PARTICIPATION OF BUFFER IN THE CATALYTIC MECHANISM OF CARBONIC ANHYDRASE

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Received 26 February 1976

1. Introduction

Enzymic reactions frequently involve the production or consumption of hydrogen ions. It is often tacitly assumed that the active site groups participating in the transfer of these hydrogen ions are in rapid equilibrium with solvent H^+_{aq} during catalysis. This assumption is probably valid in many cases, but carbonic anhydrase poses some problems because the turnover of the catalyzed reaction:

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+,$$

is extremely rapid. Thus, with HCO_3^- as substrate the turnover number of human carbonic anhydrase C is 2.5×10^5 s^{-1} at pH 7.4 and 25°C [1]. This implies that H^+ must be transported to the active site at a sufficient rate to react with this frequency. Hence, the direct combination of H^+_{aq} with the active site at pH 7.4 would require a rate constant of at least 6×10^{12} M^{-1} s^{-1}. This value exceeds that of a diffusion-controlled reaction by almost 3 orders of magnitude and, therefore, H^+ must be delivered to the active site in some other form. The most obvious alternative proton donor is the acidic form of the buffer which is generally present in kinetic turnover experiments. Khalifah [2], Lindskog and Coleman [3], and Prince and Woolley [4] have shown that such a buffer-mediated proton transfer is theoretically feasible. In this paper we present an experimental test of this hypothesis.

We find that carbonic anhydrase C is, indeed, activated by buffers. The effect appears general and has been observed with all the investigated buffer systems. The simplest interpretation of the results is that the catalytic cycle contains an obligatory proton transfer reaction between the buffer and a titratable active site group. Apparent rate constants for this reaction are about 10^8 M^{-1} s^{-1}. At sufficiently low buffer concentrations this step limits the rate of the catalyzed reaction, and equilibrium between the active site and solvent H^+_{aq} does not pertain. However, at the high buffer concentrations normally used in steady-state experiments other steps are rate limiting and the equilibrium assumption is probably a valid approximation.

2. Materials and methods

Human carbonic anhydrase C was prepared by the method of Henderson and Henriksson [5]. Enzyme concentrations were estimated spectrophotometrically at 280 nm taking $A^{1\%}_{280} = 18.7$ cm^{-1} [6] and a mol. wt. of 29 300 calculated from the amino acid sequence [7]. Diethylmalonic acid was obtained from Aldrich Chemical Co. and recrystallized from benzene. Dipropylmalonic acid was synthesized according to a procedure described by Mishin and Polonchanskaya [8] and recrystallized from chloroform. Other materials were the same as used in previous investigations [1,9].

The CO_2 hydration reaction was monitored in a Durrum-Gibson stopped-flow spectrophotometer by the 'changing pH-indicator' method [1,10]. One drive syringe contained a CO_2 solution, and the other one contained a solution of enzyme, buffer and indicator. Initial rates were estimated as described by Steiner et al. [1]. 'Buffer factors' converting absorbancy

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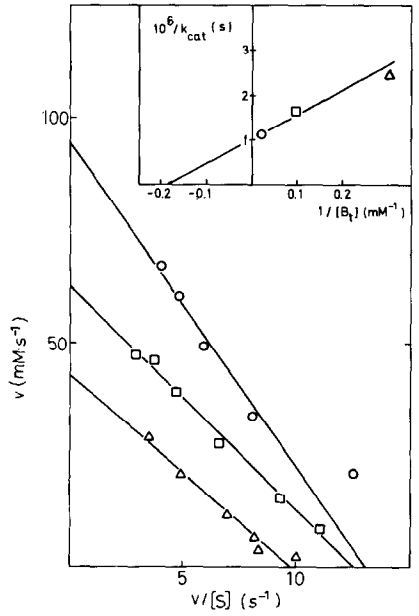

Fig.1a

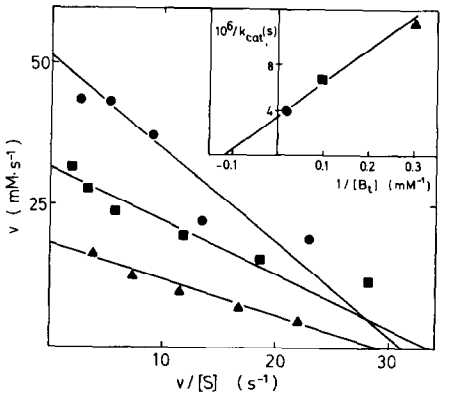

Fig.1b

Fig.1. Effects of 1,2-dimethylimidazole–H_2SO_4 buffers on the CO_2 hydration activity of human carbonic anhydrase C. (A) Solvent, ^1H_2O; pH 8.9; temperature, 25°C; ionic strength, 0.2; enzyme concentration, e=0.10 μM. Total buffer concentrations: (○) 50.3 mM; (□) 10.3 mM; (△) 3.3 mM. Insert: secondary plot where $k_{cat} = V/e$; [B_t] refers to the total buffer concentration. (B) Solvent, ^2H_2O; pH 8.9. Other conditions as in (A) except that the enzyme concentration was 0.20 μM.

changes to changes of CO_2 concentration were usually estimated according to Khalifah [10]. All measurements were performed at 25°C, and the ionic strength was kept at 0.2 with Na_2SO_4. In solutions of ^2H_2O pH was estimated by addition of 0.4 to the pH meter reading.

3. Results

In each series of experiments the catalytic rate of CO_2 hydration was estimated as a function of the initial CO_2 concentration at different, fixed buffer concentrations. Six buffer systems were investigated: 2 (*N*-morpholino) ethanesulfonic acid and 3,5-lutidine (with chlorphenol red as indicator); *N*-methylimidazole, 2,2-diethylmalonate and 2,2-dipropylmalonate (with *p*-nitrophenol); 1,2-dimethylimidazole (with metacresol purple). In all cases the same general pattern was observed. Increasing buffer concentrations have a stimulating effect on the activity, but a level of saturation appears to be reached at high concentrations of buffer. Representative examples of the results are shown in fig.1 and fig.2. The buffer activation seems to be maximally expressed in k_{cat} (= V/e)

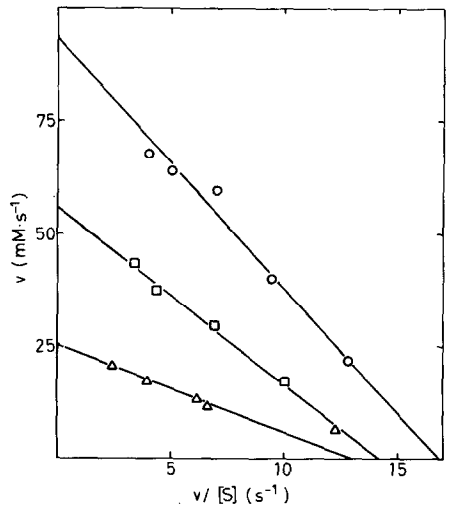

Fig.2. Effects of sodium 2,2-diethylmalonate buffers on the CO_2 hydration activity of human carbonic anhydrase C. Temperature, 25°C; ionic strength, 0.2; pH 7.8; enzyme concentration, 0.15 μM. Total buffer concentrations: (○) 50.0 mM; (□) 10.0 mM; (△) 3.3 mM.

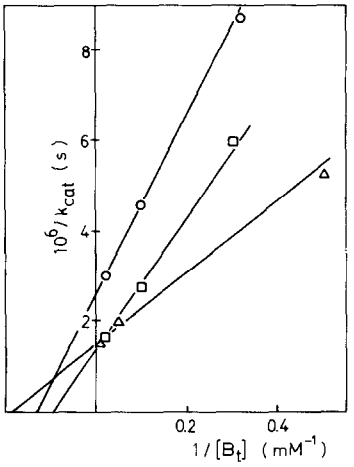

Fig.3. Effects of various buffers at pH 7.8 on the maximal rates of CO_2 hydration catalyzed by human carbonic anhydrase C. Buffer systems: (○) sodium 2,2-dipropylmalonate; (□) sodium 2,2-diethylmalonate (data from fig.2); (△) *N*-methylimidazole–H_2SO_4. Temperature, 25°C; ionic strength, 0.2.

while the effect in k_{cat}/K_M (corresponding to the intercept on the $v/[S]$-axis of fig.1 and fig.2) is smaller or, in some cases, absent. As shown in fig.1B the buffer effect is observed in ^2H_2O as well. The results suggest an approximately linear dependence of $1/k_{cat}$ with 1/[buffer] as shown in the inserts to fig.1 and in fig.3. The apparent K_M with respect to the total buffer concentration, K_{buffer}, varies between 1.7 and 11 mM for the investigated buffers as shown in table 1. Thus, at the buffer concentration of 50 mM used in previous kinetic studies [1,9,10] the catalytic rates are almost buffer independent. In most cases the extrapolated values of k_{cat}, $(k_{cat})_{max}$, correspond well to those k_{cat} values obtained in the earlier studies [1,10], but morpholino-ethanesulfonic acid and dipropylmalonate gave significantly lower values (table 1). In this connection it is interesting to note that Christiansen and Magid [11] observed significantly lower k_{cat} values in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffers than in phosphate buffers in their studies of the dehydration reaction at 2°C.

4. Discussion

The data presented in this paper show that buffers have a nonspecific, activating effect on CO_2 hydration catalyzed by human carbonic anhydrase C. Preliminary results suggest a similar effect of buffers on the human B isoenzyme. The simplest interpretation of these observations is that the buffers participate directly in the catalytic process by facilitating the transport of H^+ between the active site and the medium as pro-

Table 1
Parameters for the buffer activation of the CO_2 hydration activity of human carbonic anhydrase C

Buffer system	pK_a	pH	$(k_{cat})_{max}$ (s^{-1}) × 10^{-5}	K_{buffer} (mM)	k_B (M^{-1} s^{-1}) × 10^{-8}
2 (*N*-morpholino) ethanesulfonic acid	6.1	6.65	0.8	1.7	(0.6)
3,5-lutidine	6.2	6.65	2.0	6	0.5
N-methylimidazole	7.2	7.8	6.9	5.5	1.6
2,2-diethylmalonate	7.3	7.8	7.4	11	0.9
2,2-dipropylmalonate	7.4	7.8	3.9	8	(0.7)
1,2-dimethylimidazole (^1H_2O)	8.2	8.9	10	6	2.0
1,2-dimethylimidazole (^2H_2O)	8.7	8.9	2.9	9	0.5

The morpholinoethanesulfonic acid, diethylmalonate and dipropylmalonate buffers were made up with NaOH: other buffers with H_2SO_4. Temperature 25°C: the ionic strength was adjusted to 0.2 with Na_2SO_4. In ^2H_2O pH was estimated by adding 0.4 to the pH meter reading. The values of $(k_{cat})_{max}$ and K_{buffer} were obtained by linear extrapolation of plots of $1/k_{cat}$ versus $1/B_t]$, where B_t refers to the total buffer concentration. The apparent rate constants, k_B were calculated from the slopes of these plots (eq.4) and refer to the specific buffer activation mechanism discussed in the text (eq.1). The numbers in paranthesis represent buffer systems yielding significantly lower values of k_{cat} than obtained previously in 50 mM buffers [1].

posed earlier on theoretical grounds [2–4]. Silverman and Tu [12,13] reached similar conclusions in their studies of the effects of buffers on the carbonic anhydrase-catalyzed ^{18}O exchange at chemical equilibrium between $HC^{18}O_3^-$ and H_2O and $H^{13}CO_3^-$, respectively. They also found that a nontitratable buffer analogue, 1,3-dimethylimidazolium sulfate, had no activating effect [12].

The observed patterns of buffer activation (fig.1 and fig.2) are in reasonable accord with a proton transfer between enzyme and buffer as an obligatory step on the catalytic pathway:

$$EH + B \underset{k_{BH}}{\overset{k_B}{\rightleftharpoons}} E^- + BH^+ \qquad (1)$$

Since hydrogen ions can exchange between enzyme and solvent via reactions 2 and 3 even in the absence of buffer, E^- and EH will be in equilibrium during catalysis at sufficiently low CO_2 concentrations regardless of the buffer concentration.

$$EH + H_2O \rightleftharpoons E^- + H^+ \qquad (2)$$

$$EH + OH^- \rightleftharpoons E^- + H_2O \qquad (3)$$

Hence, buffers are not expected to have a large effect on the parameter k_{cat}/K_M in accordance with observation.

A pronounced buffer dependence is expected, of course, at saturating CO_2 concentrations when the buffer concentration is low enough to make reaction 1 rate limiting. Theoretically, reactions 2 and 3 should yield a non-zero value of k_{cat} even in the absence of buffer. For technical reasons we could not measure below 2 mM buffer, and under such conditions the contribution of these reactions should be negligible below pH 8. However, taking the forward rate constant of reaction 3 as 10^{10} M^{-1} s^{-1} (cf. [14]), we estimate that this reaction might contribute with about 20% of the observed k_{cat} in 3 mM buffer at pH 8.9 (see fig.1A).

In current steady-state kinetic schemes for carbonic anhydrase [1,9,15] CO_2 is assumed to react with E^- as well as EH of eq.1. Since these enzyme forms are not in rapid equilibrium at low buffer concentrations the rate equations with respect to CO_2 are non-hyperbolic. However, simulations with reasonable values for the rate constants suggest that the deviation from hyperbolic behavior is likely to be small and hardly detectable considering the available CO_2 concentration range and the experimental errors. Thus, the k_{cat} values obtained by linear extrapolation are probably reasonable approximations. These k_{cat} values were used to estimate values of k_B (eq.1) according to eq.4:

$$(k_{cat})^{-1} = (k_{cat})^{-1}_{max} + (k_B[B])^{-1} \qquad (4)$$

where B is the basic buffer species. These k_B values are given in table 1, and they can be compared to the value estimated by Silverman and Tu [12] for 1,2-dimethylimidazole and bovine carbonic anhydrase, $k_B \geqslant 6 \times 10^7$ M^{-1} s^{-1}, and to the rate constants for the transfer of a proton between the model compounds imidazole and *p*-nitrophenol, about 5×10^8 M^{-1} s^{-1} [14]. The k_B values are about 1 order of magnitude smaller than predicted for a diffusion-controlled process [14]. In addition, the estimated isotope effect in k_B for 1,2-dimethylimidazole is about 4 and sufficiently large to suggest that the proton transfer is not quite diffusion-controlled.

According to the simple interpretation outlined above (eq.1) the maximal k_{cat} value at a given pH should be independent of the buffer system. This is not always the case. It is difficult to explain the low k_{cat} values obtained at saturating levels of some buffers without assuming that significant binding of buffer (or of an impurity) to enzyme takes place at least in these cases, but there is no direct evidence for this. We have not observed any straightforward inhibition by any of the investigated buffer systems with the exception of dipropylmalonate, which showed a weak inhibition of the *p*-nitrophenyl acetate hydrolysis activity (about 20%) at 60 mM dipropylmalonate, pH 7.8). However, there are some published reports of buffer binding to carbonic anhydrases. Imidazole inhibits the human B isoenzyme [10], and binds in the vicinity of the metal ion [16]. In addition, imidazole buffers have been reported to affect the electron spin resonance spectrum of bovine Co^{2+}-carbonic anhydrase [17] without, apparently, inhibiting the CO_2 hydration activity [18]. If there is a significant enzyme–buffer binding the proton transfer might take place within the complex:

$$EH + B \rightleftharpoons EH\text{-}B \rightleftharpoons E^{-}\text{-}BH^{+} \rightleftharpoons E^{-} + BH^{+} \quad (5)$$

In this case the observed maximal rates could depend on the specific buffer system. One might then speculate that the 'proton transfer group', recently proposed by us [1,9] to rationalize the observed pattern of kinetic hydrogen isotope effects, is a bound buffer molecule rather than a protein group. However, more information about the interaction of the enzyme with buffers is obviously required.

Regardless of these problems it is clear that the present data do not support the hypothesis that H_2CO_3 rather than HCO_3^- is the active substrate species in the dehydration reaction. The catalytic mechanism proposed by Kaiser and Lo [19] incorporated this feature which was further elaborated by Koenig and Brown [20]. The interconversion between CO_2 and H_2CO_3 does not require a change of the ionization state of the catalytic group during turnover. Thus, there is no specific role for the buffer in this type of mechanism because the catalytic process would proceed equally well irrespective of the rate of H^+ transfer between active site groups and the medium.

Acknowledgements

We are grateful to Miss Eva-Karin Gandvik for preparing the enzyme. Financial support was obtained from the Swedish Natural Science Research Council (project no. 2911).

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