REGULATION OF GLYCOLYTIC FLUX IN *BROCHOTHRIX THERMOSPRACTA* BY THE CYTOPLASMIC pH AND ADENYLATE CHARGE: AN IN VIVO NMR STUDY

SATYA P. SINGH* AND PETER J. ROGERS

Division of Science and Technology, Griffith University, Brisbane, Q 4111, Australia

(Received October 7, 1991)

Changes in the concentration of inorganic phosphate (P_i) sugar phosphate, phosphoenol pyruvate (PEP), the adenylate charge and pH were followed by ^{31}P -NMR and assay of cell extract during glucose fermentation in *Brochothrix thermosphacta*. These changes provide an estimation of glycolytic flux via pyruvate kinase as opposed to cycling of PEP through the phosphotransferase system. There appears to be a strong causal relationship between the reduced pH, the high NTP level and the apparent reduction in pyruvate kinase activity, which support the contention that synergistic effect of pH and ATP on pyruvate kinase is an important regulatory factor in these cells.

Brochothrix thermosphacta (formerly *Microbacterium thermosphactum* (22)), a Gram-positive and facultative anaerobe, is predominantly associated with the spoilage of packaged meat products (8,9). Under anaerobic conditions this has limited ability to maintain energy reserves (8), and on this and other taxonomic grounds it relates to streptococci (9,22). To broaden the basic understanding of metabolic regulation in this organism, we have characterized carbohydrate transport (20) and focused on the regulation of certain key enzymes (18,19,21). In the present study we wished to see whether the in vitro properties of pyruvate kinase (18) could account for the apparent activity of pyruvate kinase in washed cell suspensions following glucose addition and also during the subsequent onset of starvation.

MATERIALS AND METHODS

Organism and growth conditions. *B. thermosphacta* (ATCC 11509) was anaerobically grown at 24°C in half-strength medium 56 supplemented with 0.3%

---

* Address reprint requests to: Dr. Satya P. Singh, Department of Microbiology, CBSH, Pantnagar-263145, India.

yeast extract and 0.5% glucose (8).

Assay of glycolytic intermediates. Cells were harvested in late exponential to early stationary growth phase and washed twice by resuspension in cold buffer (5 mM KH_2PO_4 , 50 mM MES, 100 mM PIPES, 200 mM KCl, 2 mM EDTA, 5 mM MgSO_4 , pH 7.4) and finally resuspended in the same buffer at 50 mg dry weight of cells per ml. Glucose was added to a final concentration of 50 mM and the cell suspension was subsequently sampled periodically. Two methods were used to extract glycolytic intermediates. In the first, 0.5 ml aliquots were treated with 0.2 vol of ice-cold perchloric acid (30%) then frozen and thawed three times before pelleting the debris at $4,000 \times g$. The supernatant was neutralized with 2 M KHCO_3 , freeze-dried and finally reconstituted with 0.5 ml of water. These extracts were assayed for glucose, lactate, ATP and ADP. For the second method, a 0.2 ml aliquot of the cell suspension was pipetted onto a membrane filter (cellulose mixed ester filter, 0.45 μm diameter) and washed with 4 ml of ice-cold suspension buffer (12). The filter was treated with $\text{CHCl}_3:\text{CH}_3\text{OH}$ (2:1) (-10°C) and processed as described earlier (12). The final clarified extract was used to assay for PEP, 2PGA, 3PGA, Fru-1,6- P_2 , Glc-6-P, triose phosphates and P_i . Fru-1,6- P_2 , G3P, DHAP, PEP, 2PGA and 3PGA were assayed by following NADH fluorescence associated with the enzymatic reactions involving these metabolites as described by Maitra and Estabrook (13). Glc-6-P was assayed by monitoring NADPH fluorescence during the oxidation of Glc-6-P catalyzed by Glc-6-P dehydrogenase. P_i was then determined by the addition of phosphorylase and phosphoglucomutase to this mixture which allowed the free P_i and glycogen to produce Glc-6-P which was then used as substrate (12). ATP levels were determined with the firefly luciferase assay using the Boehringer assay kit. Fluorescence measurements for the above reactions were performed on a Perkin-Elmer LS5 Luminometer.

pH estimation. The intracellular pH was estimated from the distribution of ^{14}C -DMO between the intracellular water space and the external medium (11). Cell suspensions (50–70 mg dry weight cells per ml) were equilibrated at 25°C and DMO (0.5 $\mu\text{Ci/ml}$; 0.5 mM) was added at -4 min. At zero time, glucose was added (usually 50 mM) and 0.2 ml aliquots were removed periodically and snap frozen in Eppendorf tubes in liquid nitrogen. At the conclusion of an experiment the aliquots were centrifuged at 4°C , which allowed the cells to pellet as the suspension thawed. The pellet was resuspended by sonication in Aquasol and counted in a Packard Series 2000 scintillation counter. By using this method we could directly relate the data of the results obtained using ^{31}P -NMR, which relied on using thick cell suspensions. The intact cell volume and the cytoplasmic volume were estimated using $^3\text{H}_2\text{O}$ (0.25 μCi per ml) and [^3H]-inulin (0.25 μCi per ml) as the penetrant and impermeable probes. There was no change in cytoplasmic volume due to the freezing and centrifuging as opposed to cell suspensions that were not snap frozen. The cytoplasmic and the cellular volumes in the chosen buffer conditions were $1.8 \pm 2 \mu\text{l}$ and $3.9 \pm 0.3 \mu\text{l}$ per mg dry weight of cells respectively (16).

^{31}P -NMR of cells. Cells were harvested and resuspended as described above.

^{31}P -NMR were performed at 80.92 MHz using an extensively modified Bruker wide-bore 200 MHz spectrometer and a 10 mm diameter probe. Free induction decays were collected in 2 min blocks with a 30° pulse and repetition times of 0.34 s or 2 s for 2 ml samples of cell suspension. Chemical shifts are quoted relative to external 85% orthophosphoric acid. To optimize signal-to-noise ratio and to follow the kinetics of glucose metabolism, spectra were normally collected with a rapid repetition time (0.45 s). We compared the effects of pulse angle and delay time on signal intensity using authentic P_i , ATP, ADP and Fru-1,6- P_2 . Cytoplasmic pH was determined from the chemical shift of the internal P_i (5,26,27) whose dependence on pH was measured by titrating cells in the presence of $80\text{ }\mu\text{M}$ CCCP. The P_i concentration was estimated by integration of the P_i peaks and comparison with standard curves obtained from spectra of P_i solutions of known concentration under comparable conditions. The estimations of sugar phosphates, P_i , NTP and NDP concentration were also based on the integration of peak areas.

Materials. Metabolites of the Embden-Meyerhof pathway were obtained from Boehringer Mannheim. Yeast extract was purchased from Difco; nucleotides, DCCD, CCCP and PEP were from Sigma.

Abbreviations. CCCP, carbonyl cyanide *m*-chlorophenyl-hydrozone; DCCD, dicyclohexyl-carbodiimide; DMO, 5,5-dimethyl-2,4-oxiazolidinedione; Fru-1,6- P_2 , fructose-1,6-bisphosphate; Glc-6-P, glucose-6-phosphate; MES, 4-morpholine-ethane-sulfonic acid; NDP, nucleoside-5-diphosphate; NTP, nucleoside-5'-triphosphate; NMR, nuclear magnetic resonance; PIPES, 1,4-piperazine diethane sulfonic acid; PGA, phosphoglyceric acids. All other abbreviations have their usual meanings.

RESULTS

The effects of ATP and pH on pyruvate kinase in vivo

Preliminary studies with *B. thermosphacta* showed that when washed cell suspensions consume glucose there is a transient alkalization of the cytoplasm (18,20). Titration with DCCD, an F_1F_0 -ATPase inhibitor, repressed the formation of a pH gradient across the cell membrane although the cells continued to ferment the added glucose. The effects of DCCD treatment were examined to determine whether cytoplasmic pH was important in the regulation of pyruvate kinase in vivo.

In washed cell suspensions there are significant amounts of 2PGA, 3PGA, PEP and hexose phosphates. The initiation of fermentation resulted in (i) the accumulation of hexose phosphates (Fig. 1a; Table 1); (ii) an increase in the ATP/ADP ratio, which lagged behind the rate of increase of the sugar phosphates (Fig. 1b); (iii) a transient increase in the cytoplasmic pH (Fig. 1c); (iv) an immediate fall in cytoplasmic PEP from 3 mM to less than 0.2 mM (Fig. 1d; Table 1); (v) a sharp decline in cytoplasmic P_i , which was inversely proportional to the rate of sugar phosphate accumulation (Fig. 1e). Comparison of Fig. 1a with 1d suggests that the residual pre-glucose PEP reserves are initially used almost exclusively for phospho-

Fig. 1. The effect of DCCD titration of cells on the kinetics of metabolic changes that accompany the glucose fermentation by washed cell suspensions. Glucose in the extracellular medium was reduced to 1 mM after 12 min.

transferase-mediated glucose transport (20). ATP accumulation as mentioned above lags behind the appearance of hexose phosphates, which is consistent with the initial diversion of PEP from the glycolytic pathway to the phosphotransferase system. The rise in the ATP/ADP ratio parallels the increase in the cytoplasmic pH and presumably signals net ATP synthesis due to pyruvate kinase mediated substrate level phosphorylation. Stimulation of pyruvate kinase activity would be favored by the drop in cytoplasmic P_i , and the rise in pH would tend to offset the inhibitory effects of ATP. The reduction in hexose phosphates corresponds to the

Table 1. Metabolite levels in washed cell suspensions of *B. thermosphacta* during the fermentation of glucose.

Time (min)	Fru-1,6-P ₂ (mM)	Glc-6-P (mM)	Triose phosphates (mM)	2PGA + 3PGA (mM)	PEP (mM)	ATP (mM)	ADP (mM)
-4	6	3	0.5	5	3.5	0.5	2
+2	11	4	1.5	2	1.5	0.5	2
+4	25	5	3	0.7	0.5	3.5	1.2
+10	12	2	1	0.8	0.25	2.5	1.5
+20	5	2.5	0.2	1	0.75	1.5	2
+30	5	2.7	0.3	1	0.75	1.5	2
+40	4.5	3	0.5	1.8	1.2	0.5	1.7

Confidence limits for these estimates are as follows: hexose phosphates, $\pm 12\%$; triose phosphates, $\pm 10\%$; 2PGA + 3PGA, $\pm 15\%$; PEP, $\pm 12\%$; ATP and ADP, $\pm 12\%$.

consumption of all the glucose and the enhanced activity of pyruvate kinase. After 30 min, PEP levels had recovered and remained fairly constant for the next 2 h (not shown). However, triose phosphates drop marginally during this period, indicating that inhibition was occurring at the pyruvate kinase step (Table 1). These changes are consistent with the observed properties of the isolated pyruvate kinase. The high P_i concentration may inhibit the enzyme despite the presence of significant amounts of Fru-1,6-P₂ in the pre-glucose cells and thus account for the residual levels of PEP (18).

Titration of cells with DCCD produced significant changes in metabolite levels following glucose addition. Firstly the rate of accumulation of sugar phosphates was reduced although the transient steady state values were about the same as in the control cells. In addition, there was a much slower reduction in sugar phosphate levels in DCCD-treated cells (Fig. 1). Net ATP synthesis proceeded at much the same rate as in the controls, but the NTP/NDP ratios reached much higher values in the treated cells and remained so for a longer period than in untreated suspensions. DCCD treatment also inhibited the alkalinization of the cytoplasm (Fig. 1). The residual PEP levels during fermentation were higher in treated cells; the minimum concentration was 1 ± 0.15 mM, which was higher than in the controls (Fig. 1). The cytoplasmic P_i concentration decreased by about the same margin in the DCCD-treated cells.

The slight reduction in the rate of sugar phosphate accumulation may be caused by a pH effect on the glucose phosphotransferase system (18). The higher NTP values are most likely due to the effect on membrane ATPase proton pumps presumably responsible for pH homeostasis (10).

³¹P-NMR of cell suspensions

³¹P-NMR spectroscopy of thick cell suspensions of *B. thermosphacta* also demonstrated a similar pattern of change in some of the glycolytic intermediates and the cytoplasmic P_i during glucose fermentation.

Fig. 2. Typical 80.92 MHz ^{31}P -NMR spectra of *B. thermosphacta* cells.

Glucose (50 mM) was added at zero time, under anaerobic conditions; the temperature was 23°C. $\text{P}_{i,\text{ex}}$ is the extracellular P_i signal and $\text{P}_{i,\text{in}}$ represents the cytoplasmic pool.

P_i , nucleoside phosphates and sugar phosphates were estimated from the ^{31}P -NMR spectra of cell suspensions (Fig. 2). After glucose addition, the sugar phosphate signal (4 ppm) increased and conversely the size of the cytoplasmic P_i peak diminished. After 4 min, signals were present at -5.5 ppm and -19 ppm, which were assigned to NDP + NTP and NTP respectively. The external P_i signal moved progressively to the right as fermentation continued and the external medium became more acidic due to lactic acid efflux from the cells. This peak eventually merged with the teichoic acid signal, although before doing so it appeared as though the integral of this peak had started to increase. The

Fig. 3. Quantitation of metabolite levels from the 80.92 MHz ^{31}P -NMR spectra of washed cell suspension of *B. thermosphacta* following glucose addition (50 mM).

P_{ex} , extracellular P_i ; P_{in} , cytoplasmic P_i .

cytoplasmic P_i signal rapidly decreased immediately after the addition of glucose, and apart from a brief shift in position to higher field strength after 2–4 min, indicative of an increase in cytoplasmic pH, it gradually drifted down and merged with the teichoic acid peak (Fig. 2). The cytoplasmic pH was calculated from the chemical shift of the P_i peak and is shown on Fig. 2 for representative spectra. In order to estimate P_i in cytoplasmic and external pools after the cessation of fermentation, the pH of the cell suspension was readjusted to 7.2 (from below pH 6) with KOH before the spectrum was collected.

Changes in metabolite levels were calculated on the basis of the integrated peak areas in the NMR spectra. The data summarized in Fig. 3 show large changes in the concentration of cytoplasmic P_i during glucose metabolism that are reciprocated by changes in NTP and sugar phosphates. Maximum sugar phosphate accumulation (24 ± 2.5 mM) preceded NTP appearance (2–3 mM, maximum attained). The NTP maximum coincided with the drop in cytoplasmic P_i concentration to minimum values (3–4 mM). By the end of fermentation the cytoplasmic P_i level had partly recovered although at 10 ± 3 mM it was considerably less than the pre-glucose value.

There was also a net reduction in total intracellular phosphorus during glucose fermentation with a corresponding increase in external P_i (Fig. 3); 10 min after glucose addition, total phosphorus increased from 25 ± 4 to 34 ± 5 μ mol (2 ml cell suspension, as depicted in Fig. 3) and finally returned to about 30 ± 4 μ mol after 30 min. This may be due to underestimation of saturation effects in pre-glucose cells or because bound P_i is released during rapid metabolism. Nevertheless the data shows that most of the participating P has been accounted for.

DISCUSSION

In vitro studies (3, 4, 18, 23, 24) have established that pyruvate kinase plays an important role in glycolytic regulation in group N streptococci and related lactic acid bacteria. There are two competing pathways from PEP to pyruvate. The pyruvate kinase catalyzed step in *Brochothrix* is susceptible to inhibition by ATP and P_i . ATP inhibition is reduced by raising pH, and Fru-1,6- P_2 serves as positive effector (18). In anaerobic cell suspensions of *B. thermosphacta* the detection of NTP corresponds with the maximum alkalinity in the cytoplasm (pH 7.8). In the case of *E. coli* (26–28), *S. faecalis* (6, 7, 17) and *S. lactis* (25) the cytoplasmic pH during anaerobic glucose metabolism can exceed 7.5. Thus in vitro response of pyruvate kinase to pH values 6–8 can legitimately be extrapolated to intact bacteria. More recently, the cell viability and cessation of growth in an alkalophilic *Bacillus* sp. has been directly linked to acidification of cytoplasmic pH (14). Glucose addition results in the accumulation of sugar phosphates and little change in the ATP/ADP ratio as PEP is recycled for glucose uptake. Eventually the drop in P_i concentration plus the saturating level of Fru-1,6- P_2 in the cytoplasm leads to pyruvate kinase activation and the net formation of ATP by substrate level phosphorylation. This also provides substrate for the ATPase proton pump which generate a more alkaline environment. The higher pH would protect pyruvate kinase from ATP inhibition. The relationship between nucleotide levels and the cytoplasmic pH is supported by the studies with DCCD-treated cells. An interplay between cytoplasmic pH and ATP levels has also been described for chemostat-grown cells of *S. cremoris* (15).

Concluding the above scenario (Fig. 4), accumulation and retention of PEP, triose phosphates and haxose phosphates may be related to the need to keep

Fig. 4. Regulation of pyruvate kinase in *B. thermosphacta*.

(a) Washed cells suspended in buffer contain significant levels of Fru-1,6- P_2 (>4 mM), but despite this, the high residual P_i concentration inhibits pyruvate kinase activity and ensures that PEP is retained to prime the PTS; (b) when glucose is added this reserve is used for the initial transport of glucose into the cells. PEP subsequently generated from glycolytic activity is directed to the PTS and as a result hexose phosphates accumulate. (c) P_i concentration decreases as more of the total phosphate pool is trapped. But because of the reduction in P_i and the high level of Fru-1,6- P_2 , pyruvate kinase is activated, and net synthesis of ATP takes place. As well as being a potent inhibitor of the pyruvate kinase, ATP serves as substrate for the ATPase proton pump. (d) The drop in proton concentration in the cytoplasm offsets the ATP-directed inhibition of pyruvate kinase; the Fru-1,6- P_2 level is sufficiently high to guarantee high catalytic activity in the presence of reduced P_i . The external glucose pool subsequently becomes depleted, the sugar phosphates diminish, P_i once again accumulates and the situation returns to the state shown in (a) above.

PEP-phosphotransferase system poised. Sugar phosphates and PEP may also mimic the function of creatine phosphate in animal cells and stabilize the ATP levels against sudden fluctuations of energy and demand (1). Thus pyruvate kinase may act as a choke, acting in response to a hierarchical chain of command, in which P_i may play the key role. The interplay between the PEP-phosphotransferase system and pyruvate kinase revolves around the fact that PEP is a common substrate to both, and that the sugar phosphates potentiate the PK activity by reducing cytoplasmic P_i concentration. The fluctuations of the glycolytic intermediate accumulation and intracellular pH as monitored in intact cells during fermentation by NMR, supports the physiological importance of the in vivo properties. This shows that the levels of the effectors that elicit a response in vitro, for low enzyme concentration, are similar to that of washed cells. Therefore, as quoted by Bosca and Corredor (2) for other enzymes, the importance of PK as a regulator of the glycolytic pathway will depend on the particular growth conditions, energy balance, sugar phosphate, P_i and pH. Possibility of other enzymes playing similar regulatory role is less likely as P_i did not modulate phospho-fructokinase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase and acetate kinase (results not included). However, a definite relationship between initial fluctuation of metabolites and alkalization of the cytoplasm would need further investigation on the mechanism of pH homeostasis in these cells.

SPS is grateful for Griffith University Post Graduate Research Award. This work was supported by an ARGs grant to PJR. Facilities by the Dean, CBS & H, Pantnagar during the preparation of this manuscript are acknowledged.

REFERENCES

- 1) Akkerman, J. W. N., Does fructose-1,6-bis phosphate reflect phosphofructokinase activity? *Trends Biochem. Sci.*, **9**, 187-188 (1985).
- 2) Bosca, L. and Corredor, C., Is phosphofructokinase a regulatory enzyme? *Trends Biochem. Sci.*, **9**, 372-373 (1984).
- 3) Collins, L. B. and Thomas, T. D., Pyruvate kinase of *Streptococcus lactis*. *J. Bacteriol.*, **120**, 52-58 (1974).
- 4) Crow, V. L. and Pritchard, G. G., Purification and properties of pyruvate kinase from *Streptococcus lactis*. *Biochim. Biophys. Acta*, **438**, 90-101 (1976).
- 5) Dawson, M. J., Gadian, D. G., and Wilkie, D. R., Contraction and recovery of living muscles studied by ^{31}P -NMR. *J. Physiol.*, **267**, 703-735 (1977).
- 6) Harold, F. M. and Altendorf, K., Cation transport in bacteria: K^+ , Na^+ and H^+ . *Curr. Top. Membr. Transp.*, **5**, 1-50 (1974).
- 7) Harold, F. M. and Kakinuma, Y., Primary and secondary transport of cations in bacteria. *Ann. N. Y. Acad. Sci.*, **456**, 375-383 (1985).
- 8) Hitchener, B. J., Egan, A. F., and Rogers, P. J., Energetics of *Microbacterium thermosphaerum* in glucose limited chemostat culture. *Appl. Environ. Microbiol.*, **37**, 1047-1052 (1979).
- 9) Hitchener, B. J., Egan, A. F., and Rogers, P. J., Characteristics of lactic acid bacteria isolated from vacuum packaged beef. *J. Appl. Bacteriol.*, **52**, 31-37 (1982).
- 10) Kobayashi, H., A proton-translocating ATPase regulates pH of the bacterial cytoplasm. *J. Biol. Chem.*, **260**, 72-76 (1985).

- 11) Krulwich, T. A. and Guffanti, A. A., Regulation of pH in acidophilic and alkalophilic bacteria. *Methods Enzymol.*, **125**, 352–365 (1986).
- 12) Mason, P. W., Carbone, D. P., Cushman, R. A., and Waggoner, A. S., The importance of inorganic phosphate in regulation of energy metabolism in *Streptococcus lactis*. *J. Biol. Chem.*, **256**, 1861–1866 (1981).
- 13) Maitra, P. K. and Estabrook, R. W., A fluorometric method for the enzymatic determination of glycolytic intermediates. *Anal. Biochem.*, **7**, 472–484 (1964).
- 14) Oshima, A. and Onoda, T., Acidification of intracellular pH results in neutral pH sensitive growth of an alkalophilic *Bacillus* sp. *J. Gen. Appl. Microbiol.*, **36**, 7–17 (1990).
- 15) Poolman, B., Smid, E. J., Veldkamp, H., and Konings, W. N., Bioenergetic consequences of lactose starvation for continuously cultured *Streptococcus cremoris*. *J. Bacteriol.*, **169**, 1460–1468 (1987).
- 16) Rottenberg, H., The measurement of membrane potential and pH in cells, organelles and vesicles. *Methods Enzymol.*, **55**, 547–569 (1979).
- 17) Simpson, S. J., Bendall, M. R., Egan, A. F., Vink, R., and Rogers, P. J., High field phosphorus NMR studies of the stoichiometry of lactate: Proton carrier in *Streptococcus faecalis*. *Eur. J. Biochem.*, **136**, 63–69 (1983).
- 18) Singh, S. P. and Rogers, P. J., Isolation and kinetic properties of pyruvate kinase from *Brochothrix thermosphacta*. *Biochem. Int.*, **25**(1), 35–45 (1991).
- 19) Singh, S. P. and Rogers, P. J., ³¹P-NMR and ¹³C-NMR studies on mannitol-1-phosphate formation in *Brochothrix thermosphacta*. Proc. 28th Annu. Conf. Assoc. Microbiologists India, Pune (1988).
- 20) Singh, S. P., Bishop, C. J., Vink, R., and Rogers, P. J., Regulation of the glucose phosphotransferase system in *Brochothrix thermosphacta* by membrane energization. *J. Bacteriol.*, **164**, 367–376 (1985).
- 21) Singh, S. P., Vink, R., and Rogers, P. J., Regulation of lactate dehydrogenase in *Brochothrix thermosphacta*. *FEMS Microbiol. Lett.*, **22**, 73–76 (1984).
- 22) Sneath, P. H. A. and Jones, D., *Brochothrix*: A new genus tentatively placed in the family lactobacillaceae. *Int. J. Syst. Bacteriol.*, **26**, 102–104 (1976).
- 23) Thompson, J., In vivo regulation of glycolysis and characterization of sugar: Phosphotransferase systems in *Streptococcus lactis*. *J. Bacteriol.*, **136**, 465–476 (1978).
- 24) Thomas, T. D., Activator specificity of pyruvate kinase from lactic streptococci. *J. Bacteriol.*, **125**, 1240–1242 (1976).
- 25) Thompson, J. and Torchia, D. A., Use of ³¹P-nuclear magnetic resonance and ¹⁴C fluorography in studies of glycolysis and regulation of pyruvate kinase in *Streptococcus lactis*. *J. Bacteriol.*, **158**, 791–800 (1984).
- 26) Ugurbil, K., Rottenberg, H., Glynn, P., and Shulman, R. G., ³¹P nuclear magnetic resonance studies of the bioenergetics and glycolysis in anaerobic *Escherichia coli* cells. *Proc. Natl. Acad. Sci. USA*, **75**, 2244–2248 (1978).
- 27) Ugurbil, K., Rottenberg, H., Glynn, P., and Shulman, R. G., Phosphorus-31 nuclear magnetic resonance studies of bioenergetics in wild type and adenosinetriphosphatase *Escherichia coli* cells. *Biochemistry*, **21**, 1068–1075 (1982).
- 28) Vink, R., Bendall, M. R., Simpson, S. J., and Rogers, P. J., Estimation of proton to adenosine 5'-triphosphate stoichiometry of *Escherichia coli* ATPase using ³¹phosphorus NMR. *Biochemistry*, **23**, 3667–3675 (1984).