Vol. 143, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 27, 1987 Pages 67-73

THE RATES OF FAST REACTIONS OF CARBON DIOXIDE AND BICARBONATE IN HUMAN ERYTHROCYTES MEASURED BY CARBON-13 NMR

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Received August 14, 1986

The application of carbon-13 nuclear magnetic resonance spectroscopy to the study of the kinetics of millisecond timescale reactions of CO_2 in human erythrocyte suspensions is described. The rates of intracellular enzyme catalyzed CO_2 hydration and HCO_3^- dehydration were quantitatively determined, as well as the rates of CO_2 diffusion into and out of the erythrocytes. The method also provides an accurate measure of the intracellular pH in the range of pH 6.0 to pH 7.0. A temperature dependence study was used to determine the thermodynamic functions for the intracellular hydration-dehydration reaction.

Inside the erythrocytes the enzyme carbonic anhydrase catalyzes the reversible carbon dioxide-bicarbonate interconversion

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

thus allowing the rapid establishment of carbon dioxide-bicarbonate equilibrium. Outside the erythrocytes this reaction is uncatalyzed and extremely slow. CO_2 diffuses rapidly into and out of the erythrocytes, while HCO_3^- anions cross the erythrocyte membrane at a much slower rate. This cycle of CO_2 and HCO_3^- transport and interconversion is illustrated in Fig. 1.

The rates of the slow processes in Fig. 1, uncatalyzed carbon dioxide-bicarbonate interconversion and the diffusion of HCO_3^- across the cell membrane, have been well characterized in previous studies (1-3). Carbon-13 NMR has previously been used to measure the rate of carbon dioxide-bicarbonate exchange in solu-

0006-291X/87 $1.50

tions of purified carbonic anhydrase (4,5) and the rate of bicarbonate exchange across the erythrocyte membrane (3). The rapid processes of catalyzed carbon dioxide-bicarbonate interconversion inside the erythrocytes (6,7) and diffusion of CO_2 across the erythrocyte membrane (7) have proven much more difficult to measure by previous methods. In this study it is shown that the bandshapes of the $^{13}CO_2$ and $H^{13}CO_3^-$ signals from an erythrocyte suspension can be used to determine the rate of intracellular carbon dioxide-bicarbonate interconversion, the rate of CO_2 diffusion across the erythrocyte membrane and the intracellular pH.

METHODS AND MATERIALS

Samples for NMR study were prepared using freshly drawn venous blood from a single donor (DWH). The blood was drawn into a heparinized collection tube and washed with 150 mM NaCl. The NMR samples were prepared by dissolving 200 mg dextran (average molecular weight = 10000) and 7 mg Na_2CO_3 (99% carbon-13 enriched) in 0.4 ml D_2O. The pH was adjusted with 1 M HCl, and $MnCl_2$ was added (10 mM). The erythrocytes then were added to bring the sample volume to 1.0 ml. The volume fraction of erythrocytes in each sample was determined using a hematocrit centrifuge after the completion of the NMR experiment.

Dextran was used in the samples to keep the erythrocytes uniformly distributed in the NMR sample tube. The purpose of the manganese is to greatly reduce the spin-spin relaxation time of the HCO_3^- outside the erythrocytes, effectively eliminating the contribution of the extracellular HCO_3^- to the observed spectrum, and thus allowing the contributions of the HCO_3^- from inside and outside the erythrocytes to be separated. The Mn^{2+} cation does not cross the cell wall on the timescale of the experiment. The spin-spin relaxation time of CO_2 is only slightly reduced by the presence of Mn^{2+}, so the observed CO_2 peak contains the mixed contributions from CO_2 inside and outside the erythrocytes.

Carbonic anhydrase inhibited erythrocytes (8) were prepared by washing the cells with 150 mM NaCl + 2 mM acetazolamide (Sigma Chemical Co.) before preparation of the NMR sample. Six hours were allowed for the acetazolamide to equilibrate across the cell membrane.

The carbon-13 NMR spectra were obtained using a Varian XL-300 spectrometer at a frequency of 75.43 MHz. A pulse angle of 30 degrees was used, with an aquisition time of 0.101 s and a recycle time of 0.2 s. Between 10000 and 15000 transients were accumulated for each spectrum. The free induction decay was multiplied by an expontial function to improve the signal to noise ratio and produce a line broadening of 10 Hz.

RESULTS

The processes by which carbon is exchanged between the four sites in Fig. 1 can be characterized by their lifetimes $\tau_{r,s}$, where $\tau_{r,s}$ is the reciprocal of the first order rate constant for the process connecting site r with site s. The effect of exchange of nuclei between the four sites on the observed CO_2 and HCO_3^- bandshapes can be calculated by modifying the Bloch equations to account for transitions between sites (9,10). The solution of the modified Bloch equations for a four site system is

$$S(\omega) \propto \mathrm{Im}(-i[\mathbf{1}][\mathbf{X}]^{-1}[\mathbf{P}]) \qquad (1)$$

where $S(\omega)$ is the signal strength at frequency ω, i is the square root of -1, $[\mathbf{1}]$ is the column vector [1,1,1,1], $[\mathbf{P}]$ is the row vector $[P_1,P_2,P_3,P_4]$ containing the populations of each of the four sites, and $[\mathbf{X}]^{-1}$ is the inverse of the four by four matrix with diagonal elements $X_{r,r} = -i(\omega_r - \omega) - 1/T_{2r} - \sum_{r \neq s}(1/\tau_{s,r})$ and off diagonal elements $X_{r,s} = (1/\tau_{s,r})$. "Im" means "imaginary part of". T_{2r} and ω_r are the effective spin-spin relaxation time and site frequency for site r in the absence of transitions between sites.

Although equation 1 contains too many unknown parameters to be determined from a single spectrum, reasonable assumptions can

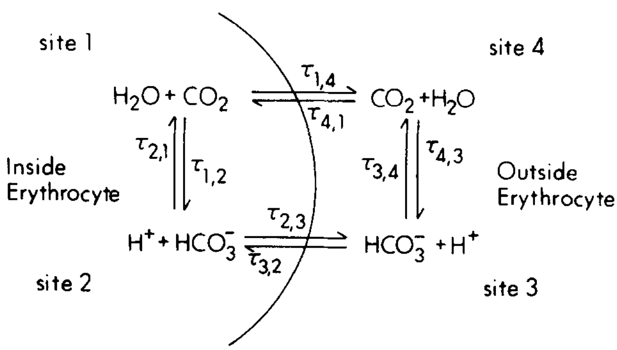

Fig. 1. The exchange and transport of CO_2 and HCO_3^- inside and outside the erythrocytes. $\tau_{r,s}$ is the lifetime of the process connecting site r with site s.

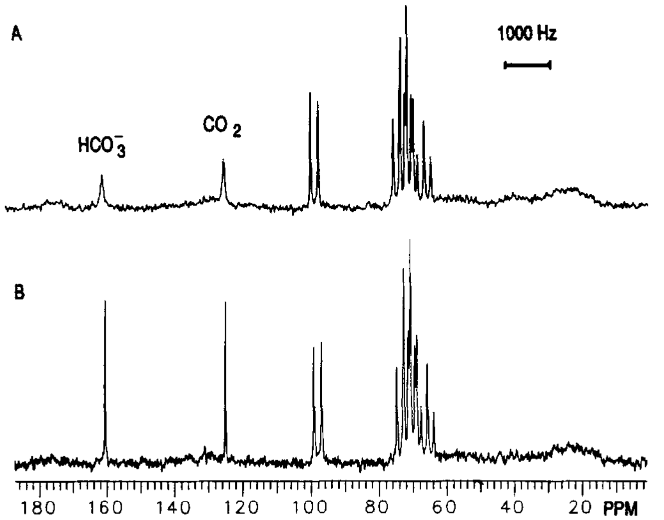

Fig. 2. The carbon-13 NMR spectra of 36 % by volume erythrocytes (Fig. 2A) and carbonic anhydrase inhibited erythrocytes (Fig. 2B), illustrating the effect of rapid intracellular carbon dioxide-bicarbonate exchange. The intracellular pH is 6.6. The extracellular solution contains 10 mM $MnCl_2$. The CO_2 and HCO_3^- peaks occur at 125.5 and 161 ppm on the TMS scale. The peaks between 60 and 100 ppm are due to the dextran in the samples. The broad peaks near 25, 135, and 175 ppm are due to intracellular hemoglobin.

be made to reduce the number of unknowns. The requirement that

$$\tau_{r,s} = (P_r/P_s)\tau_{s,r} \qquad (2)$$

at equilibrium can be used to reduce the number of unknown lifetimes from eight to four. The spin-spin relaxation times in the absence of exchange between sites were determined from the widths of the CO_2 and HCO_3^- peaks in samples prepared without erythrocytes and with carbonic anhydrase inhibited erythrocytes (Fig. 2B). Values of 16 s and 0.2 s were used (1,2) for the lifetime of uncatalyzed CO_2 hydration ($\tau_{4,3}$) and the lifetime for the diffusion of HCO_3^- out of the erythrocytes ($\tau_{2,3}$). Precise values for these lifetimes are not essential since the NMR spectrum is insensitive to processes occuring on this timescale. The intracellular and extracellular concentrations of CO_2 were assumed to be equal (2), so the ratio of CO_2(intracellular):CO_2(total) is given by the volume fraction of erythrocytes in the sample. The intracellular and extracellular concentrations of HCO_3^- were also assumed to be equal, although this would only be true in the case of equal pH inside and outside the cells. An error in this assumption would not introduce a significant error in the results, a consequence of the fact that the extracellular HCO_3^- does not contribute significantly to the HCO_3^- in the NMR spectrum due to its short spin-spin relaxation time. Some of the carbon dioxide is reversibly bound to the hemoglobin as a carbamate, however this process is much slower than the catalyzed hydration of CO_2 and the diffusion of CO_2 into and out of the cells and would not significantly influence this analysis. After making the above assumptions only three independent parameters are left to determine in equation 1: the lifetime for HCO_3^- dehydration inside the erythrocytes ($\tau_{2,1}$), the lifetime for CO_2 diffusion into the erythrocytes ($\tau_{4,1}$), and the ratio of intracellular CO_2:intracellular HCO_3^- ($P_1:P_2$).

The spectra were analyzed by digitizing the CO_2 and HCO_3^- peaks and using a nonlinear least squares procedure to fit the observed spectra to the spectra calculated from equation 1, using $\tau_{2,1}$, $\tau_{4,1}$ and $P_1:P_2$ as variables. The results of the least squares analyses are summarized in table 1, and some of the spectra from which the results were obtained are shown in Fig. 3. The intracellular pH was calculated from the ratio $P_1:P_2$, using $pH = 6.10 + \log [P_2/P_1]$ (2). An Arrhenius plot of the HCO_3^- dehydration rate is linear and indicates that at 37 C the enthalpy of activation is $\Delta H^* = 49$ kJ mole^{-1}, the entropy of activation is $\Delta S^* = -35$ J mole^{-1} K^{-1} and the Gibbs free energy of activation is $\Delta G^* = 60$ kJ mole^{-1}.

The values determined for the lifetime of CO_2 diffusing out of the erythrocytes are similar to the value of 2 ms reported by

Table 1. Rates of enzyme catalyzed hydration of CO_2 $(1/\tau_{1,2})$ and dehydration of HCO_3^- $(1/\tau_{2,1})$ inside the erythrocytes and the lifetime for the diffusion of CO_2 out of the erythrocytes $(\tau_{1,4})$. $\tau_{1,4}$ and $\tau_{4,1}$ are not independent and are related by equation 2.

	Rate of Intracellular		Lifetime for CO_2
Temp.	HCO_3^- dehydration	CO_2 hydration	Diffusion out of Cell
(C)	(s^{-1})	(s^{-1})	(ms)
13.1	110 ± 20	550 ± 150	1.1 ± 1.6
20.4	150 ± 20	790 ± 200	1.4 ± 1.1
25.8	220 ± 25	1100 ± 250	1.9 ± 0.6
31.3	350 ± 40	1750 ± 400	1.3 ± 0.4
37.1	510 ± 40	2600 ± 600	1.5 ± 0.4
41.3	750 ± 160	3800 ± 1000	1.1 ± 0.4

The erythrocyte volume fraction is 36 ± 1 %. The ratio of intracellular CO_2 to intracellular HCO_3^- is 0.20 ± 0.015 in each of the samples, indicating an intracellular pH of 6.8.

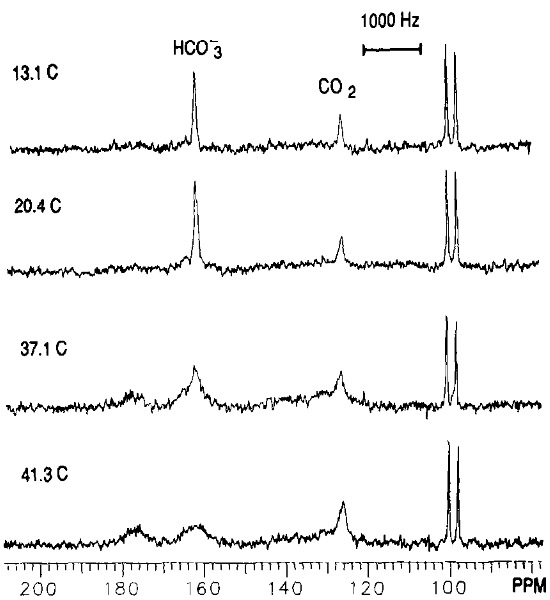

Fig. 3. Some of the carbon-13 NMR spectra used for determining the results in table 1. The samples contain 36% by volume erythrocytes. The intracellular pH is 6.8. The extracellular solution contains 10 mM $MnCl_2$.

Silverman *et al* (7) for rat erythrocytes at pH 7.4 and 25 C. No previous experimental value appears to have been reported for human erythrocytes. The values determined for intracellular CO_2 hydration and HCO_3^- dehydration are similar to the values of 0.3 ms and 4 ms reported by Silverman *et al* (7) for rat erythrocytes and the value of < 20 ms reported by Forster and Crandall (2) for human erythrocytes.

The described nuclear magnetic resonance method is well suited to the study of the kinetics of fast intracellular and extracellular reactions of CO_2 and HCO_3^- under conditions which are close to those found *in vivo*. Further investigations of the pH, temperature and concentration dependence of these processes are in progress.

ACKNOWLEDGMENT: This research was supported by grants from the National Institute of General Medical Sciences (GM 31510), the Office of Naval Research and the North Carolina Biotechnology Center.

REFERENCES

1. Silverman, D.N. (1974) Mol. Pharm., 10, 820-836.
2. Forster, R.E., and Crandall, E.D. (1975) J. App. Physiol., 38, 710-718.
3. Chapman, B.E., Kirk, K., and Kuchel P.W., (1986) Biochem. Biophys. Res. Commun., 136, 266-272.
4. Koenig, S.H., Brown, R.D., London, R.E., Needham, T.E., and Matwiyoff, N.A. (1974) Pure Appl. Chem., 40, 103-113.
5. Simonsson, I., Jonsson, B.H., and Lindskog, S. (1979) Eur. J. Biochem., 93, 409-417.
6. Forster, R.E., Dodgson, S.J., Storey, B.T., and Lin, L. (1984) Ann. NY Acad., 429, 415-429.
7. Silverman, D.N., Tu, C.K., and Roessler, N. (1981) Resp. Physiol., 44, 285-298.
8. Holder, L.B., and Hayes, S.L. (1965) Mol. Pharm., 1, 266-273.
9. Sack, R.A. (1958) Mol. Physics, 1, 163-167.
10. Binsch, G. (1975) in *Dynamic Nuclear Magnetic Resonance Spectroscopy*, M. Jackman and F. A. Cotton, Eds., pp. 45-78, Academic Press, New York. pp.45-78.