Localization of the Cl^-/HCO_3^- Anion Exchanger Binding Site to the Amino-Terminal Region of Carbonic Anhydrase II[†]

John W. Vince,[‡] Uno Carlsson,[§] 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, Ontario, Canada M5S 1A8, and IFM-Department of Chemistry, Linköping University, SE-581 83 Linköping, Sweden

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ABSTRACT: Human carbonic anhydrase II (CAII) possesses a binding site for an acidic motif (D887ADD) within the carboxyl-terminal region (Ct) of the human erythrocyte chloride/bicarbonate anion exchanger, AE1. In this study, the amino acid sequence comprising this AE1 binding site was localized to the first 17 residues of CAII, which form a basic patch on the surface of the protein. Truncation of the amino terminal of CAII by five residues resulted in a 3-fold reduction in the apparent affinity of the interaction with a GST fusion protein of the Ct of AE1 (GST-Ct) measured by a sensitive microtiter plate binding assay. Further amino-terminal truncation of CAII by 17 or 24 residues caused a loss of binding. The homologous isoform CAI does not bind AE1, despite having 60% sequence identity to CAII. One major difference between the two CA isoforms, within the amino-terminal region, is a high content of histidine residues in CAII (His3, -4, -10, -15, -17) not found in CAI. Mutation of pairs of these histidines (and one lysine) in CAII to the analogous residues in CAI (H3P/H4D or K9D/H10K or H15Q/H17S), or combinations of these various double mutants, did not greatly affect binding between GST-Ct and the mutant CAII. However, when all six of the targeted CAII residues were mutated to the corresponding sequence in CAI, binding of GST-Ct was lost. These results indicate that the AE1 binding site is located within the first 17 residues of CAII, and that the interaction is mediated by electrostatic interactions involving histidine and/or lysine residues. Further specificity for the interaction of AE1 and CAII is provided by a conserved leucine residue (L886) in AE1 that, when mutated to alanine, resulted in loss of GST-Ct binding to immobilized CAII. The binding of the basic amino-terminal region of CAII to an acidic Ct in AE1 provides a structural basis for linking bicarbonate transport across the cell membrane to intracellular bicarbonate metabolism.

Carbonic anhydrase (CA)[1] and the erythrocyte Cl^-/HCO_3^- anion exchanger (AE1) catalyze interconnected reactions important in CO_2 metabolism and bicarbonate homeostasis. Carbonic anhydrases catalyze the reversible hydration/dehydration of CO_2/HCO_3^- and are found in a variety of cell types (*1*−*3*). Human red blood cells possess CAI, CAII, and trace amounts of CAIII (*4, 5*). CAI is a homologous isoform to CAII, possessing 60% sequence identity. CAI is found in the erythrocyte in 5−6-fold excess to CAII, but its role is unclear. Humans genetically lacking CAI have no detectable clinical abnormalities (*6*). This is in contrast to CAII deficiency which is associated with osteopetrosis, renal tubular acidosis, and cerebral calcification (*7*).

AE1 (Band 3) is the Cl^-/HCO_3^- anion exchanger of red blood cells, and is a member of a family of anion exchangers (*8*−*10*). In the red blood cell, AE1 catalyzes the electroneutral exchange of chloride for bicarbonate, thus removing bicarbonate from the limited volume within the cell to be subsequently transported in the plasma (*11*−*13*).

In previous reports (*14, 15*), we identified and characterized the interaction between human AE1 and the high-activity CA isoform, CAII. In the first of these reports (*14*), we localized the human CAII binding site within the 33 residue carboxyl-terminal region (Ct) of AE1. The Ct of AE1 protrudes into the cytoplasm and is highly negatively charged (*16, 17*). We demonstrated a specific interaction between the Ct of AE1 and CAII using cosolubilization, coimmunoprecipitation, immunofluorescence, peptide competition, and a sensitive microtiter plate assay utilizing binding of a GST-fusion protein of the Ct (GST-Ct) to immobilized CAII. In a subsequent investigation of the interaction of AE1 and CAII, we made truncation and point mutations of the GST-Ct construct to discover that the CAII binding site was within a 12 residue sequence in AE1 (R879−D890) (*15*). This short sequence was adjacent to the last predicted transmembrane span and possessed a D887ADD sequence that was critical for binding. The role of charged residues in the interaction with CAII was not surprising, given that AE1 binding to CAII is pH and ionic strength dependent (*14*). In the current

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

[‡] University of Toronto.

[§] Linköping University.

[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 AE1; HEK, human embryonic kidney; Nt, amino-terminal region.

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study, we examined whether there was an oppositely charged region in CAII that could provide a complementary, electrostatic surface for the negative Ct of AE1.

Despite the high sequence similarity between CAI and CAII, only CAII is able to bind AE1 specifically. One of the main differences in the primary structure of these two proteins is a high content of histidine residues within the first 20 residues of CAII, not found in CAI (Figure 1). Based on the strong electrostatic component to the interaction and because CAI does not bind AE1, we hypothesized that these histidine residues were involved in the interaction. To test this hypothesis, we examined the ability of GST-Ct to bind to immobilized amino-terminal region (Nt) truncation mutants of CAII. Results from these experiments demonstrated that the first 17 residues of CAII were required for AE1 binding. Further experiments with point mutations in this region of the CAII sequence, replacing the basic residues in CAII with that found in CAI, indicated that histidine and lysine residues were involved in the interaction with AE1.

MATERIALS AND METHODS

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).

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 (*14*).

Truncations and Mutagenesis. Bacterial strains containing the plasmid constructs for the truncated variants of hCAII were kindly provided by Dr. B.-H. Jonsson and Dr. G. Aronsson, Umea University, Sweden. Truncation mutants lacking the first 5, 17, or 24 residues of CAII were prepared and purified as described previously (*18*). The CAII and the truncations had the mutation C206S, which does not affect the enzyme activity (*19*). The Trunc5 sequence begins at Gly6 (Figure 1). Trunc17 begins at D19 and retains an initial methionine. In the wild-type sequence and the other truncation mutants, the amino-terminal methionine is removed by methionyl aminopeptidase which excises this amino acid when the penultimate residue is small (*18, 20*).

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 have 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). Mutant pACA constructs were expressed in an identical manner; all of the constructs had esterase activity at similar levels to the wild-type sequence. Oligonucleotide-directed mutagenesis was performed with the double-stranded mutagenesis system from Clontech. For this procedure, the selection oligonucleotide, designed to eliminate a unique *Bam*HI site and introduce a unique *Sna*I site, was 5′-GCA ACT TCC CTG CGT ATA CTC AAC AAT GGT CAT GC-3′. In the first round of mutagenesis, the following oligonucleotides were also used (mutated nucleotides have been underlined): H3P/H4D, 5′-GG AGA TAT ACC ATG GCC CCT CAG TGG GGG TAC GGC-3′; K9D/H10K, 5′-C CAT CAC TGG GGG TAC GGC GCA AAG AAC GGA CCT GAG C-3′; H15Q/H17S, 5′-GC AAA CAC AAC GGA CCT GAG CAG TGG TCT AAG GAC TTC CCC-3′. Subsequent combinations of mutations were constructed using the mutant constructs as templates and the appropriate oligonucleotide listed above. Mutations were confirmed by DNA sequencing (*23*).

GST Fusion Protein Expression and Purification. The various GST-Ct constructs of human AE1 or GST were expressed in *E. coli* BL21 cells and purified using 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, 1 mM DTT, and eluted with a linear salt gradient (0−400 mM NaCl). Purity was assessed by SDS−PAGE analysis and Western blotting using a polyclonal goat anti-GST serum.

Microtiter Plate Binding Assay. This assay has been previously described (*14, 15*). Briefly, purified CAII or CAII mutants were covalently immobilized onto 96 well microtiter plates by incubating the protein in the plates with 1.25 mg mL^{-1} 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate in 150 mM NaCl, 100 mM sodium phosphate, pH 6, for 30 min at room temperature (*24, 25*). Plates were washed with antibody (Ab) buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% Triton X-100, 5 mM EDTA, 0.25% gelatin) and incubated with various concentrations of purified GST-Ct or GST-Ct mutants in Ab buffer. Bound fusion proteins were detected by incubating the plates sequentially in goat anti-GST serum, biotinylated affinity-purified rabbit anti-goat IgG, and peroxidase-labeled biotin/avidin. 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.

RESULTS AND DISCUSSION

The Ct of human AE1 contains an acidic sequence, D887ADD, required for binding of CAII (*15*). Due to the electrostatic component in the interaction between CAII and negatively charged residues on AE1 (*14*), we hypothesized that a positively charged region on CAII may be involved in the interaction. In addition, the interaction showed a marked pH dependence with increased binding under acidic conditions and a half-maximal effect at pH 7, implicating histidine residues (*14*). Human CAII binds to AE1 while the homologous isoform CAI does not. We, therefore, compared the amino acid sequences of the two enzymes in order to identify basic residues in CAII that might be involved in

A.

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                    10        20        30        40        50        60
                    |         |         |         |         |         |
Human CAII   -MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRI
Human CAI    MASPDWGYDDKNGPEQWSKLYPIANGNNQSPVDIKTSETKHDTSLKPISVSYNPATAKEI
               *  ***   **** * *  *** *  ****** *   * * **** ****  **   *

                    70        80        90        100       110       120
                    |         |         |         |         |         |
Human CAII   LNNGHAFNVEFDDSQDKAVLKGGPLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELH
Human CAI    INVGHSFHVNFEDNDNRSVLKGGPFSDSYRLFQFHFHWGSTNEHGSEHTVDGVKYSAELH
              * ** * * * *     ******    *** ********    *******  ** ****

                   130       140       150       160       170       180
                    |         |         |         |         |         |
Human CAII   LVHWN-TKYGDFGKAVQQPDGLAVLGIFLKVGSAKPGLQKVVDVLDSIKTKGKSADFTNF
Human CAI    VAHWNSAKYSSLAEAASKADGLAVIGVLMKVGEANPKLQKVLDALQAIKTKGKRAPFTNF
               ***  **     *    ***** *   *** * * **** * *  ****** * ****

                   190       200       210       220       230       240
                    |         |         |         |         |         |
Human CAII   DPRGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNGEGEPEE
Human CAI    DPSTLLPSSLDFWTYPGSLTHPPLYESVTWIICKESISVSSEQLAQFRSLLSNVEGDNAV
             **  *** *** ******** *** * ****  ** *******   ** *  * **

                   250       260
                    |         |
Human CAII   LMVDNWRPAQPLKNRQIKASFK
Human CAI    PMQHNNRPTQPLKGRTVRASF-
              *  * ** **** *   ***
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B.

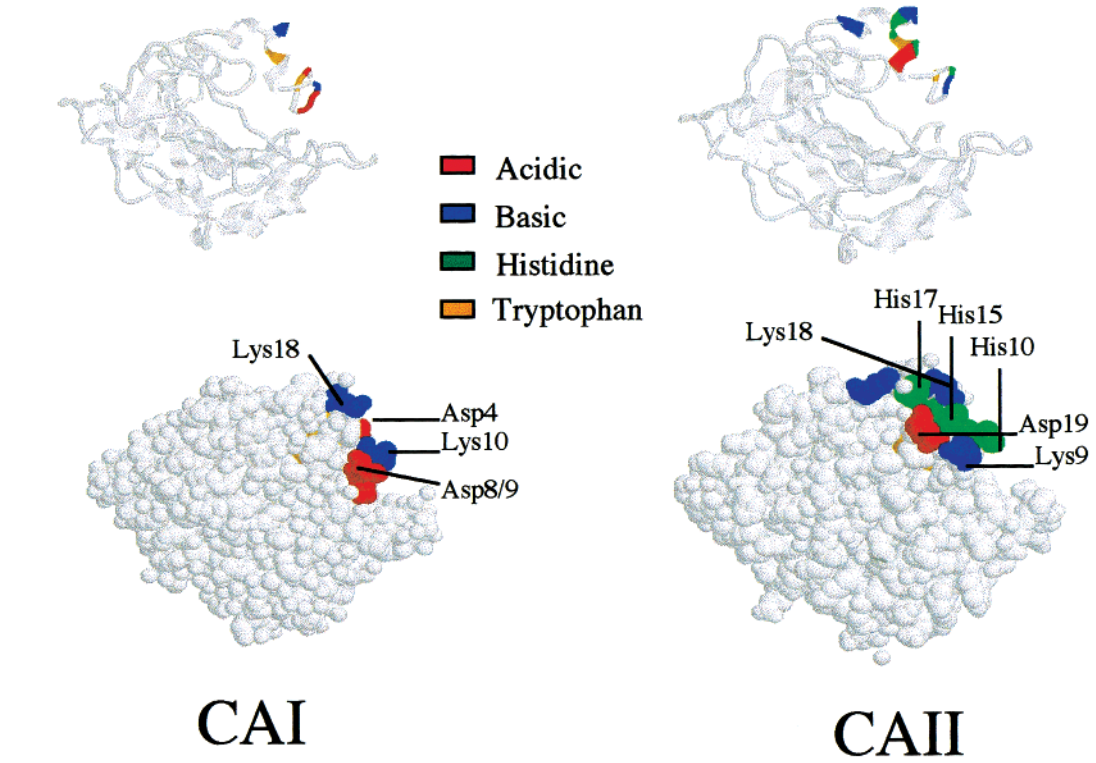

FIGURE 1: Comparison of human CAI and CAII. (A) Sequence alignment of CAI and CAII. The stars indicate identical residues. Basic residues (K, R, and H) found in CAII, but not in CAI, are highlighted. (B) Crystal structures of CAI and CAII. Structures shown as ribbon (upper) and space-filling (lower) projections. Structural data were obtained from the Protein Databank, and images were viewed using RasMol software. Selected residues have been highlighted. In the legend, basic residues include Lys and Arg; acidic residues include Asp and Glu. The Nt region of CAII is a surface-exposed helical domain containing histidine residues that form a basic stripe not found in CAI.

the interaction with the acidic tail of AE1 that are absent in CAI. The sequence analysis (Figure 1A) revealed that CAII contains a highly basic amino-terminal region (Nt), not present in CAI. The first 20 residues of the Nt of CAII have

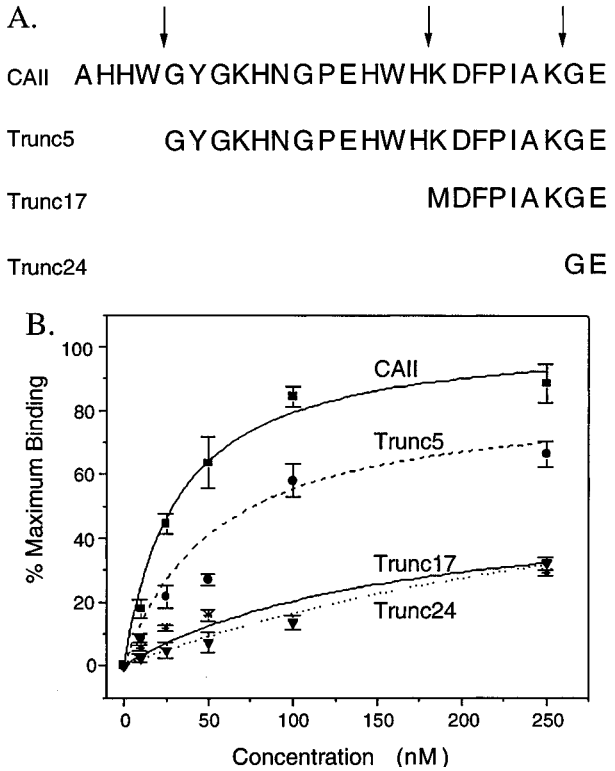

FIGURE 2: Effect of Nt truncation of CAII on binding of GST-Ct. (A) Schematic of the truncation mutants of CAII. See Materials and Methods for a complete description of Trunc mutants. (B) Binding of GST-Ct to immobilized CAII or truncation mutants of CAII. CAII wild-type and truncation sequences were expressed, purified, and immobilized onto microtiter plates as described under Materials and Methods. Immobilized CAII (squares), Trunc5 (circles), Trunc 17 (cross), or Trunc 24 (triangles) was incubated with various concentrations of GST-Ct or GST. GST binding was typically less than 10−15% of GST-Ct binding at a given concentration and has been subtracted. Standard error is indicated ($n = 4$).

50% sequence identity with the CAI sequence. Interestingly, 5 of the first 20 residues in CAII are histidine, while there are none in the homologous region of CAI. The Nt of CAII is highly exposed to solvent, folds independently of the rest of the protein (*18*), and has few contacts with the rest of the protein (*26*−*28*). The folded structure of CAII is similar to that of CAI, a protein for which a detailed analysis of the structural domains revealed few contacts between the Nt and the rest of the molecule (*29*). Additionally, previous work on CAII has shown that the protein unfolds in a three-state process and that the Nt truncations cause only a moderate destabilization of the native state (4−5 kcal/mol) relative to the intermediate state but have no effect on the stability of the intermediate state relative to the unfolded state (*18*). Our results indicate that the function of the basic Nt region of CAII is to provide a binding site for the acidic Ct of AE1.

Nt Truncations of CAII Cause Loss of AE1 Binding. Truncation of the Nt of CAII by 5, 17, or 24 residues resulted in mutant proteins that retain high enzymatic activity (*18*). We examined whether the truncated proteins retained the ability to bind the Ct of AE1 using a sensitive microtiter plate assay. Nt truncations of CAII lacking the first 5 (Trunc5), 17 (Trunc17), or 24 amino acids (Trunc24) were expressed and purified as described above (Figure 2A). The full-length and mutant CAII proteins were immobilized on microtiter plates and incubated with various concentrations of GST-Ct or GST as a control. All three of the mutant CAII proteins had similar esterase activity and coupling efficiency to the microtiter plates as the wild-type protein (data not shown). Figure 2B shows that all three of the truncated CAII proteins had altered GST-Ct binding compared to the full-length CAII. Trunc5 displayed a 3-fold decrease in the apparent affinity of the interaction with GST-Ct. Maximal binding of GST-Ct to Trunc5, similar to full-length CAII, was achieved at higher concentrations of GST-Ct. Further truncation of the Nt sequence, as in Trunc17 and Trunc24, resulted in a profound decrease in GST-Ct binding (Figure 2B). These results indicate the importance of the Nt region of CAII for the interaction with the Ct of AE1.

Mutation of CAII Residues to CAI. The first 17 residues of CAII posseses 5 histidine residues and 1 lysine that are absent in the homologous sequence of CAI (Figure 1A). To examine the role of these basic residues within the Nt region of CAII for binding AE1, the 5 histidines and 1 lysine residue in CAII were mutated to the analogous amino acids in CAI (Figure 3A). These residues were chosen based partly on the crystal structure of CAII (*26*) versus CAI (*30*), which appears to show a basic stripe, comprised of these residues (Figure 1B). In the CAII structure, the His residues 10, 15, and 17 as well as Lys9 appear to form a charged stripe. His3 and -4 are likely along this stripe as well but are not ordered enough to be seen in the crystal structure. In contrast, no such basic stripe is present in the corresponding CAI sequence.

The effect of mutation of any two of the charged residues (H3P/H4D or K9D/H10K or H15Q/H17S) was minimal (Figure 3B,C). In all cases, the esterase activity and immunoreactivity of the mutant proteins was unchanged, suggesting that they were properly folded (data not shown). GST-Ct bound to all three of the CAII mutants with similar characteristics as to the wild-type sequence (Figure 3B,C). In a subsequent round of mutagenesis, the pairs of mutants were combined to yield mutant proteins in which four of the six selected residues were mutated to the equivalent CAI residues. The results were similar; mutation of four of the six residues caused only a slight diminution on GST-Ct binding. In the final round of mutagenesis, all six residues in CAII were mutated to the CAI residues. In this case, binding of the Ct of AE1 to CAII was lost, indicating a role for these residues in the interaction (Figure 3B,C). Since the six residues in CAII were mutated to the equivalent residues in CAI and the Nt regions of the two enzymes have similar structures, it is likely that the loss of the charged domain, rather than changes in secondary structure, is responsible for the loss of binding.

Specificity of the Interaction. We previously reported that within the Ct of AE1 the D887ADD charged cluster was required for binding (*14*). Mutation of this sequence to NANN, DAAA, or AAAA resulted in loss of CAII binding. However, mutation of the sequence to DADA, DAAD, AADD, or the AE2 sequence (DANE) did not affect the interaction with CAII, suggesting that two negative charges were sufficient for the interaction. The results reported above suggest that basic residues located within the Nt of CAII provide a binding site for the acidic Ct of AE1. Electrostatic interactions are usually only one component of protein−

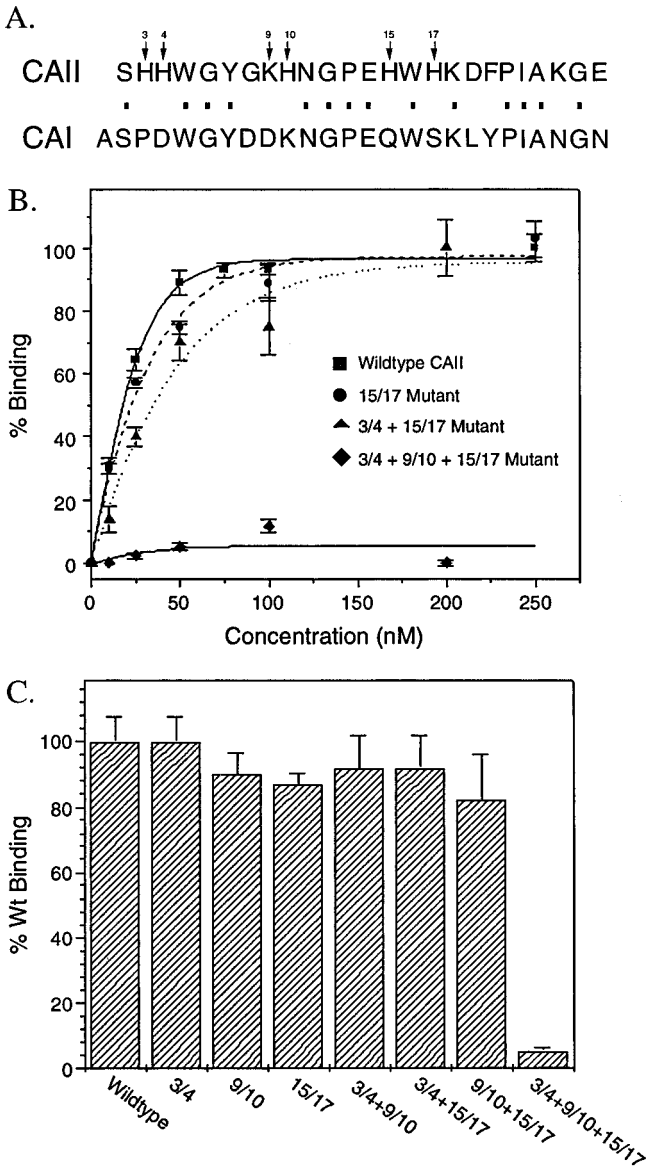

FIGURE 3: Binding of GST-Ct to mutant CAII constructs. (A) CAI and CAII amino acid sequence alignment showing identical residues (squares) and the position of the mutated residues (arrows). The residues selected for mutation (3, 4, 9, 10, 15, and 17) in the CAII sequence are numbered. (B) CAII mutants were prepared as described and treated as in Figure 2B. For clarity, the complete binding curves are shown for only a selection of the mutants, but binding assays were conducted for all mutant CAII proteins. (C) Relative level of binding of 25 nM GST-Ct to the wild-type and mutant CAII proteins.

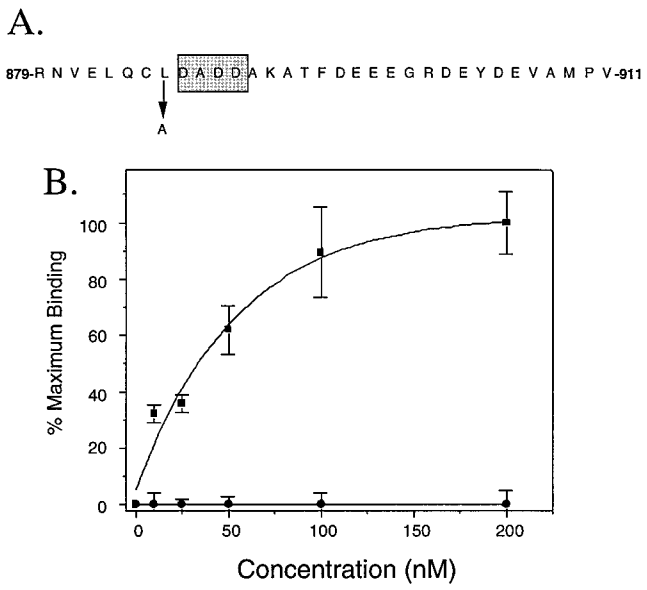

FIGURE 4: Role of Leu886 in AE1 binding to CAII. (A) Amino acid sequence of GST-Ct showing positions of the Leu 886 to Ala mutation and DADD box. (B) Binding of GST-Ct (squares) or GST-CtL886A mutant (circles) to immobilized CAII.

protein interactions, so we looked for additional residues in the Ct of AE1 which may confer specificity to the interaction with CAII. Residues E883, C885, L886, and A891 are conserved in AE1 and AE2, the two members of the AE family previously identified as binding to CAII. Mutation of Cys885 to Ala does not affect binding nor does removal of Ala891 (*15*). Mutation of Ala888 to Pro or Ser also did not affect the binding (*15*). However, as Figure 4B shows, mutation of Leu886 to Ala resulted in complete loss of binding, indicating the importance of this nonpolar residue in the interaction. It is unlikely that this loss of binding is due to changes in the secondary structure of the Ct since circular dichroism spectroscopy showed that a 33 residue peptide correspoding to the Ct of AE1 was unstructured in solution (data not shown). Thus, the interaction of AE1 with CAII depends on two types of interactions, ionic and nonpolar.

We have mapped the AE1 binding site to the Nt of CAII using truncated CAII constructs and CAII point mutations with a sensitive microtiter plate assay. Truncation of CAII by five residues results in a protein with decreased affinity for the Ct of AE1. Further truncation of 17 or 24 residues results in nearly complete loss of binding. These results suggested that residues within the extreme Nt of CAII were involved in binding to the Ct of AE1. We found this to be the case since mutation of five histidine residues and one lysine in this sequence to the corresponding residue in CAI led to a complete loss of Ct binding. It was somewhat surprising that an effect on the interaction between AE1 and CAII was seen only after removal of all of the histidine residues, since truncation of the protein by only five residues produced an effect. It is possible that the Nt of CAII, which folds independently of the rest of the protein (*18*), forms a structure important for the interaction with AE1. Removal of the first five residues may compromise this structure, while mutation of H3/H4 may not produce enough of a change in the structure to prevent binding to the Ct of AE1. The His-rich patch on CAII may serve as an "electrostatic highway" as discussed by Miles et al. (*31*). In this case, the role of the charged residues on CAII may be to funnel bicarbonate (or chloride) directly to and from the active site of CAII or Band 3. In this interpretation, these residues would not necessarily be important for the interaction, but would be in the vicinity of the binding region. Examination of the known CAII sequences revealed that histidines at positions 3, 4, and 17 are completely conserved (Figure 5). Histidine 10 is replaced by serine in rat CAII, while histidine 15 is replaced by asparagine in mouse and rat CAII. Based on our point mutations, we predict that all of the CAII sequences listed in Figure 5 should be capable of binding to the Ct of the corresponding anion exchanger since a minimum of two basic residues are required.

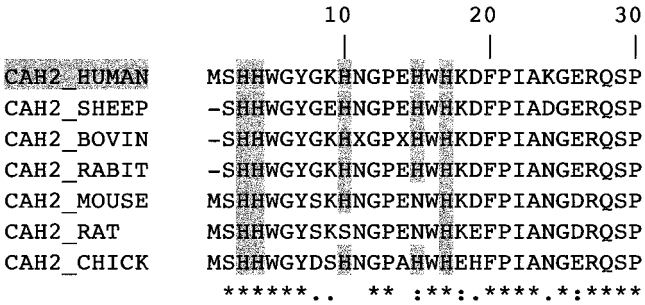

FIGURE 5: Sequence alignment of Nt regions of CAII. The five histidine residues not found in this region of CAI have been highlighted where present.

In this report we also provide evidence for a hydrophobic component in the interaction since the Leu886 to Ala mutant in GST-Ct resulted in loss of CAII binding. Interestingly, the Nt sequences of CAII contain conserved tryptophan residues at positions 5 and 16, a tyrosine at position 7, and a phenylalanine at position 20. It is possible that some of these aromatic residues contribute to CAII binding to the Ct of AE1.

We have previously speculated that an interaction between anion exchangers and CAII may form the basis for a capnometabolon (capno; Greek; smoke, CO_2). A metabolon is a sequential complex of metabolic enzymes as described by Srere (*32*, *33*). The purpose of this metabolon may be to increase the efficiency of CO_2 removal from the erythrocyte and may also be important in other tissues such as the kidney which also express CAII and AE1 isoforms. We are currently examining the effect of mutations in the Ct of AE1 that affect CAII binding on anion exchange in transfected HEK cells.

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