Enhancement of the Catalytic Activity of Carbonic Anhydrase III by Phosphates*

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Phosphate and phosphate-containing buffers of physiological interest such as ATP and 3-phosphoglycerate were found to enhance catalysis by human carbonic anhydrase III (HCA III). Addition of phosphate caused an increase in both the catalyzed rate of hydration of CO₂ at steady state measured by stopped-flow spectrophotometry and the exchange of ¹⁸O between CO₂ and water at chemical equilibrium measured by mass spectrometry. The results are consistent with a mechanism in which phosphate enhances the transfer of protons between zinc-bound water at the active site and solution. Site-directed mutations to replace lysine 64 and arginine 67 in the active-site cavity resulted in greater enhancement by phosphate when compared with wild-type HCA III and showed that these basic residues are not essential as a binding site for phosphate. Phosphate did not enhance catalysis by HCA II.

Carbonic anhydrase III is found predominantly in the cytoplasm of type I (slow-oxidative, red) skeletal muscle fibers where its physiological function has been uncertain, especially since other more active forms of carbonic anhydrase also appear in the same muscle fibers (Gros and Dodgson, 1988). With noninteracting buffers such as Mes™ and Mops at physiological pH, human carbonic anhydrase III (HCA III) catalyzes the hydration of CO₂ with a turnover number near 2 × 10⁴ s⁻¹, which is about 500 times less than that for carbonic anhydrase II, an isozyme found in red cells and secretory tissues (Sanyal et al., 1982; Engberg et al., 1985; Kararli and Silverman, 1985).

The catalysis of the dehydration of HCO₃⁻ by carbonic anhydrase occurs in two distinct and separate stages (Silverman and Lindskog, 1988). The first is the conversion of HCO₃⁻ to CO₂, which requires the zinc-bound water form of the active site (Equation 1).

\[
\text{HCO}_3^- + \text{EZnH}_2\text{O} \rightarrow \text{CO}_2 + \text{H}_2\text{O} + \text{EZnOH}^- \quad (1)
\]

The second is the regeneration of the active site by the transfer of a proton from solution to the zinc-bound hydroxide (Equation 2).

\[
\text{EZnOH}^- + \text{BH}^+ \rightarrow \text{EZnH}_2\text{O} + \text{R} \quad (2)
\]

In this reaction, BH⁺ can be buffer in solution, a residue of the enzyme itself, or water in the active site. For carbonic anhydrase II, which is among the most efficient of these isozymes, it is known that the predominant pathway in Equation 2 requires buffer in solution as the proton donor and that proton transfer from buffer to the active site is assisted by histidine 64 as a proton shuttle residue (Silverman and Lindskog, 1988; Tu et al., 1989). For carbonic anhydrase III, the buffers Mops, Hepes, and TED caused no change in the catalysis (Kararli and Silverman, 1985) and it was assumed that isozyme III was not susceptible to buffer enhancement and that in this case BH⁺ in Equation 2 was water. It was subsequently discovered by Shelton and Chegwidden (1988) that phosphate is able to enhance the dehydration of HCO₃⁻ catalyzed by HCA III at steady state. Later it was found that other small buffers of the imidazole, morpholine, and lutidine type are also able to enhance catalysis by HCA III or catalysis by some site-directed mutants of this isozyme (Tu et al., 1990).

In addition to CO₂, it is known that 4-nitrophenyl phosphate is a very poor substrate (turnover number ≈0.07 min⁻¹) for rabbit and pig carbonic anhydrase III, but is not a substrate for isozyme II (Koester et al., 1981; Pullan and Noltmann, 1985). Moreover, this phosphatase activity can be blocked by reaction of the enzyme with phenylglyoxal with no loss of CO₂ hydration activity; hence, the active site for this phosphatase activity is not the same as for the CO₂ hydration activity (Pullan and Noltmann, 1985).

We report here that phosphate and some phosphate-containing compounds were able to enhance catalysis of HCA III by facilitating the transfer of protons between enzyme and its aqueous environment. Phosphate did not enhance catalysis by HCA II. The presence of phosphate increased as much as 20-fold the proton transfer between the active site of HCA III and solution, but had no significant effect on the interconversion of CO₂ and HCO₃⁻ at chemical equilibrium. Addition of phosphates caused an increase in both the catalyzed rate of hydration of CO₂ at steady state measured by stopped-flow spectrophotometry and the exchange of ¹⁸O between CO₂ and water measured by mass spectrometry. Site-directed mutations to replace lysine 64 and arginine 67 resulted in even greater enhancement of HCA III activity by phosphate and showed that these basic residues are not essential as a binding site for phosphates.

MATERIALS AND METHODS

Enzyme A bacterial expression vector containing a wild-type HCA III gene was derived from the cDNA clone of Lloyd et al. (1986) using the pET-8 vector (a gift from Dr. F. William Studier, Brook-

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‡ The abbreviations used are: Mes, 4-morpholineethanesulfonic acid; Mops, 3-(N-morpholino)propanesulfonic acid; HCA III, human carbonic anhydrase III; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ted, 1,4-diazabicyclo[2.2.2]octane.
RESULTS

We have observed an enhancement in the catalytic activity of HCA III and some site-directed mutants of HCA III upon addition of phosphate. The addition of potassium phosphate to HCA III at pH 7.2 caused a 20-fold increase in $R_{HCO_3}$, the rate of release of $^{18}O$-labeled water from the enzyme (Fig. 1).

\[
\text{EZn}^+OH^- + BH^+ \rightleftharpoons \text{EZn}^+O\text{H}_2 + B \quad \text{(3)}
\]

Here, BH$^+$ can represent buffer in solution or water in the active site; for HCA II, BH$^+$ represents merely histidine 64. Previous reports describe in detail how these rate constants are used to obtain the rates $R_e$ and $R_{HzO}$ (Silverman, 1982; Silverman et al., 1979). Two rates for the catalysis at chemical equilibrium were determined from the $^{18}O$-exchange results. The first rate, $R_e$, is the catalyzed rate of interconversion of CO$_2$ and HCO$_3^-$ (Equation 1). The second rate, $R_{HzO}$, is the rate of release from the enzyme of $^{18}O$-labeled water (Equation 3). $R_{HzO}$ is a proton transfer-dependent process since $^{18}O$-labeled hydroxide is not expected to exchange rapidly (Silverman et al., 1979; Silverman and Lindskog, 1988).

\[
\text{EZnH}^+O + HzO + B \rightleftharpoons \text{EZn}^+O\text{H}_2 + B \quad \text{(3)}
\]

Steady-state Kinetics—The initial velocities of the catalyzed hydration of CO$_2$ and dehydration of HCO$_3^-$ were measured by a changing pH-indicator method (Khalifah, 1971) using a Durrum-Gibson stopped-flow spectrophotometer according to procedures described earlier (Rowlett and Silverman, 1982). Solutions of CO$_2$ were prepared from CO$_2$-saturated water (34 mM at 25°C; Pocker and Bjorkquist, 1977). The buffer-indicator pair used was potassium phosphate (PK, pH 5.2) in a previous report (of $^{18}O$-exchange catalyzed by bovine carbonic anhydrase III in the absence of buffers, we described the biphasic depletion of $^{18}O$ from CO$_2$ (Silverman and Tu, 1986). In this report we have measured only the slow phase of the biphasic experiment observed for wild-type HCA III and the calculation of $R_e$ and $R_{HzO}$ are based on this rate of $^{18}O$ depletion from CO$_2$. In general, biphasic behavior was not observed for the mutants of HCA III studied here.

Steady-state Kinetics—The initial velocities of the catalyzed hydration of CO$_2$ and dehydration of HCO$_3^-$ were measured by a changing pH-indicator method (Khalifah, 1971) using a Durrum-Gibson stopped-flow spectrophotometer according to procedures described earlier (Rowlett and Silverman, 1982). Solutions of CO$_2$ were prepared from CO$_2$-saturated water (34 mM at 25°C; Pocker and Bjorkquist, 1977). The buffer-indicator pair used was potassium phosphate (PK, pH 7.2) with $p$-nitrophenol (pK$_a$ 7.1). The observed wavelength in the stopped-flow experiments was 400 nm. All steady-state experiments were carried out at 25°C with the total ionic strength of solution maintained at a minimum of 0.2 M using Na$_2$SO$_4$.

**TABLE I**

Rate constants of Equation 4 for the dependence on the concentration of phosphate of $R_{HzO}$, the rate of release of H$_2$O from human carbonic anhydrase III and mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{HzO}^b$</th>
<th>$K_{HzO}^b$</th>
<th>$k_{HzO}^b/K_{HzO}^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type HCA III</td>
<td>0.78 ± 0.08</td>
<td>66 ± 23</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>K64H HCA III</td>
<td>0.37 ± 0.04</td>
<td>29 ± 10</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>R67N HCA III</td>
<td>3.0 ± 0.4</td>
<td>101 ± 26</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>K64H-R67N HCA III</td>
<td>1.4 ± 0.1</td>
<td>14 ± 5</td>
<td>10 ± 3.2</td>
</tr>
<tr>
<td>F198L HCA III</td>
<td>17 ± 1.9</td>
<td>82 ± 26</td>
<td>22 ± 3.1</td>
</tr>
</tbody>
</table>

**FIG. 1.** The dependence on the concentration of phosphate of $R_{HzO}[E]$ (○) and $R_{HzO}[E]$ (*) catalyzed by wild-type human carbonic anhydrase III (left) and the mutant R67N human carbonic anhydrase III (right). The temperature was 10°C and pH 7.2 with 25 mM total concentration of CO$_2$ and HCO$_3^-$. The total ionic strength of solution was kept at a minimum of 0.2 M by the addition of Na$_2$SO$_4$.

Experimental conditions are given in the legend to Fig. 1. The values in this table are nonlinear least squares values and standard errors in a fit to Equation 4. The values of $k_{HzO}^b$ in the absence of buffer were (in all units $^3$10$^{-9}$ s$^{-1}$ M$^{-1}$) 0.3, 2.2, 2.1, 19, and 8.1 for wild-type, K64H, R67N, K64H-R67N, and F198L HCA III, respectively.

In contrast, $R_e$, the rate of interconversion of CO$_2$ and HCO$_3^-$ at chemical equilibrium, was not affected greatly, but did show about a 2-fold increase as phosphate was added up to 400 mM (Fig. 1, left). The effect of phosphate on the mutant R67N HCA III was qualitatively similar, with R$HCO_3^-$ enhanced to a much higher magnitude upon addition of phosphate (Fig. 1, right).
nonlinear least squares fit to the data of Fig. 1 as well as data showing the enhancement by phosphate of $R_{H,O}$ catalyzed by site-directed mutants of HCA III. In each case there was no appreciable change in $R_2$ upon addition of phosphate, similar to results shown in Fig. 1.

Many phosphate containing buffers we tested had the characteristic of enhancing $R_{H,O}$ catalyzed by HCA III with only minor effects on $R_2$. Among those causing a large enhancement of $R_{H,O}$ were ATP, 3-phosphoglycerate (Table II), and 4-nitrophenyl phosphate (Fig. 2). Among these, 4-nitrophenyl phosphate is itself a substrate with maximal values of $k_{cat} = 0.07 \text{m}^{-1}$ and $K_m = 3 \text{mM}$ at pH 5.4 (Koester et al., 1981). Phosphocreatine (50 mM) and cAMP (22 mM) did not enhance $R_{H,O}$ or $R_2$ under the conditions of Fig. 1.

The effect on HCA III of 200 mM potassium phosphate over the range of pH 6 to 9 showed a 20-fold enhancement in $R_{H,O}$ at the low pH regions and no effect at pH near 9 (Fig. 2). The enhancement of $R_{H,O}$ in Fig. 2 is consistent with the ionization of a single group of $pK_a$ 6.9 suggesting that the H$_3$PO$_4$ ion ($pK_a$ 7.2) is responsible. Again, there was no significant change in the values of $R_2$ over this pH range. The enhancement of $R_{H,O}$ caused by 4-nitrophenyl phosphate ($pK_a$ 5.5 under these conditions) is shifted to lower values of pH compared with the enhancement caused by phosphate (Fig. 2).

In contrast to the results with HCA III, phosphate at pH 7.1 had an inhibitory effect on $R_2$ and $R_{H,O}$ catalyzed by HCA III from red cells. (There was evidence for a small enhancement in $R_{H,O}$, near 50 mM phosphate.) A fit of the Langmuir equation to the data showed that phosphate inhibited $R_2$ with $K_I = 207 \pm 55 \text{mM}$ and inhibited $R_{H,O}$ with $K_I = 171 \pm 50 \text{mM}$.

The hydration of CO$_2$ catalyzed by HCA III at steady state was measured by stopped-flow spectrophotometry at pH 7.0 and 25 °C. In the presence of 50 mM of the noninteracting buffer Mops, $k_{cat} = (2.1 \pm 0.5) \times 10^5 \text{s}^{-1}$ and $k_{cat}/K_m = (2.9 \pm 0.5) \times 10^6 \text{M}^{-1} \text{s}^{-1}$. Under these conditions (but in the absence of Mops), the addition of phosphate caused a saturable increase in $k_{cat}$ to a maximal value of $2.4 \times 10^6 \text{M}^{-1} \text{s}^{-1}$ with a value of $k_{cat}/K_m$ which increased from $2.6 \times 10^6 \text{M}^{-1} \text{s}^{-1}$ at 2.6 mM phosphate to a plateau near $3.5 \times 10^5 \text{M}^{-1} \text{s}^{-1}$ (Fig. 3).

**DISCUSSION**

The pattern of enhancement of catalytic activity of HCA III by addition of phosphate-containing compounds is characteristic in several ways of proton transfer between the phosphate and the enzyme. These phosphates enhance $R_{H,O}$, the rate of release of labeled water from the enzyme, with little or no effect on $R_2$, the rate of interconversion of CO$_2$ and HCO$_3^-$ (Fig. 1). This is the pattern observed for the enhancement of activity of HCA III by the buffer imidazole as proton transfer agent (Tu et al., 1990) and of HCA II by buffers (Silverman et al., 1979; Tu et al., 1989). In these cases the buffers, excluding phosphate which inhibits HCA II, act in a manner consistent with Equation 3, where BH$^+$ transfers a proton to the zinc hydroxide at the active site facilitating the exchange of zinc bound water with solvent water. This is supported by Fig. 2 in which the pH profile for the enhancement of $R_{H,O}$ is fit by a titration curve indicating that a single ionizable group, approximated in Fig. 2 with a $pK_a$ of 6.9, is responsible for the activation of catalysis. This corresponds closely to the second $pK_a$ for phosphoric acid ($pK_a$ 7.2) and suggests that the H$_2$PO$_4^-$ ion is responsible for the enhancement of activity observed at low pH in Fig. 2. Consistent with this argument, the enhancement of $R_{H,O}$ by 4-nitrophenol.

**TABLE II**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>$k_{cat}^a$</th>
<th>$K_m^a$</th>
<th>$k_{cat}^a/K_m^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>($10^4$ s$^{-1}$)</td>
<td>mM</td>
<td>($10^4$ s$^{-1}$)</td>
</tr>
<tr>
<td>Phosphate</td>
<td>7.5 ± 0.8</td>
<td>66 ± 23</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>3-Phosphoglycerate</td>
<td>5.6 ± 0.3</td>
<td>28 ± 4</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Adenosine 5'-triphosphate</td>
<td>2.8 ± 0.3</td>
<td>38 ± 13</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Imidazole</td>
<td>3.5 ± 0.2</td>
<td>92 ± 11</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

**FIG. 2.** The pH dependence of $R_{H,O}[E]$ (○) and $R_{H,O}[E]$ (●) catalyzed by HCA III in the absence of phosphate, and $R_{H,O}[E]$ catalyzed by HCA III in the presence of 200 mM phosphate (▲) or 200 mM 4-nitrophenyl phosphate (■). The experimental conditions were as described in the legend to Fig. 1. The solid line is a titration curve with a $pK_a$ of 6.9.

**FIG. 3.** Top, the phosphate dependence of $k_{cat}$ for the hydration of CO$_2$ catalyzed by HCA III at steady state. Bottom, the phosphate dependence of the logarithm of $k_{cat}/K_m$ ($\text{M}^{-1} \text{s}^{-1}$) for the hydration of CO$_2$ catalyzed by HCA III. The pH was 7.0 at 25 °C. Measurements were made by stopped-flow spectrophotometry at 400 nm. The indicator p-nitrophenol. The total ionic strength of solution was adjusted to a minimum of 0.1 M by addition of Na$_2$SO$_4$. 
phosphate, $pK_a$ 5.5, is shifted to lower values of pH (Fig. 2). The $\text{HPO}_4^{2-}$ ion with a $pK_a$ of 12 would be inefficient as a proton transfer agent since it would be required to transfer a proton resulting in a much more acidic group, zinc-bound water with a $pK_a$ below 6.

The catalytic hydration of CO$_2$ at steady state supports the role of phosphate in proton transfer with the active site. Fig. 3 shows the enhancement of $k_{cat}$ by phosphate to $2.4 \times 10^6$ s$^{-1}$; this is about 10-fold greater than the maximum of $2 \times 10^5$ s$^{-1}$ at pH 7.2 observed with noninteracting buffers such as Mops. There was no major effect on $k_{cat}/K_m$ which in the presence of phosphate has a value near $3.5 \times 10^8$ M$^{-1}$ s$^{-1}$, the same as observed with the noninteracting buffers. This is consistent with the proton transfer mechanism of Equations 1 and 2 since $k_{cat}/K_m$ contains rate constants only for the steps up to and including the first irreversible step, the release of HCO$_3^-$ that is, $k_{cat}/K_m$ contains only the steps in Equation 1 and is not affected by the proton transfer steps between enzyme and solution. The apparent second-order rate constant for the transfer of protons from HCA III to phosphate was obtained from $k_{cat}/K_m$ in Fig. 3 using Equation 4 of Jonsson et al. (1976) and was determined to be $5 \times 10^7$ M$^{-1}$ s$^{-1}$. The values of $k_{cat}/K_m$ in Table I determined by $^{18}$O-exchange are for the proton transfer in the reverse direction Shelson and Chegwidden (1988) showed the enhancement by phosphate of the dehydrogenation of HCO$_3^-$ catalyzed by HCA III at steady state and also indicate enhancement reaching a plateau at about 20 mM phosphate.

That many phosphate-containing buffers are able to activate HCA III is puzzling since the active-site cavity is sterically constrained by, among other residues, Phe-198 and Arg-67 (Eriksson, 1988) and since buffers of similar or smaller size such as Mops and Hepes have no effect (Kararli and Silverman, 1986). HCA III does not have a suitable proton shuttle residue to transfer protons between solution and the active site as has been suggested for His-64 of HCA II (Steiner et al., 1975; Tu et al., 1989). This suggests that phosphates must insert at somewhat into the active-site cavity, which is about 15 Å deep, to transfer a proton to the zinc-bound hydroxide, perhaps through intervening water bridges. The values of the apparent second-order rate constants $k_{cat}/K_m$ for proton transfer between phosphate and HCA III are much smaller than expected for a diffusion-controlled process.

The values of $k_{cat}/K_m$ in Table I determined by $^{18}$O-exchange are for the proton transfer in the reverse direction Shelson and Chegwidden (1988) showed the enhancement by phosphate of the dehydrogenation of HCO$_3^-$ catalyzed by HCA III at steady state and also indicate enhancement reaching a plateau at about 20 mM phosphate.

A source of error in some of these measurements is the biphasic depletion of $^{18}$O from CO$_2$ which was observed in the catalysis by wild-type HCA III in the absence of buffer (Silverman and Tu, 1986) or with noninteracting buffers such as Mops. This introduces an error because the calculation of $R_1$ and $R_{HCO_3}$ assumes that catalyzed depletion of $^{18}$O from CO$_2$ can be described by a single exponential. This effect was negligible with buffers which enhance activity of HCA III and was also negligible for the mutants of HCA III which have enhanced activity compared with the wild-type HCA III. In this work we have measured the slow phase of this depletion and determined $R_1$ and $R_{HCO_3}$ as if the depletion were monophasic. As a result, these rates for wild-type HCA III in the absence of buffer and at low buffer concentrations contain this error, which appears in $k_{cat}$ but not in $k_{HCO_3}$. It is difficult to determine the magnitude of this error; Silverman and Tu (1986) have shown that this effect causes an overestimate by 2-fold in $k_{cat}/K_m$ for hydration by HCA III in the absence of buffers.

We can speculate on the possible binding site of phosphate on HCA III. The prominent positions of lysine 64 and arginine 67 in the active-site cavity make them possible candidates. The very weak 4-nitrophosphatase activity of HCA III indicates a phosphate-binding site (Pullan and Noltmann, 1985). Moreover, phosphate itself is an inhibitor of this phosphatase activity with a $K_i$ near 1 mM (Koester et al., 1981). The weak phosphatase activity of pig carbonic anhydrase III was blocked by phenylglyoxal which modifies 1 arginine residue and does not affect the CO$_2$ hydration activity (Pullan and Noltmann, 1986); hence, the catalytic site for this phosphatase activity is not the same as for the CO$_2$ hydration activity. Arginine 67 is located in the active-site cavity with $\mu$-carbon about 8 Å from the zinc. Replacement of arginine 67 with asparagine by site-directed mutagenesis resulted in a mutant enzyme which had an $^{18}$O-exchange activity greatly enhanced by phosphate (Fig. 1); thus it is possible to conclude that arginine 67 is not essential for the enhancement by phosphate of the catalytic activity of HCA III. This conclusion can also be reached by similar reasoning for lysine 64 and phenylalanine 198 by noting that in which these residues are replaced there remains enhancement of activity by phosphate (Table I). This was also observed for the double mutant K64H-R67N HCA III. Thus, we have not been able to identify a binding site in HCA III for phosphate. For isozyme II, however, it is apparent from the $^{18}$O-exchange results that phosphate is an inhibitor of both $R_1$ and $R_{HCO_3}$ and probably binds to the zinc as do other anionic inhibitors of HCA II. Our results indicate that phosphate does not bind to the metal in isoziym III because we see no decrease in $R_1$ upon addition of this buffer.

The physiological significance of these results is not clear; since they indicate that activation of catalysis by HCA III at or near equilibrium is not an enhancement of the interconversion of CO$_2$ and HCO$_3^-$ but an enhancement of proton transfer between the enzyme and its aqueous environment. Although the enhancement by phosphate of this proton transfer rate is 20-fold for HCA III, the resulting enhancement of the hydration of CO$_2$ at steady state is a modest 2- or 3-fold. Moreover, the concentrations of phosphates that cause such enhancements are large compared with physiological concentrations of phosphate and ATP (at or below 5 mM). Phosphocreatine, which may attain concentrations near 30 mM in muscle cells (Kushmerick, 1983), does not enhance the activity of HCA III (this work; Shelson and Chegwidden, 1988). Thus, these phosphate effects may offer a clue to some function of carbonic anhydrase III yet undiscovered, as others have suggested (Pullan and Noltmann, 1985; Tashian et al., 1980), or we may only be observing a feature of the stereochemistry of the active-site cavity of HCA III with no physiological significance.

Phosphate and some phosphate-containing compounds of physiological significance caused an activation of the CO$_2$ hydration activity of skeletal muscle carbonic anhydrase III, a property not found for red cell carbonic anhydrase II. The characteristics of the activation are consistent with enhancement by phosphate of proton transfer between the zinc-bound water at the active site of isozyme III and solution, with phosphate having no significant effect on the actual steps involved in the interconversion of CO$_2$ and HCO$_3^-$. 

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