Biochimica et Biophysica Acta, 649 (1981) 550–556
Elsevier/North-Holland Biomedical Press

BBA 79490

TEMPERATURE EFFECTS ON KINETIC PROPERTIES OF PLASMA MEMBRANE ATPase FROM THE YEAST *SACCHAROMYCES CEREVISIAE*

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(Received June 1st, 1981)

Key words: ATPase; Temperature; Mg^{2+}; Mg · ATP; (S. cerevisiae)

The reaction of plasma membrane ATPase from yeast with Mg^{2+} and Mg · ATP was studied in a temperature range of 10–30°C. The random mechanism of activation by Mg^{2+} and the pseudocompetitive inhibition at higher concentrations was not altered when the temperature was varied, nor were the kinetic constants representing substrate binding. However, at low temperature, the affinity of the enzyme for Mg^{2+} is greatly reduced. The Arrhenius plot of log V vs. $1/T$ shows straight lines with an inflection point at 24°C, which disappears in the presence of detergent. Calorimetric studies of the plasma membranes show a transition point at the same temperature. From these findings we suppose that Mg^{2+} is bound at a regulatory site of the ATPase, which is influenced by the surrounding phospholipids.

Introduction

Several biological processes, e.g., development and ageing, are accompanied by changes of membrane structure and composition. Such alterations often influence the activity and properties of membrane-bound enzymes. Hegner and Platt [1], for instance, found, that the phospholipid content of rat liver decreases with ageing and also that the fatty acid composition is altered, with the consequence of changed allosteric properties of the $(Na^+ + K^+)$-ATPase.

Such membrane alterations, which occur in connection with biological processes, can be manipulated using microorganisms. Thus, one is able to induce defined changes and observe their influence on some membrane functions, such as enzymatic or transport processes. One strategy is to use fatty acid or sterol auxotrophic mutants [2,3], another is to use different growth conditions (temperature, supplementation of the growth medium with precursors of membrane components, harvesting in different growth phases, aerobic or anaerobic conditions, etc. [4,5]). Using a fatty acid auxotrophic mutant of *Escherichia coli*, Siñeriz et al. [6] showed, that the allosteric properties of the Ca^{2+}-ATPase was regulated by the membrane fluidity. By introducing several fluorescent substances into the plasma membrane, Overath et al. [7] found interdependences between the fluidity of the membrane and the transport of sugar. Huang et al. [8] furthermore showed that the fluidity of the membrane from *Acholeplasma laidlawaii* remained constant although the temperature was varied. In response to the alteration of the temperature, different fatty acids must have been synthesized by the microorganisms. Okuyama et al. [9] stated that the possibility of varying the chain length and the degree of unsaturation is utilized in the regulation of the fluid nature of the membrane lipids. They found that the yeast cells first produce shorter fatty acids, which are converted afterwards in part to unsaturated fatty acids, in response to variation in environmental temperature.

Abbreviation: Tricine, *N*-Tris(hydroxymethyl)methylglycine.

As functional consequence of changes in membrane structure, often just the activity of some membrane-bound enzymes under standard conditions are reported. According to the Michealis-Menten-theory, the enzyme activity is determined by several kinetic parameters, such as K_m, V, etc. Changes in the activity therefore are the result of an influence on one or more of these constants. A more sensitive insight into protein-lipid interaction could result from the investigation of the influence of the membrane alterations on the kinetic constants and on the mechanism of substrate binding and splitting at the active site.

As the most simple possibility of changing the membrane fluidity is to perform measurements at different temperatures, we investigated the reaction mechanism between the plasma membrane ATPase from *Saccharomyces cerevisiae*, Mg · ATP and free Mg^{2+} in a temperature range from 10–30°C; i.e., at each temperature, we performed [Mg · ATP] variations at several concentrations of Mg^{2+}.

Materials and Methods

Reagents. All reagents were analytical reagent grade. ATP was a product of a Boehringer (Mannheim, F.R.G.), Tris, Tricine and ascorbic acid were from Serva (Heidelberg, F.R.G.). Snail enzyme containing per ml at least 100 000 units β-glucoronidase (Fishman) and 1 000 000 units sulfatase (Roy) was from Industrie Biologique Francaise (Gennevilliers, France). Zwittergent TM 314 was purchased from Calbiochem (Lahn, F.R.G.). All other reagents were from Merck (Darmstadt, F.R.G.).

Enzyme preparation. Plasma membrane from the yeast *S. cerevisiae* (strain R XII, a kind gift from Dr. Kotyk, Prague) were obtained from protoplasts by the procedure described recently [10]. Purity of the preparation was checked by means of the plasma membrane enzymes oligomycin-insensitive ATPase and chitin-synthase, as well as by electron microscopy. Possible mitochondrial contaminations could be detected by cytochrome *c* oxidase activity.

Enzyme assays. The ATPase activity was determined by continuously recording the amount of inorganic phosphate released, as described recently [11,12]. The incubation time was 10 min. The continuous recording of the product formed during the reaction enabled us to achieve always steady-state conditions; i.e., the conversion of substrate was always less than 5–10%. The standard assay contained 0.5 mM $MgCl_2$, 0.5 mM ATP, 100 mM Tricine, pH 7.5, 80 mM KCl and 10–40 μg protein in a total volume of 5 ml. To perform variations of [Mg · ATP] at various concentrations of free Mg^{2+}, the total concentrations of ATP and $MgCl_2$ were calculated as described by Ahlers et al. [10]. The temperature dependence of the dissociation constant of the Mg · ATP complex was obtained from data given by Sillén and Martell [13].

The derivation of the reaction mechanism and of the rate equation was performed on the basis on a rapid equilibrium reaction as described recently [10,11]. The kinetic constants were calculated from the reciprocal plots by means of the direct linear plot [14] or from a computer program using the least-squares fit to the Michaelis-Menten equation [15] assuming a constant relative error [10]. All values used for kinetic characterization of plasma membrane ATPase are averages of four or more experiments performed in duplicate. To test the influence of temperature on V, substrate variations at an optimal concentration of 0.8 mM Mg^{2+} have been performed. To examine the influence of detergents on the temperature dependence of V, the plasma membrane was preincubated for 20 min at room temperature with 0.3 mg/ml Zwittergent.

Differential scanning calorimetry (DSC). 50 μl plasma membrane suspension in water (corresponding to 1 mg protein) was placed into DSC sample pans and sealed using a Perkin-Elmer sealing press. Calorimetric analyses were carried out using a Perkin-Elmer DSC-2C differential scanning calorimeter at heating rates of 5°C/min.

Results and Discussion

In order to find out whether the temperature has an influence on kinetic properties of the plasma membrane ATPase from yeast, we examined the mechanism of the reaction between enzyme, substrate and Mg^{2+} in a temperature range from 10 to 30°C. At each temperature, we performed substrate variations at several concentrations of free Mg^{2+} (0.01–10 mM). The results were plotted as $v = f(-\log Mg^{2+})$ for several substrate concentrations. In

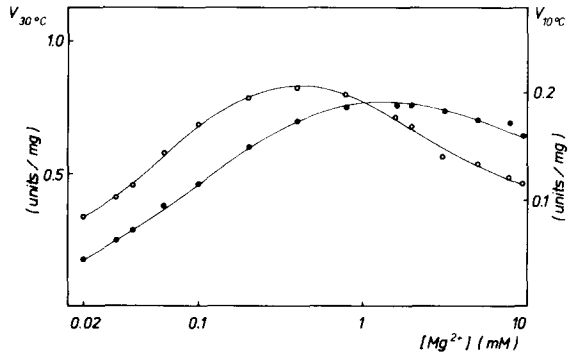


Fig. 1. Semilog plot of v vs. $[Mg^{2+}]$ for constant [Mg · ATP] at 30°C (○) and at 10°C (●). [Mg · ATP] = 160 μM, pH 7.5. Several such curves at different temperatures and substrate concentrations were the basis for the constants compiled in Table I.

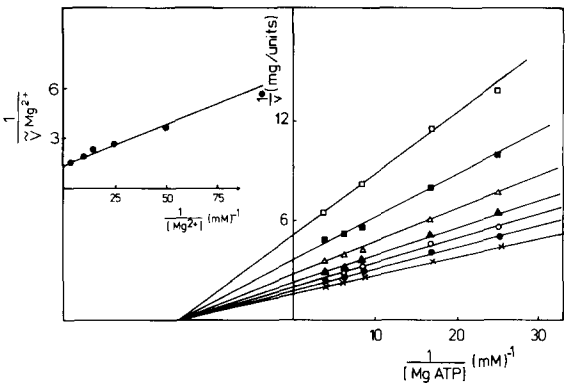


Fig. 2. $1/v$ vs. $1/[Mg \cdot ATP]$ plotted for various concentrations of Mg^{2+}. Experiments were done at 30°C, pH 7.5. $[Mg^{2+}]$ = (□) 10 μM; (■) 20 μM; (△) 40 μM; (▲) 63 μM; (○) 100 μM; (●) 158 μM; (X) 250 μM. Inset: Secondary plot of $1/\tilde{V}$ vs. $1/[Mg^{2+}]$. The values were taken from the intercepts of the straight lines of Fig. 2 with the ordinate.

all cases, we found that Mg^{2+} activates at low concentrations, whereas at higher concentrations, an inhibition takes place. As an example of these results in Fig. 1, the $[Mg^{2+}]$ variations obtained with 160 μM Mg · ATP are compared between 10 and 30°C. It can be seen that the temperature has an influence on the optimal concentration of Mg^{2+}. At the lower temperature, a 5-fold concentration of Mg^{2+} has to be employed to achieve optimal conditions. This shift of the Mg^{2+} optimum is of course the consequence of an alteration in the kinetic constants.

For further characterization, we examined the results of the activation range and those of the range of inhibition separately. At low concentration of Mg^{2+}, we obtained in the reciprocal plots (Fig. 2) straight lines with a common point of intersection on the abscissa at 10°C as well as at 30°C. This common point of intersection represents $-1/K_s$. To evaluate the other kinetic constants, a secondary plot of $1/\tilde{V}^{Mg^{2+}}$ vs. $1/[Mg^{2+}]$ was plotted (inset in Fig. 2). From the ordinate value of the straight line obtained, we get V and from its intercept with the abscissa K_a, the dissociation constant of the enzyme-activator complex. The kinetic constants obtained at 10, 20 and 30°C are compiled in Table I. The results indicate a random mechanism of reaction of substrate and activator with the enzyme, binding of substrate and Mg^{2+} occurring independently of each other [10] in the whole range of temperature.

Thus, the activation mechanism is the same as has been described recently [10]. Furthermore, the kinetic constants measured at 30°C are equal within

TABLE I

INFLUENCE OF TEMPERATURE ON THE KINETIC CONSTANTS OF ACTIVATION AND INHIBITION BY Mg^{2+}

The kinetic constants are derived according to Scheme II. The values are obtained from reciprocal and secondary plots of initial rate data. They are averages of at least four seperate determinations performed in duplicate (±S.D.)

T (°C)	Range of activation			Range of inhibition			
	$K_a = K_a'$ (mM)	$K_s = K_s'$ (mM)	V (units/mg)	K_s' (mM)	K_s'' (mM)	K_i (mM)	K_i' (mM)
30	0.04 ± 0.02	0.07 ± 0.02	1.9 ± 0.25	0.09 ± 0.01	0.56 ± 0.08	0.40 ± 0.2	1.8 ± 0.8
20	0.07 ± 0.03	0.06 ± 0.02	0.70 ± 0.15	0.10 ± 0.01	0.49 ± 0.09	1.4 ± 0.7	5.0 ± 1.5
10	0.11 ± 0.05	0.09 ± 0.04	0.20 ± 0.03	0.12 ± 0.03	0.46 ± 0.08	2.5 ± 1.1	7.6 ± 2.2

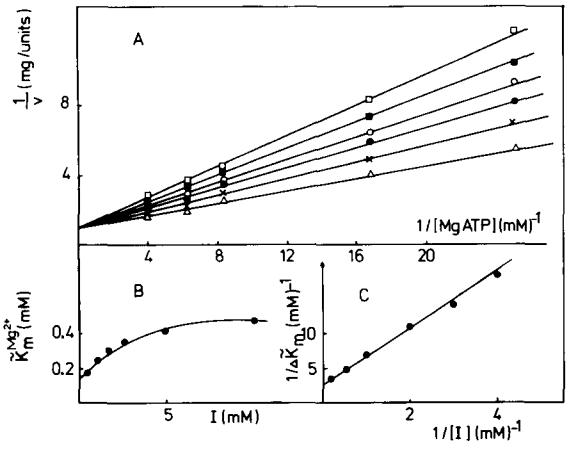
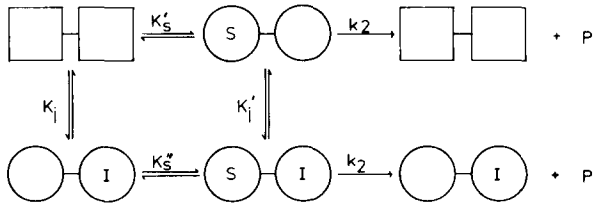


Fig. 3. (A) $1/v$ vs. $1/[Mg \cdot ATP]$ plotted for various concentrations of Mg^{2+}. Experiments were done at 30°C, pH 7.5 $[Mg^{2+}]$ = (□) 10 mM; (■) 5 mM; (○) 2.5 mM; (●) 1.6 mM; (×) 0.8 mM; (△) 0.4 mM. (B) Secondary plot of $\tilde{K}_m^{Mg^{2+}}$ vs. [I]. The values were taken from the intercepts of the straight lines of (A) with the abscissa. (C) Plot of $1/\Delta K_m$ vs. $1/[I]$. The values were taken from (B).

the limits of error with those presented in Ref. 10.

As could be seen in Fig. 1, high concentrations of free Mg^{2+} lead to an inhibition. From the results of this range again double-reciprocal plots were drawn (Fig. 3A). We obtained straight lines with a common point of intersection on the ordinate, as has been described recently [10]. However, extending the inhibitory range to 10 mM Mg^{2+}, we observed a curved line in the secondary plot of $\tilde{K}_m^{Mg^{2+}}$ vs. $[Mg^{2+}]$ (Fig. 3B). This indicates that the mechanism of inhibition cannot be competitive as has been discussed before [10], but must be more complex. A linearization could be achieved by plotting $1/\Delta K_m$ as a function of $1/[Mg^{2+}]$ (Fig. 3C). ΔK_m is the difference between the extrapolated K_m value in the absence of the inhibitory ions and that in the presence of inhibitor. These results are in agreement



Scheme I.

with the mechanism presented in Scheme I, called pseudocompetitive inhibition [15].

One sees, that inhibitor and substrate do not compete for the same site, but are bound at different sites, which interact via conformational changes. Binding of the inhibitor reduces the affinity of the enzyme for the substrate and vice versa. ES and ESI complexes are split with equal velocity [15]. The kinetic constants K_s'' and K_i' (for explanation see Scheme II) can be derived from the slope and ordinate value of the straight line of Fig. 3C. As under the assumption of rapid equilibrium the Michealis constant is identical with the dissociation constant of the ES complex, we obtain K_s' (see Scheme II) from Fig. 3B for $[I] = 0$. From thermodynamic reasons (principle of microscopic reversibility [17]), K_i can be calculated from:

$$K_i = \frac{K_s' \cdot K_i'}{K_s''}$$

Scheme II shows the complete mechanism of the reaction of E, S and Mg^{2+} and explains the meaning of the kinetic constants. It can be seen, that the enzyme possesses two independent binding sites for Mg^{2+}; an activatory and an inhibitory one.

$$\begin{array}{ccccc} & & K_s & & \\ E + S & \rightleftharpoons & ES & \not\rightarrow & \\ + & & + & & \\ Mg^{2+} & & Mg^{2+} & & \\ K_a \uparrow\downarrow & & K_s' \uparrow\downarrow & & K_a' \; k_2 \\ EMg + S & \rightleftharpoons & EMg\,S & \longrightarrow & EMg + P \\ + & & + & & \\ Mg^{2+} & & Mg^{2+} & & \\ K_i \uparrow\downarrow & & K_s'' \uparrow\downarrow & & K_i' \; k_2 \\ EMg_2 + S & \rightleftharpoons & EMg_2S & \longrightarrow & EMg_2 + P \end{array}$$

Scheme II.

In analogy to a recent discussion [10,11] and on the basis of a rapid equilibrium reaction mechanism the following rate equation was calculated for scheme II:

$$v = V \cdot [S] \Big/ \left[K_m' \left(1 + \frac{K_a}{[Mg^{2+}]}\right) \left(\frac{1 + \dfrac{[Mg^{2+}]}{K_i}}{1 + \dfrac{[Mg^{2+}]}{K_i'}} \right) + [S] \left(1 + \frac{K_m' \cdot K_a}{K_s \cdot [Mg^{2+}]}\right) \right] \quad (1)$$

At low concentrations of Mg^{2+}, Eqn. 1 simplifies to:

$$v = \frac{V \cdot [S]}{K'_m\left(1 + \frac{K_a}{[Mg^{2+}]}\right) + [S] \cdot \left(1 + \frac{K'_m \cdot K_a}{K_s[Mg^{2+}]}\right)} \quad (2)$$

representing the results of Fig. 2.

At high Mg^{2+} concentrations, Eqn. 1 simplifies to:

$$v = \frac{V \cdot [S]}{K'_m\left(\frac{1 + \frac{[Mg^{2+}]}{K_i}}{1 + \frac{[Mg^{2+}]}{K'_i}}\right) + [S]} \quad (3)$$

which is in accordance with the results shown in Fig. 3 (pseudocompetitive inhibition). At low but already inhibitory concentrations (about 0.1–1 mM), eqn. 3 further simplifies to an equation of a pure competitive inhibition. Scheme II and the reaction rate equation remain unchanged when temperature is varied from 10 to 30°C. However, some of the kinetic constants, compiled in Table I are temperature dependent. We recognize that the temperature has no significant influence on substrate binding (K_s, K'_s, K''_s), however K_a and especially K_i decreases with increasing temperature mainly between 20 and 30°C, whereas V rises. As can be deduced from Scheme II, these constants are dissociation constants of the enzyme-activator and the enzyme-inhibitor complexes. Thus from Table I we see that the temperature has an influence on binding of Mg^{2+} mainly in the inhibitory, but also in the activating ranges. At low temperatures, the affinity of the enzyme for the Mg^{2+} is reduced.

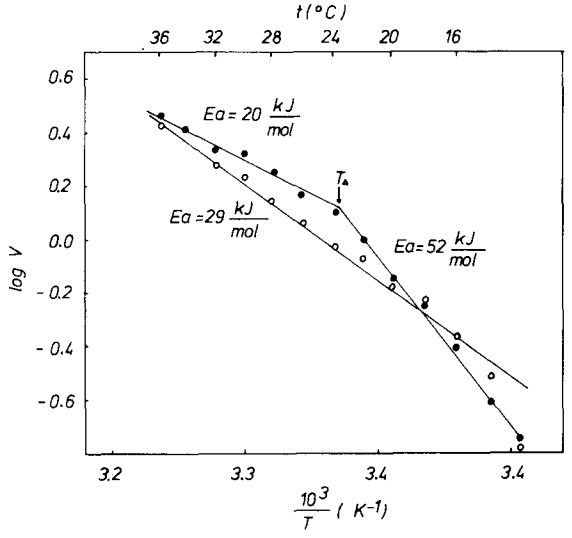


Fig. 4. Arrhenius plots for plasma membrane ATPase in the presence (○) and absence (●) of 0.3 mg/ml Zwittergent TM 314. $[Mg^{2+}] = 0.8$ mM; pH 7.5. Measurements and derivation of V have been performed as described in the text. They are averages of four separate determinations performed in duplicate. S.D. of V = 10%. T_Δ, temperature of discontinuity.

From Fig. 2, we can see that K_s is not influenced by the concentration of Mg^{2+}. As we did not detect any residual activity, Mg^{2+} must be essential for substrate splitting. Therefore, the examination of the temperature influence on V seems to be a promising possibility for obtaining more information concerning this phenomena.

Fig. 4 (closed circles) shows the Arrhenius plot, log V vs. $1/T$, for the plasma membrane ATPase from *S. cerevisiae*. We obtained two straight lines with an inflection point at 24°C. Above this temperature, the energy of activation is 20 kJ/mol and below, it is 52 kJ/mol. Such abrupt changes in the energy of activation at a certain temperature without abrupt changes in the maximum velocity can be either explained by widely different temperature dependencies of individual rate constants, or by conformational changes of the protein, which might be induced by a phase change in the lipid adjacent to the enzyme.

As pointed out by Londesborough [18] any change in the activation energy causes a relative large change in V unless it is compensated by a change in ΔS. He furthermore showed that such enthalpy/entropy compensations are quite likely as a response to a 'melting' of the surrounding lipids. If the inflection of the straight lines in the Arrhenius plot is due to phase changes of the lipids surrounding the protein molecule, one should expect an influence of detergents. Therefore, we repeated the measurement with plasma membranes which had been pretreated with 0.3 mg/ml Zwittergent for 20 min at room temperature. 1.5 mg/ml Zwittergent was used by Malpartida and Serrano [19] to solubilize the plasma membrane ATPase from *S. cerevisiae*. We chose a detergent concentration which was well below that value to avoid

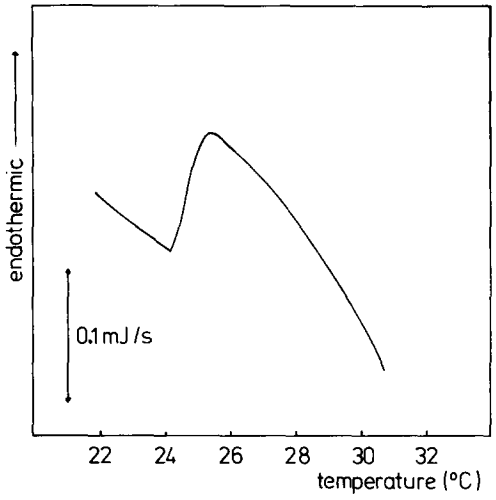


Fig. 5. Transition curve of yeast plasma membrane. Heating rate used is 5°C/min. The sample contained plasma membranes corresponding to 1 mg protein.

irreversible denaturation. From Fig. 4 (open circles) it can be seen that the inflection point disappears in the presence of the detergent. Under these conditions, the energy over almost the entire range of temperature is 29 kJ/mol, which is similar to the value obtained above 24°C in the absence of the detergent. This result supports, the view that the bend in the curves is due to the lipid surrounding of the enzyme.

To obtain more information as to whether the abrupt change of the activation energy at 24°C is accompanied by a transition of the membrane at the same temperature, calorimetric investigations of the plasma membrane from yeast have been performed. From Fig. 5 it can be seen that in the range 20–30°C, an endothermic transition occurs, with a peak temperature of 25 ± 2°C. This agrees quite well with the inflection point of 24°C observed in the Arrhenius plot of log V vs. $1/T$ (Fig. 4).

Thus, we suppose that by 'freezing' the plasma membrane, or at least of clusters around the ATPase molecule, below about 25°C, a conformation of the enzyme is induced, in which a regulatory site is altered in such a way that it is less able to bind Mg^{2+}. As Mg^{2+} is not necessary for substrate binding (Fig. 2 and Scheme II), there is no thermal effect on K_m but only on V. This hypothesis can be confirmed by the fact that the kinetic investigation of another function of the same membrane, the purine transport system, presents bends in the Arrhenius plot of log V vs. $1/T$ at 25°C as well [20]. These results indicate that the ATPase and possibly other membrane functions can be regulated by the composition of the plasma membrane as has been proposed for the (Mg^{2+} + Na^+)-ATPase of *A. laidlawii* [21]. To obtain more information about such structure-function relationships, examinations are now in progress which attempt to alter the composition of the plasma membrane by growing the yeast under varying conditions or by using fatty-acid-auxotrophic mutants and performing the kinetic analysis described above.

Acknowledgements

I thank Mrs. Eva Nehls for technical assistance. This work was supported by a research grant of the Deutsche Forschungsgemeinschaft.

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