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ON THE MEASUREMENT OF pH IN *ESCHERICHIA COLI* BY ^{31}P NUCLEAR MAGNETIC RESONANCE

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Summary

The ^{31}P high resolution NMR spectra of concentrated suspensions of *Escherichia coli* cells have been measured at 145.8 MHz. The position of the orthophosphate resonance is used as a measure of internal and external pH. In accord with Paddan, Zilberstein and Rottenberg ((1976) Eur. J. Biochem. 63, 533—541) it is shown that when properly energized the internal pH is 7.5 ± 0.1. By synchronizing the NMR data acquisition with 3-s bursts of O_2 it is possible to measure the internal pH with a time resolution of about 1 s. It is shown that at 20°C the pH remains constant for times longer than 15 s after the oxygen is discontinued and it decays in several minutes.

Introduction

Recently it has been shown that high resolution ^{31}P nuclear magnetic resonance (NMR) measurements can be used to determine intracellular pH [1—6] since the chemical shift of ^{31}P resonance of a phosphate changes appreciably as it is titrated. While inorganic phosphate (P_i) has been generally useful in determining pH values, other phosphates such as ATP [4] and phosphorylcholine [5] also have been used. These pH values have been shown to agree [3,5] with similar pH determinations measured by the distribution of weak acids [7,8]. In this paper we show how the internal pH can be measured very rapidly by ^{31}P NMR in *Escherichia coli* suspensions. The minimum time to measure an NMR spectrum by signal averaging in this type of experiments is about 0.5—1 min. However, the response of *E. coli* cells to repetitive stimuli can be followed with a time resolution of about 1 s by accumulating separately the free induction decay signals which are synchronized to the stimuli and are sampled at various time points between the repetitive stimuli. In the present

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study, a suspension of *E. coli* was stimulated by bursts of O_2. In addition to measuring the time course of the pH, it was necessary to monitor the respiratory state of these cell suspension, since high concentrations of *E. coli* cells (approx. $5 \cdot 10^{11}$ cells/ml) have been used for the NMR measurements [3]. We have estimated the O_2 consumption in those suspensions by extrapolating from the values at low cell concentrations and have measured the internal pH at various O_2 supply rates in NMR samples.

Materials and Methods

E. coli MRE 600 were grown in M9 medium supplemented with 16 mM succinate as described previously [3]. The cells were harvested during log phase growth and washed twice with M9 growth medium. The final packed cells were resuspended in known volumes of the M9 medium (containing 10% D_2O) to prepare the desired concentration of cells for NMR samples, which were approx. 1.5 ml in a 10 mm diameter NMR tube. The cell concentration was estimated by using the $A_{650} = 0.3$ at 10^9 cells/ml [6]. For NMR experiments pure gaseous O_2 was introduced through a capillary tube (2 mm diameter) to the bottom of the cell suspension. The time average O_2 flow rate, monitored by a Matheson Mass Flow meter, was up to 30 cc/min. Under these gentle bubbling conditions, *E. coli* suspensions in NMR tubes did not develop foaming and there seemed to be no appreciable amount of lysed cells after 1 h of O_2 bubbling. The O_2 supply line was regulated by an open-close operation of a solenoid switch which was controlled by the Nicolet BNC 12 computer system of the Bruker HX 360 NMR spectrometer used for these experiments. Nicolet T1 program was modified so that the O_2 bubbling pulse and RF pulse could be suitably synchronized. The NMR frequency was 145.8 MHz and the spectrometer was operated in the Fourier Transform mode with quadrature detection.

The internal pH values of *E. coli* cells were estimated from the chemical shift of the internal orthophosphate resonance using a titration curve of orthophosphate (chemical shift vs. pH) in M9 medium at 20°C. Although the pK value of orthophosphate depends on the ionic strength of the solution, the variation of the pK in physiological solvent condition is small. The chemical shifts at the end points in the titration curve (around pH 4 and 8.5) are insensitive to the ionic strength.

Results

Using this synchronized pulse technique, the NMR signal was sampled during the bubbling and non-bubbling periods separately, with typical results shown in Fig. 1. Within a cycle of O_2 bubbling on (3 s) and off (15 s) periods, the 90° radio frequency pulses were triggered at the times shown in Fig. 1 and the induced free induction decay signals were collected and stored separately in the computer. This series of free induction decays were accumulated over 50 repetitions of O_2 bubbling cycles. The timing parameters of O_2 bubbling and radio frequency pulse triggering can be varied in many ways. The resulting ^{31}P NMR signals in the orthophosphate region are also shown in Fig. 1. The number at each spectrum corresponds to the times shown in the sequence of radio fre-

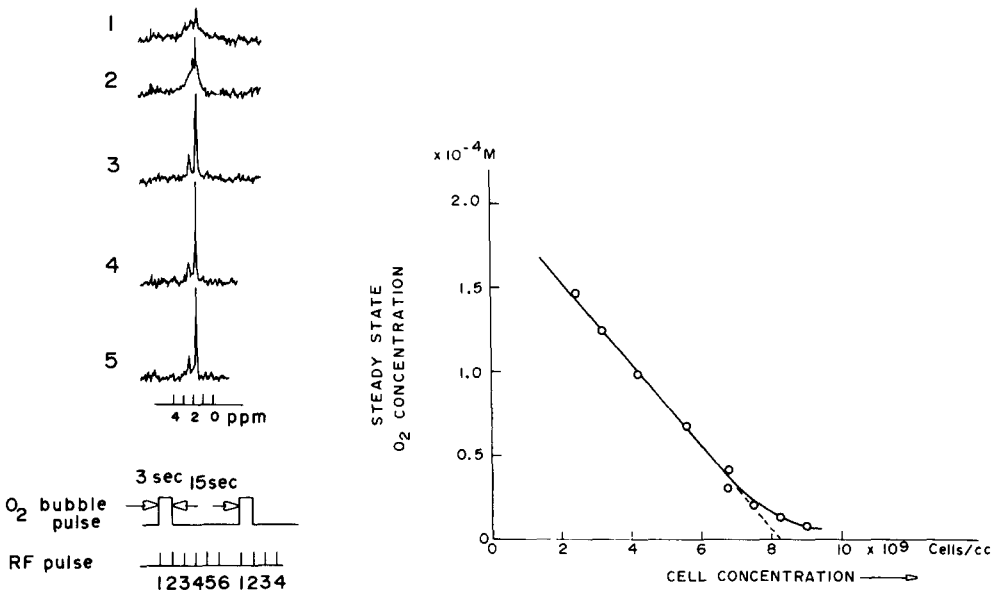

Fig. 1. 145.8 MHz ^{31}P NMR spectra of the orthophosphate peaks (85% H_3PO_4 as an external standard) in a suspension of *E. coli* cells ($6 \cdot 10^{11}$ cells/cc). Pure O_2 was bubbled for 3 s and turned off for 15 s as indicated at the bottom of the figures. The 5 spectra were obtained from radio frequency 90° pulses synchronized with the bubbling as shown at the bottom. Each spectrum is a sum of 50 individual free induction decays.

Fig. 2. Steady-state oxygen concentrations [O_2] were measured with an O_2 electrode and are plotted versus the cell concentrations which were determined as described in text.

quency pulses at the bottom. The O_2 bubbles in this test were rather large and also a residual hydrostatic gas pressure at the end of each bubbling period was present. Therefore, the disturbance of the homogeneity of the magnetic field by bubbling was very strong as seen in spectrum 1. From the broad lines it can be seen that there were residual bubbles still present at the period of the sampling of spectrum 2 which disappeared during subsequent sampling periods. The disturbance of the magnetic homogeneity by the bubbles can be reduced by making the bubbles smaller. It is possible to bubble continuously without appreciably broadening the 30 Hz wide NMR peaks generally observed, but the large bubbles were selected for the present test to show the possible inhomogeneity.

Since the concentration of *E. coli* cells in the NMR experiments was very high (approx. $6 \cdot 10^{11}$ cells/ml), the steady-state oxygen concentration even during the O_2 bubbling was expected to be low. Measurements were made of the steady state O_2 concentration with an O_2 electride (Yellow Spring) in various concentrations of *E. coli* under a constant air bubbling condition. At an air flow rate of 33 cc/min at 23°C with continuous bubbling in M9 medium containing 5 mM succinate at pH 7, the measured steady-state O_2 concentrations were plotted (Fig. 2) as a function of cell concentration. In the low concentration range there was a linear relation. Beyond the concentration of $9 \cdot 10^9$ cells/ml the oxygen concentration oscillated with time, showing the

effect of insufficient mixing of the cell suspension in which the mixing was maintained only by the movement of the bubbles. Applying a simple model of O_2 consumption by an enzymatic reaction, the steady state O_2 concentration $[O_2]$ in the bulk of the cell suspension can be obtained from the following equation:

$$\frac{d}{dt}[O_2] = A([O_2]_s - [O_2]) - \frac{Q[E][O_2]}{[O_2] + K_m} = 0$$

where $[O_2]_s$ is the O_2 concentration created by the supply gas at the surface of the bubble, A is the rate constant of O_2 transfer to the bulk of solution, Q is the maximum respiration rate of *E. coli*, $[E]$ is the *E. coli* concentration and K_m is the Michaelis-Menten constant for the oxygen-reducing enzyme. The values of A (0.6 min^{-1}) and Q (21 nmol $O_2/10^9$ cells $\cdot$ min^{-1}) were independently measured under the same solvent conditions. This respiration rate Q measured by the method described by Estabrook [10] corresponds to 340 natom oxygen/mg of protein $\cdot$ min^{-1} at 23°C and quite similar to the reported values [9,11,12]. The ratio (Q/A) calculated from Q and A was comparable to the observed value of 25 nmol $O_2/10^9$ cells as the slope of the linear portion of the plot in Fig. 2. The value of $[E]$ where $[O_2] = K_m$ is twice that of the cell concentration where the linear portion intercepts the $[O_2] = 0$ axis. From Fig. 2 this latter value of $[E]$ is $8.3 \cdot 10^9$ cells/cc. From the few points which deviated from the linear portion at higher cell concentrations, the value of K_m was estimated to be approx. $0.7 \cdot 10^{-6}$ M. When pure O_2 is used instead of air, the estimated cell concentration for which $[O_2] = K_m$ will be approx. 10^{11} cells/ml under the same bubbling condition. In typical NMR experiments the cell concentration was high as $6 \cdot 10^{11}$ cells/ml. Therefore, the respiration rate in these suspensions might be about 10 times slower than the maximum values.

In order to test the effect of varying $[O_2]$ in a NMR sample, two separate NMR experiments were conducted. In one experiment the cell concentrations were reduced from 6 to 3 and to $1.5 \cdot 10^{11}$ cells/ml, while the O_2 bubbling rate was kept constant at high flow rate (half on-half off) similar to that used during other NMR experiments. The internal pH was constant at 7.5 during this dilution, and the lowest concentration sample allowed $[O_2]$ to approach K_m.

In the second experiment the bubbling rate was varied by keeping a constant bubbling during a period of 2 s and by changing the length of the non-bubbling period from 1 to 30 s. The cell concentration was kept constant at about $3 \cdot 10^{11}$ cells/ml. NMR samplings were made during bubbling-on period and -off period and accumulated over about 25 cycles of O_2 bubbling. The bubbling off period was divided into 1 to 5 periods and during each divided period free induction decays at 1 s repetion time were summed so as to get time resolution within the bubble-off period and higher signal to noise ratio. The internal pH reached steady-state values after 10—15 min and did not change for at least an additional 30 min of bubbling. The internal pH values at those apparent steady states were plotted vs. the bubble repetition rate in Fig. 3. There are rather sharp increases of pH as the bubbling repetition rate increases from 0.03 to 0.1 s^{-1}. At higher rates the pH is independent of the rate, indicating that the cells have enough O_2 supply to maintain their internal pH even though they are

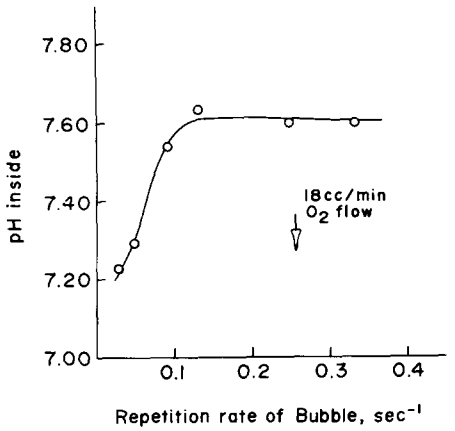

Fig. 3. The internal pH values determined from the position of the inorganic phosphate ^{31}P NMR peaks as a function of the repetition rate of a 2-s long O_2 bubbling period. During the bubbling period the gas flow was kept constant at a rate of 36 cc/min.

respiring several times slower than maximum. The arrow at 18 ml/min of average O_2 flow rate indicated the condition used in previous experiments by Navon et al. [3].

The internal pH values during the bubbling and non-bubbling periods did not show any difference for the off times shown in Fig. 3. Even when the non-bubbling period was 18 s, there was no decay of pH_{in} observed, similar to the case shown in Fig. 1. However, at longer times there is a slow rate of build up and decay of pH_{in} as O_2 is turned on and off as shown directly in Fig. 4. After the start of bubbling, there is a induction period before the pH_{in} increases to a constant value of approx. 7.5. It has been shown (Ugurbil, K., unpublished data) that the induction period is reproducible but dependent on the conditions of growth, harvesting and resuspension. After the supply of O_2 is stopped, the internal pH decays to pH 7.1 with a time constant of 2—3 min.

Discussion

We have seen how the NMR spectra can be measured within a few seconds of a stimulus which in the present case is the cessation of O_2 bubbling. By accumulating 50 of these spectra, excellent signal to noise ratios were obtained for the inorganic phosphate resonance within *E. coli* cells. It is obvious that any stimulus, either chemical or electromagnetic, can be similarly used to trigger NMR spectra. Two interesting points concerning the *E. coli* cells are illustrated by the present studies. First, it is clear that pH_{in} has reached its limiting value of approx. 7.5 at an average O_2 flow rate of approx. 9 cc/min (see Fig. 3) and that faster bubbling rate will not raise pH_{in}. From the present considerations, we have shown that, at the fastest bubbling rate shown in Fig. 3, the respiration rate of *E. coli* was about 1/6th of the maximum rate. Hence, the constant high value of pH_{in} is obtainable even at reduced respiration rates. Second, at 20°C the decay time of pH_{in} after the O_2 bubble has stopped is much slower than 15 s (Fig. 1) and is, in fact, several minutes (Fig. 4). Presumably this high internal pH, with pH gradient across the cell membrane, is maintained in part by the

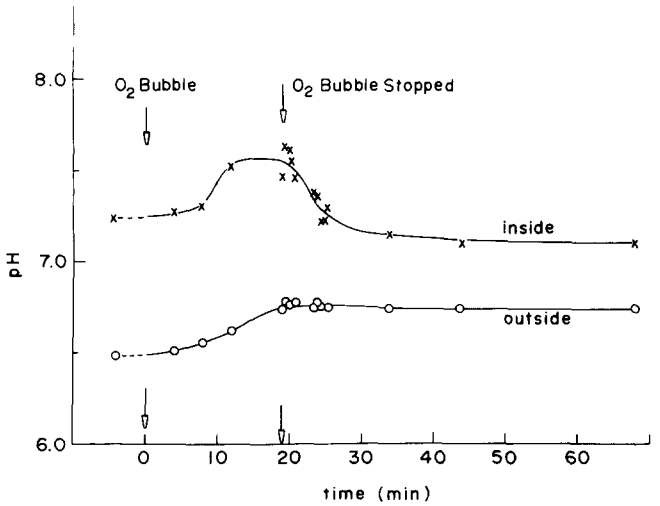

Fig. 4. Build up and decay of the internal pH with O_2 supply. To an *E. coli* suspension of approx. $4 \cdot 10^{11}$ cells/ml continuous O_2 bubbling was introduced at $t = 0$. The bubbling was stopped at the time indicated in the figure. During O_2 bubbling, NMR signals were accumulated for 2 min each and after bubbling the signal accumulation time was 0.5 min.

membrane's impermeability to protons and in part by dissipation of energy of various forms which is linked to the pH gradient and accumulated during respiration. The most direct path for the energy dissipation would be the hydrolysis of ATP by ATPase accompanied by proton translocation outward from the cell [13,14].

In these ways we see that the pH_{in} of *E. coli* cells is maintained at a physiologically meaningful condition in these concentrated samples and that changes in pH_{in} can be measured with a time resolution of a few seconds.

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