Identification of the Carbonic Anhydrase II Binding Site in the Cl^-/HCO_3^- Anion Exchanger AE1†

John W. Vince and Reinhart A. F. Reithmeier*

Medical Research Council Group in Membrane Biology, Departments of Medicine and Biochemistry, Room 7344, Medical Sciences Building, University of Toronto, Toronto, Canada M5S 1A8

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ABSTRACT: The human Cl^-/HCO_3^- anion exchanger (AE1) possesses a binding site within its 33 residue carboxyl-terminal region (Ct) for carbonic anhydrase II (CAII). The amino acid sequence comprising this CAII binding site was determined by peptide competition and by testing the ability of truncation and point mutants of the Ct sequence to bind CAII with a sensitive microtiter plate binding assay. A synthetic peptide consisting of the entire 33 residues of the Ct (residues 879−911) could compete with a GST fusion protein of the Ct (GST-Ct) for binding to immobilized CAII, while a peptide consisting of the last 16 residues (896−911) could not. A series of truncation mutants of the GST-Ct showed that the terminal 21 residues of AE1 were not required for binding CAII. Removal of four additional residues (887−890) from the Ct resulted in loss of CAII binding. Acidic residues in this region (D887ADD) were critical for binding since mutating this sequence in the GST-Ct to DAAA, AAAA, or NANN caused loss of CAII binding. A GST-Ct construct mutated to D887ANE, the homologous sequence in AE2, could bind CAII. AE2 is a widely expressed anion exchanger and has a homologous Ct region with 60% sequence identity to AE1. A GST fusion protein of the 33 residue Ct of AE2 could bind to CAII similarly to the Ct of AE1. Tethering of CAII to an acidic motif within the Ct of anion exchangers may be a general mechanism for promoting bicarbonate transport across cell membranes.

The erythrocyte Cl^-/HCO_3^- anion exchanger (AE1)[1] and carbonic anhydrase (CA) catalyze interconnected processes involved in blood gas exchange (*1*−*3*). In the peripheral tissues, CO_2 diffuses into the erythrocyte and is hydrated by CA and the resulting bicarbonate is transported into the blood plasma by AE1. In the lungs, the process is reversed: bicarbonate enters the red cell via AE1 and is dehydrated by CA, and the resulting CO_2 diffuses out of the red cell. This process of CO_2 and bicarbonate exchange across the membrane is described by the Jacobs−Stewart cycle (*4*).

Human erythrocytes contain two major isoforms of CA. CAI comprises 85% of the total red blood cell CA, while the remainder is predominantly CAII, a higher activity isoform (*1*). Patients lacking CAI have no serious medical disorders (*5*), while CAII deficiency is associated with osteopetrosis, renal tubular acidosis, and cerebral calcification (*6*). Human AE1 (band 3) is a 911 amino acid polytopic membrane protein and a member of a family of anion exchangers that catalyze the electroneutral exchange of Cl^- for HCO_3^- (3, 7−10). AE1 possesses a 360 residue amino-terminal domain that mediates protein−protein interactions with a number of cytoskeletal proteins and glycolytic enzymes (*11*). This domain can be removed from the membrane-spanning domain of the protein without affecting anion transport (*12*). The membrane domain is predicted to possess 12−14 transmembrane segments (*13*, *14*) and terminates in an acidic 33 residue cytoplasmic carboxyl-terminal region (Ct) (*15*, *16*).

Erythrocyte membranes have been shown to bind CAII and to stimulate its enzymatic activity (*17*). It was later shown that the rate of reaction of CAII with a fluorescent inhibitor was perturbed when AE1 was modified with a stilbene disulfonate inhibitor (*18*). This finding suggested some form of interaction between AE1 and CAII since the effect of an AE1 inhibitor binding extracellularly affected CAII intracellularly. We showed recently that AE1 does possess a binding site for CAII and that this binding site is contained within the 33 residue Ct of AE1 (*19*). In our study, immunofluorescence was used to show that lectin-mediated agglutination of AE1 in ghost membranes caused a similar redistribution of CAII. This finding, similar in concept to that reported by Solomon's group (*18*), suggested a physical link between AE1 and CAII. Furthermore, a fraction of the erythrocyte CAII remained associated with the cell membrane and CAII could be coimmunoprecipitated with solubilized AE1. A sensitive microtiter plate assay with immobilized CAII was developed to localize the region on AE1 responsible for binding CAII. With this assay it was shown that both intact AE1 and the membrane domain of AE1 (which lacks the first 360 residues) could bind CAII similarly, indicating that the binding site was not in the amino-terminal domain. Finally, a GST fusion protein of the 33 residue Ct of AE1 (GST-Ct) could specifically bind CAII. Ligand

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* To whom correspondence should be addressed: Tel (416) 978-7739; Fax (416) 978-8765; E-mail r.reithmeier@utoronto.ca.

[1] Abbreviations: AE, anion exchanger; BSA, bovine serum albumin; CA, carbonic anhydrase; Ct, carboxyl-terminal region; GST, glutathione-S-transferase; GST-Ct, glutathione-S-transferase fusion protein of the 33 residue carboxyl-terminal region of human anion exchanger 1.

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affinity blotting of immobilized CAI and CAII with the GST-Ct demonstrated that the interaction was specific to the CAII isoform. These findings demonstrated that a membrane transporter, like AE1, could be directly and specifically coupled to an enzyme producing its substrate.

In the present study, the residues within the Ct of AE1 important for CAII binding were determined. We predicted that acidic clusters on the Ct of AE1 would be important for the interaction since the binding of the Ct to CAII was ionic strength- and pH-dependent (*19*). The rate of binding of GST-Ct to CAII was enhanced by acidic pH, with a half-maximal effect at pH 7, and by low ionic strength, with a half-maximal effect at 70 mM NaCl (in 50 mM Tris-HCl, pH 7). To test the role of acidic clusters in the Ct, binding of a series of truncation mutants of the Ct sequence to immobilized CAII was measured. The results showed that the Ct sequence 879−890 of human AE1 was sufficient to bind CAII. This region of the Ct is predicted to protrude into the cytosol from the last putative transmembrane segment of AE1, suggesting that CAII is bound to AE1 in close proximity to the membrane. Point mutations of the Ct further showed that an acidic cluster of residues (D887ADD) was required for CAII binding. Finally, it was discovered that AE2, another member of the AE1 family of transporters, could also bind to CAII. Since AE2 and CAII are found together in a number of cell types, this suggested that an interaction between anion exchangers and CAII may be important in tissues other than the red cell and may contribute to the efficiency or regulation of HCO_3^- transport and metabolism.

EXPERIMENTAL PROCEDURES

Materials. The following is a list of products and their suppliers (in parentheses): *Escherichia coli* strains DH5α and BL21, pGEX-5x-1 plasmid, DEAE-Sepharose 4B, glutathione−Sepharose 4B, goat anti-GST serum and the T7 sequencing kit (Amersham Pharmacia Biotech); *E. coli* strain BL21 (DE3) (Novagen); bovine serum albumin (BSA), glutathione, *o*-phenyldiamine, and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (Sigma); restriction enzymes, calf intestinal phosphatase (New England Biolabs); secondary antibodies and the biotinylated peroxidase/avidin system (Vector Laboratories); Transformer site-directed double-stranded mutagenesis kit (Clontech); rabbit anti-human CAII serum (Serotec); chemiluminescence kit (Roche−Boehringer Mannheim). A Ct peptide of the last 33 residues of human AE1 (879−911) with the mutation C885A (to prevent disulfide cross-linking) and a peptide consisting of the last 16 residues of human AE1 (896−911) were synthesized by solid-phase Fmoc chemistry and purified by HPLC (HSC Biotech Services).

Plasmid Construction. A pBluescript II SK(+) vector (Stratagene) containing the entire human AE1 sequence was the generous gift of Drs. A. M. Garcia and H. Lodish, Whitehead Institute. The construction of GST-Ct in the pGEX-5x-1 vector has been previously described (*19*).

The 33 residue Ct of human AE2 was subcloned from a pSP6/T7-AE2-1 plasmid that was the generous gift of Dr. Heribert Appelhans, Max-Planck-Institut für Biophysik. The Ct residues were amplified by PCR using two primers designed to generate *Bam*HI sites at each end of the sequence: 5′-CCC GGA TCC TCT AGA CAT CCC ACC AGC CTG TCCC-3′ and 5′-CCG GAT CCT CAC CGA CCG AGA GAT GAA ATG-3′. The amplified sequence was gel-purified, digested with *Bam*HI, and ligated into the pGEX-5x-1 vector that had been treated with *Bam*HI and calf intestinal phosphatase. Clones containing the insert were identified by restriction enzyme digestion with *Bam*HI and the correct orientation was confirmed by DNA sequencing (*20*).

Mutagenesis. Oligonucleotide-directed mutagenesis was performed with the double-stranded mutagenesis system from Clontech. Each GST-Ct truncation mutant was made by mutating the endogenous codon to a stop codon at the appropriate location in the Ct sequence. Mutations were confirmed by DNA sequencing (*20*).

GST Fusion Protein Expression and Purification. The various GST-Ct mutants or GST were expressed in *E. coli* BL21 cells and purified on glutathione−Sepharose. The peak fractions were pooled and further purified on DEAE-Sepharose 4B. DEAE-bound fusion protein was washed with 50 mM Tris-HCl, pH 7.4, and 1 mM DTT, and eluted with a linear salt gradient (0−400 mM NaCl). Purity was assessed by SDS−PAGE analysis and Western blotting with a polyclonal goat anti-GST serum.

CAII Purification. The pACA plasmid containing the human CAII gene was a generous gift of Dr. C. Fierke (Duke University). The expression of recombinant CAII in *E. coli* BL21 (DE3) cells and subsequent purification has been described (*21*). The enzymatic activity of the purified protein was tested by an esterase assay (*22*) and found to be equal to that of commercially available enzyme (Sigma).

Microtiter Plate Binding Assay. As previously described (*19*), purified CAII (200 ng/well) was covalently immobilized onto 96 well microtiter plates by incubating the protein in the plates with 1.25 mg/mL 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate in 150 mM NaCl and 100 mM sodium phosphate, pH 6, for 30 min at room temperature (*23*, *24*). The plates were washed extensively with phosphate-buffered saline (PBS; 150 mM NaCl and 5 mM sodium phosphate, pH 7.5) and then blocked for 2 h at room temperature in PBS supplemented with 2% BSA. Plates were washed with antibody (Ab) buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 1 mM DTT, and 0.25% gelatin) and incubated with the GST-Ct, GST-Ct truncations, GST-Ct point mutants, or GST as a control, for 18 h at room temperature. Our previous study (*19*) showed that under these conditions the binding reaction was nearly complete after 18 h. The plates were incubated sequentially in goat anti-GST serum [1:5000 dilution (v/v), 2 h], biotinylated affinity-purified rabbit anti-goat IgG (1:5000, 2 h), and then avidin/biotinylated peroxidase (1:10 000, overnight). This was followed by incubation with the peroxidase substrate *o*-phenyldiamine and detection of enzymatic activity at 450 nm in a ThermoMax microplate reader (Molecular Devices) connected to a Macintosh workstation. The linear, nonspecific binding of the GST control was subtracted from the curves. To determine the capacity of the wells on the microtiter plate for CAII, various amounts of the enzyme (0−400 ng) were immobilized and detected with an anti-CAII serum followed by development as described above.

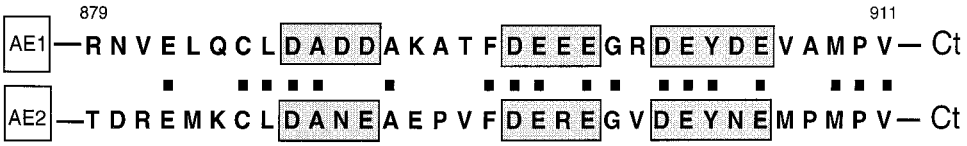

FIGURE 1: Amino acid sequences of the 33 residue Ct of human AE1 and AE2. The shaded areas indicate the three acidic clusters in both proteins. The box to the left of each sequence represents the end of the last transmembrane segment. Solid squares indicate conserved residues. The residue numbers for the first and last amino acids in the Ct of human AE1 are indicated.

Peptide Competition Studies. Competition was performed by mixing GST-Ct or GST control (25−50 nM) in Ab buffer with increasing concentrations of the 16 (residues 896−911) or the 33 residue peptide (residues 879−911) corresponding to the Ct sequence of human AE1. Samples were added to microtiter plates and incubated as described above.

RESULTS

Experimental Rationale. In the present study, residues within the Ct of human AE1 that are important for binding CAII were identified. The Ct of AE1 contains three acidic clusters (Figure 1). We hypothesized that one or more of these acidic clusters would be involved in binding CAII, since our previous work showed that the interaction between AE1 and CAII was pH- and ionic strength-dependent (*19*). To investigate the interaction between the Ct and CAII, a solid-phase binding assay was used in which CAII was immobilized on microtiter plates. The immobilized CAII was incubated with various concentrations of GST-Ct fusion proteins and the amount of bound GST-Ct was determined by using an anti-GST antibody. The binding reaction occurred with a half-time of 12 h and required an 18 h incubation period to reach completion under conditions of physiological ionic strength and pH (*19*). The binding assay is an enzyme-linked immunosorbent assay (ELISA) and therefore does not measure true equilibrium binding but rather the amount of fusion protein tightly bound to immobilized CAII. To examine the role of specific residues in the Ct in binding to CAII, a series of GST-Ct truncation and point mutants were constructed and their ability to bind CAII was tested by this assay under identical buffer conditions. As a control, the binding of GST was determined in parallel and was typically less than 15% that of GST-Ct.

Competition with Synthetic Peptides. A competition experiment with either a 33 residue synthetic peptide encoding the entire Ct (879−911) or a synthetic peptide of the last 16 residues of the Ct (896−911) is shown in Figure 2. The 33 residue peptide contains all three acidic clusters, D887ADD, D896EEE, and D902EYDE, while the 16 residue peptide possesses only the last two. The peptide encoding the entire Ct was able to compete with GST-Ct for CAII binding with a half-maximal concentration of 100 μM. The 16 residue peptide could not compete with GST-Ct for CAII binding at up to 10 000-fold molar excess. This suggested that the region containing the last two acidic sequences was not sufficient for binding CAII.

The value of 100 μM for the apparent K_i of the intact peptide was much higher than the half-maximal concentration of 20−100 nM previously determined for AE1 or GST-Ct binding to immobilized CAII (*19*). This difference may be because the Ct is structured in the context of intact AE1 or GST-Ct while having a different conformation in the isolated peptide. In support of this, CD and NMR spectroscopy

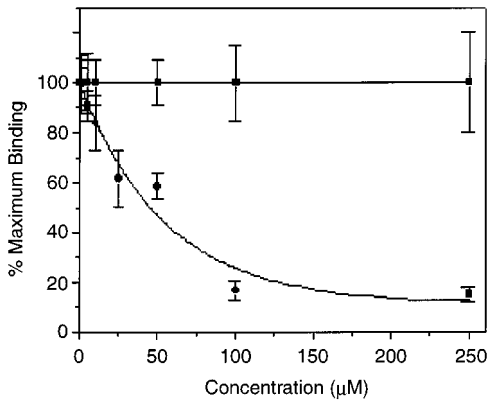

FIGURE 2: Competition of GST-Ct binding to immobilized CAII by Ct peptides. Squares (■) show that the binding of 25 nM GST-Ct to CAII was not affected by the presence of 0−250 μM of a 16 residue Ct peptide (residues 896−911 of human AE1). Circles (●) show a decreased binding of 50 nM GST-Ct to CAII caused by increasing amounts (0−250 μM) of a peptide encoding the entire 33 residues of the Ct (879−911).

indicated that the 33 residue peptide was unstructured in aqueous solution (data not shown). Alternatively, the higher affinity binding of GST-Ct and AE1 may be due to an avidity effect. GST-Ct and AE1 are dimers while the peptide is a monomer. The dimeric nature of GST-Ct may allow a two-point attachment to immobilized CAII if the density of CAII on the plate is sufficient. Binding of a single Ct to CAII could exhibit weak binding, while binding of the second Ct in the dimer to a second immobilized CAII would result in an enhanced interaction. To test whether avidity was a factor, the binding of GST-Ct to various amounts of immobilized CAII (0−400 ng/well) was determined. The amount of CAII immobilized on the plate was measured by direct antibody absorption. The binding of antibody to immobilized CAII increased with increasing amounts of CAII applied to the plate and reached a maximum level above 100 ng/well (Figure 3). This indicates that there was saturable binding of CAII to a limited number of activated sites on the microtiter plate surface. The binding of GST-Ct to CAII showed a sigmoidal relation, with binding minimal at low amounts of CAII but then rising rapidly to a maximal level (Figure 3). These results indicate that the binding of GST-Ct for CAII is likely enhanced by a two-point attachment to immobilized CAII. This avidity effect displayed by dimeric GST fusion proteins is advantageous for examining weak interactions between proteins that might otherwise be difficult to detect by other types of assays.

Truncation Mutants. The sequence within the Ct of AE1 responsible for the interaction with CAII was investigated by constructing a series of GST-Ct truncation mutants. Five mutants of GST-Ct lacking the last 5 (Trunc5), 10 (Trunc10), 16 (Trunc16), 21 (Trunc21), or 25 (Trunc25) residues of the Ct were made (Figure 4, top). These mutants were expressed

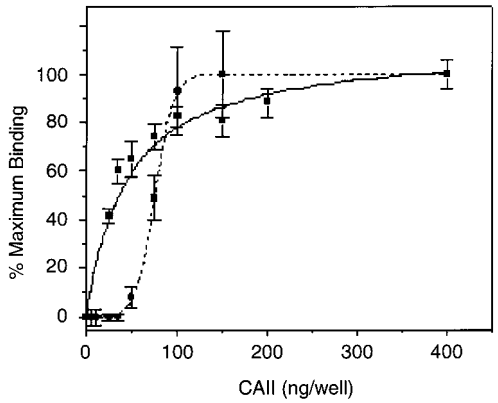

FIGURE 3: Comparison of anti-CAII serum (■) and GST-Ct (●) binding to increasing amounts of immobilized CAII. Binding of the antibody shows saturable binding, while GST-Ct binding follows a sigmoidal curve.

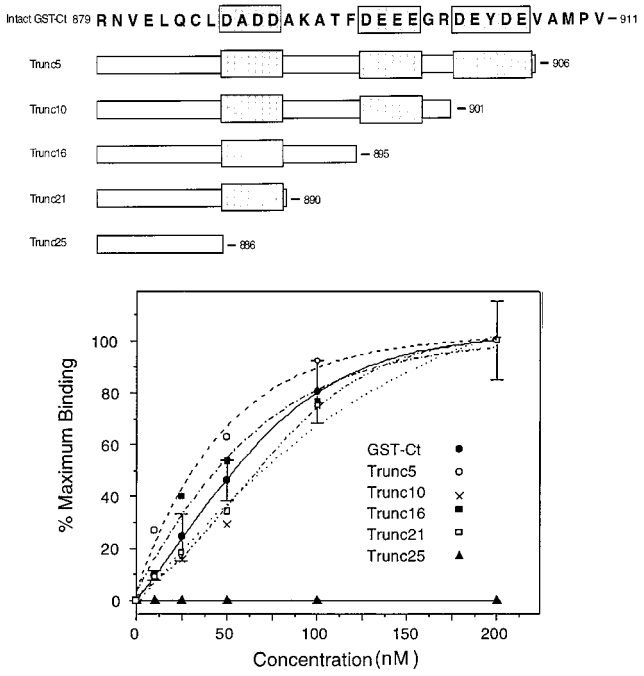

FIGURE 4: (Top) Truncation mutants of GST-Ct were constructed by inserting stop codons into the GST-Ct sequence. The acidic patches are shaded and the residues remaining in each truncation are shown in schematic form. (Bottom) Concentration dependence of binding of GST-Ct and a series of GST-Ct truncation mutants to immobilized CAII. Various concentrations of GST-Ct (●), Trunc5 (○), Trunc10 (×), Trunc16 (■), Trunc21 (□), or Trunc25 (▲) were incubated with immobilized CAII as described under Experimental Procedures. For clarity, error bars (standard error) are shown only for GST-Ct; similar results were obtained for the truncated proteins.

in *E. coli* and purified in an identical manner to the GST-Ct construct. The immunoreactivity of these mutant proteins to the anti-GST sera was unchanged relative to GST-Ct (not shown). GST-Ct truncation mutants lacking the last 5, 10, 16, or 21 residues all retained the ability to bind to CAII (Figure 4, bottom). Although Figure 4 (bottom) shows some differences in the curves, the half-maximal binding values were similar and within experimental errors. These results support the peptide competition data shown in Figure 2 and further indicates little role for the distal part of the Ct of AE1 in CAII binding. Removal of a further four residues (Trunc25) resulted in a complete loss of binding (Figure 4, bottom). The loss of binding of Trunc25 to CAII was not a result of a change in the ionic strength dependence of the interaction, since this mutant still was unable to bind CAII under conditions of low ionic strength (data not shown). These results indicated that a 12 residue domain (residues 879−890) was sufficient for binding to CAII. Since these 12 residues are close to the end of the last transmembrane segment of AE1, this suggests that bound CAII is in very close proximity to the inner membrane surface of the erythrocyte.

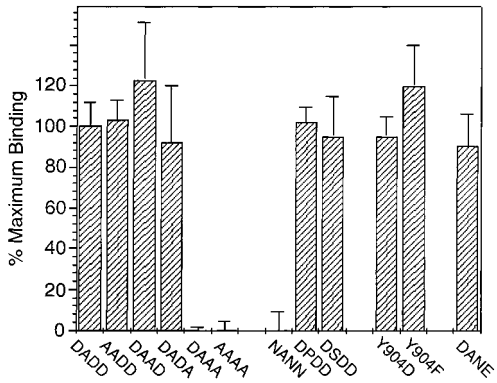

FIGURE 5: Relative binding for 25 nM GST-Ct (DADD) or various point mutants to immobilized CAII. Signals are plotted as a percentage of the wild-type sequence (DADD) binding. Binding of DAAA, AAAA, and NANN was not significant and was similar to background (GST) levels.

Point Mutants. The results from the peptide competition experiment and from the GST-Ct truncation mutants showed that residues 879−890 were sufficient for the interaction with CAII. Since removal of the D887ADD sequence resulted in a loss of binding and the interaction with CAII has an electrostatic component, this acidic region was targeted for mutagenesis. Figure 5 shows the relative binding to CAII of various D887ADD point mutants in GST-Ct at a concentration (25 nM) near the half-maximal binding value. Complete binding curves, similar to those shown in Figure 3, were done and indicated similar results. Figure 5 shows that loss of one negative charge in the DADD sequence had no effect since the mutants DADA, DAAD, and AADD all bound to CAII similarly to the wild-type sequence. This indicated that no individual acidic residue was essential for binding. However, elimination of two Asp residues (DAAA) or all three (AAAA) resulted in a complete loss of CAII binding. These results indicate that the charge on the DADD sequence was specifically involved in binding CAII and that the other distal, charged clusters at D896EEE and D902EYDE could not substitute in its absence.

The D887ADD sequence is predicted to form a reverse turn (*3*, *25*). Substitution of Asp by Ala not only eliminated a charge but also reduced the propensity for a turn (*25*). Table 1 shows the turn propensities of the various mutants, with a value of 0.5×10^{-4} being the minimum propensity to form a turn. To examine the role of the putative turn on binding, a mutant (N887ANN) was constructed in which the charge was eliminated while the turn propensity was predicted to be preserved. It was found that the NANN mutant did not bind CAII (Figure 5). In contrast, as shown in Figure 5, a mutant that was not predicted to form a turn (DAAD) retained the ability to bind CAII. To further explore the role of the putative turn in the interaction, the Ct mutants A888S

Table 1: Properties of CAII Binding Site Mutants

mutant	charge	turn propensity[a] ($\times 10^4$)	binding[b]
D A D D	−3	1.6	+
A A D D	−2	0.6	+
D A A D	−2	0.3	+
D A D A	−2	1.1	+
D A A A	−1	0.2	−
A A A A	0	0.1	−
N A N N	0	2.1	−
D P D D	−3	6.4	+
D S D D	−3	3.0	+
D A N E	−2	1.3	+

[a] Turn propensities derived from Chou and Fasman (*25*). The cutoff for a reverse turn is 0.5×10^{-4}. [b] + indicates similar binding to CAII as the wild type (DADD) sequence; − indicates no CAII binding.

and A888P were made. If the turn were important for the interaction, the mutation A888P might increase the binding of the Ct to CAII, since proline should increase the rigidity of the turn. However, Figure 5 shows that mutations A888P or A888S had no enhanced effect on the interaction as determined by the microtiter plate binding assay.

Role of Phosphorylation. Tyrosine 904 is known to be phosphorylated in AE1 (*26*). To examine whether this modification affected the ability of the Ct to bind CAII, Y904D and Y904F mutants were constructed. Neither mutation Y904D, to mimic the negative charge of a phosphate group, nor mutation Y904F affected CAII binding (Figure 5). When the GST-Ct construct itself was in vitro phosphorylated with Abl kinase, no change in binding to immobilized CAII was observed (data not shown). These results, using the microtiter plate binding assay, indicate that phosphorylation may not profoundly influence CAII binding and further supports the finding of little if any role of the distal Ct region of AE1 in the interaction with CAII.

AE2 Binding. AE2 possesses 60% sequence identity with AE1 in the Ct including the presence of three similar charged clusters (Figure 1). The corresponding D887ADD sequence in AE1 is DANE in AE2. As Figure 5 shows, mutation of D887ADD in the GST-Ct of AE1 to DANE did not affect binding to CAII, indicating that this motif in the context of AE1 was also capable of being recognized by CAII. To further confirm that the Ct of AE2 could bind to CAII, a GST fusion protein containing the 33 residue Ct of AE2 was constructed. This AE2 fusion protein could bind to CAII in a manner similar to the Ct of AE1 (Figure 6). This result suggests that binding of CAII to anion exchangers may be a general phenomenon occurring in cells that contain AE1 or AE2 and CAII.

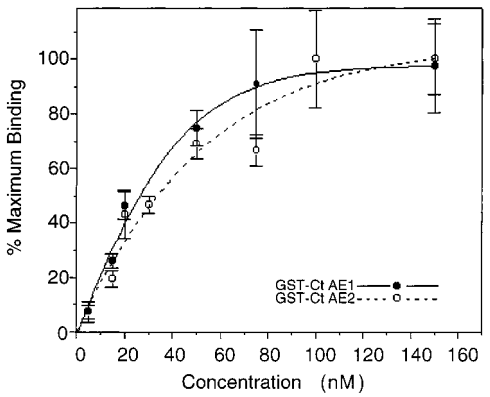

FIGURE 6: Comparison of binding of GST-Ct fusion proteins of AE1 (●) and AE2 (○) to immobilized CAII.

DISCUSSION

Identification of the CAII Binding Site on AE1. In this study, we identified the sequence D887ADD in the 33 residue Ct of human AE1 responsible for binding CAII. Within this sequence at least two negatively charged residues in the charged cluster were required for CAII binding. The ability of small motifs such as DADD to specifically mediate protein interactions is not uncommon. SH2 domains on some members on the Src family recognize a four residue motif of the type pTyr-Glu-Glu-Ile (*27*). In vesicle trafficking a DXE diacidic motif is required on the cytoplasmic tail of cargo molecules in order for them to be efficiently recruited to vesicles mediating export from the ER (*28*). Another example involving AE1 is seen in the binding of an IRRY motif in protein 4.1 to a LEEDY sequence in AE1 (*29*). In each of these three examples, a short, linear sequence of charged residues mediates a specific protein−protein interaction.

Conservation of the CAII Binding Site. The acidic cluster at D887ADD is conserved in most of the known AE1 protein sequences. Along with human AE1, mouse (DGDD), rat (DGDD), chicken (DTDD), and bovine (DADD) AE1 proteins possess DXDD sequences at this position. Trout AE1 possesses the sequence DASD, which retains two negative charges and therefore should bind CAII. Human, mouse, rat, and guinea pig all possess the DANE sequence in their AE2 proteins. Nearby residues E882, L886, and A891 are also conserved in the AE gene family. Since all of these species possess CAII isoforms, binding of anion exchangers to CAII may occur in these organisms as it does in human. AE2 and CAII are both widely expressed proteins found together in a number of cell types involved in bicarbonate absorption and secretion. For example, CAII and AE2 immunoreactivity colocalize in the same region of the corneal endothelium (*30*). In gastric parietal cells a pH-dependent association of CA with gastric light microsomal membranes has been observed (*31*). Like the interaction between the Ct of AE1 and CAII, this interaction is increased by acidic pH. It is possible that this interaction of CAII with the gastric membranes is mediated through the Ct of AE2.

Interestingly, the DADD sequence is also found in the cytosolic Ct of an unrelated erythrocyte membrane protein, the water channel (AQP1). It is possible that this protein also serves a role in sequestering CAII to the membrane. Additionally, it has recently been observed that AQP1, as well as facilitating water transport, may also serve as a conduit for CO_2 entry into the cell (*32*−*34*). The binding of CAII to AQP1 would bring CAII close to the transport pathway for CO_2 and H_2O. CO_2 and H_2O that enter the red cell via AQP1 might be the preferred substrates for membrane-bound CAII.

The CAII Binding Site Is Proximal to the Membrane. Truncation of the Ct of the GST-Ct fusion protein showed that 12 residues (879−890) were sufficient for binding CAII. Since the last transmembrane segment of AE1 is predicted to end at position 877 (*13*), the sequence 879−890 is immediately adjacent to the lipid bilayer. As an extended chain, this sequence is less than or equal to 40 Å from the

membrane, suggesting that CAII is tethered to AE1 in immediate proximity to the inner cell membrane surface. This location may ideally position CAII to hydrate incoming CO_2 and directly supply the AE1 transporter. The partition coefficient for CO_2 in the erythrocyte membrane at 37 °C is around 1.6 (*35*). CO_2 partitioning through the membrane would be rapidly hydrated by CAII, thus supplying AE1 with a localized substrate pool. In the lungs, bicarbonate that enters the red cell through AE1 could be immediately dehydrated by bound CAII.

A Capno (CO_2) Metabolon. The interaction between AE1 and CAII may be an example of a metabolon, a weakly associated complex of sequential metabolic enzymes as described in ref *36*. Examples of metabolic channeling have been documented for the enzymes involved in the urea cycle (*37*−*39*), and those comprising the Krebs cycle (*40*, *41*). In these examples, sequential enzymes or a metabolon may be important for channeling the products of one enzyme reaction to the next enzyme in a metabolic sequence (*42*).

One of the difficulties in determining the constituents of a metabolon is that the interacting enzymes are often only weakly associated (*39*). For example, there is evidence that the urea cycle enzymes carbamoyl phosphate synthetase 1 and ornithine transcarbamoylase are associated with the inner mitochondrial membrane, but this interaction is lost by mild detergent treatment or dilution (*38*). We found a weak interaction ($K_i = 100\ \mu M$) for a Ct peptide with CAII. This value was initially surprising since our previous work had suggested half-maximal binding occurred between 20 and 100 nM for the interaction between GST-Ct or AE1 and CAII (*19*). The reason for this difference appears to lie in an avidity effect observed in our microtiter plate assay. The higher value for the synthetic peptide may reflect the different oligomeric state of the peptide, which is monomeric, to that of AE1 and the GST fusion proteins, which are dimeric. The interaction of one Ct tail of the GST-Ct dimer may be weak and undetectable in the microtiter plate assay. Binding of the second Ct tail of the GST-Ct dimer to a second immobilized CAII would greatly enhance the interaction. This avidity effect proved advantageous for examining the interaction of GST-Ct with CAII since it served to increase the sensitivity of the assay. Since the concentration of CAII within the human erythrocyte is 20 μM (*43*), an affinity in this range would be significant in vivo. Our previous studies (*19*) support this conclusion and indicated that only a fraction of the CAII remains associated with AE1 after preparation of ghost membranes and solubilization of AE1.

A capno (Greek: smoke, CO_2) metabolon would involve a functional complex between AE1 and CAII in which the product of one step in the metabolic sequence of CO_2/HCO_3^- cycling is channeled to the next step. This complex forms a structural basis for the Jacobs−Stewart cycle, which describes the equilibrium of CO_2/HCO_3^- across the red cell membrane (*4*). CO_2 enters the red cell by diffusion (or by AQP1) and is hydrated to form HCO_3^- by CAII. The bicarbonate product is then directly channeled to AE1 and is transported out of the erythrocyte. The cycle completes itself in the lungs with HCO_3^- entering the cell through AE1, being channeled directly to CAII for dehydration and the CO_2 product exiting through the membrane. We are currently studying the effect of Ct mutants of AE1 that do not bind CAII on the rate of HCO_3^-/Cl^- exchange in transfected cells.

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