Purification and Characterization of a Carbonic Anhydrase II Inhibitor from Porcine Plasma†

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ABSTRACT: Plasma from many vertebrates, including pigs, contains a soluble component that inhibits the CO_2 hydrase activity of carbonic anhydrase (CA). This activity was purified to homogeneity (~4000-fold) from porcine plasma using a combination of DEAE-Affi-Gel Blue chromatography and carbonic anhydrase II-affinity chromatography, yielding 16 mg of inhibitory protein/L of plasma. This protein, porcine inhibitor of carbonic anhydrase (pICA), is a monomeric protein with an apparent molecular mass of 79 kDa, as determined by electrospray mass spectrometry. As isolated, pICA contains about 3 kDa of N-linked glycosylation removable by peptide-N-glycosidase F. pICA inhibits CA reversibly with a 1:1 stoichiometry. pICA is a potent and specific inhibitor of the CA II isozyme, with $K_i < 0.1$ nM for porcine CA II at pH 7.4. Although the K_i is dependent on the CA isozyme type (CA II ≪ CA IV ≪ CA III ≈ CA I), it is relatively insensitive to the species source, as long as it is mammalian. The K_i is pH dependent with log K_i decreasing linearly as the pH decreases, implicating at least one ionizable group with the $pK_a \leq 6.5$ in the binding interaction. The isozyme and species dependence of the inhibition suggest that pICA interacts with amino acids on the surface of CA II.

Carbonic anhydrase (CA)[1] (EC 4.2.1.1.) is a zinc metalloenzyme which catalyzes the reversible hydration of carbon dioxide to bicarbonate and a proton (Silverman & Lindskog, 1988). This enzyme is ubiquitous in living systems, playing roles in CO_2 transport, secretory processes, calcification, and photosynthesis (Tashian et al., 1990). Seven different mammalian isozymes (CA I to CA VII) have been identified with varying activity, tissue specificity, and physiological roles (Tashian et al., 1990). Two isozymes, CA I and CA II, are found in high concentrations in red blood cells and in lower concentrations in the cytosol of many tissues of mammals (Dodgson, 1991). Small amounts of acetozolamide-inhibitable CO_2 hydrase activity have been observed in human serum, although this may be attributable to red blood cell lysis (Roughton, 1935; Pihar & Svorc, 1966). A CA II-deficiency syndrome exists in humans; its symptoms include osteopetrosis, renal tubular acidosis, mental retardation, growth retardation, and cerebral calcification (Sly, 1991). CA III is present mainly in the cytoplasm of muscle tissues; CA IV is bound to the plasma membrane of cells of various organs, including lung, brain, and kidney tissue; and other isozymes are confined to mitochondria (CA V) or salivary glands (CA VI, CA VII) (Dodgson, 1991).

The sera of many mammals, including pigs, rats, and cats, inhibit the CO_2 hydrase activity of carbonic anhydrase (Booth, 1938). Inhibitory activity has also been measured in the plasma of sheep (Leiner et al., 1962), dogs (Rispens et al., 1985; Hill, 1986), rabbits (Hill, 1986), trout (Haswell & Randall, 1976), and eel (Haswell et al., 1983), although it has not been observed in the plasma of bowfin (Heming & Watson, 1986), pigeons (Booth, 1938), ducks (Booth, 1938), and humans (Booth, 1938; Hill, 1986). Initial studies, including partial purification of the inhibitory activity from sheep and eel sera (Leiner et al., 1962; Haswell et al., 1983), suggest that the inhibitor is a protein. Gel filtration of eel plasma indicates that the inhibitor from this species has a molecular mass in the range of 10 000–30 000 Da (Haswell et al., 1983). The physiological function of this inhibitor is unknown. Among other possibilities, it may play a role in protecting capillary walls from tissue damage due to transient pH changes caused by carbonic anhydrase activity in plasma (Roughton, 1935) or in preventing CA II that has leaked into plasma from being filtered by the kidneys into the urine (Appelgren et al., 1989).

In order to identify a specific CA inhibitor and illuminate the biochemical properties of this inhibitor, our laboratory has purified it from porcine plasma using a combination of chromatography on DEAE-Affi-Gel Blue and carbonic anhydrase–Sephadex affinity resins. This 4000-fold purification yields a 79-kDa N-linked glycoprotein that exists as a monomer and binds carbonic anhydrase in a 1:1 complex. This protein, which we have named porcine inhibitor of carbonic anydrase (pICA), is a very efficient inhibitor of porcine carbonic anhydrase II (PCA II), with a K_i of 0.5 nM at pH 8.0. pICA specifically inhibits CA isozyme II. K_i increases for other isozymes in the order CA II ≪ CA IV ≪ CA III ≈ CA I, exhibits only a 2-fold increase in specificity between porcine CA II (PCA II) and human CA II (HCA II), and is pH dependent. The isozyme and species specificity suggest that pICA interacts with amino acids on the surface of CA as opposed to the active site.

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[1] Abbreviations: pICA, porcine inhibitor of carbonic anhydrase; CA, carbonic anhydrase; HCA II, human carbonic anhydrase isozyme II; PCA II, porcine carbonic anhydrase isozyme II; PNGase F; peptide-N-glycosidase F; BSA, bovine serum albumin; CNBr, cyanogen bromide; Tris–SO_4, Tris buffered with sulfuric acid; NaSCN, sodium thiocyanate; $(NH_4)_2SO_4$, ammonium sulfate; Na_2SO_4, sodium sulfate; KH_2PO_4, monobasic potassium phosphate; DTT, dithiothreitol; PNPA, *p*-nitrophenyl acetate; DEAE, (diethylamino)ethyl; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MW, molecular weight; V_e, volume eluted; V_o, void volume.

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MATERIALS AND METHODS

Materials. Whole blood was collected from the arteries and veins of the neck of a pig (Garrard Sausage Co., Durham, NC) into a 4-L jar containing 42.8 mL of 0.5 M EDTA, pH 8.0, and cooled on ice. Plasma and cellular fractions were separated by centrifugation for 15 min at 3000*g* at 4 °C and stored at −20 °C for future use in the purification of pICA and porcine carbonic anhydrases, respectively.

HCA II–Sepharose resin was prepared by the method of Porath (Porath, 1974), with the following exceptions: the CNBr-activated Sepharose CL-4B was purchased from Sigma, all coupling and blocking steps were done for 24 h at 4 °C, and the glycine wash of the resin was omitted. Coupling efficiency, as determined by absorbance of the protein solution at 280 nm before and after incubation with activated resin, was routinely greater than 95%. Using 5 g of resin and 100 mg of HCA II, this procedure yields approximately 15 mL of resin with 6.7 mg of HCA II coupled/mL of resin as determined by assays of HCA II-catalyzed *p*-nitrophenyl acetate (PNPA) hydrolysis.

Recombinant HCA II (from *Escherichia coli*) (Fierke et al., 1991) and CA I and CA II from porcine, ovine, and chicken red cells were purified using sulfonamide affinity resin (Osborne & Tashian, 1975). HCA I, SDS–PAGE high molecular weight markers, horse heart myoglobin, and column chromatography molecular weight markers were obtained from Sigma. Bovine carbonic anhydrase, bovine serum albumin (BSA), endoglycosidase F, and peptide-N-glycosidase F (PNGase F) were purchased from Boehringer Mannheim. DEAE-Affi-Gel Blue agarose and SDS–PAGE low molecular weight markers were purchased from Bio-Rad. Coomassie protein reagent was obtained from Pierce. Defibrinated blood of chicken and sheep was procured from Colorado Serum Co. A Trio 2000 single-quadrupole mass spectrometer and software were graciously lent by Fisons-VG. Porcine CA IV, HCA III, and bovine CA III were generous gifts from Lee Cody of Dr. William Sly's laboratory, Dr. David Silverman, and Dr. Roger Rowlett, respectively. A Leu204 → Arg mutant of HCA II in *E. coli* crude extract was courteously provided by Joseph Krebs of our laboratory. All other chemicals were reagent grade from standard suppliers.

Purification of Porcine Carbonic Anhydrase Inhibitor (pICA). Frozen porcine plasma (≈2 L) was thawed and diluted to 16 L with 1 mM EDTA, pH 8.0, at 4 °C, and then applied to a DEAE-Affi-Gel Blue agarose column (20 cm × 8 cm) at a flow rate of 4 L/h. The column was washed with 2 L of buffer A (10 mM Tris–SO_4, pH 8.0), and pICA was eluted with 2 L of buffer A with 25 mM $(NH_4)_2SO_4$. This fraction was split into two parts, and each part was applied directly to a 15-mL HCA II–Sepharose column (2.6 cm × 3 cm) at a flow rate of 300 mL/h. The affinity column was then washed with 3 volumes each of buffer A, buffer A with 100 mM $(NH_4)_2SO_4$, and buffer A with 500 mM $(NH_4)_2SO_4$. The inhibitor was then eluted with 3 volumes of buffer A containing 500 mM NaSCN, and the column was washed with 30 mL of buffer A. These fractions were collected into a stirred beaker containing 6 volumes of buffer A in order to decrease the concentration of the chaotrope. This eluate was dialyzed 2 × 25-fold against buffer A, concentrated 15-fold by filtration against an Amicon YM-30 membrane, and then redialyzed 4 × 400-fold against buffer A to remove residual NaSCN. pICA activity was monitored during the purification procedure by measuring inhibition of CO_2 hydrase activity of HCA II using a pH-indicator spectrophotometric assay (Brion et al., 1988) at 4 °C on either a Shimadzu 265 or a Cary 219 spectrophotometer. pICA may be stored at 4 °C for up to 2 months with no loss of activity.

Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was done using a discontinuous buffer system (Laemmli, 1970). Gels were routinely stained with Coomassie Brilliant Blue R-250 to detect the presence of protein (Weber & Osborn, 1969). A periodic acid–Schiff's reagent technique (Glossmann & Neville, 1971) was used to detect the presence of carbohydrates using α-2-macroglobulin as a positive control and BSA and HCA II as negative controls.

Determination of Protein Concentration. The concentration of purified pICA was determined by stoichiometric titration of HCA II-catalyzed PNPA hydrolysis (Pocker & Stone, 1967) with pICA. PNPA hydrolysis was measured by the change in A_{348} per minute at 0.5 mM PNPA, 100 mM Tris–SO_4, pH 8.0, 25 °C. Concentrations of pICA were also estimated using a Coomassie Blue Dye binding assay (Bradford, 1976) with BSA as a standard curve and were found to be within 10% of the concentration as determined by titration. An extinction coefficient of 99 000 $M^{-1}\cdot cm^{-1}$ for pICA was derived by measuring the absorbance at 280 nm of a 1 μM sample of pICA. The concentration of HCA II was determined from the ϵ_{280} of 54 000 $M^{-1}\cdot cm^{-1}$ (Coleman, 1967) or by stoichiometric titration with acetazolamide of fluorescence of the HCA II–dansylamide complex (excitation wavelength = 280 nm, emission wavelength = 465 nm) (Chen & Kernohan, 1967).

Determination of Molecular Mass of pICA. The molecular mass of pICA was estimated by comparison to a standard curve (fitted by linear regression) of molecular mass standards using SDS–PAGE (Laemmli, 1970; Shapiro et al., 1967). A more precise determination of molecular mass was made by electrospray mass spectrometry, conducted by Dr. Robert Stevens of the Department of Pediatrics, Division of Genetics and Metabolism, Duke University Medical Center. Samples of pICA were dialyzed against ultrapure (resistance = 18 MΩ) water. A 3 pmol/μL solution of the protein containing 0.01% HCl was prepared in the carrier solvent stream (1:1 mixture of water and HPLC-grade acetonitrile containing 1% formic acid). The sample (10 μL) was introduced into the carrier solvent stream (flow rate = 2.0 μL/min) of an electrospray ionization source of a Fisons-VG Trio 2000 single quadrupole mass spectrometer calibrated with horse heart myoglobin (MW 16951.48). The spectrometer was scanned over a range of 1450–1850 *m/e*, and the results were analyzed using the software provided by the manufacturer.

The subunit composition of native pICA was characterized by size-exclusion chromatography using a 23 cm × 1.6 cm column of Sepharose CL-4B resin equilibrated with buffer A containing 100 mM $(NH_4)_2SO_4$. The apparent molecular weight (MW) of pICA was estimated from the ratio of volume eluted/void volume (V_e/V_o) compared to a plot of log MW of protein standards versus V_e/V_o (Andrews, 1964).

Deglycosylation of pICA by PNGase F. pICA samples (50 μg) were boiled for 3 min in 1% SDS, 10% β-mercaptoethanol, diluted 2-fold with 10% octyl β-glucoside, and cooled on ice for 5 min. The sample was then added to a solution of 100 mM KH_2PO_4, pH 8.5, 50 mM EDTA, with or without 60 microunits/mL PNGase F (Tarentino et al., 1985). Samples were incubated overnight at 37 °C and assayed for deglycosylation by electrophoresis on an 8% SDS–polyacrylamide gel. Changes in molecular mass were determined by comparison to molecular mass standards.

Assay of Carbonic Anhydrase Inhibition. CO_2 hydrase activity of carbonic anhydrase was measured using a pH

Table I: Purification of pICA from Porcine Plasma[a]

step	volume (mL)	[protein][b] (mg/mL)	activity		sp act. (units/mg)	purification (x-fold)	yield (%)
			units/μL[c]	total units			
(1) porcine plasma	1800	180	0.96	1.7×10^6	5.3	1	100
(2) DEAE-Affigel Blue[d]	2000	15	0.92	1.8×10^6	63	12	106
(3) HCA II–Sepharose[e]	13	2.2	46	6.0×10^5	2.1×10^4	3880	35

[a] From one representative purification procedure. [b] Estimated from absorbance at 280 nm (Layne, 1957) assuming the absence of nucleic acids in the solution. [c] One unit of pICA is defined as the amount of pICA required to produce 50% inhibition of 2 pmol of HCA II in the colorimetric hydrase assay of Brion et al. (1988). [d] pICA was eluted with 25 mM $(NH_4)_2SO_4$ as described in Materials and Methods. [e] Two affinity columns were run as described in the text. Then, the eluate was dialyzed and concentrated.

colorimetric assay (Khalifah, 1971) in a stopped-flow spectrophotometer from KinTek Instruments. Assays were conducted at 25 °C using a ratio of 5:2 for enzyme:substrate mixing. The enzyme concentration varied from 2 nM (HCA II) to 2.5 μM (CA III), such that the enzyme-catalyzed rate was at least twice the uncatalyzed CO_2 hydration rate. The following indicator–buffer pairs were used either at 50 mM buffer, $\mu = 0.1$ with Na_2SO_4 or 10 mM buffer, 1 mM EDTA: pH 7.7–8.5, *m*-cresol purple and either 1,2-dimethylimidazole or Tris–SO_4; pH 6.9–7.7, *p*-nitrophenol and 1-methylimidazole; and pH 6.6–6.9, bromocresol purple and 2,4-lutidine. The uncatalyzed rate of CO_2 hydration was decreased by lowering the buffer concentration and adding EDTA (Pocker & Bjorkquist, 1977).

Inhibition of CO_2 hydration by pICA was measured by preincubating pICA and carbonic anhydrase in the colorimetric buffer at room temperature for at least 1 min, diluting with CO_2-saturated water, and then measuring the CO_2 hydration rate in the stopped-flow spectrophotometer as described. The rate was measured as a function of pICA concentration before dilution and the K_i was determined by fitting the data to the positive solution to

$$[E]^2 + (K_i + [I_{tot}] - [E_{tot}])[E] - [E_{tot}]K_i = 0 \quad (1)$$

where [E] = free enzyme, $[E_{tot}]$ = total enzyme, and $[I_{tot}]$ = total inhibitor (Morrison, 1969). [E] is calculated from the initial velocity of the reaction in the presence of inhibitor as a function of the velocity of the reaction in the absence of inhibitor. For proper fitting of the data from porcine carbonic anhydrase IV, it was necessary to introduce an additive constant *k*, representing enzyme activity insensitive to pICA, to the equation. All data were fitted using the computational program SYSTAT (SYSTAT Inc., Evanston, IL).

RESULTS

Porcine plasma was initially reported to contain one of the highest levels of carbonic anhydrase inhibitory activity (Booth, 1938), making it an excellent starting source for purification of this inhibitor. We have determined that 1 μL of porcine plasma diluted into 1 mL of assay buffer inhibits 50% of the CO_2 hydrase activity of 2 nM HCA II. Assuming a 1:1 stoichiometry between the inhibitor and HCA II, the concentration of inhibitor in porcine plasma is about 1 μM. We have developed the following two-step method to purify to homogeneity milligram quantities of the carbonic anhydrase inhibitory activity in porcine plasma, which we have named porcine inhibitor of carbonic anhydrase (pICA).

Purification of pICA. The first step in the purification of pICA from porcine plasma is a DEAE Affi-Gel Blue agarose (a complex chromatography matrix containing both (diethylamino)ethyl groups and Cibracron F3GA dye molecules coupled to agarose) column. This procedure was developed to facilitate purification of large quantities of inhibitor using the ion-exchange properties of the matrix. Additionally, this step separates albumin from pICA due to the extraordinarily strong affinity of Cibacron F3GA dye for serum albumin (Travis & Pannell, 1973). This could not be accomplished using conventional anion-exchange resins. pICA binds to this resin at low ionic strength ($\mu \approx 0.02$) which is achieved by an 8-fold dilution of plasma into cold 1 mM EDTA, pH 8.0. After the resin is washed with 10 mM Tris-SO_4, pH 8.0, pICA is eluted via a step gradient by the addition of 25 mM $(NH_4)_2SO_4$ to the buffer. Overall, this column results in at least a 10-fold purification of pICA with little or no loss in inhibitory activity, as summarized in Table I.

The final step for purification of pICA is a HCA II–Sepharose column prepared by cross-linking recombinant HCA II to CNBr-activated Sepharose CL-4B under mild conditions. After elution from the DEAE-Affi-Gel Blue column, the inhibitory activity was applied directly to the HCA II–Sepharose column. The resin was rinsed with a low salt buffer ($\mu \approx 0.01$) and washed with a high salt buffer ($\mu \approx 1.5$) to remove nonspecifically bound proteins, and then the inhibitor was eluted with 0.5 M NaSCN, a highly chaotropic salt as well as a CA inhibitor (Tibell et al., 1984). The eluate was immediately diluted into buffer to decrease the chaotrope concentration in order to minimize loss of pICA activity, followed by exhaustive dialysis to lower the NaSCN below inhibitory concentrations. We were unable to elute pICA from the HCA II–Sepharose resin with other carbonic anhydrase inhibitors such as sodium azide or acetazolamide (Pocker & Stone, 1967), or with 1% Triton-X-100, 1 M $(NH_4)_2SO_4$, or 100 mM dithiothreitol. pICA was eluted from the affinity resin with 4 M urea or 6 M guanidine hydrochloride, but with complete loss of activity (data not shown). The affinity resin step purified pICA approximately 400-fold, with an average yield of 35%. This low yield is due to a combination of factors including saturation of the affinity column with pICA, denaturation of the proteins at high thiocyanate concentrations, and incomplete release of pICA from HCA–Sepharose. After affinity purification, the inhibitor is a protein with a purity of ≈98%, as assayed by SDS–PAGE (Figure 1). Table I is a chart of a representative pICA purification showing a 4000-fold increase in specific activity over the starting plasma and an overall yield of 35%. Using this procedure, we can routinely purify to homogeneity 15 mg of pICA/L of porcine plasma.

Physical Properties of pICA. The molecular mass of pICA was estimated from its electrophoretic mobility on an 8% polyacrylamide gel under denaturing conditions by comparison to molecular mass standards on the same gel (Figure 2). A standard curve of log M_r versus R_f (Shapiro et al., 1967) was fitted by linear regression, giving an apparent mass of 83 ± 3 kDa. Additional characterization using electrospray mass spectrometry gave a molecular mass of 79 035 ± 18 amu for the major peak of our protein (Figure 3). In this analysis, a second smaller peak (≤5% total protein) was detected with

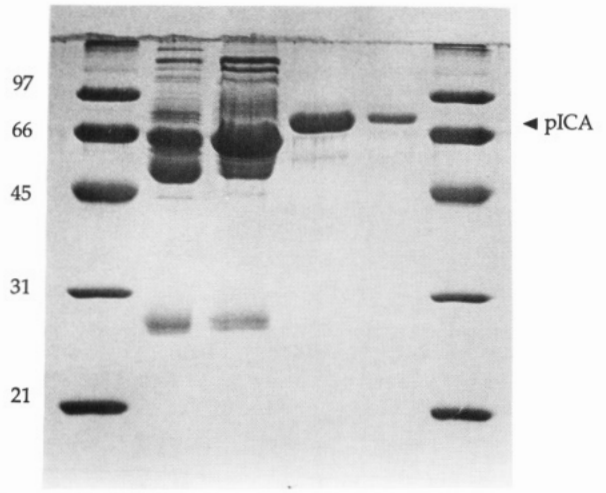

FIGURE 1: Purification of pICA, 12% SDS–PAGE gel. Lanes 1 and 6: low molecular weight markers (Bio-Rad) phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor. Lane 2: 18 μg of porcine plasma. Lane 3: 29 μg of 25 mM $(NH_4)_2SO_4$ wash of the DEAE-Affi-Gel Blue column. Lane 4: 5 μg of purified pICA. Lane 5: 1.2 μg of purified pICA.

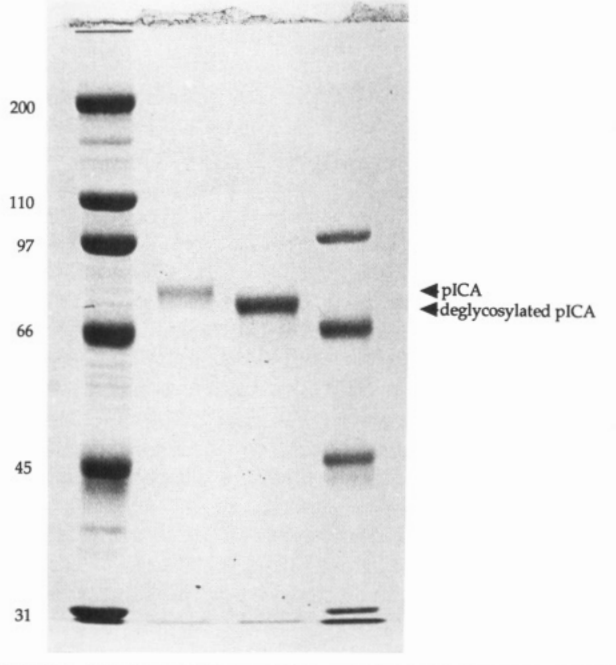

FIGURE 2: Deglycosylation of pICA by PNGase F, 8% SDS–PAGE gel. Lane 1: high molecular weight markers (Sigma) myosin, β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, and carbonic anhydrase. Lane 2: 2.5 μg of pICA control. Lane 3: 5 μg of deglycosylated pICA, prepared as described in the Materials and Methods section. Lane 4: low molecular weight markers (Bio-Rad) phosphorylase B, bovine serum albumin, ovalbumin, and carbonic anhydrase (soybean trypsin inhibitor and lysozyme were eluted from the gel).

a mass approximately 560 amu larger than the primary peak. This peak may result from inhomogeneities in posttranslational modification of pICA.

The subunit composition of pICA was determined by size-exclusion chromatography using Sepharose CL-4B resin. The apparent molecular weight of pICA was estimated from the ratio of volume eluted/void volume (V_e/V_o) compared to a plot of log molecular weight of protein standards versus V_e/V_o as fitted by linear regression (Andrews, 1964). Using this method the apparent molecular mass of the native protein was estimated to be 65 ± 15 kDa, indicating that pICA is a monomeric protein (data not shown).

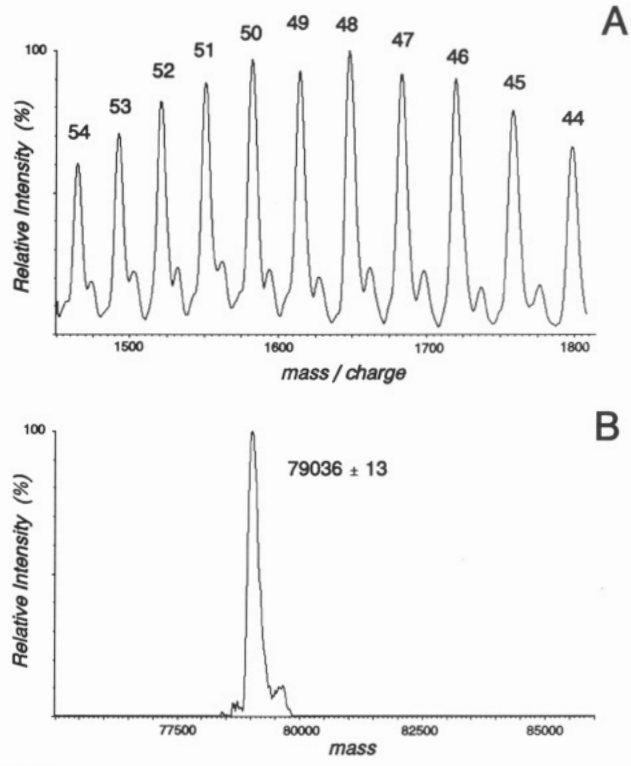

FIGURE 3: Electrospray mass spectrometry of pICA. (A) The electrospray mass spectrum of pICA. The number on top of each peak represents n, the charge number, in the formula $(M + nH)^{n+}$ for a particular charged ion (Edmonds & Smith, 1990). Once n is assigned for a given peak, M can be computed for each peak in the spectrum and an average molecular mass value can be calculated. (B) The m/e data transformed to a molecular mass scale. Note the secondary peak (approximately 5% of total protein) of MW approximately 79 600.

To reveal whether pICA is glycosylated, a SDS–PAGE gel of the inhibitor was stained using a periodic acid–Schiff's reagent technique (Glossmann & Neville, 1971). pICA gave a positive reaction in this assay, indicating the presence of covalently attached carbohydrates. Deglycosylation of pICA by PNGase F, a glycosidase specific for N-linked carbohydrates, reduces the apparent molecular mass as determined by SDS–PAGE by approximately 4 ± 2 kDa (Figure 2). Other N-linked glycosidases, such as endoglycosidase F and glycopeptidase A, have no effect on the apparent molecular mass of pICA. However, these glycosidases tend to be less effective than PNGase F at removing complex carbohydrate structures from large protein substrates (Tarentino et al., 1985; Taga et al., 1984).

Inhibition of CA. The stoichiometry of the inhibition of HCA II by pICA (Figure 4) was determined by titration of the PNPA esterase activity of HCA II (Pocker & Stone, 1967) by pICA at a concentration of HCA II (300 nM) well above the K_i under these conditions (see Figure 5). Linear regression analysis of the data in Figure 4 indicates that 310 ± 40 nM pICA should completely inhibit 300 nM HCA II, giving a stoichiometry of 1.0 ± 0.1 for the pICA–HCA II interaction.

The inhibition of HCA II by pICA is rapidly reversible; incubation of 100 nM HCA II with 200 nM pICA in 10 mM Tris–SO_4, pH 8.0 (such that >99% of HCA II is inhibited) followed by 100-fold dilution into 20 mM imidazole, 5 mM Tris–SO_4, 0.2 mM *p*-nitrophenol, pH 8.0, 4 °C, restores 60% of the CO_2 hydrase activity (Brion et al., 1988) within 10 s. This activity is consistent with the apparent K_i measured under these conditions.

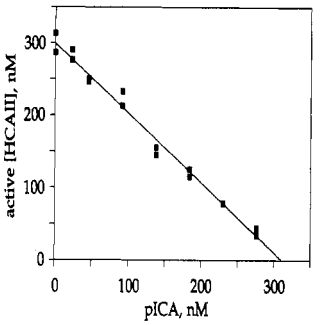

FIGURE 4: Stoichiometric inhibition of HCA II by pICA. The PNPA esterase activity of 300 μM HCA II was measured at pH 8.0, as described in the Materials and Methods Section, at varying concentrations of pICA. The concentration of pICA was determined using a Coomassie Blue-binding assay (Bradford, 1976). The free enzyme concentration was calculated from the percent of activity. The slope = 0.96 ± 0.03 as determined by linear regression, indicating a 1:1 stoichiometry for the inhibition of HCA II by pICA.

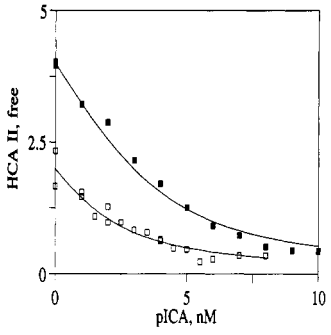

FIGURE 5: Determination of K_i for HCA II by pICA. The initial rate for CO_2 hydration activity of HCA II was measured using a stopped-flow CO_2 hydrase assay (Khalifah, 1971) with 14 mM 1,2-dimethylimidazole, pH 8.0, 24 μM *m*-cresol purple, and 1 mM EDTA at 25 °C. The HCA II concentration was either 2 nM (□) or 4 nM (■). Free HCA II was calculated from percent activity and the data set was fitted to eq (1) as described in the Materials and Methods Section.

Table II: Inhibition of Carbonic Anhydrase Isozymes by pICA[a]

isozyme	K_i (M)	isozyme	K_i (M)
isozyme II		other isozymes	
porcine CA II	0.6 (±0.4) × 10^{-9}	porcine CA IV	1.5 (±0.5) × 10^{-7} [c]
human CA II	1.0 (±0.3) × 10^{-9}	human CA III	2.3 (±0.1) × 10^{-5}
sheep CA II	2.9 (±0.1) × 10^{-9}	bovine CA III	≥7.0 × 10^{-5} [d]
chicken CA II	≥1.0 × 10^{-5} [b]	human CA I	≥5.0 × 10^{-5} [e]
		porcine CA I	≥2.0 × 10^{-5} [f]

[a] All inhibition constants were determined as described in the legend of Figure 5. [b] Activity inhibited <10% at 1 μM pICA. [c] 50% of acetazolamide-inhibitable hydrase activity not inhibited by pICA. [d] Activity inhibited 15% at 20 μM pICA. [e] Activity inhibited ≤10% at 5 μM pICA. [f] Activity inhibited <10% at 2 μM pICA.

The K_i for inhibition of HCA II by pICA was determined from the decrease in CO_2 hydrase activity as the concentration of pICA increases (Figure 5). The CO_2 hydrase activity was measured at pH 8.0 using a stopped-flow pH indicator assay (Khalifah, 1971) at the lowest feasible enzyme concentration. The K_i was calculated to be 1.15 ± 0.14 nM at 2 nM HCA II and 0.98 ± 0.09 nM at 4 nM HCA II by fitting the data to the positive solution to the quadratic equation (eq 1). The independence of K_i on HCA II concentration indicates that we are accurately measuring K_i.

The stopped-flow pH indicator assay was used to determine the K_i of pICA for several carbonic anhydrase isozymes from different species, as listed in Table II. For the most part, the isozymes can be divided into two categories: high affinity and low affinity. All tested species of the CA II isozyme, except

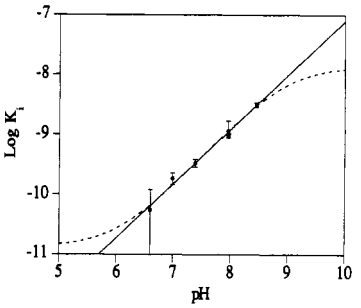

FIGURE 6: Dependence of K_i on pH. Inhibition constants for pICA were determined from the inhibition of CO_2 hydrase activity of HCA II at 25 °C, 1 mM EDTA, [buffer] = 14 mM, in the pH range 6.5–8.5 using buffer–indicator pairs described in the Materials and Methods section. The K_i was determined at each pH by fitting to eq 1 (Materials and Methods). Linear regression of K_i versus pH gave a slope = 0.9 ± 0.04. The dotted line illustrates the observed change in K_i as a function of pH assuming that protonation of a single ionization group with pK_a = 6.0 decreases K_i 1000-fold.

chicken, fall into the high-affinity group characterized by complete inhibition of CA activity with nanomolar inhibition constants. The dependence of K_i on the mammalian species of CA II is small; pICA inhibits HCA II only 2-fold less efficiently and sheep CA II 6-fold less efficiently than its presumed physiological target, PCA II. The low-affinity group, containing all CA I and III species tested as well as chicken CA II, showed little or no inhibition at experimentally obtainable concentrations of pICA. Inhibition constants were estimated to be >10 μM (Table II). In our assays, porcine CA IV behaves anomalously. Although the apparent K_i (150 nM) is fairly low when compared to members of the low-affinity group, only 50% of the activity was inhibited. This partial inhibition may be due to heterogeneity of the porcine CA IV and/or the presence of detergent (required for solubility of the membrane-bound CA IV) in the preparation (Zhu & Sly, 1990).

To further characterize the interaction between pICA and CA II, we determined K_i as a function of pH. Recombinant HCA II was used for these experiments because of its ready availability and lack of contamination with other CA isozymes. Figure 6 shows that log K_i increases linearly as pH increases from 6.5 to 8.5 with a fitted slope of 0.9 ± 0.04. This indicates that protonation of at least one group on either pICA or HCA II increases the pICA–HCA II interaction. Assuming that only a single ionizable group is involved, this data indicates that the pK_a of this group is ≤6.5 and that the HCA II–pICA interaction is increased at least 100-fold by protonation. A similar effect has been seen with the PCA II–pICA interaction (data not shown). At physiological pH (pH 7.4), the K_i for PCA II is <0.1 nM and for PCA I is 21 μM).

DISCUSSION

This paper describes a simple and rapid two-step procedure for purifying the carbonic anhydrase inhibitory activity in porcine plasma (pICA) with a yield of 16 mg/L plasma and an overall efficiency of 35%. Biochemical characterization of this inhibitor indicates that it is a 79-kDa monomeric glycoprotein which specifically and reversibly inhibits mammalian CA II with a 1:1 stoichiometry. The K_i for inhibition of other carbonic anhydrase isozymes by pICA increases in this order: CA II ≪ CA IV < CA I ≈ CA III. These data suggest that pICA is similar in nature to other mammalian inhibitors of carbonic anhydrase, as far as they have been characterized (Booth, 1938; Leiner et al., 1962). However, due to differences in apparent molecular mass, we would

suggest that the inhibitor of carbonic anhydrase found in eel plasma (Haswell et al., 1983) is substantially different from mammalian inhibitors.

Although the physiological substrate of pICA is unknown, our results suggest that CA II, CA IV, and/or a CA II-like protein is the likely target of the inhibitor. pICA in the plasma has access to CA I and CA II released from red cells and membrane-bound CA IV in various organs including lung, brain, and kidney (Dodgson, 1991). The concentration of pICA in plasma, 1 μM, is more than 1000 times the K_i for PCA II at physiological pH, indicating that PCA II in the plasma will be inhibited unless the concentration exceeds 1 μM. More than 15% of a pig's red blood cells must lyse to achieve this concentration (Tanis et al., 1970). The pICA concentration is also higher than the K_i for PCA IV, suggesting that a significant portion of the membrane-bound CA IV activity may also be inhibited. As confirmation of this hypothesis, Heming's laboratory has demonstrated inhibition of carbonic anhydrase activity (presumably CA IV) by pICA in vivo in perfused rat lung (T. Heming, personal communication). The K_i values for two other isozymes, CA I and CA III, are larger than the pICA concentration, indicating that little inhibition of these isozymes would occur in plasma.

The function of a carbonic anhydrase inhibitor in plasma was initially proposed to prevent acidosis of the plasma from equilibration of CO_2 and HCO_3^-, thus preventing damage to pH-sensitive tissues during capillary transit (Roughton, 1935). Recent data suggest that pICA may play a role in recycling CA II released from cells. The half-life for radiolabeled CA II in rat plasma is longer than for other CA isozymes and, in addition, CA II is taken up by the liver while other CA isozymes are taken up by the kidneys (Appelgren et al., 1989). Superoxide dismutase, a protein of similar size (MW = 32 000) and Stoke–Einstein radius (25 Å), is quickly filtered from the plasma (half-life $\approx$ 6 min) and localized into the kidney and urinary tract (Odlind et al., 1988; Bayati et al., 1988). Therefore, it is possible that the role of pICA is to prevent CA II from being filtered through the 40-Å pores of the glomerular capillary membrane of the kidneys by formation of a large protein complex (Maack et al., 1979). Finally, the main physiological function of pICA may be inhibition of CA in tissues other than plasma. The decreased inhibitory activity in the plasma of several species, including humans (Booth, 1938; Hill, 1986; Heming & Watson, 1986), may be consistent with this hypothesis. This question will be resolved only by further studies on the tissue localization of pICA.

Several lines of evidence suggest that pICA does not interact directly with the active site of carbonic anhydrase. We have found that pICA cannot be eluted from CA–Sepharose by carbonic anhydrase inhibitors, such as acetazolamide, which bind to the active site zinc of CA (Ericksson et al., 1988). Thiocyanate is an inhibitor of carbonic anhydrase (K_i for HCA II $\approx$ 3 mM at pH 8.0) (Tibell et al., 1984), but the concentration of thiocyanate required to elute pICA from CA–Sepharose is 10^3 greater than the K_i, suggesting that the chaotropic properties of thiocyanate (Scopes, 1987) are responsible for disrupting the pICA–CA II interaction. Finally, we have been unable to observe significant changes in the fluorescence spectra of dansylamide bound to HCA II (Chen & Kernohan, 1967) in the presence of pICA (data not shown) suggesting that pICA may neither displace nor significantly affect the environment of bound dansylamide.

To further define a possible pICA binding site on CA II, we have compared the known primary sequences of high-affinity enzymes (HCA II, sheep CA II) to those of low-affinity enzymes (HCA I, HCA III, and chick CA II) (Hewett-Emmett & Tashian, 1991) to identify amino acid residues that are specific to mammalian CA II. Residues in the active site cleft near the catalytic zinc atom are highly conserved in all CA isozymes, again suggesting that this region is not the site of the specific interaction between CA II and pICA. However, several amino acids on the surface of CA are completely conserved only in mammalian CA II. These residues include Asp 19, Asp 34, Lys 112, Asp 130, Leu 204, and Glu 238. Four of these residues, Asp 19, Asp 130, Leu 204, and Glu 238 are on the front surface of HCA II (Figure 7). Three additional residues on the front surface, Arg 58, Ser 173, and Asp 175, are conserved in all known mammalian CA II sequences except mouse. We propose that some or all of these residues, rather than active site amino acid residues, form a portion of the pICA binding site on CA. By binding over the active site cavity of CA, pICA would prevent diffusion of substrate to the catalytic zinc. This proposal is consistent with the large size of the inhibitor.

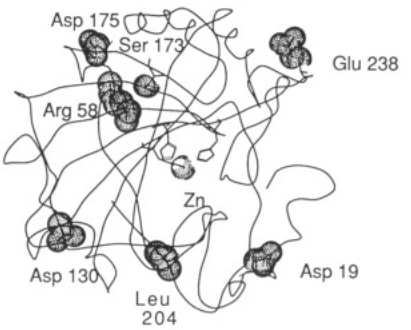

FIGURE 7: Ribbon structure of HCA II. Surface amino acid residues that are conserved in mammalian CA II and are also on the same face as the active site pocket are highlighted by dot surfaces. Illustrated amino acid residues include Asp 19, Asp 130, Leu 204, Glu 238, Arg 58, Ser 173, and Asp 175. This plot was generated using the molecular graphics program SYBYL (Tripos Associates, Inc.) from a refined structure of recombinant HCA II (Alexander et al., 1991).

To begin testing this model, we have determined that the K_i for a single amino acid mutant in HCA II, Leu204 $\rightarrow$ Arg, is not significantly different than that for wild type (data not shown), indicating that this residue at the outer edge of the active site cavity (Figure 7) does not interact with pICA. The pH dependence of K_i suggests that protonation of a group with a $pK_a \leq 6.0$ on either CA II or pICA increases the pICA–CA II interaction. Possible candidates on CA II for the observed pH dependence include Asp 19, Asp 130, Glu 238, and Asp 175. Future studies to test our proposed binding mode include measuring the K_i for additional CA species and site-specific mutants and pursuing structural studies of the CA II–pICA complex.

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