# THE MEMBRANE ADENOSINE TRIPHOSPHATASE OF *CLOSTRIDIUM PASTEURIANUM*

## Effects of key intermediates of glycolysis on its ATP phosphohydrolase activity

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### 1. Introduction

*Clostridium pasteurianum* is an obligately fermentative anaerobic bacterium that is not known to undertake any type of electron transport-linked phosphorylation. It seems likely that its $Mg^{2+}$-dependent, membrane ATPase [1–4] will in vivo serve solely as a proton-translocating ATP phosphohydrolase maintaining the transmembrane protonmotive force which the organism normally requires for ionic regulation and the active transport of certain nutrients [5,6]. Some of the factors which in this organism can regulate the fermentative supply of ATP have been elucidated [7]. Yet, although maintenance of the transmembrane proton motive force could account for the consumption of a major portion of the ATP synthesised by a fermentative anaerobe [8], we at present do not know whether, or by what means, this expenditure of ATP may also be controlled, other than possibly by the prevailing adenylate energy charge [4,9].

It is the chief purpose of this communication to report that in *Cl. pasteurianum* key intermediates in the fermentative route of ATP generation can considerably influence the activity of its membrane ATP phosphohydrolase.

*Abbreviations:* ATPase, the complete ($BF_0F_1$) membrane-associated adenosine triphosphatase complex (EC 3.6.1.3); $[S]_{0.5V}$, the concentration of substrate at which the velocity of the enzymic reaction is one half that ($V_{max}$) when the enzyme is saturated with the substrate: if the enzyme demonstrates Michaelis-Menten kinetics $[S]_{0.5V}$ is the Michaelis constant $K_m$

### 2. Experimental procedures

The origin, maintenance and growth of *Clostridium pasteurianum* 6013 on a glucose minimal medium, have been described [10]. *Escherichia coli* K12 was grown aerobically at 37°C in 'R broth' [11], whilst *Streptococcus faecalis* NCIB 8256 was grown anaerobically under $N_2$ plus $CO_2$ (95:5, v/v) at 37°C in 'KTY medium' [12]. Protein was assayed by the Lowry method [13] using bovine serum albumin as standard.

#### 2.1. *Preparation of cell membrane fraction*

The cell membrane fraction was recovered as in [14] from lysozyme (EC 3.2.1.17)-prepared protoplasts of *Cl. pasteurianum* harvested from early exponential phase batch culture in glucose minimal medium [10]. Cell membrane preparations were similarly obtained from *E. coli* and *Strep. faecalis*. All membrane preparations were washed and resuspended at 4°C in 90 mM Tris–$H_2SO_4$ buffer (pH 8) containing 6 mM $MgSO_4$, 20 mM 2-mercaptoethanol and 1 mM ATP.

#### 2.2. *Assay of ATP phosphohydrolase activity*

Three methods were employed:

(i) Release of inorganic phosphate assayed by the method in [15] as modified [1];
(ii) Release of protons assayed by the method in [16];
(iii) Release of ADP assayed spectrophotometrically in the presence of excess pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase

(EC 1.1.1.27), phosphoenolpyruvate and NADH, as in [3].

In each case the unit of ATP phosphohydrolase activity was defined as 1 μmol product formed/min at 37°C.

## 3. Results

### 3.1. *ATP phosphohydrolase activity in the absence of effectors*

The $Mg^{2+}$-dependence of the membrane ATPase of *Cl. pasteurianum* has been reported [1,3], as has its slight activation by additional $NH_4^+$ [4] and its broad pH optimum around pH 7.5–8 [3,4]. The ATP phosphohydrolase activity of the enzyme complex was therefore initially assayed (by release of $P_i$ from ATP, or by the associated release of protons) in Tris–HCl buffer (pH 7.8) containing 5 mM $NH_4Cl$. Under these conditions the app. $[S]_{0.5\,V}$ for Mg-ATP (supplied as an equimolar mixture of disodium ATP and $MgSO_4$) could only be approximately determined (as 8.3 mM) since a sigmoidal relationship was obtained between enzymic activity and substrate concentration (fig.1a). This was in contrast to the behaviour of the membrane ATPases of both *E. coli* and *Strep. faecalis* which displayed values of $[S]_{0.5\,V}$ for ATP of $< 2$ mM (fig.1b).

### 3.2. *Phosphoenolpyruvate as an effector of the ATP phosphohydrolase of* Cl. pasteurianum

When studies similar to those in section 3.1 were undertaken using the spectrophotometric, linked-enzyme procedure in section 2.2.iii to follow release

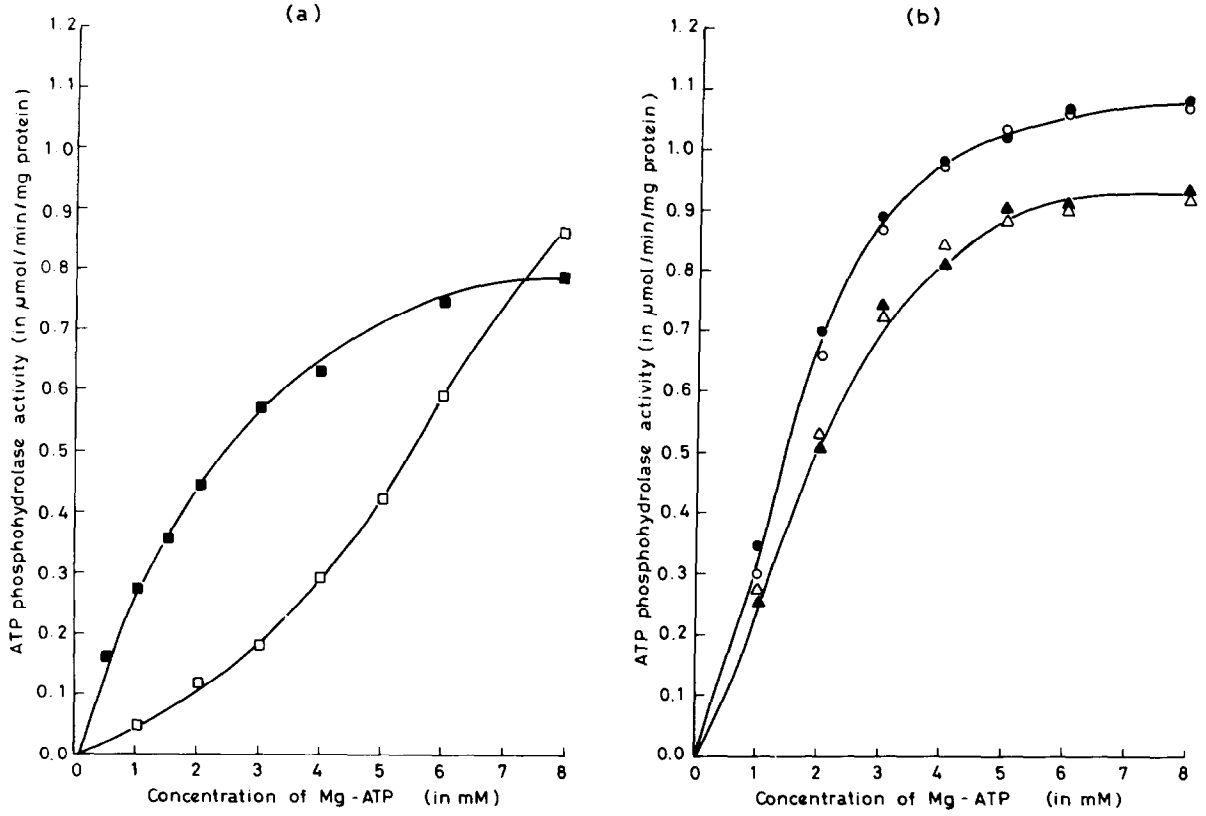


Fig.1. Effect of phosphoenolpyruvate on the substrate concentration versus enzyme activity relationships of membrane ATP phosphohydrolases of: (a) *Cl. pasteurianum* (□, ■); (b) *E. coli* (△, ▲) and *Strep. faecalis* (○, ●). The rate of hydrolysis of ATP was assayed at 37°C, by the proton release procedure, at various concentrations of Mg-ATP in the presence (■, ▲, ●) or absence (□, △, ○) of 2 mM phosphoenolpyruvate.

of ADP, strikingly different results were obtained with the *Cl. pasteurianum* ATP phosphohydrolase. A rectangular hyperbolic plot was now obtained between enzyme activity and substrate concentration, with a $[S]_{0.5V}$ value for ATP of 1.83 mM which was similar to that value (1.3 mM ATP) reported [3]. Investigation of the cause of the discrepancy between the results obtained with the different assay procedures revealed that the phosphoenolpyruvate employed in the procedure for ADP determination had a considerable modifying effect on the behaviour of the *Cl. pasteurianum* ATP phosphohydrolase. When added (at that concentration employed in the ADP assay) to the reaction mixtures used for the measurement of phosphate or proton release, phosphoenolpyruvate provoked a decrease in both the $V_{max}$ and in the $[S]_{0.5V}$ for ATP, with the enzyme now displaying Michaelis-Menten kinetics (fig.1a). The same concentration of phosphoenolpyruvate had no effect on the activities of the membrane ATP phosphohydrolases of *E. coli* or *Strep. faecalis* (fig.1b).

### 3.3. *Effects of other glycolytic intermediates*

The ATP phosphohydrolase of *Cl. pasteurianum* was affected by low concentrations of phosphoenolpyruvate, i.e., < 1 mM (fig.2). In a survey of other phosphorylated intermediates of glycolysis only fructose 1,6-diphosphate and 2,3-diphosphoglycerate proved similarly potent effectors of the *Cl. pasteurianum* enzyme. Both of these compounds acted like phosphoenolpyruvate to decrease the $[S]_{0.5V}$ value for ATP (fig.2) and the $V_{max}$ of the enzyme (table 1). Other intermediates in the glycolytic sequence (e.g., glucose 6-phosphate, fructose 6-phosphate, 3-phosphoglycerate) were considerably less effective, as illustrated in table 1 which gives the concentration at which each of these compounds displayed its maximum effect on the $[S]_{0.5V}$ value for ATP.

### 3.4. *Modification of the pH-activity profile of the* Cl. pasteurianum *enzyme*

In the absence of added phosphoenolpyruvate (or fructose 1,6-diphosphate) the ATP phosphohydrolase of *Cl. pasteurianum* showed little activity at pH values more acid than pH 6.5 (fig.3a). In the presence of relatively low concentrations of these effectors the pH-activity curve of the enzyme was greatly extended

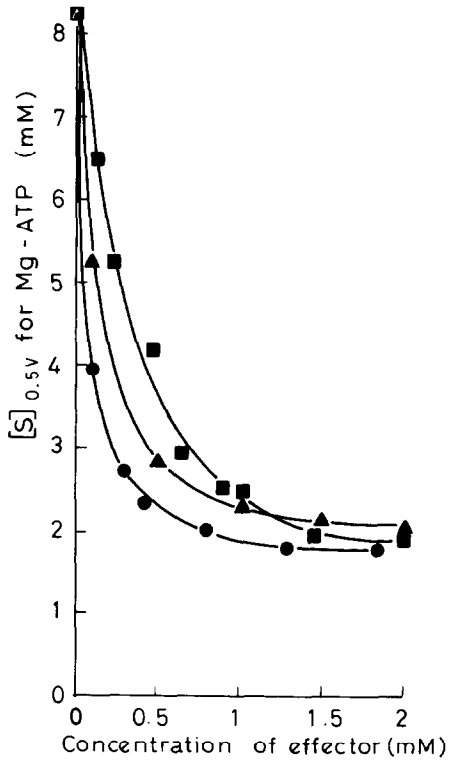


Fig.2. Effect of some key glycolytic intermediates on the $[S]_{0.5V}$ value for ATP of the membrane ATP phosphohydrolase of *Cl. pasteurianum*. The proton release procedure was used to obtain values of $[S]_{0.5V}$ for ATP in the presence of the indicated concentrations of phosphoenolpyruvate (■), or fructose 1,6-diphosphate (▲), or 2,3-diphosphoglycerate (●).

so that significant activity was now measurable at pH 5.5 (fig.3b).

## 4. Discussion

The initial in vitro findings that the ATPase of *Cl. pasteurianum* displayed little activity as an ATP phosphohydrolase at pH < 6.5 and possessed the relatively high $[S]_{0.5V}$ value of 8.3 mM ATP, were difficult to reconcile with the essential role of this enzyme in an organism wherein during growth in glucose minimal medium the internal pH falls progressively to pH 5.9 [5] and the intracellular ATP is normally ≯ 2 mM [4,7]. This paradox was satis-

Table 1
Action of various effectors on the membrane ATP phosphohydrolase of *Clostridium pasteurianum*

| Effector | Effector (mM) | $[S]_{0.5V}$ (mM ATP) | $V_{max}$ (units/mg protein) |
|---|---|---|---|
| $NH_4Cl$ | 5.0 | 8.33 | 2.55 |
| Fructose 6-phosphate | 2.8[a] | 4.55 | 1.48 |
| Glucose 6-phosphate | 2.8[a] | 5.00 | 1.55 |
| 3-Phosphoglycerate | 1.7[a] | 4.50 | 1.36 |
| Phosphoenolpyruvate | 2.0 | 1.84 | 0.78 |
| Fructose 1,6-diphosphate | 2.0 | 2.05 | 0.68 |
| 2,3-Diphosphoglycerate | 2.0 | 1.75 | 0.76 |

[a] Concentration of effector giving maximum decrease in $[S]_{0.5V}$ for ATP

Assays were performed at 37°C and pH 7.8 by the proton release procedure using washed membrane preparations

factorily explained by the discovery that certain intermediates in glucose fermentation could, at low concentrations, act as effectors of the enzyme, serving to extend its pH range of activity and substantially to decrease the $[S]_{0.5V}$ for ATP. That these compounds also decreased the $V_{max}$ of the enzyme would seem relatively inconsequential in circumstances wherein the substrate ATP was < 2 mM (fig.1a). Thus at pH 6.5 and 2 mM Mg-ATP, addition of 2 mM phosphoenolpyruvate to a membrane ATPase preparation from *Cl. pasteurianum* increased its ATP phosphohydrolase activity 10-fold, i.e., from 0.04–0.39 units.mg protein$^{-1}$.

Similar effects were not observed with the membrane ATPases of anaerobically grown *Strep. faecalis* or of aerobically grown *E. coli* and they may therefore be peculiar to the *Cl. pasteurianum* ATPase complex. As with ATPases from other bacteria and mitochondria [17] the ATP phosphohydrolase activity of this complex is subject to inhibition by ADP and

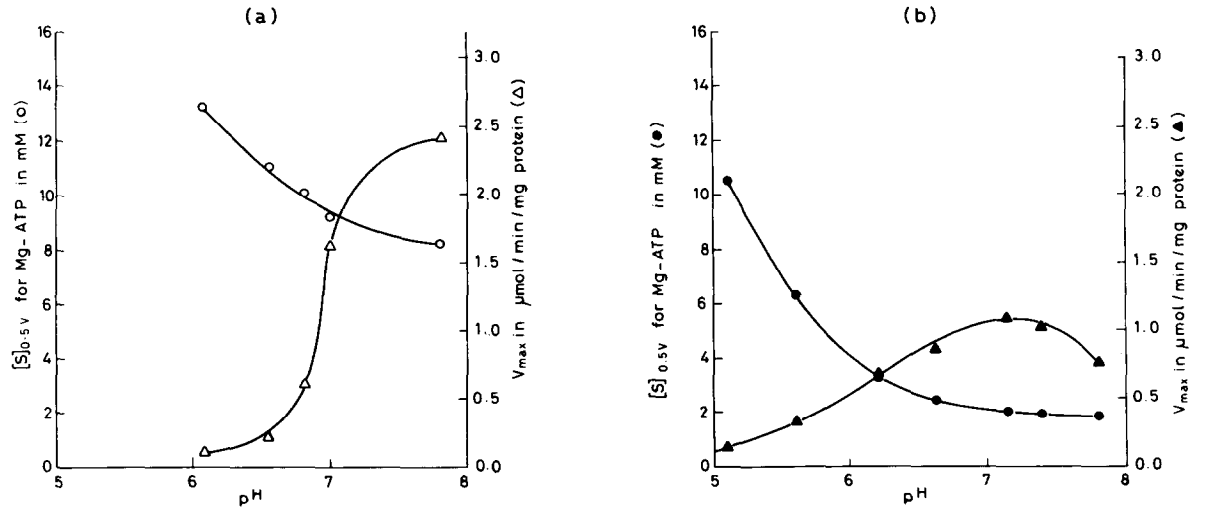


Fig.3. Variation with pH of the membrane ATP phosphohydrolase activity of *Cl. pasteurianum* in: (a) the absence of effectors; (b) the presence of 2 mM phosphoenolpyruvate.

by $P_i$ [4] and is therefore subject to fine control by a number of interactive factors. It is conceivable that the regulation by intermediates in the primary route of ATP generation could serve to prolong conservation of ATP and hence adenylate charge under conditions of 'energy starvation' i.e., deprivation of exogenous energy sources.

## Acknowledgement

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