Proton Transfer between Hemoglobin and the Carbonic Anhydrase Active Site*

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The exchange of $^{18}$O between CO$_2$ and water (type I) and the exchange of $^{18}$O between $^{12}$C- and $^{13}$C-containing species of CO$_2$ (type II) at 25° near neutral pH have been taken as a measure of the hydration activity of bovine red cell carbonic anhydrase. The rate constant for type I exchange increased sharply by 70% upon addition of bovine oxyhemoglobin and reached a plateau at $5 \times 10^{-6}$ M hemoglobin. The rate constant for type II exchange decreased sharply and leveled off at $5 \times 10^{-6}$ M oxyhemoglobin. In the same range of concentration, L-histidine had a similar effect on type I and II exchange. Carbonic anhydrase was present at 2 or 3 $\times 10^{-6}$ M. In analogy with the interpretation of similar observations at $5 \times 10^{-6}$ M of buffers such as imidazole and 2,4-lutidine, it is proposed that hemoglobin enhances catalytic activity of bovine carbonic anhydrase by donating protons to and accepting protons from the enzyme active site. The observation that hemoglobin causes a maximal increment of hydration activity at concentrations 1000-fold less than for imidazole and many other buffers of low molecular weight cannot be explained solely by summing the expected proton transfer capabilities of residues on the surface of the protein. Furthermore, this effect need not be related to surface properties or the large size of hemoglobin since rate constants for type I and II exchange also level off at concentrations near $5 \times 10^{-6}$ M of L-histidine. In these equilibrium experiments, it is possible that there is cycling of protons between hemoglobin, or L-histidine, and the carbonic anhydrase active site during which more than 1 atom of $^{18}$O is exchanged from CO$_2$ to water. Hemoglobin and carbonic anhydrase are the two most abundant proteins in red cells and we anticipate that proton transfer between them, including possibly the Bohr proton, occurs in part as a result of the direct encounter of hemoglobin with carbonic anhydrase in the cell.

The catalytic activity of carbonic anhydrase is consistent with a mechanism in which the unprotonated form of an ionizable group in the active site catalyzes the hydration of CO$_2$ and the protonated form of this group catalyzes the dehydration of HCO$_3^{-}$. Consequently, repeated carbonic anhydrase-catalyzed hydration reactions require proton transfer from the active site to the environment of the enzyme to regenerate the unprotonated form.

$\text{CO}_2 + E + H_2O \rightleftharpoons EH^+ + HCO_3^-$

To account for the very high turnover number for the hydration of CO$_2$ catalyzed by this enzyme (about $10^8$ s$^{-1}$), it has been proposed that the proton transfer is facilitated by buffers in solution (1-3). This hypothesis has been supported by observations of an increase in the catalytic hydration activity as the concentration of buffers is increased, the activity reaching a maximum near $5 \times 10^{-6}$ M for buffers such as imidazole (pK$_a \sim 7.0$) and 2.4-lutidine (pK$_a \sim 6.8$) (4-6). These buffers act as proton transfer agents which, when present at concentrations large compared to [H$_2$O$^+$] or [OH$^-$], enhance catalytic activity by donating protons to or accepting protons from the activity-controlling group at the active site, which has a pK$_a$ near 7 for bovine and human C carbonic anhydrase (1). It is not known whether this proton transfer occurs directly or through intervening amino acid side chains or water bridges.

The purpose of this work is to determine how the activity of carbonic anhydrase is affected by the main buffer of the red cells, hemoglobin.

EXPERIMENTAL PROCEDURES

Materials - Carbonic anhydrase was obtained from freshly drawn bovine erythrocytes. The erythrocytes were washed three times with isotonic saline and then lysed by addition of a volume of water equal to the volume of packed cells. The membranes were separated by centrifugation and the hemolysate was placed in contact with an affinity gel prepared by the coupling of a carbonic anhydrase inhibitor, p-aminomethylbenzene sulfonamide, to an agarose matrix CM-Bio-Gel A (Bio-Rad) (7). The affinity gel with carbonic anhydrase bound was washed thoroughly and carbonic anhydrase was eluted from the affinity gel with 0.4 M NaNO$_3$. This enzyme was extensively dialyzed against water, lyophilized, and stored at temperatures less than 0°. Prior to each run the carbonic anhydrase was again dialyzed and the concentration of this enzyme in solution was determined by a changing pH method (8).

The kinetic technique used to measure carbonic anhydrase activity in this report is very sensitive; consequently, to determine accurately the effect of hemoglobin on the catalytic hydration rate it was necessary to achieve an efficient separation of carbonic anhydrase from hemoglobin. Two procedures to purify hemoglobin were followed. 1) Bovine red cell hemolysate was passed through the aforementioned sulfonamide-coupled affinity gel six times. After
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RESULTS

In each experiment the rate constants $\theta_{cat}$ and $\phi_{cat}$ were determined as a function of concentration of imidazole, L-histidine, human serum albumin, and bovine hemoglobin but at a constant concentration of bovine carbonic anhydrase. Experiments were performed at least four times with each of these buffers or proteins, except albumin for which experiments were performed twice. Results are given in Figs. 1 to 4. Fig. 1 illustrates the changes observed in $\theta_{cat}$ and $\phi_{cat}$ as a function of the concentration of the buffer imidazole at a constant concentration of bovine carbonic anhydrase. Patterns of $\theta_{cat}$ and $\phi_{cat}$ similar to Fig. 1 have been observed with other small-sized buffers such as 2,4-dinitrophenol (15) and 1,5-dimethylimidazol (pK$_a$ ~ 8.3) (6). $\theta_{cat}$ is the catalyzed component of the first order rate constant for the exchange of $^{16}$O between CO$_2$ and water, and $\phi_{cat}$ is the rate constant for the catalyzed component of the exchange of $^{16}$O between $^{13}$C- and $^{13}$C-containing species of CO$_2$. The results of Fig. 2 are for oxyhemoglobin, obtained by bubbling oxygen through the sample. Results indistinguishable from those of Fig. 2 were obtained using carbon monoxide hemoglobin or partial (50%) deoxygenated hemoglobin. When the $^{16}$O exchanges were carried out in a solution containing both 25 mm imidazole and 10 m oxyhemoglobin, values of $\theta_{cat}$ and $\phi_{cat}$ were identical to those shown in Fig. 1 for 25 mm imidazole alone.

The hemoglobin sample contained a very small impurity of carbonic anhydrase (see "Methods"). The value of $\theta_{cat}$ due to the presence of 3.0 x 10$^{-9}$ m bovine carbonic anhydrase is then obtained from each hemoglobin concentration by subtraction of $\theta$ before addition of enzyme from $\theta$ obtained after addition of enzyme. The result of this procedure is the subtraction from $\theta$ of the value of $\theta_{cat}$ resulting from the small carbonic anhydrase impurity and the value of $\phi_{cat}$. The value of $\theta_{cat}$ under the conditions of these experiments is 7.6 x 10$^{-4}$ s$^{-1}$. Because of the carbonic anhydrase impurity in our hemoglobin sample, a solution 10$^{-6}$ M in hemoglobin had $\theta = 8.7$ x 10$^{-4}$ s$^{-1}$ and a solution 5 x 10$^{-5}$ M in hemoglobin had $\theta = 1.32$ x 10$^{-3}$ s$^{-1}$ before formal addition of enzyme in the experimental

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C^{16}O^{18}O + H_{2}^{16}O \rightarrow H^{16}CO^{18}O + HO^{16}O + H_{2}^{16}O
\]

and is catalyzed by carbonic anhydrase. Type I exchange in the absence of enzyme occurs as a result of the chemical reaction between CO$_2$ and CO$_2$ which is catalyzed by carbonic anhydrase (6, 12, 14). Eight milliliters of a solution containing buffer or protein, 16O-labeled bicarbonate, and 13C-labeled bicarbonate, making a total concentration of 0.015 M in all species of CO$_2$ were placed in the inlet vessel at 25$^\circ$C. Ionic strength was maintained at 0.2 with Na$_2$SO$_4$ and pH was adjusted with 0.1 M NaOH or H$_2$SO$_4$. The inlet vessel had as its base a membrane permeable to CO$_2$ which allowed the measurement of isotopic content of CO$_2$ by a mass spectrometer. This apparatus is described in Ref. 12 under the heading "Low pH Range." Following a period of 2 or 3 min to approach chemical equilibrium between CO$_2$ and HCO$_3$-, rates of $^{16}$O exchange were measured from the atom fraction of $^{16}$O in 13C- and 13C-containing CO$_2$. These procedures are described in Ref. 12. $\theta$ is the first order rate constant for type I exchange, obtained as the slope of a plot of the logarithm of the atom fraction of $^{16}$O in all CO$_2$ against time. $\phi$ is the first order rate constant for the exchange of $^{16}$O between 13C- and 13C-containing CO$_2$. Type II exchange in the competitive exchange of CO$_2$ and water, and $\phi_{cat}$ is the rate constant for the catalyzed component of the exchange of $^{16}$O between 13C- and 13C-containing species of CO$_2$. The results of Fig. 2 are for oxyhemoglobin, obtained by bubbling oxygen through the sample. Results indistinguishable from those of Fig. 2 were obtained using carbon monoxide hemoglobin or partial (50%) deoxygenated hemoglobin. When the $^{16}$O exchanges were carried out in a solution containing both 25 mm imidazole and 10 m oxyhemoglobin, values of $\theta_{cat}$ and $\phi_{cat}$ were identical to those shown in Fig. 1 for 25 mm imidazole alone.

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Fig. 2. The same as in Fig. 1 with rate constants measured at various concentrations of bovine oxyhemoglobin instead of imidazole. The points are mean values shown with standard deviations from five experiments.

Fig. 3. The same as in Fig. 1 with rate constants measured at various concentrations of human serum albumin instead of imidazole.

Fig. 4. The rate constants for the catalyzed exchange of $^{18}$O between CO$_2$ and water, $\theta_{\text{cat}}$ (○); and the rate constants for the catalyzed exchange of $^{18}$O between $^{14}$CO$_2$ and $^{14}$CO$_2$-containing species of CO$_2$, $\theta_{\text{cat}}$ (○) at pH 7.1, 25°, and various concentrations of L-histidine. The concentration of bovine carbonic anhydrase was 2.0 x 10$^{-3}$ M and the total concentration of all species of CO$_2$ was 1.5 x 10$^{-2}$ M. Ionic strength was held constant at 0.2 with Na$_2$SO$_4$, and the $^{13}$C content of CO$_2$ was 43 to 45%. Rate constants for the uncatalyzed exchanges are not affected by these buffer concentrations: $\theta_{\text{uncat}} = 1.4 \times 10^{-3}$ s$^{-1}$, $\phi_{\text{uncat}} = 1.4 \times 10^{-4}$ s$^{-1}$. Under these conditions with 25 mM imidazole replacing L-histidine $\theta_{\text{cat}} = 1.1 \times 10^{-2}$ s$^{-1}$ and $\phi_{\text{cat}} = 2.0 \times 10^{-2}$ s$^{-1}$.

The observations in Fig. 1 have been interpreted in terms of the catalytic mechanism (5, 6, 12). Most notably, the pattern for $\theta_{\text{cat}}$ is indicative of a change in rate-determining step: as the concentration of proton-transfer agent increases the catalysis moves from a region in which buffer-assisted proton transfer is rate-limiting to a region in which another step in the catalysis is rate-limiting. The pattern for the variation of $\phi_{\text{cat}}$ is symmetrical with that for $\theta_{\text{cat}}$. It can be accounted for by the hypothesis that the residence time for $^{18}$O in the active site is roughly the same order of magnitude as the time for one catalytic cycle (6, 12); this results in the catalytic reaction of $^{18}$O with $^{13}$CO$_2$. As the concentration of proton transfer agent increases, the residence time for $^{18}$O in the active site decreases leading to the decrease in $\phi_{\text{cat}}$. Treating the data of Fig. 1 in a manner described earlier (5), it is possible to estimate the bimolecular rate constant for the proton exchange between imidazole and the carbonic anhydrase active site: $k_2 \approx 1 \times 10^4$ M$^{-1}$ s$^{-1}$. This proton transfer is probably diffusion-controlled or close to it.

The data for the changes in $\theta_{\text{cat}}$ and $\phi_{\text{cat}}$ with concentration of bovine oxyhemoglobin, human serum albumin, and L-histidine (pK$_a$ ~ 6.0) at a constant concentration of bovine carbonic anhydrase are shown in Figs. 2, 3, and 4. These data display the same symmetrical pattern for $\theta_{\text{cat}}$ and $\phi_{\text{cat}}$ and the same evidence for a change in rate-determining step as observed for buffers of low molecular weight. Also, the magnitude of the increase in $\theta_{\text{cat}}$ upon addition of hemoglobin or L-histidine is similar to that observed for buffers of low molecular weight. Consequently, it is a reasonable hypothesis that the changes in the $^{18}$O exchange rates upon addition of hemoglobin and histidine result from their action as proton transfer agents.
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Human serum albumin, although similar to hemoglobin in molecular weight, contains 16 histidines out of about 610 residues and requires about 12 eq of acid to be titrated from pH 8 to 6. Other amino acid side chains containing carboxyl (pKₐ ~ 4.5), e-amino (pKₐ ~ 10), or phenolic (pKₐ ~ 10) groups would not be expected to exert a proton transfer effect as great as the imidazole (pKₐ ~ 6 to 7) side chain of histidine under the conditions of these experiments. Consider a carbamylate anion as a proton acceptor and the enzyme active site as donor (pKₐ ~ 7). Such a proton transfer is a positive free energy process and would occur with a bimolecular rate constant several orders of magnitude below that for a diffusion-controlled process. On the other hand proton transfer from the carbonylic acid moiety to the enzyme active site would be expected to proceed with a rate constant close to a diffusion-controlled process. The order of magnitude is a conservative estimate since these arguments have not yet considered the large size of hemoglobin since L-histidine exhibits a smaller diffusion coefficient than buffers of small molecular weight. Even if all titratable groups on hemoglobin are considered, the factor of 1000 is not met: it requires about 170 eq of hydrogen ions to titrate 1 mol of bovine hemoglobin from pH 13 to 1.5. Hence, the second order rate constant for the transfer of protons between hemoglobin and the carbonic anhydrase active site, calculated from the data of Fig. 2, is greater by roughly a factor of 10 than can be accounted for on the basis of residues of hemoglobin which are buffers near neutral pH.

Considering these facts and the data of Figs. 1 and 2 it appears that the efficiency of hemoglobin as a proton transfer agent in activating carbonic anhydrase is greater than the sum of the expected effects of the individual buffering groups on the surface of the hemoglobin molecule. Bovine hemoglobin activates carbonic anhydrase at concentrations 1000-fold less than the concentrations required of imidazole and certain other buffers of low molecular weight (4, 5), although the maximal activities attained are nearly the same with hemoglobin and imidazole. Even if all titratable groups on hemoglobin are considered, the factor of 1000 is not met: it requires about 170 eq of hydrogen ions to titrate 1 mol of bovine hemoglobin from pH 13 to 1.5. Hence, the second order rate constant for the transfer of protons between hemoglobin and the carbonic anhydrase active site, calculated from the data of Fig. 2, is greater by roughly a factor of 10 than can be accounted for on the basis of residues of hemoglobin which are buffers near neutral pH. This order of magnitude is a conservative estimate since these arguments have not yet considered that hemoglobin has a smaller diffusion coefficient (6 x 10⁻⁷ cm²/s) than buffers of small molecular weight (about 5 x 10⁻⁶ cm²/s).

The low concentration at which hemoglobin acts as a proton transfer agent with the carbonic anhydrase active site in these experiments, then, is due not only to the buffering groups on the surface of hemoglobin but also to additional factors. Whatever the nature of these additional factors, they need not rely on the large size of hemoglobin since L-histidine exhibits a rate constant of 1 x 10⁻⁷, similar to that observed with hemoglobin, as evident in comparing Figs. 2 and 4. L-Histidine exemplifies the nature of the problem and is more simple to consider than hemoglobin. From Fig. 4, the increase in θₑ reacted caused by the addition of histidine is about 3.0 x 10⁻⁷ s⁻¹, corresponding to an increase in the rate of the catalyzed hydration reaction at equilibrium of 1.4 x 10⁻⁸ M s⁻¹ (calculated using Equation 8 of Ref. 5). The rate constant k₂ for proton transfer between the imidazole group of histidine and the enzyme active site must be large enough to account for this increase in rate.

\[ k[B][E_{cat}] \frac{[H^+]^2}{[H]^+ + K_a} \geq 1.4 \times 10^{-4} \text{ mol}^{-1} \text{s}^{-1} \]

where [H⁺] = 10⁻¹ to 10⁻⁴ M and \( K_a = 10^{-6} \) M, the ionization constant of the activity-controlling group in the enzyme active site. \( B \) is the concentration of the buffer histidine. The value of \( k_2 \) obtained in this calculation is larger by a factor of 10⁶ to 10⁸ than expected for a diffusion-controlled proton transfer between a small molecule and a macromolecule (20). The rate constants for the transfer of protons between the carbonic anhydrase active site and buffers such as 2,2-diethylmalonate, N-methylimidazole, and imidazole are near 10⁴ M⁻¹ s⁻¹ (4, 5).

Assuming that the changes in the rate constants for \(^{18}O\) exchange observed in these experiments are due to proton transfer effects, the efficiency of micromolar concentrations of histidine and hemoglobin in enhancing the activity of carbonic anhydrase can be explained. One possibility is that more than one intermolecular proton transfer step occurs for each encounter of hemoglobin or histidine with carbonic anhydrase. Since these are equilibrium experiments, it is possible that there is cycling of protons between these proton transfer agents and the active site during which more than one catalytic hydration-dehydration cycle occurs and more than 1 atom of \(^{18}O\) is exchanged from CO₂ to water. This implies that histidine provides more efficient proton cycling than imidazole alone or His 63 in the active site of carbonic anhydrase. This hypothesis requires binding of hemoglobin and histidine to carbonic anhydrase of duration comparable to the time for many catalytic cycles. An association or binding of hemoglobin with carbonic anhydrase has not been demonstrated to this date. If this explanation that hemoglobin and histidine cycle protons during the catalytic hydration-dehydration cycle is correct, we would not expect to observe the 1000-fold greater efficiency of these proton transfer agents compared to imidazole under initial velocity conditions for which there is unidirectional catalysis. Work to investigate this point is in progress.

The plateau regions of Figs. 2 to 4 show that neither bovine hemoglobin nor human serum albumin nor histidine inhibit bovine carbonic anhydrase significantly at the concentrations used. Furthermore, the maximal value of θₑ max is nearly as great in Fig. 2 as in Fig. 1. This exchange relies on the entry into the active site of \(^{18}O\)-labeled substrate. Hence, it appears that the accessibility of CO₂ and HCO₃⁻ into the carbonic anhydrase active site is not significantly hampered by the presence of hemoglobin. From Fig. 4, a similar result was obtained for histidine. The maximal increment in θₑ max caused by the presence of human serum albumin, Fig. 3, is not as great as that observed with hemoglobin, an observation which we cannot explain at this time.

About half of human oxyhemoglobin is dissociated into dimers at concentrations of 10⁻⁶ M; a smaller fraction is dissociated at higher concentrations (21). At concentrations as low as those of Fig. 2, dissociation of hemoglobin occurs and will result in exposure of more residues to the solvent. This may cause a small effect on the rate of proton transfer involving hemoglobin. On the other hand, carbamate formation is not expected to influence the rate constants obtained from Fig. 2. First, the ratio of hemoglobin to carbon dioxide concentrations is low (10⁻⁶ to 10⁻⁵); and second, the mechanism of carbamate formation is such that it is not expected to cause

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1 As quoted in Ref. 17.
an exchange of oxygen between species of CO₂ or between CO₂ and water. We conclude that hemoglobin is an efficient proton transfer agent for the carbonic anhydrase active site; in these equilibrium experiments it is more efficient than can be accounted for by summing the expected effects of the buffering groups on its surface. It follows that, under non-equilibrium conditions, hemoglobin can act as an acceptor of protons for the carbonic anhydrase active site during the catalytic hydration of CO₂ and as a donor of protons during catalytic dehydration of HCO₃⁻. Furthermore, these protons can be transferred at diffusion-controlled rates as a result of the encounter of hemoglobin with carbonic anhydrase, although this transfer may be mediated by water bridges or amino acid side chains. Hemoglobin and carbonic anhydrase are the two most abundant proteins in red cells, the former present at about 5 mM and the latter at about 150 μM; hence we anticipate that proton transfer occurs between them in red cells. This may have significance in the Bohr shift, the effect of pH on the affinity of hemoglobin for O₂. Forster and Steen showed that in red cells the Bohr off-shift in response to an increase in external (H⁺) relies on the production of protons in the cell by the hydration of CO₂ catalyzed by carbonic anhydrase. In the presence of carbonic anhydrase inhibitors, the rate of the Bohr shift is dramatically decreased in the same experiment, demonstrating a link in the functions of carbonic anhydrase and hemoglobin. The work reported here points out that the transfer of the Bohr proton at diffusion-controlled rates may occur, at least in part, as a result of the direct encounter of hemoglobin with carbonic anhydrase.

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