhibitor to band 3 perturbs the fluorescence of labeled bovine CA, suggesting there is a physical link between the two enzymes (28).

In the present study, we tested the hypothesis that band 3 contains a binding site for CAII. We demonstrate a direct interaction between CAII and band 3 and localize the binding site for CAII to the Ct 33 residues of band 3. By binding CAII, the bicarbonate supplier is colocalized with the red cell chloride/bicarbonate exchanger. This may be an example of metabolic channeling in which sequential enzymatic steps are compartmentalized (29, 30). Membrane-associated CAII is ideally positioned to catalyze CO₂ hydration and supply band 3 with bicarbonate.

EXPERIMENTAL PROCEDURES

Materials—The following is a list of products and their suppliers. Escherichia coli strain DH5α, pGEX-5x-1 plasmid, DEAE-Sepharose 4B, glutathione-Sepharose 4B, goat anti-GST serum, protein G-Sepharose, T7 Sequencing Kit, and isopropyl-β-D-thiogalactopyranoside (Amersham Pharmacia Biotec); glutathione, human CAI and CAII, bovine serum albumin (BSA), o-phenylenediamine, protease inhibitors, and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (Sigma); C₁₂E₈ (Niko); restriction enzymes and T4 DNA ligase (New England Biolabs); secondary antibodies, biotinylated tomato lectin, and the biotinylated peroxidase/avidin system (Vector Laboratoires); rabbit polyclonal anti-human CAII serum (Serotec); rabbit polyclonal anti-Ct serum raised against a synthetic Ct peptide (16), mouse monoclonal anti-Ct serum (31), and rabbit polyclonal serum that recognizes the amino-terminal cytosolic domain (32, 33) have been described. For immunofluorescence experiments, anti-human CAII serum was affinity purified by incubating a 1:100 dilution of the serum with a...
nitrocellulose membrane on which purified CAII had been immobilized. Adsorbed anti-human CAII was eluted from the membrane using Immunopure Ab elution buffer (Pierce) and then diazoylated against distilled water and concentrated using a centrifugal concentrator (Amicon). The anti-Ct of band 3 serum used for immunofluorescence was generated from a peptide comprising the last 16 residues of band 3. The BSA conjugate of this peptide was coupled to CNBr-activated Sepharose (0.5 ml) (Pharmacia). After adsorbing the anti-Ct serum to the BSA, it was eluted and concentrated as above. Plasmid Construction—The pBluescript II SK(+) vector (Stratagene) containing the human band 3 sequence was the generous gift of Drs. A. M. Garcia and H. Lodish (Whitehead Institute). The vector was linearized with Smal and used in a polymerase chain reaction with oligonucleotide primers 5′-GACGATCGGCAAGGTTGGAGCTTCAG-3′ and 3′-CCATGATCCGCAAGCTCG-5′ designed to amplify residues 879–911 of the Ct region of band 3 (RNVELQCLDADDAKATFDEEEGRDEYDEVAMPV) plus 95 base pairs of 3′-untranslated sequence and to insert BamHI sites onto both ends of the DNA. The polymerase chain reaction product was digested with BamHI, purified by agarose gel electrophoresis, and ligated into pGEX-5x-1 plasmid that had been linearized with BamHI and treated with calf-intestinal phosphatase. Competent E. coli DH5α were transformed with the ligation reaction products. Clones containing the insert were identified by restriction enzyme digestion with BamHI, and the correct orientation was confirmed by DNA sequencing. Bacterial Expression and Protein Fusion Purification—GST or GST-Ct was expressed in E. coli DH5α cells and purified according to the supplier (Pharmacia) using glutathione-Sepharose. The peak fractions were pooled and further purified on DEAE-Sepharose 4B. DEAE-bound fusion protein was washed with 50 ml Tris-HCl, pH 7.4, 1 mM dithiothreitol, and protease inhibitor mixture and eluted with a linear salt gradient (0–400 mM NaCl). Purity was assessed by size-exclusion high pressure liquid chromatography and SDS-polyacrylamide gel electrophoresis. High pressure liquid chromatography size-exclusion chromatography revealed that the fusion protein, like native GST, was dimeric. Aliquots (100 μl) were frozen in a dry ice/ethanol bath and stored at −20 °C. Samples were stable for at least 6 months. There was no difference in the reactivity of GST-Ct to the goat anti-GST serum compared with GST control. Purification of Band 3 or the Membrane Domain—Band 3 and the membrane domain were purified from KI-stripped inside-out vesicles (35), solubilized in C12E8, and chromatographed by published protocols (35). Immunofluorescence—Erythrocyte ghost membranes were prepared from normal band 3–deficient human red cells. In both cases, packed membranes (50 ml) were diluted to 500 μl in 5 mM sodium phosphate, pH 8 (5P8) and incubated with or without the addition of 0.2 mg/ml 1-cyclohexyl-3-(2-morpholinopyridine) carbodiimide metho-p-toluenesulfonate in 150 mM NaCl, 100 mM sodium phosphate, pH 6, for 30 min at room temperature (37, 38). The plates were washed extensively with PBS (150 mM NaCl, 5 mM Tris-HCl, pH 7.5) or 5P8 and then blocked for 2 h at room temperature in PBS supplemented with 2% BSA. The bound CAII was shown to be active by an esterase assay (39). Plates were washed with Ab buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% C12E8, 5 mM EDTA, 0.25% gelatin) and incubated with purified band 3 or the band 3 membrane domain. Bound protein was detected by incubating the plates first with a biotinylated tomato lectin that binds to the band 3 oligosaccharide and then with peroxidase-labeled biotin/avidin. This was followed by incubation with the peroxidase substrate o-phenylenediamine and detection of enzymatic activity at 450 nm in a Thermomax microplate reader (Molecular Devices) connected to a Macintosh workstation. For purified GST-Ct or GST, bound protein was detected by incubating the plates sequentially in goat anti-GST serum, biotinylated affinity-purified rabbit anti-sheep IgG (Precision Biologics), and a biotinylated tomato lectin. The buffer used for incubations of GST-Ct or GST with immobilized CAII was Ab buffer containing 0.05% Triton X-100 or as described in the figure legends. Subsequent washes and antibody incubations were done in Ab buffer in all cases. Competition Studies—Band 3 or the membrane domain in 0.1% C12E8 was mixed with rabbit polyclonal anti-Ct of band 3 serum or rabbit pre-immune serum and then incubated with immobilized CAII. Competition of GST-Ct with antibodies was done in the same way. For competition of GST-Ct with band 3 or the membrane domain, various concentrations of GST-Ct were mixed with either band 3 or the membrane domain and then incubated with immobilized CAII. Bound protein was detected as described above. Solid Phase Binding Assay—Purified GST-Ct or GST was immobilized on the Laminex gel spin system (36) and then transferred to nitrocellulose. Nitrocellulose transfers were blocked (0.25% gelatin, 10% ethanolamine, 50 mM Tris-HCl, pH 9.0) and then washed in Ab buffer containing 0.05% Triton X-100. Transfers were incubated with 0.2–2.0 μg/ml GST-Ct or GST for 18 h at room temperature. Transfers were washed in Ab buffer and then incubated with goat anti-GST serum (1:500 dilution in 50 mM Tris-HCl, pH 7.5–8.0, 50 mM NaCl). To 10-μl aliquots of Sepharose washed, bound protein was detected by incubation with biotinylated affinity-purified rabbit anti-CAII IgG (1:10,000). Further amplification was achieved by a subsequent incubation with biotinylated peroxidase-avidin (1:10,000). Reactive bands were visualized by ECL as described by the manufacturer (Boehringer Mannheim). Affinity Chromatography—Purified GST-Ct or GST was immobilized on glutathione-Sepharose 4B (25–100 μg of protein/ml of resin) in 10 ml of 50 mM Tris-HCl, pH 7.5–8.0, 50 mM NaCl. To 10-μl aliquots of Sepharose, were added various concentrations of purified CAII to a total volume of 100 μl. The mixtures were incubated for 15–18 h at 4 °C and pelleted by centrifugation, and the supernatant was then removed. The resin was then washed with 1 ml of buffer. Bound protein was eluted with 90 μl of Laemmli sample buffer and electrophoresed. Densitometry of the Coomassie Blue-stained gel was used to determine the ratio of bound to either GST-Ct or GST control. GST-Ct, GST-Ct, and CAII bound similar amounts of Coomassie Blue dye. Data Analysis—Binding curves were plotted using Microcal Origin 4.0. Absorbance values were converted to percent maximum binding by determining the maximum signal obtained in the assay and calculating the other values relative to it. RESULTS Band 3 and CAII Are Colocalized—Since band 3 and CAII catalyze interconnected processes, we set out to determine whether the two proteins form a complex in the erythrocyte. To test this hypothesis, we examined blood distributed at room temperature and band 3 and CAII in the membrane following treatment of intact ghost membranes with tomato lectin. Tomato lectin specifically binds to the repeating poly lactosaminoglycan structure on band 3 in erythrocyte membranes (40) and causes their clustering within the plane of the membrane. If CAII is attached to band 3 on the cytoplasmic side of the membrane, it should also redistribute in...
a similar fashion as a consequence of lectin treatment. Band 3 is the predominant tomato lectin-binding protein in the erythrocyte membrane, although the glucose transporter (GLUT1) and the water channel (AQP1) also possess polylactosaminoglycan. Red cell ghosts were prepared and incubated with or without tomato lectin as described under “Experimental Procedures.” The background immunofluorescence of membranes not incubated with primary antibodies was barely detectable (Fig. 1, top panels). Immunofluorescence of non-lectin-treated samples showed that both band 3 and CAII were present diffusely in ghost membranes (Fig. 1, middle panels). In samples treated with tomato lectin, band 3 was distributed in clusters (Fig. 1, bottom panels). The confocal view of these agglutinated red cells showed patches of immunofluorescence (see inset), due to band 3 aggregation in the plane of the membrane. The effect on CAII was nearly identical, with the redistribution of this enzyme also occurring after addition of lectin. This suggests that band 3 is physically tethered to CAII in intact red cell membranes. Similar results were obtained using red cell ghost smears obtained from an individual with CAI deficiency.

Because it seemed that band 3 and CAII were interacting in situ, we used Western immunoblotting with a polyclonal anti-CAII serum to determine whether CAII copurified with band 3. After lysing erythrocytes with 20–40 volumes of 5P8, a significant fraction (5–10%) of the total red cell CAII (Mr = 29,000) remained tightly associated with the ghost membranes and was only partially removed by subsequent incubation with PBS (pH 8) (Fig. 2A). Red cells from a CAI-deficient individual showed that this bound fraction was the low abundance isoform CAII. After detergent solubilization of band 3, most of the membrane-bound CAII was found in the supernatant with band 3, rather than associated with the pelletel cytoskeleton. When the supernatant was incubated with antiserum recognizing the amino terminus of band 3, CAII was coimmunoprecipitated with band 3 (Fig. 2B). However, when band 3 was incubated with an antiserum to the Ct or with pre-immune rabbit IgG (data not shown), CAII did not coimmunoprecipitate. The inability of anti-Ct serum to coimmunoprecipitate CAII suggests that CAII may be associated with the Ct of band 3. Band 3-CAII complexes would not be accessible to the anti-Ct serum since a free Ct is required for antibody binding to this sequence (16, 19).

Band 3 Binding to Immobilized CAII—A solid phase binding assay was developed to examine the interaction between band 3 and CAII in greater detail. CAII immobilized on microtiter plates possessed enzymatic activity indicating it had retained a native conformation (data not shown). The binding of both intact band 3 and the membrane domain of band 3 to immobilized CAII was examined. The membrane domain of band 3

Fig. 1. Immunofluorescence of red cell ghost membranes. Top panels, control experiment showing no immunofluorescence in the absence of primary antibodies. Left, probed with secondary fluorescein isothiocyanate anti-rabbit IgG; right, probed with secondary biotinylated anti-rabbit IgG then rhodamine-avidin. Middle panels, red cell ghost smears probed with either anti-band 3 (left) or anti-CAII (right). Inset shows the diffuse staining pattern present in single ghosts in both samples. Bottom panels, red cell ghost smears treated with tomato lectin prior to antisera incubation. Inset shows that clustering of band 3 in the plane of the membrane (left) causes a similar redistribution of CAII (right) indicating a physical association.

Fig. 2. Association of CAII with solubilized band 3. A, immunoblot of various ghost membrane fractions using an anti-CAII serum. Lane 1, ghost membranes; lane 2, PBS supernatant of ghost membranes; lane 2, PBS-washed ghosts; lane 5, pellet of PBS-washed ghosts solubilized with 1% C12E8. Arrowheads show positions of molecular mass markers in kDa. The cross-reactive band at 45 kDa in lanes 1 and 2 is band 6. B, immunoblot of immunoprecipitated band 3 fractions probed with anti-CA serum. Detergent-solubilized membranes (Fig. 1A, lane 4) were incubated with antibodies to either the Ct or amino-terminal domain of band 3. Immunoprecipitates were collected as described under “Experimental Procedures” and probed with an antibody to CAII. Lane 1, CA marker (50 ng); lane 2, anti-amino-terminal immunoprecipitate; lane 3, anti-Ct immunoprecipitate.
lacks the 360-amino acid amino-terminal domain but retains the ability to transport anions. Fig. 3 shows that both band 3 ($K_{1/2} = 70 \text{ nM}$) and the membrane domain ($K_{1/2} = 100 \text{ nM}$) bound saturably to immobilized CAII. Under identical conditions (24-h incubation, 22 °C), no binding to immobilized CAI was observed (data not shown), suggesting a specificity of band 3 for the high activity isoform, CAII. The ability of both band 3 and the membrane domain of band 3 to bind CAII with similar $K_{1/2}$ indicated that the binding site for CAII did not require the amino terminus of band 3. The binding curve for the membrane domain differed from band 3 following a sigmoidal relationship and suggesting a cooperative interaction (Fig. 3).

The location of the interaction between band 3 or the membrane domain with immobilized CAII was shown by competition with a Ct antibody (Fig. 4). When the anti-Ct serum was incubated with band 3 or the membrane domain, binding to immobilized CAII was decreased in a dose-dependent manner, suggesting that the Ct was the main site of interaction. Pre-immune serum did not affect the interaction of band 3 and CAII. A GST-Ct fusion protein (see below) was also able to compete with band 3 for binding to CAII (data not shown).

Detection of a GST-Ct/CAII Interaction by Affinity Blotting—To directly demonstrate the role of the Ct in CAII binding, we constructed a GST-fusion protein encoding the Ct region of band 3 (residues 879–911) to use as a probe in an affinity blotting assay. Binding of the GST-fusion protein (GST-Ct) or GST control to proteins immobilized on nitrocellulose membranes was detected using an anti-GST serum. When erythrocyte hemolysate was tested, GST-Ct bound to a protein identified by its electrophoretic mobility as carbonic anhydrase, consisting of isoforms I and II (Fig. 5A). When equivalent amounts of GST control were used to probe nitrocellulose membranes, much lower levels of binding were observed (Fig. 5A). When hemolysate from a CAI-deficient patient was tested, a similar result was obtained (data not shown). Because band 3 and the membrane domain seemed only to bind CAII, purified CAI and CAII were tested using the same assay. GST-Ct bound to CAII, and only a low level of binding of the GST control to CAII was detected (Fig. 5B). Both GST-Ct and GST bound similarly at low levels to CAI (data not shown). This indicated that GST-Ct specifically recognized the high activity isoform, CAII.

Characterization of Band 3 Ct Binding to CAII—To characterize the interaction between GST-Ct and CAII under native conditions, the solid phase binding assay was used. The binding of GST-Ct to immobilized CAII saturated with half-maximal binding occurring at 20 nM (Fig. 6). Under these conditions, GST did not bind, thereby demonstrating a requirement for the Ct in CAII binding. This $K_{1/2}$ value, 20 nM, is comparable with those obtained for band 3 (70 nM) and the membrane domain (100 nM). This suggests that the Ct is sufficient for binding to CAII.

The binding of GST-Ct to immobilized CAII was slow, with a $t_{1/2}$ of 12 h under conditions of physiological ionic strength and pH (Fig. 7A). A similar time course was found for band 3 or the
membrane domain binding to immobilized CAII. As the Ct of band 3 is enriched in acidic residues, the effects of ionic strength and pH on binding CAII were examined. GST-Ct or GST was incubated for 18 h with CAII in the presence of various concentrations of KCl or NaCl. The binding was inhibited by high concentrations of KCl or NaCl with a half-maximal decrease in the signal seen at approximately 100 mM (Fig. 7B).

The effect of NaHCO₃, a band 3 substrate, was also examined. A concentration of 25 mM sodium bicarbonate was sufficient to reduce binding to half-maximal (data not shown). The greater inhibitory effect of bicarbonate over chloride may reflect a regulatory role of bicarbonate on the interaction.

The effect of pH on the binding of CAII to GST-Ct was dramatic, with the rate of binding being greater at acidic pH (Fig. 7C). A sharp pH dependence was observed with half-maximal binding observed at pH 7.0. This pH dependence of the interaction may partly explain why only a fraction of the erythrocyte CAII remained associated with membranes after lysis of red blood cells in 5P8. The enhanced binding seen with acidic pH and low ionic strength are on the time course of the interaction; both factors decrease the time required to reach saturation (data not shown). The effects of low ionic strength and acidic pH occur at the initial binding step since subsequent washes and antibody incubations are performed in Ab buffer (100 mM NaCl, pH 7.5). This may indicate that the interaction between GST-Ct and CAII is promoted by low ionic strength and acidic pH and that, once “locked on,” the interaction is very strong.

**Inhibitor Effects**—Inclusion of acetazolamide (100 μM), an inhibitor of CAII that binds to the enzyme’s active site, did not affect binding of GST-Ct to immobilized CAII. Furthermore, GST-Ct had no effect on the esterase activity of soluble CAII (data not shown). Therefore, the Ct sequence of band 3 does not seem to perturb the enzymatic function of CAII. Prelabeling band 3 or the membrane domain with H₂DIDS, a potent inhibitor of anion transport, did not prevent binding of the modified protein to CAII (data not shown).

**Competition of GST-Ct Binding to Immobilized CAII**—Antibodies recognizing the Ct of band 3 blocked GST-Ct binding to CAII (Fig. 8). Both a monoclonal and a polyclonal antibody to the Ct were able to decrease binding of GST-Ct to immobilized CAII. As the Ct of band 3 is enriched in acidic residues, the effects of ionic strength and pH on binding CAII were examined. GST-Ct or GST was incubated for 18 h with CAII in the presence of various concentrations of KCl or NaCl. The binding was inhibited by high concentrations of KCl or NaCl with a half-maximal decrease in the signal seen at approximately 100 mM (Fig. 7B). The effect of NaHCO₃, a band 3 substrate, was also examined. A concentration of 25 mM sodium bicarbonate was sufficient to reduce binding to half-maximal (data not shown). The greater inhibitory effect of bicarbonate over chloride may reflect a regulatory role of bicarbonate on the interaction.

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CAII in a dose-dependent manner. This confirmed that the Ct sequence on the GST-Ct was responsible for the interaction with CAII. Band 3 ($K_i = 15 \text{ nm}$) and the membrane domain ($K_i = 100 \text{ nm}$) also competed with GST-Ct for binding to CAII (Fig. 9). This confirmed that the binding site for CAII was localized within the Ct of the membrane domain.

**Affinity Chromatography Using Immobilized GST-Ct**—To examine whether soluble CAII could bind to immobilized GST-Ct with similar characteristics and to determine the stoichiometry of the interaction, an affinity chromatography protocol using glutathione-Sepharose was developed. GST-Ct or GST was immobilized on glutathione-Sepharose and incubated with various concentrations of purified CAII for 15 h at 4 °C. Bound CAII and GST-Ct or GST were eluted from the resin in Laemmli sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. Coomassie-stained gels were scanned, and the resulting ratios of bound CAII to GST-Ct or GST were plotted (Fig. 10A). CAII bound to immobilized GST-Ct in a saturable manner, whereas binding to immobilized GST was much lower and increased linearly. A Scatchard plot (Fig. 10B) of the specific binding component revealed that the binding had an apparent $K_d$ of 110 nm and a stoichiometry of approximately 1:1.

**Discussion**

**Band 3 and CAII Form a Complex in the Erythrocyte**—CAII and band 3 catalyze sequential processes in CO$_2$ removal from the erythrocyte. Previous work has suggested that CAII and band 3 form a complex (28). We have tested this hypothesis and discovered that CAII binds specifically to the Ct region of band 3.

Immunofluorescence showed that clustering of polylactosaminoglycan-containing proteins in ghost membranes by tomato lectin caused a similar clustering of CAII. Band 3 is the predominant tomato lectin-binding protein in the membrane (40), and this finding suggests clustering of band 3 results in redistribution of CAII as a result of a linkage between the two enzymes. It is not possible to rule out an indirect interaction or CAII redistributing as a result of a linkage between the two polylactosaminoglycan-containing proteins; however, the data in this report imply a specific interaction with band 3.

Band 3 and CAII could be communoprecipitated from solubilized erythrocyte membranes, suggesting a direct link between these proteins exists in situ. Interestingly, an antibody recognizing amino-terminal epitopes, but not one recognizing the Ct region of band 3, was able to communoprecipitate CAII. These findings suggested that the interaction involved the Ct region of band 3.

Both band 3 and the membrane domain of band 3 could bind saturably to immobilized CAII in the solid phase assay and were blocked by a Ct antibody. This indicates that the amino-terminal domain of band 3 is not required for the interaction and again suggested that the Ct region was the site of the interaction. The binding curves for band 3 and the membrane domain differed, with binding of the membrane domain following a sigmoidal relationship. The differences in the two curves may be due to the oligomeric states of detergent-purified band 3 and membrane domain (41, 42). The affinity of the interaction of both band 3 and the membrane domain was similar, with $K_{1/2}$ values of 70 and 100 nm. This level of affinity is common among proteins that bind to band 3. Phosphofructokinase, aldolase, band 4.2, and ankyrin all have affinities for band 3 of between 50 and 100 nm (12, 43–45).

**Localization of the CAII Binding Domain to the Ct Region of Band 3**—We examined directly whether the 33-residue Ct region of band 3 was capable of binding CAII by using a GST-Ct fusion protein. The GST-fusion protein system has been used extensively in binding experiments to discover and examine protein-protein interactions (46–48). Affinity blotting, the solid phase assay, and affinity chromatography all showed that GST-Ct bound specifically to CAII, indicating that the Ct was sufficient for band 3 interaction with CAII. A maximal stoichiometry of approximately 1:1 was determined. Given the near equivalent copy number of band 3 and CAII in the erythrocyte, there are sufficient band 3 sites to bind all of the CAII. The identification of the CAII binding site within the Ct region is novel as all previously characterized band 3-binding proteins have been found to interact with the amino-terminal domain (9). Several lines of evidence had indicated that the Ct region of band 3 is involved in protein binding. Antibody binding (16, 19), carboxypeptidase Y digestion (20), and trypsin digestion (21) of the Ct are each hindered if extrinsic membrane proteins are

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*Note:* The text above is a summary of the content of the image, including equations, figures, and tables.
Carbonic Anhydrase Binds to Band 3

FIG. 10. Affinity chromatography of CAII binding to immobilized GST-Ct. A, saturation curve showing specific binding of CAII to GST-Ct and nonspecific, linear interaction with control GST. B, Scatchard plot on CAII binding to GST-Ct (corrected for nonspecific GST binding). The curve indicates an apparent $K_d$ of 110 nM and a stoichiometry of approximately 1:1 ($n = 0.9$).

not removed by high salt or alkaline pH treatment.

Binding Determinants—No significant binding of GST-Ct to CAI was detected, despite the 60% amino acid sequence identity between the two isoforms. The most notable difference between the two sequences seems to be the concentration of histidine residues in the amino terminus of CAII (5 out of the first 16 residues) that is absent from the corresponding sequence of CAI. These histidines, along with lysine and arginine residues, form a basic patch on the surface of CAII that may interact with the acidic Ct of band 3. Neither band 3 nor CAII contain canonical protein-protein interaction motifs such as SH2 or PDZ domains; however, electrostatic interactions between proteins can be highly specific (49). For example, binding of aldolase, phosphofructokinase, and glyceraldehyde-3-phosphate dehydrogenase occurs by electrostatic interactions with the extreme amino terminus of band 3 (10–12). Despite the non-physiological low salt and acidic pH conditions required to demonstrate binding of these glycolytic enzymes in vitro, immunofluorescence has shown that glyceraldehyde-3-phosphate dehydrogenase is membrane bound in vivo (50).

Unlike the binding of glycolytic enzymes, a significant association between GST-Ct and CAII was observed under near physiological buffer conditions. Half-maximal binding was obtained at pH 7.0 and at 100 mM NaCl (plus 50 mM Tris-HCl). The slow kinetics of the band 3-CAII interaction, measured in the solid phase assay, are similar to binding of band 4.2 or ankyrin to band 3. For example, saturation of band 4.2 binding to band 3 required 6 h (44). Binding of ankyrin required a 60–90-min incubation at 0 °C to reach saturation (45). Binding of a low (10 nM) concentration of GST-Ct to CAII had a $t_{1/2}$ of 12 h. Since the concentration of CAII in the erythrocyte is approximately 20 µM (24) and $k_{asso}$ rates are concentration dependent, the interaction in vivo may be more rapid. A conformational change or dependence on intracellular factors missing from the in vitro system may also be responsible for the slow binding. The highly acidic Ct is likely to have an extended structure in solution; binding to CAII may require the Ct to fold into a discrete structure. The binding may take place through a two-state type of model, initially low affinity binding followed by a conformation change allowing a higher affinity interaction.

Beyond the Erythrocyte—A complex between CAII and band 3 may have significance beyond the red cell. CAII and band 3 are also found in the a-intercalated cells of the mammalian kidney (51, 52). The human kidney expresses an alternatively spliced version of band 3 (AE1b) that is missing the first 65 residues found in erythrocyte band 3 but is otherwise identical, including the Ct region (53). In these cells, basolateral band 3 extrudes bicarbonate while an apical proton-ATPase extrudes acid. Interaction of CAII with the Ct region of band 3 suggests that CAII may also form a complex with the kidney isoform of band 3 on the basolateral membrane. The Ct region of AE2 shares 60% sequence identity with AE1. We are presently testing whether CAII can also form a complex with AE2, a widely expressed anion exchanger.

Tethering CAII to the Ct region of band 3 may be another example of metabolic channeling (29, 30). By physically linking two enzymes, substrate diffusion requirements are decreased, and high local concentrations of substrate are produced for efficient enzyme function (29). Metabolic channeling, in the form of ATP compartmentalization, has been demonstrated in the red cell (54). The membrane-bound glycolytic enzymes produce ATP that becomes compartmentalized at the membrane surface. This pool of ATP is used to fuel the sodium-potassium ATPase (55, 56).

Our findings suggest that CAII is tethered to the Ct region of band 3 at the membrane surface. The partition coefficient for CO$_2$ in the erythrocyte membrane at 37 °C is around 1.6 (57). The concentration of CO$_2$ in the membrane provides greater availability of CO$_2$ for the membrane-associated CAII and consequently bicarbonate for band 3. This substrate pool would presumably allow maximum efficiency for removing HCO$_3^-$ from the red blood cell in the peripheral tissues. In the lungs, this process would be reversed, and band 3-bound CAII would dehydrate incoming HCO$_3^-$ to CO$_2$, which would then diffuse out of the erythrocyte. Therefore, binding of CAII to the Ct region of band 3 may facilitate the efficient transport and removal of CO$_2$ from the tissues to the lungs.

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