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^{31}P nuclear magnetic resonance measurements of ATPase kinetics in aerobic *Escherichia coli* cells

(saturation transfer/dicyclohexylcarbodiimide)

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ABSTRACT **We have measured the *in vivo* unidirectional rates between the terminal phosphate of ATP and intracellular inorganic phosphate (P_i^{IN}) in aerobic suspensions of *Escherichia coli* cells using ^{31}P nuclear magnetic resonance saturation transfer techniques. Typically, the measurements consisted of saturating the ATPγ resonance and observing a 20 ± 5% reduction in the intensity of the P_i^{IN} resonance. No saturation transfer was observed after incubation with 1 mM dicyclohexylcarbodiimide, an ATPase inhibitor. From the measured decrease in intensity of P_i^{IN} coming from saturation transfer, the apparent unimolecular rate constant of P_i^{IN} to ATP was calculated to be 0.6 ± 0.15 sec^{-1}.**

The direct measurement of *in vivo* chemical rates has been an important and difficult problem for many years. We report here the application of a standard nuclear magnetic resonance (NMR) technique, saturation transfer (1–3), to the determination of the *in vivo* exchange rates between inorganic phosphate (P_i) and the terminal phosphate of ATP (ATPγ) in suspensions of aerobic *Escherichia coli* cells. Recently, high-resolution ^{31}P NMR spectra of suspensions of cells (4, 5) and of muscles (6, 7) have been obtained, showing that it is possible to measure time-dependent concentration changes of different phosphate metabolites.

In the present experiments, the saturation transfer technique has allowed us to measure unidirectional rates under steady-state conditions. To measure these rates, the spin magnetization of one chemical species is perturbed from its thermal equilibrium value and the appearance of the nonequilibrium spin magnetization at the second species is monitored to determine the enzymatic reaction rate. In contrast to ^{31}P measurements of net changes in concentrations with a resolution time of minutes, spin magnetization transfer techniques measure the unidirectional rates under equilibrium or steady-state conditions with resolution times of 1 sec or less. The experiments reported in this paper consist of saturating the NMR peak of ATPγ and observing the decrease in magnetization at the peak previously assigned to the intracellular inorganic phosphate (P_i^{IN}) (5). This transfer of saturation does not occur in the presence of dicyclohexylcarbodiimide (DCCD), an inhibitor of ATPase activity (8).

All measurements were made at 25°C in a 10-mm tube with a Bruker HX-360 NMR spectrometer operating at 145.7 MHz in the Fourier transform mode. An independent frequency synthesizer was used to drive the decoupling channel in the homonuclear decoupling mode at the ATP resonance frequency. *E. coli*, strain MRE 600, were grown in M9 minimal medium with 20 mM glucose as the carbon source. The cells were harvested in midlogarithmic phase and resuspended in 15 mM Na_2HPO_4/15 mM KH_2PO_4/100 mM 1,4-piperazinediethanesulfonic acid/30 mM 4-morpholinoethanesulfonic acid/80 mM NaCl at pH 6.5. NMR samples were 2.5 ml in volume. Ten minutes prior to the start of the experiment, 8 mM glucose was introduced into the NMR samples and the cells were oxygenated by bubbling O_2 through the suspension at the rate of 20 ml/min. By the start of the NMR measurement, the glucose content was exhausted and the cells were respiring entirely on endogenous carbon sources.

Typical NMR spectra are shown in Fig. 1, with details of the spectra given in the legend. The resonance at 5.0 ppm, labeled P^{γ}-NTP, arises from the total nucleotide triphosphate pool, approximately 50% of which is ATP (5, 9). For simplicity, this resonance will be referred to as ATPγ.

As determined from the P_i^{IN} and extracellular inorganic phosphate (P_i^{EXT}) chemical shifts, in the presence of O_2 the intracellular pH was maintained at 7.55 while the external pH was 6.6. The irradiating pulses, shown by the arrows, were positioned either on the ATPγ resonance frequency (Fig. 1*B*) or half way between ATPγ and P_i^{IN} resonance frequencies (Fig. 1*A*). To eliminate possible systematic effects such as changes in metabolite concentrations and extrenal pH drift, the irradiating pulses were switched between these two frequencies every 30 sec, and the two free induction decays were accumulated separately, for a total of ~25 min (4000 scans each). Fig. 1*C* is the difference between spectra *A* and *B* and shows clearly the decrease in magnetization of the P_i^{IN} peak at −2.95 ppm, when the ATP γ peak is irradiated. Note that any direct saturation of the P_i^{IN} peak due to the irradiating pulses would be larger under the conditions of Fig. 1*A* than under those of Fig. 1*B* because the irradiating frequency is then closer to the P_i^{IN} frequency. If this were occurring, the P_i^{IN} intensity difference would be opposite in sign to that observed. Furthermore, note that the P_i^{EXT} peak, which is closer to the saturating frequency, shows no change.

From the integrals of Fig. 1 we estimate that the magnetization of P_i^{IN} is reduced by 20 ± 5% when the ATPγ phosphate is saturated. Note also that there is no sign of saturation transfer to any other metabolite in Fig. 1*B*. Immediately after the spectra shown in Fig. 1 were obtained, 1 mM DCCD was added in order to inhibit the ATPase reaction. After 10 min of incubation at 25 °C, the spectra of Fig. 2 were taken under the same conditions. As can be shown in the difference spectrum (Fig. 2*C*), no saturation transfer was detectable within our signal-to-noise ratio, indicating that the DCCD-sensitive pathway, presumably catalyzed by ATPase, dominates the observed exchange under the conditions of Fig. 1.

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Abbreviations: NMR, nuclear magnetic resonance; P_i, inorganic phosphate; ATPγ, terminal phosphate of ATP; P_i^{IN}, intracellular P_i; DCCD, dicyclohexylcarbodiimide; P_i^{EXT}, extracellular inorganic phosphate.

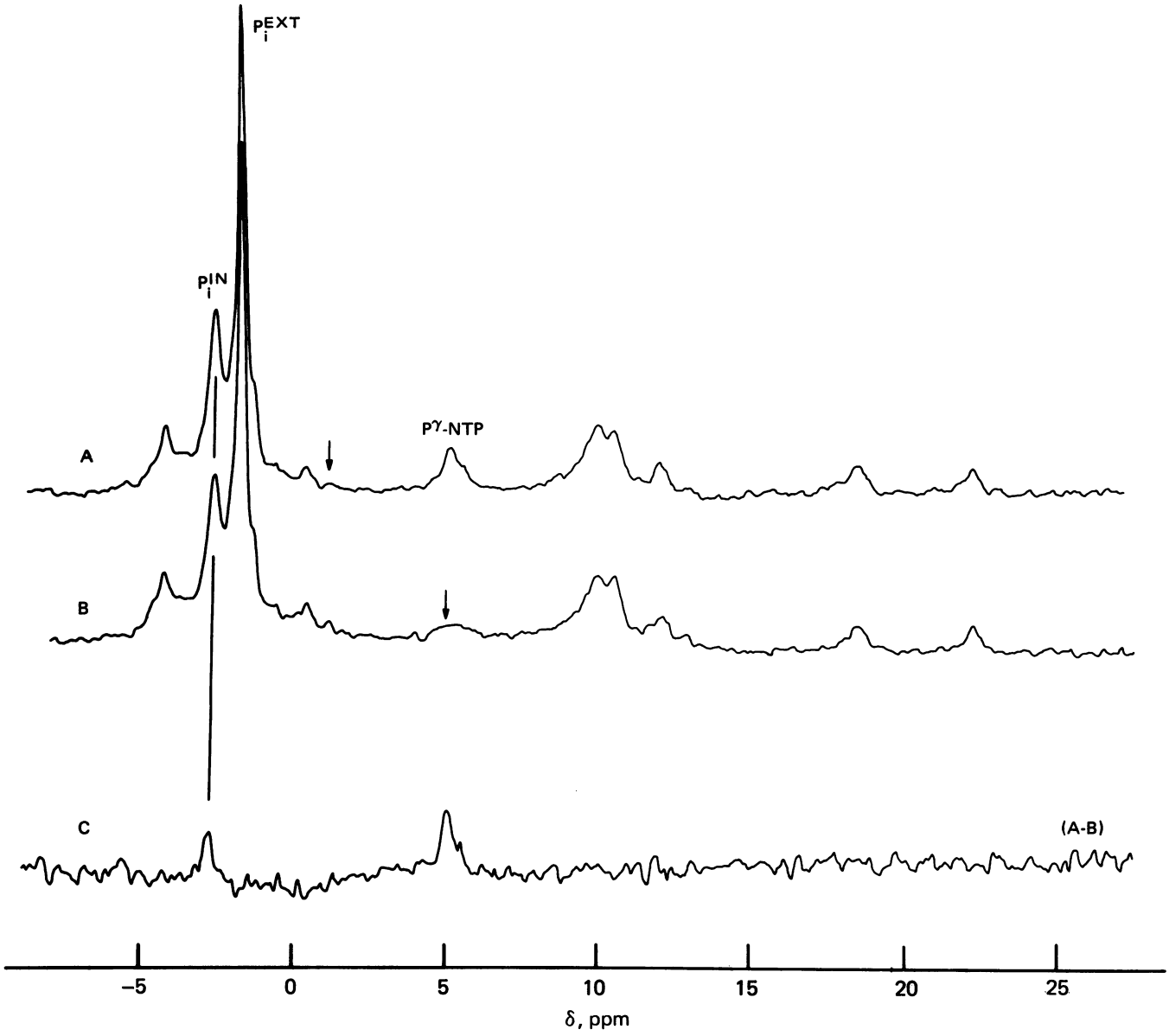

FIG. 1. ^{31}P NMR spectra of aerobic *E. coli* at 25°C. The samples contained ~5×10^{11} cells per ml. The arrows indicate the frequencies of the low-power pulses used in *B* to saturate the P^{γ}-NTP peak. The repetition time was 0.17 sec and the pulse angle was 60°. The spectra consist of 4000 scans each, taken in alternate 30-sec intervals. The peaks labeled P_i^{IN} and P_i^{EXT} correspond to intracellular and extracellular inorganic phosphate, respectively. The peak identified as P^{γ}-NTP consists of approximately 50% ATP and 50% non-adenine nucleotide triphosphates. The high-field shoulder on this peak is due to the β phosphates of the nucleoside diphosphates. The P_i^{IN}-to-ATP ratio derived from these intensities is 20 ± 5.

We attempted to observe, in a separate sample, the inverse exchange in which we saturate P_i^{IN} and measure the decrease in magnetization of the ATPγ. A reduction of 25–37% in the overall NTPγ intensity (equivalent to 50–75% of the ATPγ magnetization) was observed. The large range stems from the poor signal-to-noise ratio of the difference spectrum. Furthermore, these experiments showed no sign of exchange between P_i^{IN} and any other observable phosphates. We also measured the spin lattice relaxation times, T_1, of similar cellular suspensions both with and without DCCD. They were 0.24 ±0.02 and 0.38 ± 0.05 sec without DCCD for the ATPγ and P_i^{IN} resonances, respectively. With the addition of 1 mM DCCD, the low concentration of ATPγ made the measurement of its T_1 difficult. However, we could determine, that T_1 was still in the range of 0.2 sec. T_1 of the P_i^{IN} resonance was 0.43 ± 0.08 sec under these conditions.

In calculating the actual exchange rates from the data, we assumed that the T_1 values measured in the presence of DCCD can be used as single spin relaxation times with no complications due to possible relaxation pathways via other species. Such an assumption appears plausible but we have been unable to prove it conclusively. With such an assumption, the analysis of our data reduces to analyzing two-component exchanges between P_i^{IN} and ATPγ. In this case, it can be shown that the effect of saturating one of the components is to reduce the magnetization of the other by $k(1/T_1 + k)^{-1}$ in which k is the unidirectional rate from the unsaturated to the saturated component and T_1 is the spin-lattice relaxation time of the *unsaturated* component in the absence of the exchange reaction.

Using a value of 0.4 sec for the T_1 of P_i^{IN} in the presence of DCCD, together with the reduction of magnetization from Fig. 1*C* of 20%, we obtain a unidirectional rate from P_i^{IN} to ATP of 0.6 sec^{-1} in the absence of DCCD. From the lack of any observed transfer (Fig. 2*C*) we infer that this rate is decreased

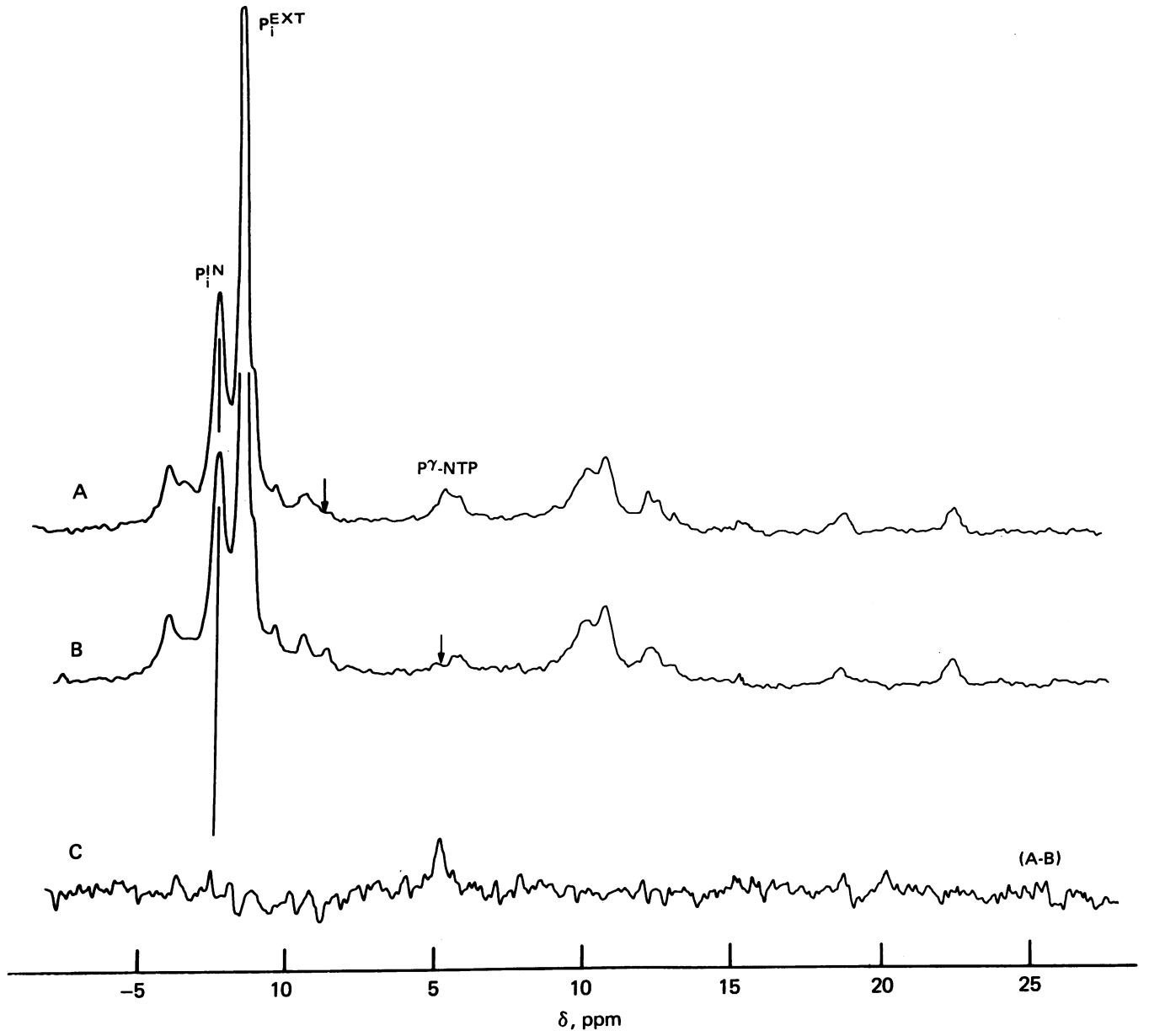

FIG. 2. ^{31}P NMR spectra of the sample in Fig. 1 taken 10 min after the addition of 1 mM DCCD. All other conditions are as in Fig. 1.

at least 3-fold in the presence of 1 mM DCCD. With a T_1 of 0.2 sec for the ATP peak and an observed reduction in its magnetization of 50–75% when P_i^{IN} is saturated, we calculate an apparent unimolecular rate constant for ATP to P_i^{IN} of 5–15 sec^{-1}. Assuming that we can compare the two separate samples, this gives a concentration ratio of P_i^{IN} to ATP of 10–30, which is consistent with our experimentally observed ratio of 20 ± 5. We would like to emphasize that these unidirectional rates represent the one-way flow of the ATPase and are different from net velocites measured by either ATP pool changes or O_2 consumption rates (10, 11).

In conclusion, we have determined the *in vivo* apparent unimolecular exchange rates between P_i^{IN} and ATP as catalyzed by the DCCD-sensitive ATPase in *E. coli*. Under our aerobic conditions they are 0.6 ± 0.15 sec^{-1} for P_i^{IN} to ATP and 20 ± 10 sec^{-1} for ATP to P_i^{IN} at 25°C.

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