Kinetics of the membrane-bound inorganic pyrophosphatase from *Rhodospirillum rubrum* chromatophores

Effect of the transmembrane electrical potential on the rate constants

Åke Strid, Pål Nyrén, Jeff Boork* and Margareta Baltscheffsky

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

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The behaviour of the membrane-bound proton-translocating pyrophosphatase (H^+-PPase) in *Rhodospirillum rubrum* chromatophores upon application of an electrochemical potential is studied. The rate constants are shown to be affected in an asymmetric fashion. The forward rate constant (PP_i synthesis) is shown to be at least 45-times larger during illumination than when there is no proton-motive force. The hydrolysis rate is increased maximally 8-times when the potential is dissipated. The effect of the electrical field gradient is thus mainly to increase the forward rate of the reaction. The H^+-PPase also seems to be a functionally simpler enzyme than the H^+-ATPase, lacking the hydrolysis activation step during energization found in the latter.

(Rhodospirillum rubrum) *Inorganic pyrophosphatase* *Photophosphorylation* *Enzyme kinetics* *Membrane potential* *Proton pump*

1. INTRODUCTION

The membrane-bound proton-translocating pyrophosphatase (H^+-PPase) of *Rhodospirillum rubrum* is an enzyme which can hydrolyze PP_i while pumping protons across the bacterial membrane in which the enzyme is sited. This proton flow through the enzyme creates an electrochemical gradient ($\Delta\mu H$) across the membrane. The gradient is the sum of two components, one electrical ($\Delta\psi$) and one chemical (ΔpH). According to the chemiosmotic theory [1], the electrochemical gradient, the proton-motive force, can be transformed by some enzymes into high-energy compounds, for example ATP synthesized by the H^+-ATPase. The pyrophosphatase can also utilize such an electrochemical gradient to make pyrophosphate from two phosphate ions. The reaction catalyzed by this enzyme is given by:

$$2P_i + n(H^+_{in})_v + x(H^+_{out})_s \underset{k^*_{-1}}{\overset{k^*_1}{\rightleftharpoons}} PP_i + H_2O + n(H^+_{out})_v$$

where k^*_1 and k^*_{-1} are the overall forward rate and overall reverse rate constants, respectively, the vectorial and scalar protons being denoted by v and s. The equilibrium constant for the reaction is defined as:

$$K_{eq}(\Delta\psi) = [PP_i][H^+_{out}]^n_v / [P_i]^2 [H^+_{in}]^n_v [H^+_{out}]^x_s$$

The scalar protons are present in the bulk phase

* Present address: Alfa Laval AB, Industrial Biotechnology, Integrated systems, PO Box 500, S-147 00 Tumba, Sweden

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; APS, adenosine 5'-phosphosulphate

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and their concentration is thus constant in this medium. The active site of centration is therefore constant in this medium. The active site of the H^+-PPase in a chromatophore is also in contact with the bulk phase. The outside pH therefore affects the equilibrium constant for the reaction and the V_{max} of the enzyme but has no effect on the shape of the control curve [2]. We have previously [2] analyzed, on a theoretical basis, the effect of transmembrane electrical gradients on the rate constants for reactions of membrane-bound enzymes. We related this effect to the spatial location of the enzymes active site within the membrane. The equilibrium constant is:

$$K_{eq}(\Delta\psi) = k_f/k_r$$

where f denotes the forward and r the reverse direction. The expressions for the different rate constants can be written [2]:

$$k_f(\Delta\psi) = k_f^\circ \exp(-\alpha ne\Delta\psi/kT)$$

$$k_b(\Delta\psi) = k_b^\circ \exp((1-\alpha)ne\Delta\psi/kT)$$

where k_f° and k_b° are the rate constants for the homogeneous reaction. $\Delta\psi$ is negative and α is the factor determined by the position of the active site within the transmembrane electrical field gradient.

We have also shown [2] that the forward reaction is the one that will be enhanced if the active site is situated on the outside of the membrane ($\alpha = 1$ gives $k_f > k_f^\circ$ and $k_b = k_b^\circ$), as is the case for the H^+-ATPase in chromatophores from *R. rubrum* [3]. If, on the other hand, the active site of the enzyme is located at the higher potential, the reverse reaction would be slowed down ($k_f = k_f^\circ$ and $k_b < k_b^\circ$ when $\alpha = 0$).

Here, we present results from studies on the kinetics of the H^+-pyrophosphatase in *R. rubrum* chromatophores and relate these results to the effect on the rate constant of the enzyme.

2. MATERIALS AND METHODS

The chromatophores used were prepared from *R. rubrum*, strain S-1, as in [4] except that the cells were suspended in 0.2 M glycylglycine (pH 7.5) and broken in a Ribi cell fractionator.

Determination of PPase activity was carried out in the following medium: 70 mM glycylglycine (pH 7.5), 2 mM P_i, 0.65 mM $MgCl_2$, 10 μg oligomycin (to prevent any ATP formation from possible endogenous ADP present), 0.2 mM $^{32}P^{32}P_i$ (16.7 mCi/mmol), chromatophores corresponding to 2 μM Bchl, in a final volume of 1 ml.

The P_i was added to prevent the produced $^{32}P_i$ being lost in large amounts in resynthesis of PP_i. The loss of radioactive P_i is in this way kept below 2%.

The PP_i synthesis medium was as follows: 70 mM glycylglycine (pH 7.5), 7 mM $MgCl_2$, 10 μg oligomycin, 3 mM $^{32}P_i$ (15.0 mCi/mmol), 3 mM ATP, 0.2 mM sodium succinate, 0.3 mM APS, 0.3 U ATP-sulphurylase (EC 2.7.7.4), and chromatophores corresponding to 20 μM Bchl, in a final volume of 1 ml.

APS and ATP-sulphurylase were included to convert the $^{32}PP_i$ produced into ATP, labelled in the β or the γ position, according to:

$$^{32}PP_i + APS \rightleftharpoons [^{32}P]ATP + SO_4^{2-}$$

This reaction has recently been described in connection with a new method for measuring PP_i synthesis [5] developed in our laboratory. The reaction has a $\Delta G^{\circ\prime}_{obs} = -11.4$ kcal/mol in the direction of ATP formation [6], which clearly shows that virtually all PP_i is converted to ATP. In this way we avoided having to separate P_i from PP_i. Separation of PP_i and P_i has been described [7] but the accuracy and reproducibility were low in our hands. To prevent hydrolysis of the ATP formed by ATP-sulphurylase, unlabelled ATP and oligomycin were included.

Reaction were started by the addition of the premixed substratres, $^{32}PP_i$ and P_i in hydrolysis experiments and of PP_i, $^{32}P_i$ and ATP in synthesis experiments, and terminated by adding trichloroacetic acid to a final concentration of 5%. Saturating illumination was applied by two 100 W tungsten lamps. To keep a constant temperature (23°C) during illumination, the tubes containing the reaction mixtures were immersed in water and a $CuSO_4$ solution was placed in front of the lamps as an IR filter. FCCP (2 or 20 μM) or Triton X-100 (0.5%) was added to make the membranes of the chromatophores permeable to H^+, thus dissipating the proton-motive force.

PP_i synthesis, the forward reaction, was studied

by measuring $^{32}PP_i$ formed, while the hydrolysis of PP_i, the reverse reaction, was determined by counting $^{32}P_i$ released. Separation of PP_i from P_i in hydrolysis experiments was accomplished by the method of Keister et al. [7]. Separation of ATP from P_i was achieved according to the procedure for separation of PP_i from P_i [7] with the exception that cold P_i was added to a final concentration of 1.6 mM after two extractions. The water phase was then further extracted at least 5 times. This was done to minimize the contamination of radioactive phosphate giving high blanks since only about 2% of the P_i was converted to PP_i and further to ATP. In the extraction medium, toluene was used instead of benzene.

Xylene-based scintillation liquids (Lumagel) from Lumac/3M were used when counting the radioactivity. The radionuclides used were supplied by Amersham International (England) and APS, FCCP, Triton X-100 and ATP-sulphurylase were obtained from Sigma (St. Louis, MO).

3. RESULTS AND DISCUSSION

We have reported [3] that the H^+-ATPase from chromatophores of *R. rubrum* is affected by the proton-motive force in an asymmetric way, i.e. the forward and reverse rates are not altered by the same factor during a change in the proton-motive force, as would be expected for ions freely diffusing in an electrical field. The reason for this behaviour may be ascribed to an asymmetry related to the architecture of ion-translocating enzymes [2].

Here we have studied the H^+-PPase from the same type of chromatophores. Comparing the values in table 1 for illuminated samples and samples lacking an electrochemical gradient (dark + FCCP) it can be seen that the rate of PP_i synthesis is increased drastically ($10 \pm 1 : 0.0 \pm 0.2 > 45$) while that of hydrolysis of PP_i is decreased by a factor of between 4 and 8 when a proton-motive force is built up.

If one proton is needed to synthesize one molecule of PP_i and the proton-motive force is altered from 0 to 180 mV, then the equilibrium constant would be shifted by a factor of 10^3 according to the equation [8]:

$$K'_{eq}/K_{eq} = 10^{(\Delta\psi/(RT \ln 10/nF))}$$

Table 1

Rates of synthesis and hydrolysis of PP_i

Conditions	μmol PP_i formed/10 min per μmol Bchl	μmol PP_i hydrolysed/10 min per μmol Bchl
Light	10.0 ± 1.0	3.8 ± 0.6
Dark	0.2 ± 0.2	10.0 ± 1.0
Light + FCCP		
2 μM	2.0 ± 1.0	12.5 ± 2.0
20 μM	0.0 ± 0.2	12.5 ± 1.0
Dark + FCCP		
2 and 20 μM	0.0 ± 0.2	21.0 ± 4.0
Triton X-100	0.0 ± 0.2	n.d.

n being number of protons and $\Delta\psi$ being given in mV. From this value we can estimate that the forward rate is increased between 125- and 250-times (1000:8 and 1000:4, respectively) when a proton-motive force is applied.

This much larger effect of the proton-motive force on the forward rate compared to the reverse rate indicates that the H^+-PPase is an enzyme very much like the H^+-ATPase with a proton channel crossing the membrane and a catalytic centre localized on the side of the membrane that becomes negative during energization.

It can also be seen from table 1 that the amount of FCCP needed to dissipate the proton-motive force is higher while illuminating than during PP_i hydrolysis in the dark. This indicates that the proton pumping of the electron transport chain is faster than that of the H^+-PPase when both systems are working at their maximal rate.

An interesting difference between the hydrolysis of ATP by the H^+-ATPase and of PP_i by the H^+-PPase is that an activation step takes place only in the former [3,9–11]. This activation under high proton-motive force subsequently gives a more rapid hydrolysis of ATP in the dark and during illumination than under uncoupled conditions [3]. The lack of an activation step would indicate that the H^+-PPase is a simpler enzyme system than the H^+-ATPase.

Fig.1 shows a possible reaction scheme for the H^+-PPase. It has been reported [12] that oxygen exchange between P_i and water is not strongly dependent upon an energized membrane and is only slightly enhanced by light [12]. The rate of

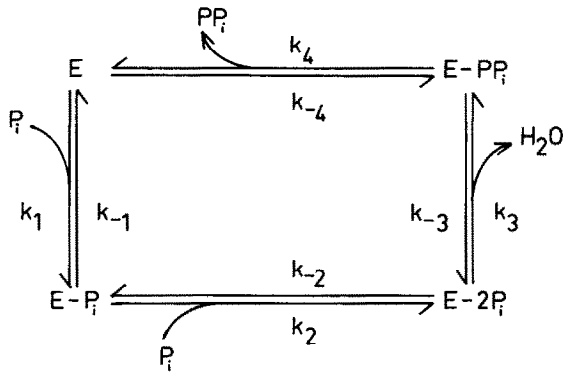

Fig.1. A possible reaction scheme for the H^+-PPase.

formation of free PP_i is too slow to account for the oxygen exchange. However, enzyme-bound PP_i might be formed from P_i, and subsequently cleaved to P_i sufficiently rapidly to account for most of the oxygen exchange. Once PP_i is formed, it is more likely to be cleaved than to appear as free PP_i. Therefore, the main contribution of a membrane potential for the synthesis of PP_i would occur in k_4 (fig.1) significantly increasing the rate of release of PP_i formed.

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