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SIMULTANEOUS AND INDEPENDENT VERSUS ANTAGONISTIC INHIBITION OF MUSCLE CARBONIC ANHYDRASE (CA III) BY ACETAZOLAMIDE AND CYANATE*

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Abstract—The inhibition by cyanate and acetazolamide of pig muscle carbonic anhydrase III (CA III) CO_2 hydratase activity was studied in order to explore mechanistic features possibly unique to the muscle isoenzyme. The turnover number for CO_2 hydration was found to be 6000 sec^{-1} with a K_m of 83 mM for CO_2. Cyanate inhibition (K_i, 3 μM) and acetazolamide inhibition (K_i, 44 μM) were both found to be noncompetitive with respect to CO_2. Significantly, acetazolamide and cyanate displayed non-exclusive binding to pig muscle carbonic anhydrase. The similarity of mode and degree of inhibition of muscle carbonic anhydrase by cyanate as compared with the inhibition of the erythrocyte isoenzymes suggests the existence of a similar metal environment. However, the observation that cyanate and acetazolamide bind simultaneously to CA III and the comparatively large K_i for acetazolamide *per se* appear to be more compatible with a different mode of coordination of the zinc with the sulfonamide, thus supporting a five-coordinant zinc in the catalytic mechanism of CO_2 hydration for CA III.

Muscle carbonic anhydrase (CA III)§, first described in 1977 by our laboratory [2] and also by Holmes [3], differs from the erythrocyte isoenzymes in its lower CO_2 hydratase and esterase activities and in its greater resistance to inhibition by the classic carbonic anhydrase inhibitor acetazolamide. The hydratase turnover numbers for CA III are 2400 sec^{-1} in rabbit muscle‖, 4200 sec^{-1} in cat muscle, 8900 sec^{-1} in human muscle, and 4200 sec^{-1} in bovine muscle [4] with K_m values of 95 mM for rabbit muscle‖, 37 mM for cat muscle and 45 mM for human and bovine muscle [4]. The esterase activity for the rabbit muscle CA III is about 0.06% that of rabbit erythrocyte CA II [5].

Monovalent anions have long been known to be inhibitory to carbonic anhydrase. The strongest anionic inhibitors are the "metal poisons" SH^-, CN^-, and NCO^- with K_i values of 10^{-5} to 10^{-6} M [6]. X-ray diffraction studies of the Cl^-, Br^-, I^- and SCN^- complexes indicate that the anions bind to the metal ion in erythrocyte carbonic anhydrase [7]. Usually one-to-one complexes are formed. The anion binding is pH dependent with the strongest binding occurring to the protonated form of the ionizing group of the enzyme [8].

The mechanism of anion inhibition has been re-examined recently. Pocker and Deitz [9], in a thorough study in which, however, a sulfate buffer system was used that is questioned by others [10], demonstrated that at low pH the inhibition by anions of the CO_2 hydration is noncompetitive. The inhibition diminishes at high pH with a transition to linear, uncompetitive kinetics. To explain this mode of anion inhibition, the authors proposed an optional fifth coordination site on the zinc. They suggested furthermore that the OH^- formed by general base catalysis is at the fifth site to enable attack on the CO_2 [9]. Maren and Couto [11] found the inhibition by iodide and cyanate to be "mixed but favoring noncompetitive" with respect to CO_2.

Certain sulfonamides, long known as anti-bacterial compounds, were found to function as carbonic anhydrase inhibitors as early as 1940 [12], their inhibition being noncompetitive with respect to CO_2 [13]. X-ray diffraction studies on erythrocyte carbonic anhydrase showed the sulfonamides to be liganded to the metal [14]. Anionic inhibitors of erythrocyte carbonic anhydrase were found to be displaced by addition of sulfonamides [15]. The binding of sulfonamide proved to be pH dependent, the interaction requiring the ionized form of the sulfonamide group and the protonated form of the enzyme [16]. In the X-ray diffraction patterns the acetamido group and the ring of acetazolamide were located in a hydrophobic part of the active site [14].

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§The nomenclature used in this paper for the description of carbonic anhydrase isoenzymes is that of Tashian and Carter [1].

‖Values of 2400 sec^{-1} and 95 mM were determined recently for the turnover number and K_m, respectively, of the rabbit muscle enzyme (L. M. Pullan and E. A. Noltmann, unpublished experiments, 1982). The value of 1000 sec^{-1} previously referred to as the maximal rate [2] was based on data obtained from assays under standard conditions without conversion to true V_{max} conditions.

Bovine CA III was sequenced by Tashian *et al.* [17], who found residue 91 to be an arginine, reflecting a non-conservative substitution for the phenylalanine of human CA I and for the isoleucine of human CA II which are postulated to be in contact with acetazolamide when the latter is present [14]. This substitution might affect the affinity of the aromatic or heterocyclic portion of the sulfonamide for muscle carbonic anhydrase (CA III). However, Lindskog [16] suggested that the guanidino group is likely to be oriented away from the hydrophobic region of the active site and that the substitutions of the bulkier phenylalanine and isoleucine in CA III for leucine 198 and valine 207, respectively, in human CA I and CA II may be responsible for the the lesser affinity of acetazolamide for the muscle enzyme.

It was of interest, therefore, to examine whether a study of the anion and acetazolamide inhibitors with muscle carbonic anhydrase would shed new light on some of these proposals. A preliminary account of this work has been given previously [18].

MATERIALS AND METHODS

Carbonic anhydrase III was isolated from pig skeletal muscle by the procedure previously described for rabbit muscle [2, 5]. Potassium cyanate of analytical reagent grade was obtained from Mallinckrodt. Acetazolamide was obtained from Lederle Laboratories as Diamox, and chlorzolamide was provided by E. R. Swenson. Diethylmalonic acid, 95%, was obtained from Aldrich and sodium barbital from Sigma. All solutions were made with doubly glass-distilled, deionized water. A Beckman research grade pH meter was used for all pH measurements.

For the study of the inhibition patterns of cyanate and sulfonamides prevailing for their interaction with pig carbonic anhydrase III, the CO_2 hydratase activity was monitored at various concentrations of substrate (CO_2) and inhibitor(s) by using a modification of the Wilbur–Anderson method [19] introduced by Rickli *et al.* [20]. Enzyme units were defined as the liberation of 1 μmole H^+/sec. Enzyme and CO_2 are added immediately before each assay to the buffer containing the inhibitor. There was no need to preincubate enzyme with any of the inhibitors since anions have rapid association and dissociation rates [21], and preincubation of the sulfonamides was found to have no effect. All assays were performed in random order with the assay tubes kept in an ice bath. For the competition of cyanate and acetazolamide, both inhibitors were present in the buffer with the maximum concentration of CO_2 being used.

The data points were subjected to line fitting employing the process of linear least squares regression analysis. Data so obtained for triplicates of the uncatalyzed samples were subtracted from the means of triplicates of samples containing the enzyme. The resultant velocities corresponding to the enzyme catalyzed process were then plotted accordingly with use of linear regression analysis.*

RESULTS

The objective of this study was to identify the mode of inhibition of muscle carbonic anhydrase III caused by two inhibitors known for the erythrocyte carbonic anhydrases to have different but overlapping points of binding and inhibition, with the goal to understand the similarities and differences in catalysis and inhibition between the muscle and erythrocyte isoenzymes. Muscle carbonic anhydrase III is a less efficient catalyst of CO_2 hydration and ester hydrolysis and is more resistant to inhibition by acetazolamide than the erythrocyte carbonic anhydrases. Yet the muscle enzyme apparently has substantial structural homology and its study may offer new information for any proposed generalized carbonic anhydrase mechanism.

Cyanate inhibition of CA III. Cyanate is one of the strongest monovalent anion metal-binding inhibitors of erythrocyte carbonic anhydrase and has long been studied as a marker of the coordination state of the enzyme and of its mechanism of catalysis [22]. Cyanate was found to be a good inhibitor of the CO_2 hydratase activity of pig muscle CA III as also reported by Sanyal *et al.* [4] for cat muscle CA III. A plot (Fig. 1) of the reciprocal of velocity versus the reciprocal of substrate concentration at various cyanate concentrations ranging from 0 to 4.7 μM cyanate, with each line fitted independently, gives an apparent common intercept just below the x-axis, indicative of almost totally noncompetitive inhibition

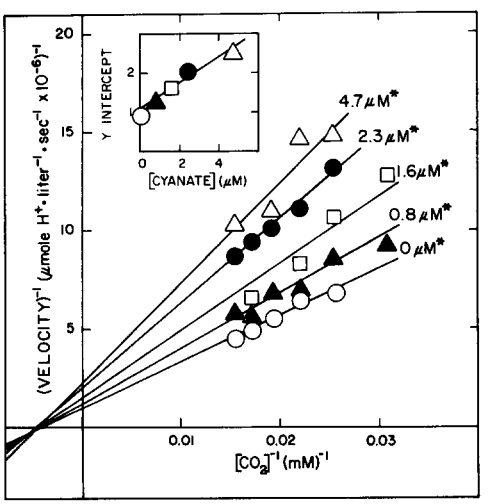

Fig. 1. Cyanate inhibition of muscle carbonic anhydrase III. A plot of the reciprocal of velocity against the reciprocal of substrate concentration is shown. Experimental conditions: 0.08 μM pig muscle carbonic anhydrase III, 12.5 mM sodium barbital, pH 8.2, 0°. Key: (○) no cyanate; (▲) 0.8 μM cyanate; (□) 1.6 μM cyanate; (●) 2.3 μM cyanate; and (△) 4.7 μM cyanate. The asterisks indicate concentration of cyanate. Inset: y-intercept as a function of cyanate concentration.

*It should be noted that the conditions required for these experiments (variation of CO_2 concentration over ranges at which *absolute* rates are comparatively low and therefore significantly affected by the corresponding blank rates) yielded some scatter and, additionally, required rather long extrapolation through CO_2 levels at which no actual measurements can be made.

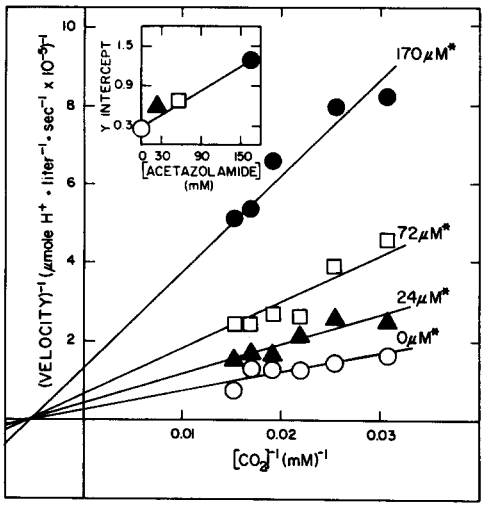

Fig. 2. Acetazolamide inhibition of muscle carbonic anhydrase III. A plot of the reciprocal of velocity against the reciprocal of substrate concentration is shown. Experimental conditions: 0.03 μM pig muscle enzyme, 12.5 mM sodium barbital, pH 8.2, 0°. Key: (○) no acetazolamide; (▲) 24 μM acetazolamide; (□) 72 μM acetazolamide; and (●) 170 μM acetazolamide. The asterisks indicate concentration of acetazolamide.

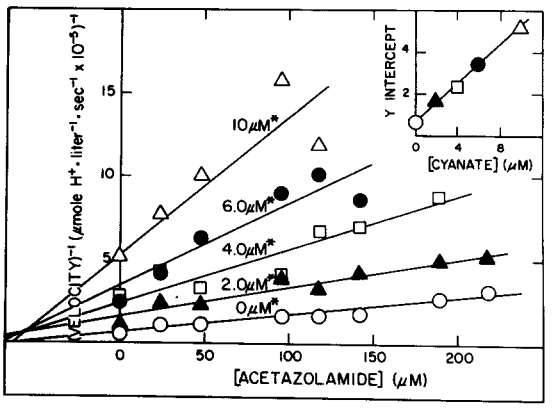

Fig. 3. Competition of cyanate and acetazolamide for muscle carbonic anhydrase III. A plot of the reciprocal of velocity versus acetazolamide concentration in the presence of various cyanate concentrations is shown. Experimental conditions: 0.03 μM pig muscle carbonic anhydrase III, 12.5 μM sodium barbital, pH 8.2, 0°. Key: (▲) 2.0 μM cyanate; (□) 4.0 μM cyanate; (●) 6.0 μM cyanate; and (△) 10 μM cyanate. The asterisks indicate concentration of cyanate. Inset: y-intercept as a function of cyanate concentration.

with respect to CO_2. The K_i, as obtained from linear regression of the intercepts on the y-axis versus cyanate concentration (Fig. 1, inset), was found to be 3 μM.

Acetazolamide inhibition of CA III. Acetazolamide is the most extensively studied of the sulfonamide inhibitors of carbonic anhydrase and, for the erythrocyte isoenzymes, is known to be a potent inhibitor with its binding dependent both on the nature of the active-site metal and the presence of a hydrophobic pocket. A plot (Fig. 2) of the reciprocal of the velocity versus the reciprocal of the CO_2 concentration at acetazolamide concentrations ranging from 0 to 170 μM, with each line fitted independently, was found to give a common intercept near the x-axis, suggesting noncompetitive inhibition. The K_i, as calculated from the linear regression of the y-intercepts versus the acetazolamide concentration (Fig. 2, inset), was found to be 44 μM. The K_m for CO_2 corresponding to the x-axis intercept of Fig. 2, in the absence of acetazolamide, extrapolated to 83 mM. The k_{cat} was calculated to be 5970 sec^{-1}.

Competition between acetazolamide and cyanate. Incubation of an enzyme with two inhibitors, each at various concentrations, can determine whether the two inhibitors bind simultaneously or whether each can only bind exclusive of the other [23]. Experimentally, the velocity of enzyme catalysis is determined for samples with different concentrations of each of the two inhibitors. Parallel lines, one line for each concentration of one inhibitor, will result from a plot of the reciprocal of the velocity versus the concentration of the other inhibitor if the two inhibitors cannot bind simultaneously but act exclusively of one another [24]. A plot (Fig. 3) of the reciprocal of CA III velocity versus acetazolamide concentration at various cyanate concentrations ranging from 0 to 10 μM yielded a set of non-parallel lines when each line was independently fitted. The K_i obtained from the intercept of the x-axis with the line in the absence of cyanate was found to be 53 μM. A K_i of 1.4 μM was calculated for cyanate from linear regression analysis of the y-intercepts versus the cyanate concentration (Fig. 3. inset). The intercept of the non-parallel lines of Fig. 3 is very near the x-axis, indicating no difference in the mode of inhibition by acetazolamide in the presence or absence of cyanate.

DISCUSSION

This study, the first on pig muscle carbonic anhydrase III, confirms the low hydratase activity of muscle CA III isoenzymes in general as compared with that of the erythrocyte carbonic anhydrases. The pig muscle turnover number of 6000 sec^{-1} is comparable with those reported for other species of the muscle isoenzyme, but the K_m of 83 mM is somewhat larger [4, 5] and similar to that found for the enzyme from rabbit muscle (see ‖ footnote at the beginning of the paper).

Cyanate is an inhibitor of muscle carbonic anhydrase with potency comparable to that observed for the erythrocyte isoenzymes. Our data on the inhibition of pig muscle carbonic anhydrase III by cyanate show a K_i of about 3 μM, similar to that reported for cat muscle, 0.52 μM [3], and erythrocyte carbonic anhydrase I and II, 0.8 and 20 μM respectively [11]. Our study shows that cyanate gives almost totally noncompetitive inhibition with respect to CO_2 for muscle carbonic anhydrase III as it does for the erythrocyte isoenzymes [9, 11]. Thus, based upon the similarity of its behaviour towards cyanate as an inhibitor of the muscle isoenzyme, there ap-

pears to be little difference in the metal environment at the active site of carbonic anhydrases I, II and III.

The relative resistance to acetazolamide inhibition by muscle carbonic anhydrase III has been of considerable historical importance. In fact, it discouraged isolation and identification of the muscle enzyme as long as Roughton's paradigm that in this tissue "carbonic anhydrase would be an enemy of the organism. . ." [24] was taken literally. We believe that the resistance to acetazolamide inhibition is also indicative of important but as yet unknown structural differences in the muscle isoenzyme as compared with the erythrocyte enzyme species. For pig muscle carbonic anhydrase III, the K_i of acetazolamide is about 44 μM, the inhibition following a noncompetitive pattern with respect to CO_2. The K_i is comparable to that estimated by our laboratory in 1978 for rabbit muscle carbonic anhydrase III [5] and to that reported recently for cat muscle [4]. However, it is much larger than the K_i values for erythrocyte carbonic anhydrases [25]. Despite the large difference in inhibition levels, the sulfonamide is noncompetitive with respect to CO_2 for both the muscle and the erythrocyte isoenzymes [13]. The similarity of this inhibition type for the muscle enzyme may be taken to suggest some similarity in the sulfonamide binding to the erythrocyte carbonic anhydrases.

From his early studies with human erythrocyte carbonic anhydrases, Coleman [26] concluded that a single site is involved in the inhibition by anions and sulfonamides with displacement of acetazolamide occurring by NCO^-, N_3^-, SH^- and CN^-. For the muscle enzyme the difference in inhibitor strength suggested the need to test whether anions and sulfonamide were indeed exlusive of each other with respect to their binding. It was found that acetazolamide and cyanate were clearly non-exclusive as indicated by the non-parallel lines at various cyanate concentrations in the plot of the reciprocal of velocity versus acetazolamide concentration. Chlorzolamide, a sulfonamide with K_i of 0.27 μM for cat muscle carbonic anhydrase [4] also gave the impression of non-exclusive inhibition with regard to cyanate. The lack of difference in the K_i of acetazolamide in the presence or absence of the anion may be taken to imply that the mechanism of acetazolamide binding is similar in both instances.

Simultaneous binding of the two inhibitors at the active site of muscle carbonic anhydrase requires that the binding of one of the inhibitors differs from the mode of binding seen with the erythrocyte isoenzymes which have been found to display mutual exclusiveness of the two types of inhibitors. Since the level and type of inhibition of CA III by cyanate closely parallel the data typical for carbonic anhydrases I and II, cyanate binding would have to be substantially unaltered. On the other hand, acetazolamide yields noncompetitive inhibition of pig muscle carbonic anhydrase III, presumably binding to the metal because acetazolamide does not possess a preferential affinity for the hydrophobic region. Indeed, in carbonic anhydrase III the amino acid substitutions in the area of the hydrophobic pocket are expected to reduce the affinity in this location. Acetazolamide must bind in the vicinity of the metal but, presumably with altered coordination, must also allow simultaneous binding of the cyanate anion. Taylor and Burgen [21] discussed, and rejected, the possibility of an intermediate with both sulfonamide and anion on the metal coordination sphere of erythrocyte carbonic anhydrase. Acetazolamide is bidentate when bound to either carbonic anhydrase I or II. The nitrogen of the sulfonamide group replaces water at the fourth site of coordination of the zinc and an oxygen of the sulfonamide group is located at its fifth coordination site [14]. Perhaps, for muscle carbonic anhydrase, acetazolamide utilizes only one ligand to the zinc. For copper-substituted bovine and human erythrocyte carbonic anhydrases, Bertini *et al.* [27] found that the metal coordination of sulfonamide ligands, irrespective of whether coordination occurred with one or two ligands of the sulfonamide, depended on both the type of sulfonamide and on the particular isoenzyme. Certainly, some change in the metal coordination by sulfonamides must occur to explain the simltaneous binding of cyanate and acetazolamide to carbonic annydrase III. It seems difficult to conceive of a four-coordinate zinc ion in the active site of CA III to which both ligands simultaneously bind noncompetitively with respect to CO_2. We, therefore, feel that the inhibition patterns of muscle carbonic anhydrase III lend support to a five-coordinate zinc in the catalytic mechanism of CO_2 hydration.

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