We report three experiments which show that the hydrolysis of 4-nitrophenyl acetate catalyzed by carbonic anhydrase III from bovine skeletal muscle occurs at a site on the enzyme different than the active site for CO₂ hydration. This is in contrast with isozymes I and II of carbonic anhydrase for which the sites of 4-nitrophenyl acetate hydrolysis and CO₂ hydration are the same. 1) The pH profile of k₄₅/K₉₅ for hydrolysis of 4-nitrophenyl acetate was roughly described by the ionization of a group with pKₐ 6.5, whereas k₄₅/K₉₅ for CO₂ hydration catalyzed by isozyme III was independent of pH in the range of pH 6.0–8.5. 2) The apoenzyme of carbonic anhydrase III, which is inactive in the catalytic hydration of CO₂, was found to be as active in the hydrolysis of 4-nitrophenyl acetate as native isozyme III. 3) Concentrations of N₅ and OCN⁻ and the sulfonamides methazolamide and chlorzolamide which inhibited CO₂ hydration did not affect catalytic hydrolysis of 4-nitrophenyl acetate by carbonic anhydrase III.

Carbonic anhydrase in mammals occurs in at least three genetically distinct forms, designated isozyme I (formerly B), II (formerly C), and III (1). Each is a zinc-containing enzyme of molecular weight near 30,000 with 50–60% homology between the residues in any pair of the bovine isozymes (2). Carbonic anhydrase III from bovine skeletal muscle has been fully or partially sequenced by two research groups, with some differences obtained in cysteine content (2, 3). The maximal turnover number for hydration of CO₂ catalyzed by bovine isozyme III, 3 x 10⁵ s⁻¹ (3), is 500 times less than that of bovine isozyme II and 70 times less than that of human isozyme I. These steady-state rate constants for CO₂ hydration catalyzed by isozyme III are independent of pH in the region of pH 6–9, very different from the properties of isozymes I and II (1). Moreover, isozyme III is not sensitive to inhibition by acetazolamide, which is a potent inhibitor (Kᵢ ~ 10⁻¹²–10⁻¹⁰ M) of isozymes I and II (3, 4).

Both carbonic anhydrases I and II catalyze the hydrolysis of various aryl carboxylate esters and the hydration of aliphatic aldehydes, among other reactions (5). These catalytic properties occur at the same active site as the hydration of CO₂, based on similarities in pH rate profiles and inhibition by anions and sulfonamides (5). Carbonic anhydrase III from rabbit muscle is known to catalyze the hydrolysis of 4-nitrophenyl acetate, but with a very low activity (6); the activity at pH 7.2 is 0.06% of that catalyzed by rabbit isozyme II, and is 0.5% of that catalyzed by rabbit isozyme I. Engberg et al. (3) found that cyanate (OCN⁻), a potent anionic inhibitor of CO₂ hydration catalyzed by bovine isozyme III, had no effect on the 4-nitrophenyl acetate hydrolytic activity of bovine isozyme III. This suggests that for this isozyme the CO₂ hydration and 4-nitrophenyl acetate hydrolysis sites are different. We have confirmed this suggestion by measuring three catalytic properties of bovine isozyme III. 1) The pH rate profile for hydrolysis of 4-nitrophenyl acetate was different than that for hydration of CO₂ catalyzed by isozyme III. 2) A sample of bovine carbonic anhydrase III with zinc partially removed was very much less active in the hydration of CO₂ but retained its full catalytic activity in the hydrolysis of 4-nitrophenyl acetate. 3) Catalysis of 4-nitrophenyl acetate hydrolysis was not inhibited by concentrations of N₅, OCN⁻, and certain sulfonamides that inhibit CO₂ hydration and ¹⁸O exchange.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hepes, 1 M, hexamethylenetetramine, and phenol red were obtained from Sigma and used without further purification. 4-Nitrophenyl acetate was recrystallized from acetone. 2-Carboxy-1,10-phenanthroline was the generous gift of Drs. P. Engberg and S. Lindskog (Umeå University, Sweden). NaN₅ and KOCN were obtained from Aldrich and methazolamide and chlorzolamide from the Lederle Laboratories (Pearl River, NY). Distilled, deionized water was passed through an ion exchange column prior to use (Cole-Palmer 1506-35) and glassware was rinsed with 1 mM EDTA followed by thorough rinsing with water. ⁴⁰O-labeled bicarbonate was prepared by equilibrating KHCO₃ in ⁴⁰O-enriched water and then removing the water by vacuum distillation.

**Enzyme**—Carbonic anhydrase III was obtained from bovine flank muscle by two procedures. One procedure used two affinity chromatographic resins as described by Sanyal et al. (4), the first is specific for red cell carbonic anhydrase and the second binds carbonic anhydrase III in a different manner. This method was not as efficient for bovine isozyme III as was described by Sanyal et al. (4) for cat isozyme III. Consequently, the enzyme sample was also applied to an Ultrogel column (ACa 44, LKB) and eluted with a solution containing 10 mM Tris and 100 mM Na₂SO₄ at pH 7.6. A second purification procedure used a combination of ion exchange and gel filtration chromatography. One kilogram of flank muscle from a freshly killed bull was homogenized in a Waring blender with about 1.5 liters of 10 mM Tris buffer at pH 6.0. The resulting material was filtered through coarse cotton gauze followed by filtration through glass wool or, in some instances, centrifuged. Six hundred grams/liter ammonium sulfate was used to precipitate the soluble protein which was then collected and dialyzed against several changes of distilled water. After filtration through Whatman No. 1 filter paper, the clear liquid was concentrated to about 40 ml by ultrafiltration. After dialysis against a solution of 0.025 M Tris and 0.1 M sodium sulfate at pH 8.0, the sample of approximately 1 g of total protein was eluted from a gel filtration column (Ultrogel ACa 44, LKB) (5 x 50 cm) using the dialysis buffer. The active fractions were pooled and concentrated as previously described to about 20 ml.

*The abbreviations used are: Hepes, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid.
and approximately 300 mg of total protein. This was followed by ion exchange chromatography (DEAE-Sephacel, Pharmacia) utilizing discontinuous step elution on a 2.5 × 50-cm column. The equilibrated sample was first eluted with 0.05 M Tris sulfate at pH 8.5, then 0.050 M Tris sulfate at the same pH. Three fractions were found to have relatively equal carbonic anhydrase activity. Polyacrylamide sodium dodecyl sulfate electrophoresis of the three active fractions isolated indicated each to have one major band corresponding to a molecular weight of 30,000. The second and by far the major fraction to be eluted was used in these experiments.

Concentrations of bovine carbonic anhydrase III were estimated using a molar absorptivity at 280 nm of 6.4 × 10^4 M^-1 cm^-1 (7). The apoenzyme of bovine carbonic anhydrase III was prepared by addition of the chelating agent 2-carboxy-1,10-phenanthroline followed by dialysis in a procedure described by Engberg and Lindskog (7).

Stopped-flow Measurement of CO2 Hydration—The initial velocity of CO2 hydration was determined by measuring the rate of change of absorbance of a pH indicator as described by Khalil et al. (8). In this study, the buffer-indicator pair Hepes/phenol red was used at pH 7.5. Both Hepes and phenol red have pH; 7.5 and phenol red was observed at 557 nm, where it has a molar absorptivity of 5.59 × 10^4 M^-1 cm^-1. Saturated solutions of CO2 (33.8 mM at 25°C) were prepared by bubbling CO2 gas into water. Dilutions were made by coupling two syringes. Total ionic strength of the solution was maintained at 0.2 by adding the appropriate amount of Na2SO4; buffer concentration were determined at the substrate and buffer concentrations given in the legend to Fig. 1; the uncatalyzed rates of hydrolysis have been measured by the methods of Verpoorte et al. (9), and its catalysis by carbonic anhydrase has been described (10). The exchange of O from CO2 to H2O is caused by the hydration-dehydration cycles at chemical equilibrium.

CO2O + H2O = HCO3O^- + H+ → CO2 + H2O

The second step is considered irreversible because H2O cannot be so greatly diluted by H2O in solution. This exchange was measured by pico-18O-labeled bicarbonate into solution and waiting an appropriate time for the approach to chemical equilibrium. Then the depletion of 18O from CO2 in solution was measured by using a membrane inlet to a mass spectrometer; the membrane is permeable to CO2 and allows a continuous measurement of the 18O content of CO2 in solution (11). The measured variable is a, the atom fraction of 18O in CO2.

\[ a = \frac{46 + 2(48)}{2(44 + 46 + 48)} \]

where 44, 46, and 48 represent the heights of the mass peaks of 12C18O16O, 13C18O16O, and 13C18O18O. The analysis of this 18O exchange has been fully described (9-12). a decreases in a first-order manner with rate constant 18.

\[ \alpha = \alpha_0 e^{-kt} \]

The rate constant 18 can be described as the sum of uncatalyzed and catalyzed processes, \( \alpha = \alpha_0 + \alpha_{cat} \). At pH 7.4, 18max = 7.0 × 10^-4 s^-1.

Into the membrane vessel was placed 8.0 ml of solution containing a varied concentration of CO2-HCO3 and sufficient Na2SO4 to make a total ionic strength of 0.2; no buffers were used. After measurement of the uncatalyzed exchange, carbonic anhydrase III was added in a volume less than 0.1 ml. During an experiment, pH remained constant to within 0.02 pH unit and temperature was 25.0 ± 0.2°C.

Measurement of Ester Hydrolysis—The catalysis by bovine carbonic anhydrase III of the hydrolysis of 4-nitrophenyl acetate was measured by the methods of Verpoorte et al. (13), in which the inorganic phosphate absorbance was followed at 348 nm, the isosbestic point of nitrophenol and the conjugate nitrophenolate ion using the molar absorptivity 5.0 × 10^4 M^-1 cm^-1 (this value is corrected for a small absorbance of p-nitrophenol acetate itself, see Ref. 13). A Gilford 2400 spectrophotometer interfaced with a Digital MINC 11 computer and a Beckman DU 7 spectrophotometer were used. Initial velocities were determined at the substrate and buffer concentrations given in the legend to Fig. 1; the uncatalyzed rates of hydrolysis have been subtracted from all data presented here. After we had determined that we were observing the region of the velocity versus substrate curve that is first order in substrate, we were able to determine Kcat by dividing the observed initial velocity of hydrolysis by E[S]. (That is, kcat = kcat/Km for hydrolysis.)

RESULTS

The steady-state constants for the hydration of CO2 catalyzed by bovine carbonic anhydrase III are independent of pH in the range of pH 6-8.5, as has already been reported for cat isozyme III (14) and bovine isozyme III (3). We have also obtained this result for bovine isozyme III with steady-state constants in agreement with those of Engberg et al. (9) at 25°C. Our values are as follows (mean and standard deviation): \( k_{cat} = (3.5 ± 1.5) × 10^8\) M^-1 s^-1; \( k_{cat}/K_m = (3.2 ± 1.0) × 10^6\) M^-1 s^-1. These standard deviations are large because they were calculated from constants obtained in the pH range from 6.0 to 8.5 using five different buffers. Unlike the catalyzed hydration of CO2, which is independent of pHe, the hydrolysis of 4-nitrophenyl acetate resembles roughly the form of the pH titration of a single ionizable group with a pKa of 6.5 and a maximum at alkaline pH (Fig. 1). A similar result with a higher pKa of about 8 was obtained by Koester et al. (6) for rabbit muscle isozyme III.

Both native bovine isozyme III and a sample treated with chelator (2-carboxy-1,10-phenanthroline) to remove zinc had the same activity in the hydrolysis of p-nitrophenyl acetate (Table I). In the 18O exchange assay, the enzyme treated with chelator was found to be 10% as active as native enzyme when measured before the esterase experiment and 28% as active after the esterase experiment, the difference presumably being due to contamination by adventitious zinc.

The inhibition by N2 of the hydration of CO2 catalyzed by bovine isozyme III was uncompetitive with respect to CO2 with \( K_i = 9.6 \mu M \) at pH 7.5 (Fig. 2). The cause of the uncompetitive nature of this inhibition has been discussed earlier (15). Isozyme III, like isozyme II (16), is believed to be limited in rate of CO2 hydration by a proton transfer occurring outside the steps involved in the interconversion of CO2 and HCO3-. Uncompetitive inhibition arises because anions bind to a form of enzyme appearing after product release but before the rate-limiting step (16). This value of \( K_i \) can be compared with 14 \mu M measured at 0°C (4) and 25 \mu M at 25°C (15), both for cat isozyme III, and 5 \mu M for the bovine isozyme III at 25°C (3). Table II also gives values for \( K_i \) for cyanate, melhazolamide, and chlorozolamide in the inhibition of CO2 hydration at steady state; comparable values measured with cat isozyme III are given by Sanyal et al. (4) measured at 0°C

![Fig. 1. The variation with pH of the second-order rate constant kcat for the hydrolysis of 4-nitrophenyl acetate catalyzed by bovine carbonic anhydrase III (1.5 × 10^-4 M). 4-Nitrophenyl acetate was present at 1.0 mM, and 33 mM of the following buffers were used: ○ hexamethylenetetramine, ● Mes, ● Hepes. Solutions also contained 33 mM Na2SO4. Temperature was 22°C. Points are means with standard deviations of three experiments. The line is the calculated titration curve for a single ionizable group with pKa = 6.5 and maximum at 11.3 M^-1 s^-1.](image-url)
TABLE I

The catalytic activity of native bovine carbonic anhydrase III and bovine carbonic anhydrase III with zinc removed

<table>
<thead>
<tr>
<th></th>
<th>Hydrolysis of 4-nitrophenyl acetate</th>
<th>Exchange of ¹⁸O between CO₂ and H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \nu_{\text{cat}} ) (catalytic rate, ( \text{ms}^{-1} ))</td>
<td>( \theta_{\text{cat}} ) (rate constant for ( \text{¹⁸O} ) loss from CO₂, ( \text{s}^{-1} ))</td>
</tr>
<tr>
<td>Native carbonic anhydrase III</td>
<td>(1.6 ± 0.1) ( \times 10^{-7} )</td>
<td>(14.0 ± 0.2) ( \times 10^{-8} )</td>
</tr>
<tr>
<td>Enzyme treated with chelator*</td>
<td>(1.4 ± 0.1) ( \times 10^{-7} )</td>
<td>(1.4 ± 0.1) ( \times 10^{-3} )</td>
</tr>
<tr>
<td>Measured before esterase assay</td>
<td></td>
<td>(3.6 ± 0.3) ( \times 10^{-3} )</td>
</tr>
<tr>
<td>Measured after esterase assay</td>
<td></td>
<td>(12.6 ± 0.2) ( \times 10^{-3} )</td>
</tr>
<tr>
<td>Enzyme treated with chelator then add excess ZnSO₄</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Bovine carbonic anhydrase III was treated with the chelating agent 2-carboxy-1,10-phenanthroline and then dialyzed to remove the chelator and zinc according to the procedure of Engberg and Lindskog (7).

The inhibition by \( \text{NaN₃} \) of the exchange of \( \text{¹⁸O} \) between CO₂ and H₂O catalyzed by bovine carbonic anhydrase III (1.4 \( \times 10^{-4} \) M) and measured by membrane-inlet mass spectrometry at chemical equilibrium. [5] is the first-order rate constant for \( \text{¹⁸O} \) depletion from all species of CO₂. The pH was 7.4 and temperature 25 °C. The concentrations of \( \text{NaN₃} \) were none, \( \text{NaN₃} \), 0.4 mM; \( \text{KOCN} \), 0.4 mM; and 1.0 mM. No buffers were used, and total ionic strength of solution was 0.2 achieved by addition of Na₂SO₄. The lines were calculated assuming uncompetitive inhibition and \( k_{\text{cat}} = 3.0 \times 10^3 \text{s}^{-1} \), \( K_a = 0.025 \text{M} \), and \( (K_{\text{Hg}})_\text{Hg} = 9.6 \text{mM} \).

**TABLE II**

Inhibition constants \( K_I \) of bovine carbonic anhydrase III from skeletal muscle by the anions azide and isocyanate and the sulfonamides methazolamide and chlorzolamide

Values were measured at 25 °C in the presence of sufficient Na₂SO₄ to give a total ionic strength of solution of 0.2. Further details are described in text. The values in parentheses are the pH of the measurements.

<table>
<thead>
<tr>
<th></th>
<th>CO₂ hydration at steady state</th>
<th>( ^{18} \text{O} ) exchange at equilibrium</th>
<th>Hydrolysis of 4-nitrophenyl acetate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu \text{M} ) (pH)</td>
<td>( \mu \text{M} ) (pH)</td>
<td>1000 (7.6)</td>
</tr>
<tr>
<td>Na₃N</td>
<td>9.6 (7.5)</td>
<td>350 (7.4)</td>
<td>&gt;1000 (7.6)</td>
</tr>
<tr>
<td>KOCN</td>
<td>1.4 (7.2)</td>
<td>15 (7.4)</td>
<td>&gt;1300 (7.5)</td>
</tr>
<tr>
<td>Methazolamide</td>
<td>100 (7.5)</td>
<td>&gt;1000 (7.5)</td>
<td></td>
</tr>
<tr>
<td>Chlorzolamide</td>
<td>0.3 (7.6)</td>
<td>20</td>
<td>&gt;130 (7.5)</td>
</tr>
</tbody>
</table>

* No inhibition of the hydrolysis of 4-nitrophenyl acetate, within our experimental errors, was detected at the concentrations of inhibitor listed in this column.

**DISCUSSION**

Our main conclusion is that the catalysis of hydrolysis of 4-nitrophenyl acetate by bovine isozyme III occurs at a site on the enzyme different than the active site for CO₂ hydration. This conclusion is based on three results, the interpretation of which is discussed in more detail below. (1) The pH profile of the activity of catalytic hydrolysis was different than that for catalytic hydration. (2) The apoenzymes, which have no catalytic hydrolysis of CO₂, was found to be as active as catalytic activity of this ester as native carbonic anhydrase III. (3) Concentrations of N₂ and OCN⁻ and certain sulfon-
amides which inhibited CO$_2$ hydration did not affect catalytic hydration of this ester.

The catalytic site for CO$_2$ hydration catalyzed by isozymes II and I has convincingly been shown to be the zinc-bound hydroxyl group located at the bottom of a funnel-shaped cavity about 14 Å from the surface of the protein (1). The catalysis by isozymes II and I of the hydrolysis of esters, as well as the hydration of aliphatic aldehydes, occurs at the same zinc-bound hydroxyl as determined by the similar pH rate profiles, susceptibility to inhibitors, and lack of activity of apoenzyme (5). There are some notable exceptions to this comment: the hydrolysis of phenyl N-methyl acetimidate catalyzed by bovine carbonic anhydrase II is not inhibited by sulfonamides and anions which inhibit the CO$_2$ hydration and 4-nitrophenyl acetate hydrolysis (17). Also, there is a large component at pH near 10 of the hydrolysis of 2,4-dinitrophenyl cyclobutane carboxylate catalyzed by bovine isozyme II which is not inhibited by potent sulfonamides and is also catalyzed by the apoenzyme (18). Bovine isozyme II when inhibited by acetazolamide also shows hydrolysis activity toward 4-nitrophenyl propionate at pH > 10 (19). Our results with isozyme III are more like these exceptions for isozyme II, although in none of these cases has the identity of the second catalytic site been determined.

**pH Rate Profile**—Our profile for catalysis of 4-nitrophenyl acetate by bovine isozyme III has a pK$_a$ close to 6.5 (Fig. 1) but appears too complex to be explained by the ionization of a single group. The steady-state constants for hydration of CO$_2$ catalyzed by bovine isozyme III are independent of pH from pH 6 to 8.5 (Ref. 3 and this work). The catalytic constants describing CO$_2$ hydration by cat isozyme III at chemical equilibrium are also independent of pH in this region (14). We conclude that the catalyses of CO$_2$ hydration and 4-nitrophenyl acetate hydrolysis depend on different groups on the enzyme. As an example, CO$_2$ hydration could be catalyzed by zinc-bound hydroxide with a pK$_a$ < 6 and ester hydrolysis by a general base mechanism involving a surface group such as a histidine.

**Catalysis by Enzyme with Zinc Removed**—For isozymes II and I, removal of zinc abolishes CO$_2$ hydration activity (1), as we also observed with isozyme III, although we had contamination by adventitious zinc. The binding of zinc to the active site of isozyme III may be more tight than for isozymes II and I, as judged by the observation that chelators which render isozymes I and II into the apoenzyme form have no effect on isozyme III (3). The results in Table I indicate that the hydrolysis of 4-nitrophenyl acetate does not depend on the zinc site of the bovine enzyme III.

**Inhibition**—Concentrations of azide, cyanate, methazolamide, and chlorozolamide which are clearly inhibitory in the hydration of CO$_2$ catalyzed by isozyme III do not affect the hydrolysis of 4-nitrophenyl acetate (Table II). This has already been pointed out for OCN$^-$ by Engberg et al. (3) who, on this basis, suggested that the catalytic site for hydrolysis of this ester is different from the active site for CO$_2$ hydration. (Their rate constant for hydrolysis of 4-nitrophenyl acetate at pH 7.5 and 20 °C, $k_{\text{max}} \approx 1 \times 10^{-9}$ s$^{-1}$, is considerably below our value of $k_{\text{max}} = 10^{-4}$ m$^{-1}$ s$^{-1}$ in Fig. 3 for reasons as yet undetermined.) Concentrations of these inhibitors which do not affect the hydrolysis block also catalytic $^{18}$O exchange at equilibrium (Table II). These data are included to support our conclusion in view of the following argument: the maximum velocity of CO$_2$ hydration catalyzed by isozyme III appears limited in rate, at least in part, by a proton transfer step (15), most likely the step which forms zinc-bound hydroxide from zinc-bound water. Consequently, like isozyme II (16, 20), isozyme III may have an accumulation of the species preceding the rate-limiting step, an accumulation of the form of enzyme with zinc-bound water at the active site at steady state. The form of carbamic anhydrase with zinc-bound water is the form to which anions and sulfonamides bind most tightly (16). This explains in Table II the observation that $K_I$ measured at steady state is lower than $K_I$ measured at chemical equilibrium in CO$_2$ hydration. The experiments using $^{18}$O exchange have the equilibrium ratio of zinc-bound water to zinc-bound hydroxide, a ratio which is very low because the corresponding pK$_a$ is less than 6. Then at equilibrium there is a small fraction of enzyme in the form to which inhibitors bind most tightly. In steady state the zinc-bound water form accumulates to which inhibitors bind tightly, resulting in lower values of $K_I$.

The catalysis of the hydrolysis of 4-nitrophenyl acetate is so slow that the ionization states of the active site are at or very near equilibrium. The data in Table II show that even if this is the case, the concentrations of N$_3$ and OCN$^-$ which block catalysis at equilibrium do not affect the ester hydrolysis.

**Acknowledgments**—We thank Drs. S. Lindskog and P. Engberg for a generous gift of 2-carboxy-1,10-phenanthroline and Michael Hule for assistance with the esterase runs.

**REFERENCES**