MOLECULAR PHARMACOLOGY, 22:211-220

The Carbon Dioxide Hydration Activity of Skeletal Muscle Carbonic Anhydrase

Inhibition by Sulfonamides and Anions

GAUTAM SANYAL, ERIK R. SWENSON, NESS I. PESSAH, AND THOMAS H. MAREN

Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Gainesville, Florida 32610

Received January 8, 1982; Accepted February 25, 1982

SUMMARY

The cytoplasmic carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) isozyme III has been purified from the skeletal muscle of cat, using an affinity chromatography technique followed by gel filtration. The turnover number (k_{cat}) and the Michaelis-Menten constant (K_m) of this enzyme have been determined for carbon dioxide hydration at 1° using a changing pH method. The numbers at a mean pH of 7.6 are 1000 sec^{-1} for k_{cat} and 40 mM for K_m. We also determined the kinetic parameters for 25° and pH 7.5 using stop-flow spectrophotometric technique. Because of the lower solubility of CO_2 at 25°, the range of substrate concentrations used is limited to values less than the K_m. Our estimates are 4200 sec^{-1} for k_{cat} and 37 mM for K_m. Thus, the cat muscle carbonic anhydrase shows a 200-fold lower k_{cat} and a 4-fold higher K_m as compared with the high-activity human red cell carbonic anhydrase isozyme II. The inhibitory activities of several sulfonamides against the muscle enzyme are 700–30,000 times lower than their activities against the human red cell isozyme II. On the other hand, the muscle enzyme is more sensitive to anion inhibition than is carbonic anhydrase isozyme II and, in this regard, is closely comparable to isozyme I. We have also determined the turnover numbers for CO_2 hydration by human and bovine muscle cytoplasmic carbonic anhydrase at 10° and pH 7.5 using the stop-flow method. Like the cat muscle enzyme, both enzymes are characterized by a large K_m for CO_2 (45 mM). The human enzyme has somewhat higher k_{cat} (8900 sec^{-1}) as compared with the bovine enzyme (4200 sec^{-1}) under our experimental conditions, but this turnover number is still 50-fold lower than that of human carbonic anhydrase II.

INTRODUCTION

The presence of CA[1] in skeletal muscle cytoplasm has been discovered in the last few years by several groups (1–3). This enzyme, which has been designated by Tashian as carbonic anhydrase III (CA III), originates from a genetic locus distinct from those responsible for the synthesis of the mammalian red cell isozymes CA I and CA II (4, 5). CA III differs from CA I and CA II in having significantly lower CO_2 hydratase and esterase activities (6, 7) and greater resistance toward inhibition by acetazolamide (8). The complete amino acid sequence of bovine CA III has been reported, and some critical differences from CA I and CA II are found (5).

Although CO_2 hydratase assays have shown the relatively poor activity of CA III (6), no determination of the critical kinetic parameters, namely, the turnover number (k_{cat}) and the Michaelis-Menten constant (K_m), has so far been reported. Also, the sulfonamide and anion inhibitors of CA I and CA II have not been systematically tested for activity against CA III. We felt that a quantitative study of the kinetics and inhibition of CA III was necessary before one could approach the fundamental problem of the physiological role of this enzyme. Questions as to the role of carbonic anhydrase in muscle (9) cannot be answered without a quantitative knowledge of the sensitivity of the enzyme to inhibition. We therefore undertook to determine the Michaelis-Menten kinetic parameters for the purified cat skeletal muscle CA III and the inhibitory activities of several anions and sulfonamides against this enzyme. By using a stop-flow spectrophotometric technique (10), we also measured the k_{cat} and K_m for the purified bovine and human muscle CA III.

This work was supported by National Institutes of Health Grant HL-22258.

[1] The abbreviations used are: CA, carbonic anhydrase (carbonate hydrolase, EC 4.2.1.1); PAMBS, *p*-aminomethylbenzenesulfonamide; CM, carboxymethyl.

MATERIALS AND METHODS

Purification of CA III. Cat muscle was obtained from the hind limbs of fully anesthetized animals (pentobar-

0026-895X/82/040211-10$02.00/0

bital, 25 mg/kg i.v.). Perfusion with cold 0.9% NaCl solution was continued, after administration of heparin, 3000 units/kg i.v., until the muscles were visibly pale and the venous effluent was colorless. The muscle sample was homogenized with water and the homogenate was then subjected to differential centrifugation in order to separate the cytoplasm from the membrane fractions, the latter being discarded. The supernatant was further filtered through glass wool and then treated with a mixture of chloroform and ethanol according to the method of Tashian *et al.* (11). The ethanol layer was dialyzed against 0.1 M Tris-sulfate/0.25 M sodium sulfate buffer (pH 7.5).

This muscle cytoplasmic fraction was then mixed with an affinity chromatographic resin containing CM-Bio-Gel A (Bio-Rad Laboratories, Richmond, Calif.) coupled to PAMBS prepared according to the method of Khalifah *et al.* (12) and equilibrated with 0.1 M Tris-sulfate/0.25 M sodium sulfate buffer (pH 7.5). This affinity gel is specific for adsorption of red cell isozymes CA I and CA II (12), but did not bind cat muscle CA III (see Results). Consequently, this procedure was well-suited to eliminating the possibility of any red cell CA contamination not removed by perfusion. The gel and muscle supernatant were stirred gently together for 2 hr. The CA activity of the supernatant was checked initially and at several time intervals during mixing. The supernatant was then separated from the gel on a Buchner funnel.

The affinity gel that was used to bind the CA III from cat muscle was prepared exactly the same way as PAMBS affinity gel (12), except that PAMBS was replaced by 2-(4-aminobenzene)-sulfonamido-1,3,4-thiadiazole-5-sulfonamide (*p*-aminobenzolamide: CL 13475). The *p*-aminobenzolamide affinity resin was also equilibrated with 0.1 M Tris-sulfate/0.25 M sodium sulfate buffer (pH 7.5). The cat muscle supernatant that had been earlier treated with PAMBS gel was now treated with the *p*-aminobenzolamide affinity gel. The binding of CA III to the gel was followed by measuring the decrease in CO_2 hydration activity of the supernatant. After all of the measurable activity disappeared from the supernatant, the gel was poured into a column and washed thoroughly with 0.1 M Tris-sulfate buffer (pH 7.5) until no more protein was eluting (~1 liter), as monitored by $A_{280\ nm}$ measurement. To elute CA III, 0.1 M Tris-sulfate buffer (pH 7.4) containing 0.4 M sodium azide was used.

The isolated CA III isozyme was subsequently passed through a Sephadex G-100 column (2.5 cm × 35 cm) equilibrated with 0.05 M Tris-sulfate/0.025 M sodium sulfate buffer (pH 7.5). The same buffer was used for elution. The monomeric enzyme fractions were pooled and dialyzed extensively against 1 mM diothiothreitol and stored at 4° in the same disulfide-reducing medium. The approximate molecular weight of the monomeric enzyme was obtained upon calibrating the same column with horse heart cytochrome *c* (mol wt = 12,400), bovine carbonic anhydrase (mol wt = 29,000), and bovine serum albumin (mol wt = 67,000). All of these reference proteins were obtained from Sigma Chemical Company (St. Louis, Mo.).

Polyacrylamide gel electrophoresis. Electrophoresis of CA III was carried out using 5% polyacrylamide gels. The electrophoresis equipment was obtained from Bio-Rad Laboratories. The buffer contained 0.2 M Tris/0.2 M glycine (pH 8.9). For staining, Coomassie Brilliant Blue was used. Destaining was done in 10% acetic acid containing 10% isopropyl alcohol.

Zinc determination. The zinc content of cat CA III was analyzed by means of atomic absorption spectrophotometry on a Perkin-Elmer Model 303 spectrophotometer.

Amino acid analysis. Hydrolysis of enzyme aliquots was carried out at 110° in 6 N HCl in duplicates for 24, 48, and 72 hr. Extrapolation to zero-time hydrolysis was carried out to yield the maximal recovery for serine and tyrosine. To obtain the value for half-cystine, a sample was oxidized with performic acid and hydrolyzed for 24 hr. Tryptophan was determined by the UV spectrophotometric method of Edelhoch (13).

Inhibitors and inhibition studies. All sulfonamide inhibitors of carbonic anhydrase were gifts from the Lederle Laboratories (Pearl River, N. Y.), with the exception of PAMBS, sulfanilamide, and ethoxzolamide, which were obtained from Sigma Chemical Company, Fisher Chemical Company (Fair Lawn, N. J.), and The Upjohn Company (Kalamazoo, Mich.), respectively. The properties of these sulfonamide drugs have been discussed (14). All other chemicals used were of analytical reagent-grade.

The inhibition of CA III by some sulfonamides and anions was studied by monitoring their effects on the enzyme-catalyzed CO_2 hydration rate at 1°, according to the method of Maren and Couto (15). The CO_2 and barbital concentrations were 72 mM and 14 mM, respectively, and the mean pH of the assay was 7.6. The I_{50} values for all inhibitors except KCNO and 2-chlorophenyl-1,3,4-thiadiazole-5-sulfonamide (chlorzolamide: CL 13580) were determined from data at several different concentrations, assuming noncompetitive inhibition. K_i was then calculated as $I_{50}-0.5\ E_0$. For the stronger inhibitors KCNO and chlorzolamide, K_i was determined by Easson-Stedman plotting of data (16) as described by Maren *et al.* (17).

Enzyme concentration. The concentration of cat muscle CA III was determined in purified solutions of the monomeric enzyme containing 1 mM dithiothreitol by using the method of Lowry *et al.* (18) for protein determination.

The concentration of CA III was also independently determined from the intercept of the Easson-Stedman plot obtained by active-site titration of an enzyme sample with chlorzolamide (17).

We calculated the molar extinction coefficient of the bovine and the human muscle CA III, according to the method of Wetlaufer (19), using the known number of tryptophan, tyrosine, and disulfide bonds as given by Tashian *et al.* (5) and Carter *et al.* (7). These values were then used to determine the respective enzyme concentrations.

UV spectra were recorded on a Beckman UV 5260 spectrophotometer.

Kinetic methods. The CO_2 hydration kinetics of purified cat muscle CA III at 1° was studied according to the method of Maren and Couto (15), using a 14 mM final concentration of barbital buffer, the mean reaction pH being 7.6. Barbital was found to have no inhibitory effect

on the enzyme at concentrations up to 28 mM. The initial rates of the enzyme-catalyzed reactions were used to calculate K_m and V_{max}, using a weighted least-squares analysis with constant absolute error in rate determinations (20).

Kinetic studies of the cat CA III at 25° and of bovine and human CA III at 10.5° were carried out by using a Durrum-Gibson stopped-flow spectrophotometer (Model D-110), in conjunction with a Nicolet digital oscilloscope (Model 206) provided with floppy storage disc. Data were then computed on Hewlett-Packard 9835 B computer. Initial rates were calculated (using, respectively, for the 10.5° and 25° runs, the first 3–5% and 10% of the reaction traces) by employing linear least-squares over 100–200 data points. Correlation coefficients ranged from 0.94 to 0.99. At each substrate concentration, initial rates were calculated for four reaction traces. Barbital and phenol red were used as the buffer-indicator pair, since at the low ionic strengths of our experiments, they both have about the same pK_a values (21), thereby minimizing the pH dependence of the "buffer factor" (10). The initial decrease in absorbance of phenol red at 557 nm was monitored and then converted to the initial rate by multiplication by the buffer factor. K_m and V_{max} were calculated by the weighted least-squares method described above.

RESULTS

Affinity chromatography. Figure 1 shows the lack of binding of cat muscle cytosolic CA activity by the PAMBS gel and the rapid adsorption of the same enzymatic activity by the *p*-aminobenzolamide gel. Over a period of 90 min, there was no appreciable loss of the cytosolic activity to the PAMBS gel. Subsequent to PAMBS gel treatment, when the same cytosol sample was mixed with the CM-Bio-Gel A coupled to *p*-aminobenzolamide, 98% of the CO_2 hydration activity was adsorbed by the gel within 25 min. Compared with the total activity prior to mixing of the cytosol with the PAMBS, there was a 9% decrease in activity immediately (assayed within 30 sec) following the mixing. This rapid initial binding of CA activity by PAMBS could indicate incomplete perfusion leading to a very small amount of red cell CA in the crude cytosol. The subsequent lack of binding of the enzymatic activity by the PAMBS saturated gel is consistent with our finding that concentrations of sulfanilamide up to 5 mM do not inhibit cat CA III. This observation is in contrast with an earlier report by Holmes (1), who used PAMBS in the affinity chromatographic purification of chicken and sheep muscle CA. Unfortunately, Holmes (1) did not report the K_i of either PAMBS or sulfanilamide against the chicken and the sheep enzymes. Holmes eluted these muscle enzymes by using 100 mM Tris-sulfate/200 mM sodium sulfate buffer, after washing the column with 200 mM Tris-sulfate buffer. It is not clear whether sulfate was used as an enzyme inhibitor or, in effect, to form an ionic-strength gradient. In our purification method, cat CA III eluted as a single peak off the *p*-aminobenzolamide bound CM-Bio-Gel A with 0.4 M NaN_3. Both *p*-aminobenzolamide and NaN_3 are strong inhibitors of this enzyme (see Table 2).

Gel filtration. When subjected to Sephadex G-100 chromatography, the cat muscle CA III showed evidence of the presence of both monomeric and dimeric forms. Register *et al.* (3) previously found that rabbit muscle CA dimerizes in the absence of a sulfhydryl-reducing agent, indicating that dimerization occurs through intermolecular disulfide bond formation. We routinely separated the monomeric form from the dimeric species, then dialyzed the monomer extensively against 1 mM dithiothreitol and stored it at 4° in the same medium. Such enzyme preparations eluted as a single peak off the Sephadex G-100 column when 1 mM dithiothreitol was used in the elution buffer and also yielded a single band in polyacrylamide gel electrophoresis.

The gel filtration method yielded an approximate mo-

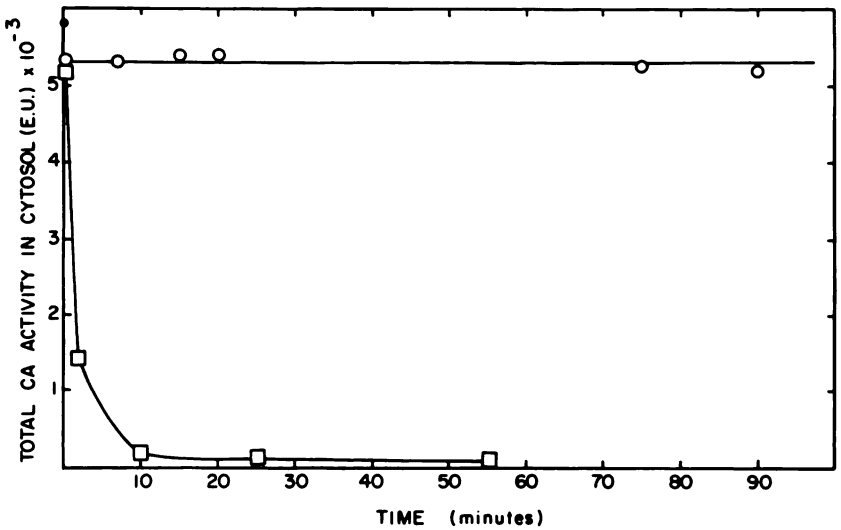

FIG. 1. *Treatment of cat skeletal muscle supernatant with sulfonamide-bound CM-Bio-Gel A*

The total CA activity of the supernatant was measured (●) before mixing with gel. The cytosol was then mixed with CM-Bio-Gel A coupled to PAMBS at 25° in 0.1 M Tris-SO_4/0.2 M Na_2SO_4 buffer (pH 7.5) and the CA activity (○) not adsorbed by the gel was followed as a function of time. The PAMBS-treated supernatant was then mixed with aminobenzolamide-bound gel and the time course of adsorption of CO_2 hydration activity (□) was followed.

lecular weight for cat CA III. Relative to the void volume (V_0) of the gel bed as determined by using Blue Dextran, the ratios of elution volumes (V_e/V_0) for the standard proteins were 1.28 for bovine serum albumin (mol wt = 67,000), 1.94 for bovine red cell CA (mol wt = 29,000), and 2.28 for horse cytochrome *c* (mol wt = 12,400). The cat muscle CA III eluted with a V_e/V_0 of 1.96, in agreement with a mol wt of 28,500. CA III from several species has been reported to have the same molecular weight as CA I and CA II (1, 3, 7), although the sheep muscle enzyme has been reported to have a slightly higher mol wt of 34,000 (1).

Polyacrylamide gel electrophoresis. The homogeneity of our cat muscle CA III monomer preparation was verified by polyacrylamide gel electrophoresis as shown in Fig. 2. If the enzyme is stored in the absence of dithiothreitol, evidence of possible dimerization appears on the gel. It is interesting to note that Jeffery and Carter (6) found, for the human muscle enzyme, reappearance of a triple-banded electrophoretic pattern by removing mercaptoethanol and adding oxidized glutathione or iodoacetic acid to a monomer sample.

Amino acid composition. Table 1 compares the amino acid analysis of cat muscle CA III with the bovine CA III composition, taken from the complete amino acid sequence reported by Tashian *et al.* (5). One significant difference between cat CA III and bovine CA III appears in the tryptophan content. We determined tryptophan

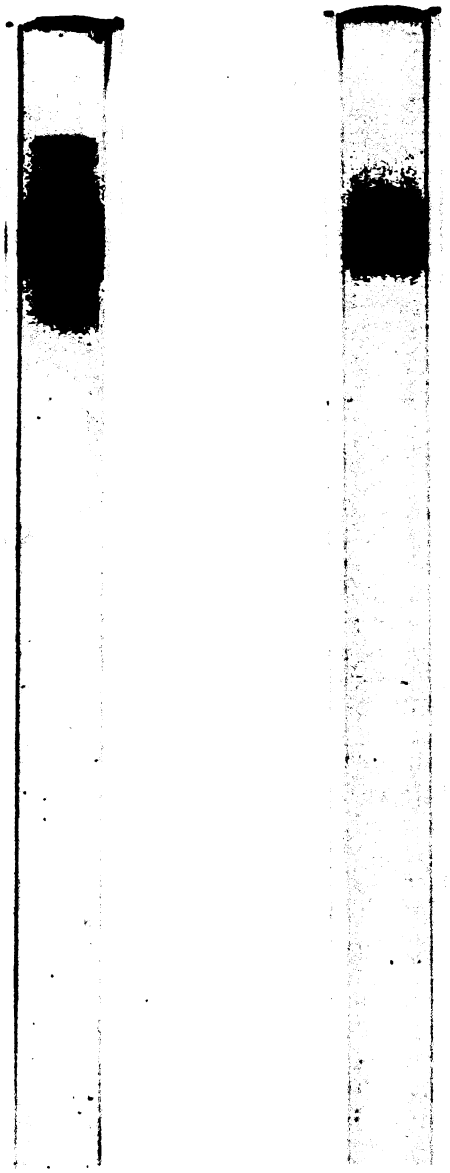

FIG. 2. *Polyacrylamide gel electrophoresis of cat skeletal muscle CA III*

Electrophoresis was carried out using 5% polyacrylamide gel, and the buffer contained 0.2 M Tris/0.2 M glycine (pH 8.9). The single-banded electrophoretic pattern represents the Sephadex (G-100)-separated monomer (36 μg) stored in 1 mM dithiothreitol. Evidence of heterogeneity appears in the other monomer sample (38 μg), which was stored for 48 hr without dithiothreitol after Sephadex chromatography.

TABLE 1

Comparison of amino acid composition of cat muscle CA III with bovine muscle CA III

Amino acid residue	Cat[a] CA III	Bovine[b] CA III
Lysine	16.6	20
Histidine	8.0	12
Arginine	10.5	11
Aspartic acid	22.2	29
Threonine	8.5	12
Serine	14.7	19
Glutamic acid	21.4	19
Proline	18.9	20
Glycine	17.6	17
Alanine	17.4	20
Valine	14.6	13
Methionine	3.1	1
Isoleucine	9.7	12
Leucine	23.8	24
Tyrosine[c]	7.9	9
Phenylalanine	7.9	9
Tryptophan[c]	5.1	8
Cysteine[d]	3.8	3

[a] Numbers of residues are calculated for a molecular weight of 28,500. Values represent an average of eight analyses of individually hydrolyzed protein samples, the standard deviations being within ±5%. Values of serine and tyrosine are calculated by extrapolation to zero-time hydrolysis from a hydrolysis time study.

[b] Data are taken from the complete sequence reported by Tashian *et al.* (5).

[c] Tryptophan content was determined according to the method of Edelhoch (13). Tyrosine content was also obtained from this experiment and was identical with the number determined by hydrolysis.

[d] Cysteine was determined as cysteic acid after performic acid oxidation of the monomer sample.

and tyrosine for cat CA III by the method of Edelhoch (13), using 6 M guanidine hydrochloride and 12 mM dithiothreitol at pH 6.5. Sufficient time was allowed for the denaturation of the enzyme, and no time-dependent change in the UV spectra was observed. The reference cuvette contained 6 M guanidine hydrochloride and 12 mM dithiothreitol for baseline correction. The number of tyrosine residues thus obtained was the same as that determined by the conventional amino acid analysis. The relatively low tryptophan content of cat CA III is consistent with the lower extinction coefficient of this enzyme (see below) as compared, for example, with the reported absorptivity of the rabbit muscle enzyme containing 9 or 10 tryptophans (3). CA III differs from both CA I and CA II in having a higher cysteine content.

UV spectrum. The UV spectrum of CA III enzyme is a typical tryptophan-tyrosine spectrum with the maximum 281 nm and a shoulder at 290 mM. Upon calibration against the protein determination of Lowry *et al.* (18), an average extinction coefficient of 1.67 was obtained for a 0.1% solution of the monomeric cat muscle CA III. This is equivalent to a molar extinction coefficient value of 4.76×10^4 M^{-1} cm^{-1}, for a molecular weight of 28,500. The A_{280}/A_{260} ratio is 1.93. The dimer has a subunit molar extinction coefficient of 4.65×10^4 M^{-1} cm^{-1}, assuming a molecular weight of 57,000. By using the method of Wetlaufer (19), we calculate, for five tryptophans, eight tyrosines, and one disulfide bridge, a molar extinction coefficient of 3.86×10^4 M^{-1} cm^{-1} at 278 nm for the monomeric cat muscle enzyme. The ratio of the observed to the calculated value at 278 nm is 1.20. For the determination of enzyme concentration, we used our observed molar extinction coefficient.

For the bovine muscle CA III, we calculate, using the method of Wetlaufer (19), an extinction coefficient value of 5.66×10^4 M^{-1} cm^{-1} at 278 nm based on eight tryptophans, nine tyrosines, and one disulfide linkage (8). For the human muscle, the calculated value is 6.23×10^4 M^{-1} cm^{-1} for ten tryptophans, five tyrosines, and one disulfide bridge (6). These numbers were used to calculate the respective enzyme concentrations.

Active-site titration. Figure 3 shows the determination of cat CA III concentration in a purified monomeric enzyme sample by titration of the enzymatic activity with the specific inhibitor chlorzolamide. The linear regression slope of the Easson-Stedman plot yields a K_i of 0.27 μM, the 95% confidence interval being 0.13–0.40 μM. The intercept of the same plot yields 0.91 μM for the enzyme concentration used in this experiment, the 95% confidence range being 0.61–1.2 μM. This agrees with the spectrophotometrically determined concentration of 0.90 μM. According to this active-site titration experiment with chlorzolamide, 1 enzyme unit in our changing pH assay system is equivalent to 0.52 μM of the active enzyme. This is the enzyme concentration required to halve the uncatalyzed time using 72 mM CO_2 at 1° in 7 ml of reaction volume.

Enzyme inhibition. Table 2 gives the K_i values of seven sulfonamide inhibitors and five anionic inhibitors against cat muscle CA. For comparison, K_i values for the same inhibitors against human red cell isozymes CA I and CA II and male rat liver CA are given. The sulfonamide data for male rat liver enzyme are taken from King *et al.* (22).

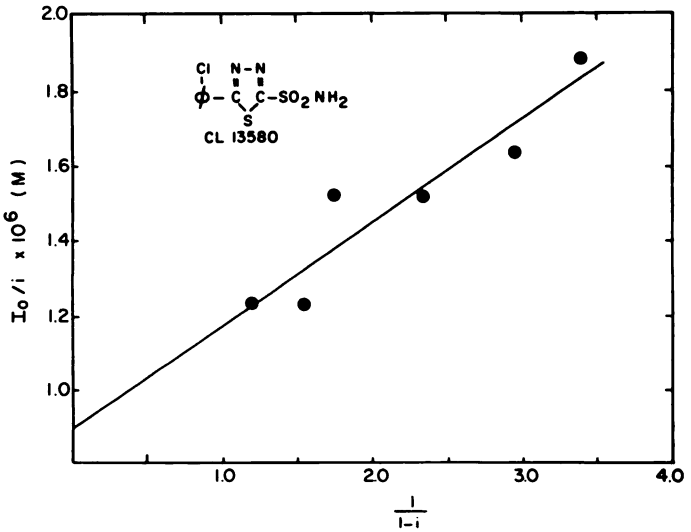

FIG. 3. *Active-site titration of monomeric cat skeletal muscle CA III with chlorzolamide (CL 13580)*

The inhibition of CO_2 hydration activity by adding different concentrations of CL 13580 was assayed at 1° according to the changing pH method described in the text. I_0 is the total inhibitor concentration, and i represents fractional inhibition. The slope of this plot yields the K_i for chlorzolamide, and the y intercept gives the enzyme concentration. The *solid line* is drawn by linear regression; correlation coefficient = 0.936.

TABLE 2

Inhibition of cat muscle CA III by sulfonamides and anions: comparison with other CA isozymes

All data are for inhibition of CO_2 hydration at temperature $\simeq 0°$ with the exception of the anion data on CA I and CA II, which were obtained at 25°. No measurable incubation time was required for muscle CA III with any inhibitors with the exception of ethoxzolamide, benzolamide, and aminobenzolamide.

Inhibitor	K_i			
	Cat muscle CA III	Male rat[a] Liver CA	Human[b] CA I	Human[b] CA II
		μM		
Acetazolamide	306	100	0.20	0.01
Methazolamide	96	15	0.01	0.01
Ethoxzolamide	54[c]	150	0.002	0.002
Chlorzolamide	0.27	0.80	0.001	0.0004
Benzolamide	3.1[d]	4.0	0.002	0.0004
Aminobenzolamide	3.7[d]	—	—	
Sulfanilamide	>5,000	>1,000	50	2.0
KCNO	0.52	0.70	0.70	20
NaCN	2.3	—	0.38[e]	17[e]
NaI	1,100	1,000	300	26,000
NaCl	5,910	11,000	6,000	200,000
NaN_3	14	—	—	—

[a] Sulfonamide inhibition data are taken from ref. 22. Anion inhibition data were obtained on well-perfused, male rat liver supernatant (unpurified enzyme) and represent I_{50} values.

[b] Sulfonamide and anion data are taken from refs. 24 and 15, respectively.

[c] Two minutes of incubation were required for maximal effect.

[d] Four minutes of incubation were required for maximal effect.

[e] Present data.

The I_{50} values of all inhibitors against the muscle and the male rat liver enzyme are very similar. We also looked at the anion inhibition of the crude male rat liver supernatant and obtained I_{50} values of 11 mM for chloride and 1.1 mM for iodide, which are very similar to the values reported by Garg (23) with purified male rat liver enzyme. However, we differ with Garg (23) on cyanate, and obtain an I_{50} value of 0.7 μM for cyanate inhibition of the CO_2 hydration activity of male rat liver supernatant. The strongest inhibitors of the muscle enzyme are chlorzolamide and cyanate. The K_i values for these were determined by using the Easson-Stedman plot (16), as shown for chlorzolamide in Fig. 3. Among the monovalent anionic inhibitors, chloride and cyanate have the same K_i against CA III and CA I. Iodide and cyanide are, respectively, 4 and 6 times weaker against CA III, compared with their inhibitory activity against CA I. CA II is significantly less sensitive toward anions than either CA I or CA III. The sulfonamides that we tested are less active against muscle CA than they are against either CA I or CA II (24). However, the range of activities of different heterocyclic sulfonamides varies over more than 3 orders of magnitude. The strongest inhibitor chlorzolamide has a K_i against muscle CA III which is about 700-fold higher than its K_i against CA II. On the other end of the spectrum, acetazolamide exhibits a 30,000-fold higher K_i against the muscle enzyme compared with CA II. For acetazolamide inhibition of rat CA III, an I_{50} value of about 10^{-4} M has been reported by Carter *et al.* (8), which is in agreement with I_{50} values of acetazolamide against CA III from bovine and rabbit muscle (5, 25).

CO_2 hydration kinetics. The activity of the monomeric cat muscle enzyme is much higher than the dimeric enzyme. Using the changing pH assay system (15) at 1°, we obtain on an average 23 and 0.9 enzyme units/mg of protein for the monomer and dimer, respectively. All of our kinetic work was done using fresh Sephadex G-100-separated monomeric enzyme which was dialyzed against 1 mM dithiothreitol.

Figure 4A shows a typical Lineweaver-Burk plot for the dependence of initial enzymatic rate of cat CA III on the CO_2 concentration at 1° and a mean pH of 7.6. The data plotted in Fig. 4A yield a K_m of 33 mM and a k_{cat} of 900 sec^{-1}, using the weighted least-squares calculation (20) described under Materials and Methods. The mean values of K_m and k_{cat} are compared with the corresponding values for human red cell CA I and CA II (26) in Table 3. CA I and CA II have turnover numbers, respectively, 29-fold and 236-fold higher under identical experimental conditions. The highest CO_2 concentration in these experiments was 72 mM, which is the CO_2 solubility at 1° (27). Therefore, the accuracy of determining a large K_m value was best at this low temperature.

For 25° kinetics, we used the stop-flow method for accurate determinations of faster reaction rates. One inherent difficulty in determining the K_m at 25° is the low solubility (34 mM) of CO_2 (27). In the stop-flow mixing chamber, the maximal CO_2 concentration was thus only 17 mM. Figure 4B shows a Lineweaver-Burk plot of the initial enzymatic rate data at 25°. The uncatalyzed rate contributed 26–32% of the over-all rate in presence of the enzyme at 1.1 μM concentration. The buffer factor necessary to convert the initial rate of absorbance change to the actual rate of concentration change was calculated using equation 5 of the appendix of reference 10. Because of the closeness of the pK_a values of barbital and phenol red (21) at the low ionic strength (0.005–0.01) of our studies, the pK_a- and pH-dependent contributions toward the buffer factor were limited to a factor of 1.06. The validity of our buffer

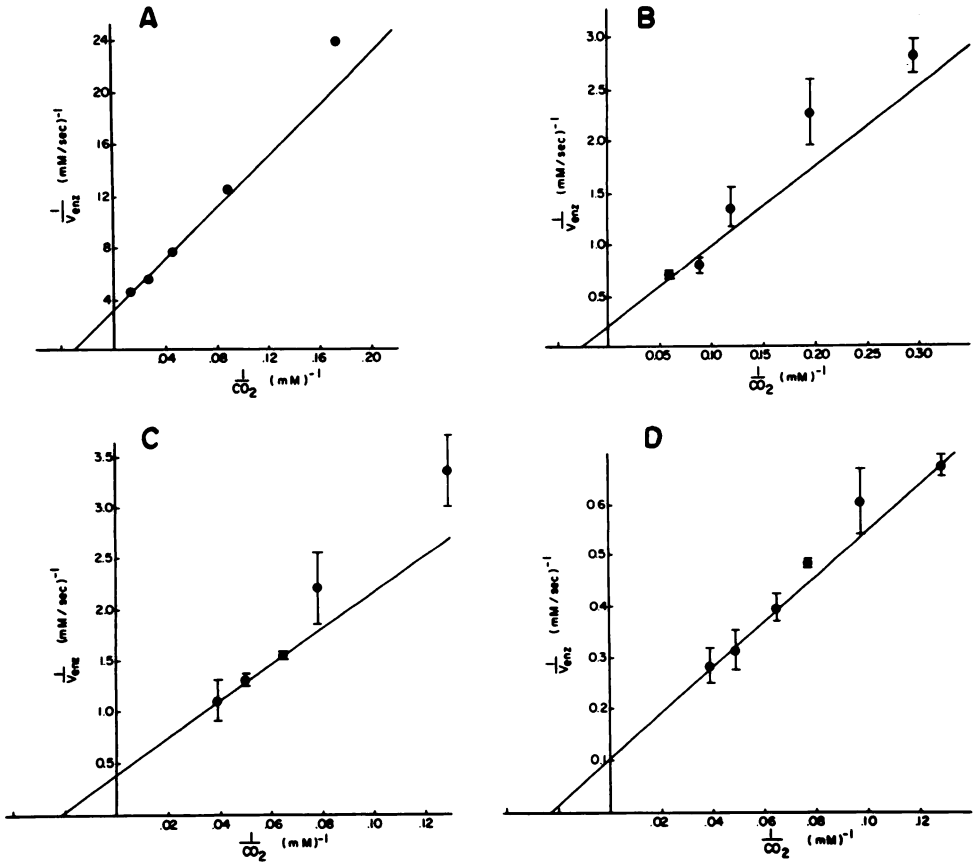

FIG. 4. *Double-reciprocal plots of initial enzymatic rates versus CO_2 concentrations for cat, bovine, and human skeletal muscle CA*

The K_m and V_{max} values in each case were determined numerically by weighted least-squares analysis of the data is described in the text. The *solid lines* were drawn according to the calculated K_m and V_{max} values. *Error bars* in B, C, and D represent standard deviations of the mean rates for four determinations at each substrate concentration.

A. Determination of K_m and V_{max} for CO_2 hydration by monomeric cat muscle CA III at 1°, using the changing pH method described in the text. The enzyme concentration was 0.35 μM, and the barbital buffer concentration was 14.3 mM. The mean pH was 7.6.

B. Stop-flow kinetic determination of K_m and k_{cat} for cat muscle CA III at 25° (±0.15°). The final concentrations (in the mixing chamber) of enzyme barbital buffer, phenol red, and dithiothreitol were, respectively, 1.1 μM, 13.3 mM, 1.1 μM, and 0.5 mM. The pH was 7.50 ± 0.02.

C. Stop-flow kinetic determinations of K_m and k_{cat} for bovine muscle CA III at 10.5° (± 0.2°). The final concentrations (after mixing) of the enzyme, barbital buffer, phenol red, and dithiothreitol were, respectively, 0.61 μM, 25 mM, 30 μM, and 0.5 mM. The pH was 7.48 ± 0.02.

D. Stop-flow kinetic determinations of K_m and k_{cat} for human muscle CA III at 10.5° (± 0.2°). The final concentrations (after mixing) of the enzyme, barbital buffer, phenol red, and dithiothreitol were, respectively, 1.1 μM, 22 mM, 30 μM, and 0.5 mM. The pH was 7.48 ± 0.02.

factor calculation was evident in the determination of uncatalyzed CO_2 hydration rate at all CO_2 concentrations shown in Fig. 4B. For the average rate constant, we obtained a value (0.040 ± 0.006 sec^{-1}), in good agreement with the value found by Khalifah (10) of 0.037 sec^{-1}. The standard deviations for the enzymatic initial rates were between 2.8% and 10% of the mean of four reaction traces at each substrate concentration. The calculated values of K_m and k_{cat} are compared in Table 3 with the corresponding values for the two red cell isozymes. Relative to CA I and CA II, respectively, the turnover number of cat muscle CA III at 25° is 12 and 200-fold lower at pH 7.5. The differences in activities between CA III and the red cell isozymes CA I and CA II become even more pronounced when the k_{cat}/K_m ratios are compared.

Kinetics of bovine and human CA III. The kinetics of bovine and human CA III was analyzed by using the stop-flow method at 10.5° and pH 7.5. The saturating CO_2 solubility at this temperature is 51.5 mM (27), so that 27.75 mM was our maximal substrate concentration. Figure 4C and D shows the double-reciprocal plots of enzymatic rate versus CO_2 concentration. Standard deviations ranging from 0.05 to 14% ($n = 4$) for the human CA III runs and from 2.9 to 20% ($n = 4$) for the bovine CA III runs were obtained for enzyme-catalyzed initial rates at one substrate concentration. In the human CA III experiments, the uncatalyzed rates were 6.8–8.8% of the over-all rates in presence of 1.1 μM enzyme. For bovine CA III, the uncatalyzed rates contributed 25–28% to the over-all rates, the enzyme concentration being 0.61 μM. The mean uncatalyzed rate constant at 10.5° was 0.013 sec^{-1} with a standard deviation of 0.0005 sec^{-1}. A value of 0.010 sec^{-1} was obtained in our earlier work (26), where a different method was employed. The important kinetic parameters, K_m and k_{cat}, for both bovine and the human CA III are given in Table 3. The bovine and the human CA III have the same K_m for CO_2, but the turnover number of the human enzyme is twice that of the bovine enzyme. These numbers are compared in Table 3 with the 13° kinetic parameters for CA I and CA II, reported

TABLE 3

CO_2 hydration kinetic parameters: comparison of CA III with CA I and CA II

All data are for pH 7.5–7.6. Human CA I and CA II values are from ref. 26 and represent three to six independent experiments. The values for cat CA III at 1° and 25° represent, respectively, three and two separate experiments. Bovine and human CA III data are from single experiments. In each experiment several velocity determinations were made for each CO_2 concentration, standard deviations being 0–20% of the mean. Temperatures were constant to ±0.2°.

Temperature	Enzyme	K_m	$k_{cat} \times 10^{-3}$	$(k_{cat}/K_m) \times 10^{-6}$
		mM	sec^{-1}	$M^{-1}\ sec^{-1}$
1°	Cat CA III	40 ± 7	1.0 ± 0.2	0.025
	Human CA I	4.4 ± 1.4	29.0 ± 0.05	6.6
	Human CA II	10.5 ± 1.3	236 ± 8	23
25	Cat CA III	37 ± 2	4.2 ± 0.1	0.11
	Human CA I	4.8 ± 1.3	52 ± 1.6	11
	Human CA II	12.7 ± 1.7	860 ± 6.5	68
10.5	Bovine CA III	46	4.2	0.09
	Human CA III	45	8.9	0.20
13	Human CA I	4.9 ± 0.005	37.4 ± 0.05	7.6
	Human CA II	8.8 ± 0.005	458 ± 6.5	52

earlier (26). The human CA III has a 50-fold lower turnover number and a 5-fold higher K_m as compared with human CA II. Assuming a linear Arrhenius relationship, we calculate a turnover number of 1800 sec^{-1} at pH 7.5 for cat CA III at 10.5° from our 1° and 25° k_{cat} values. The human CA III enzyme is therefore probably 5 times as active as cat CA III.

DISCUSSION

We have confirmed that the soluble skeletal muscle CA is the lowest-activity isozyme of carbonic anhydrase known so far. Although the K_m (CO_2) values are about the same for cat, bovine, and human muscle enzymes, the turnover numbers are somewhat different. The basis of these differences is difficult to assess unless structural information is available. No crystal structure data are available for CA III. The complete amino acid sequence of bovine CA III has been reported (5), and partial sequencing of human CA III shows very good agreement with the primary structure of the bovine enzyme.[2] However, binding experiments show a higher affinity of neoprontosil toward human CA III as compared with bovine CA III.[3] Chicken CA III shows an even higher neoprontosil affinity than does human CA III.[3] We also note that Holmes (1) purified chicken CA III using an affinity chromatographic column containing PAMBS, whereas we show (Fig. 1) that cat CA III does not exhibit any detectable binding to PAMBS-saturated agarose gel. Since sulfonamide binding to CA I or CA II is known to be an active-site event (28), it is conceivable that the same subtle structural difference which may be responsible for these differing sulfonamide affinities may also be causing the differences in k_{cat} values among cat, bovine, and human muscle CA III. It is worth noting that, compared with CA I or CA II, the lower activity of CA III is accompanied by an increased resistance toward sulfonamides. Since we have not determined the pH dependence of K_m and k_{cat}, we cannot rule out the possibility that enzyme activity is linked to the ionization of an active-site group (*e.g.*, in CA I and CA II) and that the differences in turnover numbers at pH 7.5 reflect the differing ionization constants of this critical group in the enzymes from three different species.

Not all sulfonamides are poor inhibitors of cat muscle CA III. Table 2 shows that benzolamide is as active and chlorzolamide is 10 times as active against CA III as sulfanilamide is against CA II. Chlorzolamide would probably be suitable for physiological studies. Its K_i is 0.27 μM at 1°, and it should be possible to achieve more than 100-fold this concentration for *in vivo* experiments. Another important finding of our study is that CNO^- is a very potent inhibitor of CA III. Cyanate may be very useful as a physiological inhibitor of muscle enzyme, since it is relatively nontoxic (29) and is 40 times more active against CA III than it is against red cell CA II isozyme. Thus cyanate may be suitable in discriminating between muscle and red cell CA inhibition. The muscle CA III and male rat liver CA show a very similar pattern of inhibition by sulfonamides and anions. This is consistent, since male rat liver and rat muscle CA have been found to be indistinguishable by partial sequencing of the respective cyanogen bromide fragments (7).

The physiological function of muscle CA remains unresolved. Holmes (1) proposed that this enzyme may function as a CO_2/HCO_3^- binding and transport protein. However, our consistent finding of a rather high K_m (CO_2) does not support this hypothesis. Register *et al.* (3) showed that CA III has very weak activity as an esterase; ~0.06% that of enzyme C. It is also active as a *p*-nitrophenyl phosphatase (30), a property not shared by the other two isozymes of CA. However, this latter activity (k_{cat}) is extremely low. The K_m increases and the k_{cat} decreases with increasing pH. At the lowest pH of the experiment (5.35), K_m is 4.2 mM, but k_{cat} is only 0.0012 sec^{-1} at 30° for rabbit muscle CA (30).

Although skeletal muscle CA III has relatively poor intrinsic activity for CO_2 hydration, the enzyme is present in high concentration in muscle and thus may still function as a CA. From our data on cat whole muscle supernatant, we calculate the endogenous enzyme concentration in muscle to be ~100 μM. The total tissue activity can then be roughly calculated by using the Michaelis-Menten equation:

$$= \frac{k_{cat} \cdot [E] \cdot [CO_2]}{[CO_2] + K_m}$$

V and E being the enzymatic rate for CO_2 hydration and the enzyme concentration, respectively. At 0° and a CO_2 concentration of 1 mM or 33 mm Hg pCO_2, human red cells (CA I = 125 μM, CA II = 25 μM) contain carbonic anhydrase activity (V) of 1000 mM sec^{-1}, whereas cat muscle cytosol has a total CO_2 hydration activity of 2.5 mM sec^{-1}. This low activity could still be consistent with a role of carbonic anhydrase, since greater than 99% inhibition of red cell activity is necessary before any

[2] Personal communication from Dr. Richard E. Tashian, of the University of Michigan at Ann Arbor.

[3] Personal communication from Dr. William R. Osborne, of the University of Washington at Seattle.

profound changes occur in the total CO_2 output by lung (31) or before secretion is affected in certain tissues[4] (14).

There are two definite directions in which the investigation of the possible physiological role of muscle CA may be made. One is the mechanism of intracellular buffering during acidosis or alkalosis. However, there is some experimental evidence that this mechanism may not necessarily require carbonic anhydrase. For example, Bettice *et al.* (32) and Strome *et al.* (33) showed that cardiac muscle has considerable buffering capability, but Moynihan (34) was unable to demonstrate any CA activity independent of red cell contamination, and Carter *et al.* (7) showed only trace amounts of immunoreactive CA III in this tissue. In view of our finding of the low activity of skeletal muscle CA, the possibility of any intrinsic CA activity of cardiac muscle remaining undetected cannot be ruled out. Furthermore, the effects of CA inhibitors on myocardial buffering are yet to be studied. There are other studies reported in literature showing little or no intracellular buffering in skeletal muscle (35, 36).

A second and more plausible function for muscle CA would appear to be respiratory. There is considerable evidence that the high-activity (Type II) CA enzyme augments or facilitates total CO_2 movement through tissues and solution (37, 38). This theory of facilitated CO_2 diffusion may be applicable to muscle, despite the classic admonition by Roughton (39) that "in such a location carbonic anhydrase would be an enemy to the organism, rather than a friend: in its absence the CO_2 is able to diffuse away rapidly without loitering appreciably by the wayside in the form of bicarbonate ions." It is possible that CO_2 transport in buffered solutions is accelerated by utilizing HCO_3^- ion which diffuses as readily as CO_2. At the point of production, CO_2 is hydrated rapidly to HCO_3^- and the H^+ is buffered by phosphate or myoglobin. The reverse would occur at the cell-capillary interface, whence CO_2 would diffuse the short distance into blood. This process would thus permit greater CO_2 exchange over smaller pCO_2 gradients. Kawashiro and Scheid (9) actually showed experimental and theoretical evidence for facilitated CO_2 diffusion in muscle, but did not study the effects of CA inhibitors. Effros and Weissman (40) showed that 90 μM acetazolamide in the bloodless perfused hind limb of cat caused a large decrease in bicarbonate space of resting muscle. They interpreted their findings to be a reflection of capillary endothelial CA inhibition. The latter is a high-activity, sulfonamide-sensitive enzyme found by Ryan *et al.* (41) in cultured pulmonary capillary endothelial cells and shown histologically by Lönnerholm (42) in lung and by Ridderstrale (43) in skeletal muscle. Knowing the K_i (3×10^{-4} M) of acetazolamide against cat muscle CA III, we can now calculate the extent of inhibition of this enzyme in the experiments of Effros and Weissman (40). Their perfusate contained bovine serum albumin, 5 gm/liter, and assuming 50% drug binding to albumin, they had only 13% inhibition of muscle CA III. Therefore, their conclusion appears to be justified. A similar experiment was carried out in dog by Enns and Hill (44) with the added feature that spinach CA was added to the perfusate to replace the loss of endothelial enzyme during inhibition. Spinach CA is a high-activity enzyme, yet resistant to acetazolamide (45). Unlike Effros and Weissman (40), Enns and Hill (44) concluded that the observed reduction in bicarbonate output from the perfusate was due to inhibition of tissue (muscle?) enzyme. Further experiments are surely necessary in which the pharmacological effects of selective red cell and muscle CA inhibitors on both resting and exercising muscle should be studied.

Finally, we wish to emphasize that any meaningful study of the physiological function of CA in skeletal muscle would have to include mitochondrial CA. Mitochondrial CA activity has been detected in guinea pig muscle (46). The kinetic nature of this enzyme or its inhibition properties is unknown, and so is its concentration in mitochondria. Experiments to characterize muscle mitochondrial CA are currently in progress in our laboratory.

[4] The 99% inhibition of an enzymatic rate of 1000 mM sec^{-1} yields 10 mM sec^{-1}. It may appear then that an activity of 2.5 mM sec^{-1} is inadequate for function. However, this may be resolved in the following way. If we assume that skeletal muscle has a maximal blood volume of 5 ml/100 g (22), then the total CA activity of blood enzyme in 1 kg of muscle is 1000 mM sec^{-1} × 0.05 liter = 50 mmole·sec^{-1} whereas that of muscle enzyme is 2.5 mM sec^{-1} × 0.95 liter = 2.3 mmole·sec^{-1}. Thus 99% inhibition of 50 mmoles·sec^{-1} yields 0.5 mmole·sec^{-1}, a value approximating that of muscle. Since we used total blood volume rather than capillary volume, the activity of blood is overestimated. However, even in this example, the apparent excess in red cell disappears and the 2.5 mM sec^{-1} rate could be meaningful.

ACKNOWLEDGMENTS

We are grateful to Dr. Richard E. Tashian for his gift of purified bovine and human muscle CA, and for many valuable discussions. We thank Dr. Chingkuang Tu for providing us with the least-squares program for calculation of stop-flow data, and Dr. William R. Osborne for communicating results prior to publication. Amino acid analyses were performed by Dr. David Bevan and Ms. Blanche Combs, of Virginia Polytechnic Institute. Zinc determination was done at the Soil Testing Laboratory of the University of Florida. We thank Ms. Joyce Hearn for expertly typing the manuscript.

REFERENCES

1. Holmes, R. S. Purification, molecular properties and ontogeny of carbonic anhydrase isozymes: evidence for A, B and C isozymes in avian and mammalian tissues. *Eur J. Biochem.* **78**:511–520 (1977).
2. Tashian, R. E., S. K. Strout, Y.-S. L. Yu, and D. Hendriksson. Primary structure of an isozyme of carbonic anhydrase isolated from bovine skeletal muscle. *Fed. Proc.* **37**:1797 (1978).
3. Register, A. M., M. K. Koester, and E. A. Noltmann. Discovery of carbonic anhydrase in rabbit skeletal muscle and evidence for its identity with "basic muscle protein." *J. Biol. Chem.* **253**:4143–4152 (1978).
4. Tashian, R. E. Evolution and regulation of the carbonic anhydrase isozymes, in *Isozymes: Current Topics in Biological and Medical Research* (M. C. Rattazzi, J. G. Scandalios, and G. S. Whitt, eds.), Vol. 2. Alan R. Liss, New York, 21–62 (1977).
5. Tashian, R. E., D. Hewett-Emmett, S. K. Stroup, M. Goodman, and Y.-S. L. Yu. Evolution of structure and function in the carbonic anhydrase isozymes of mammals, in *Biophysics and Physiology of Carbon Dioxide* (C. Bauer, G. Gros, and H. Bartels, eds.). Springer-Verlag, New York, 165–176 (1980).
6. Jeffery, S., and N. Carter. A comparison of carbonic anhydrase III isozymes from Human, baboon, pig and sheep muscle. *Comp. Biochem. Physiol B Comp. Biochem.* **66**:439–441 (1979).
7. Carter, N., S. Jeffrey, A. Shiels, Y. Edwards, T. Tipler, and D. A. Hopkinson. Characterization of human carbonic anhydrase III from skeletal muscle. *Biochem. Genet.* **17**:837–854 (1979).
8. Carter, N. D., D. Hewett-Emmett, S. Jeffrey, and R. E. Tashian. Testosterone-induced, sulfonamide-resistant carbonic anhydrase isozyme of rat liver is indistinguishable from skeletal muscle carbonic anhydrase III. *F. E. B. S. Lett.* **128**:114–118 (1981).
9. Kawashiro, T., and P. Scheid. Measurement of Krogh's diffusion constant of CO_2 in respiring muscle at various CO_2 levels: evidence for facilitated diffu-

sion. *Pfluegers Arch. Gesamte Physiol. Menschen Tiere* **362**:127–133 (1976).
10. Khalifah, R. G. The carbon dioxide hydration activity of carbonic anhydrase: stop-flow kinetic studies on the native human isozymes B and C. *J. Biol. Chem.* **246**:2561–2573 (1971).
11. Tashian, R. E., S. K. Riggs, and Y.-S. L. Yu. Characterization of a mutant human erythrocyte carbonic anhydrase: carbonic anhydrase Ic Guam: the amino acid substitution and carboxyl esterase and hydratase activities. *Arch. Biochem. Biophys.* **117**:320–327 (1966).
12. Khalifah, R. G., D. J. Strader, S. H. Bryant, and S. M. Gibson. Carbon-13 nuclear magnetic resonance probe of active-site ionizations in human carbonic anhydrase B. *Biochemistry* **16**:2241–2247 (1977).
13. Edelhoch, H. Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry* **6**:1948–1954 (1967).
14. Maren, T. H. Carbonic anhydrase: chemistry, physiology and inhibition. *Physiol. Rev.* **47**:595–781 (1967).
15. Maren, T. H., and E. Couto. The nature of anion inhibition of human red cell carbonic anhydrases. *Arch. Biochem. Biophys.* **196**:501–510 (1979).
16. Easson, L. H., and E. Stedman. The absolute activity of cholineesterase. *Proc. R. Soc. Lond. B Biol. Sci.* **127**:142–164 (1936).
17. Maren, T. H., A. L. Parcell, and M. N. Malik. A kinetic analysis of carbonic anhydrase inhibition. *J. Pharmacol. Exp. Ther.* **130**:389–400 (1960).
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
19. Wetlaufer, D. B. Ultraviolet spectra of proteins and amino acids. *Adv. Protein Chem.* **17**:303–390 (1962).
20. Cornish-Bowden, A. *Principles of Enzyme Kinetics.* Butterworths, London (1976).
21. Kortüm, G., W. Vogel, and K. Andrussow. *Dissociation Constants of Organic Acids in Aqueous Solution.* Butterworths, London (1961).
22. King, R. W., L. C. Garg, J. Huckson, and T. H. Maren. The isolation and partial characterization of sulfonamide-resistant carbonic anhydrases from the liver of the male rat. *Mol. Pharmacol.* **10**:335–343 (1974).
23. Garg, L. C. Inhibition of carbonic anhydrases of rat liver by halides. *J. Pharmacol. Exp. Ther.* **200**:434–438 (1977).
24. Sanyal, G., N. I. Pessah, and T. H. Maren. Kinetics and inhibition of membrane-bound carbonic anhydrase from canine renal cortex. *Biochim. Biophys. Acta* **657**:128–137 (1981).
25. Koester, M. K., A. M. Register, and E. A. Noltmann. Basic muscle protein: a third genetic locus isoenzyme of carbonic anhydrase. *Biochem. Biophys. Res. Commun.* **76**:196–204 (1977).
26. Sanyal, G., and T. H. Maren. Thermodynamics of carbonic anhydrase catalysis: a comparison between human isoenzymes B and C. *J. Biol. Chem.* **256**:608–612 (1981).
27. Linke, W. F. (Ed.). *Solubilities of Inorganic and Metalorganic Compounds*, Vol. 1. D. Van Nostrand, Princeton, N. J. (1958).
28. Kannan, K. K., I. Vaara, B. Nostrand, S. Lövgreen, A. Borell, K. Fridborg, and M. Petef. Structure and function of carbonic anhydrase: comparative studies of sulfonamide binding to human erythrocyte carbonic anhydrases B and C, in *Drug Action at the Molecular Level* (G. C. K. Roberts, ed.). University Park Press, Baltimore, 73–91 (1977).
29. Cerami, A., T. A. Allen, J. H. Graziano, F. G. de Furia, J. M. Manning, and P. N. Gillette. Pharmacology of cyanate: general effects on experimental animals. *J. Pharmacol. Exp. Ther.* **185**:653–666 (1973).
30. Koester, M. K., L. M. Pullan, and E. A. Noltmann. The *p*-nitrophenyl phosphatase activity of muscle carbonic anhydrase. *Arch. Biochem. Biophys.* **211**:632–642 (1981).
31. Swenson, E. R., and T. H. Maren. A quantitative analysis of CO_2 transport at rest and during maximal exercise. *Respir. Physiol.* **35**:129–159 (1978).
32. Bettice, J. A., B. C. Wang, and E. B. Brown. Intracellular buffering of heart and skeletal muscles during the onset of hypercapnia. *Respir. Physiol.* **28**:89–98 (1976).
33. Strome, D. R., R. L. Claney, and N. C. Gonzalez. Myocardial CO_2 buffering: role of transmembrane transport of H^+ or HCO_3^- ions. *Am. J. Physiol.* **230**:1037–1041 (1976).
34. Moynihan, J. B. Carbonic anhydrase activity in mammalian skeletal and cardiac muscle. *Biochem. J.* **168**:567–569 (1977).
35. Arieff, A. I., A. Kernian, S. G. Massry, and J. Delima. Intracellular pH of brain: alterations in acute respiratory acidosis and alkalosis. *Am. J. Physiol.* **230**:804–812 (1976).
36. Aickin, C. C., and R. C. Thomas. Microelectrode measurement of the intracellular pH and buffering power of mouse soleus muscle fibers. *J. Physiol.* (Lond.) **267**:791–810 (1977).
37. Gros, G., W. Moll, H. Hoppe, and H. Gros. Proton transport by phosphate diffusion: a mechanism of facilitated CO_2 transfer. *J. Gen. Physiol.* **67**:773–790 (1976).
38. Gutknecht, J., M. A. Bisson, and D. C. Tosteson. Diffusion of carbon dioxide through lipid bilayer membranes. *J. Gen. Physiol.* **69**:779–794 (1977).
39. Roughton, F. J. W. Recent work on carbon dioxide transport by the blood. *Physiol. Rev.* **15**:241–296 (1935).
40. Effros, R. M., and M. W. Weissman. Carbonic anhydrase activity of the cat hind leg. *J. Appl. Physiol.* **47**:1090–1098 (1979).
41. Ryan, U. S., P. L. Whitney, and J. W. Ryan. Pulmonary endothelial cells possess carbonic anhydrase. *Circulation [Suppl. II]* **60**:416 (1979).
42. Lönnerholm, G. Carbonic anhydrase in the lung. *Acta Physiol. Scand.* **108**:197–199 (1980).
43. Ridderstrale, Y. Observations on the localization of carbonic anhydrase in muscle. *Acta Physiol. Scand.* **106**:239–290 (1979).
44. Enns, T., and E. P. Hill. Facilitation of carbon dioxide transport by bicarbonate in the lung and leg tissue, and the role of carbonic anhydrase. *Fed. Proc.* **39**:1094 (1980).
45. Pocker, Y., and J. S. Y. Ng. Plant carbonic anhydrase: hydrase activity and its reversible inhibition. *Biochemistry* **13**:5116–5120 (1974).
46. Dodgson, S. J., R. E. Forster II, B. T. Storey, and L. Mela. Mitochondrial carbonic anhydrase. *Proc. Natl. Acad. Sci. U. S. A.* **77**:5562–5566 (1980).

Send reprint requests to: Dr. Gautam Sanyal, Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Box J-267, JHM Health Center, Gainesville, Fla. 32610.