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Journal of Molecular Catalysis B: Enzymatic 10 (2000) 555–563

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Enzymatic activity of an extremely halophilic phosphatase from the Archaea *Halobacterium salinarum* in reversed micelles

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Received 10 June 1999; accepted 14 January 2000

Abstract

Alkaline *p*-nitrophenylphosphate phosphatase (*p*NPPase) from the halophilic archaeon *Halobacterium salinarum* (previously *halobium*) was solubilized in reversed micelles of cetyltrimethylammonium bromide (CTAB) in cyclohexane with 1-butanol as cosurfactant. The hydrolysis reaction appears to follow Michaelis–Menten kinetics. The dependency of the maximum reaction rate (V_{max}) on the water content θ (% v/v) (or ω_0 value: molar ratio of water to surfactant concentrations) showed a bell-shaped curve for 0.3 M CTAB, but not for 0.2 M CTAB. The enzyme activity increased with the surfactant concentration at a constant ω_0 value (10.27). When the surfactant concentration was increased at a constant θ, the enzyme activity decreased. The enzyme was more stable in reversed micelles than in aqueous media.

Keywords: Archaea; Halophilic enzyme; Alkaline phosphatase; CTAB; Reversed micelles

1. Introduction

Alkaline *p*-nitrophenylphosphate phosphatase (*p*NPPase) from the halophilic archaeon *Halobacterium salinarum* (previously *halobium*) was encapsulated in the reversed micelles system. The phenomenon of enzyme catalysis in non-polar organic media using reversed micelles is currently the focus of intense research [1–4]. Reversed micelles consist of three phases: amphiphilic molecules, water, and non-polar organic solvent. The polar heads of the surfactant molecules are directed toward the interior of a water-containing sphere, while the aliphatic tails of the surfactant are oriented toward the non-polar organic phase. It is believed that this system reliably resembles the microenvironment that enzymes find in the cells, because the properties of the water in the reversed micelles resemble the properties of water closely associated with the cell [5–7].

Enzymes solubilised in reversed micelles have been proposed to fall into two categories: those which interact with the micellar membrane and those which are solubilised within the aqueous phase and are not associated with the micellar matrix [5]. In order to study the nature of this interaction, the dependence of enzymatic activity on micellar concentration (surfactant concentration at constant surfactant-to-water ratio) has

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1381-1177/00/$ - see front matter
PII: S1381-1177(00)00009-6

been studied. Membrane active enzymes typically show decreasing catalytic activity as the micellar concentration increases, while no changes are evident for enzymes which do not interact with membranes. However, the mechanism responsible for this behaviour is not well understood [3].

Several groups of researchers have been investigating the hydrolysis of *p*-nitrophenylphosphate (*p*NPP) catalysed by different alkaline phosphatases in reversed micellar systems [8–12]. *p*NPP is generally considered as the standard substrate for alkaline phosphatase (EC 3.1.3.1) activity [13]. *H. salinarum p*NPP phosphatase (*p*NPPase) had a typical halophilic behaviour [14], requiring high concentrations of salt for maximal activity (more than 0.6 M KCl or 1.2 M NaCl). This *p*NPPase shows an absolute requirement of high ionic strength for stability. With very few exceptions, the halophilic enzymes require high salt concentration to remain stable. At low salt concentrations (<2 M NaCl), they denature. This property of halophilic enzymes was a problem in the reversed micelles system because the high ionic strength makes the formation of reversed micelles very difficult. For these reasons, it was necessary to adjust the conditions of the system for this *p*NPPase from *H. salinarum*. Reversed micelles were formed using the cationic surfactant, cetyltrimethylammonium bromide (CTAB); 1-butanol as cosurfactant; and cyclohexane as organic solvent.

The main objective of this work was to study the influence of the system parameters on the enzymatic activity of *p*NPPase from *H. salinarum* in organic medium with CTAB/1-butanol/cyclohexane reversed micelles system.

2. Materials and methods

2.1. Materials

p-Nitrophenylphoshate (disodium salt) was supplied by Boehringer-Mannheim. All salts employed were of analytical grade. Cations were used as chlorides. CTAB was obtained from Fluka and organic solvents, cyclohexane and 1-butanol, were purchased from SDS (analytical grade).

2.2. Purification and enzymatic activity of pNPPase from H. salinarum

*p*NPPase from *H. salinarum* (a colourless mutant NRC 36014) was purified 440-fold according to Ref. [14]; the final preparation was not completely homogeneous by electrophoretic criteria, but only minor contaminants were present. The *p*NPPase activity was dialysed for 24 h at 4°C against 200 vol of 100 mM Tris–HCl buffer pH 8.5, 4 M NaCl, 20 mM 2-mercaptoethanol, and 5 mM $MnCl_2$. Protein concentration was determined by the Bradford [15] method. *p*NPPase was assayed according to Ref. [14]. An activity unit (U) was defined as the amount of enzyme (g) that catalysed the formation of 1 nmol of *p*-nitrophenol (*p*NP) per minute under the conditions of standard assay. The molar absorption coefficient (ε) of *p*NP at 400 nm was taken as 18,300 M^{-1} cm^{-1}.

2.3. Preparation of reversed micelles containing pNPPase from H. salinarum

*p*NPPase from purified *H. salinarum* was concentrated by centrifugation at $3000 \times g$ using Centricon 10 (Amicon, Danvers, MA) ultrafiltration membranes with a molecular weight cut-off of 10,000. Reversed micellar solutions were prepared by injecting controlled amounts of both buffer without NaCl (100 mM Tris–HCl buffer pH 8.5, 20 mM 2-mercaptoethanol, and 5 mM $MnCl_2$) and buffered enzyme solution into the desired mixture of cyclohexane/1-butanol (cosurfactant concentration was 1 M) containing the appropriate concentration of CTAB. The final buffer concentration in the reversed micelles was 100 mM Tris–HCl buffer pH 8.5, 0.85 M NaCl, 20 mM 2-mercaptoethanol, and 10 mM $MnCl_2$ (Buffer A). The ω_0 value of a

reversed micellar system is defined as the molar ratio of water to CTAB. This value was adjusted by adding a suitable amount of buffer (0.85 m NaCl). The θ value of a reversed micellar system is defined as the percentage of water determined by Karl–Fisher (METTLER DL18).

2.4. pNPPase from H. salinarum activity assay in reversed micelles

The reaction was started by mixing 1 ml of reversed micelles of *p*NPPase from *H. salinarum* with 1 ml reversed micelles formed with different *p*NPP concentrations ranging from 5.33 to 106.7 dissolved in 100 mM Tris–HCl pH 8.5, 0.85 M NaCl, 20 mM 2-mercaptoethanol, and 10 mM $MnCl_2$. Reversed micelles from the enzyme and the *p*NPP had the same value of ω_0. The rate of hydrolysis of *p*NPP was followed at 40°C by monitoring the increase in absorbance at 400 nm in the Shimadzu UV-160 spectrophotometer.

The molar extinction coefficient (ε) of *p*NPP, in reversed micelles, did not depend on ω_0 or [CTAB]; however, it depended on the temperature. Its values were 12.8×10^3, 12.2×10^3, 11.8×10^3, 11.3×10^3, 10.7×10^3 and 10.1×10^3 M^{-1} cm^{-1} at 10°C, 20°C, 30°C, 40°C, 50°C and 60°C, respectively. The ε at 40°C in CTAB reversed micelles was 11.3×10^3 M^{-1} cm^{-1}. All kinetic measurements were carried out three times.

2.5. Determination of enzyme kinetic parameters

The kinetic results were processed using the Michaelis–Menten equation: $v = V_{max}[S]/(K_m + [S])$. The values of V_{max} and K_m were determined by non-linear regression analysis of the corresponding Michaelis–Menten curves (v vs. [*p*NPP]) using the algorithm of Marquart–Lvenberg with the SigmaPlot program (Jandel Scientific, v. 1.02). The $[S]$, V_{max}, and K_m values were referred to the aqueous solution volume of the system because the hydrolysis of *p*NPP took place in this phase [16].

3. Results and discussion

3.1. Effect of protein concentration

The effect of the enzyme concentration on the reaction rate was studied in reversed micelles at different CTAB concentrations (0.1, 0.2, and 0.3 M CTAB), as it can be seen in Fig. 1. A linear dependence was observed in all cases, confirming an enzymatically controlled reaction and no mass transfer limitation on *p*NPPase activity in reversed micelles.

3.2. Effect of water content on the enzyme-catalysed reaction

The dependence of activity of *p*NPPase from *H. salinarum* on the water content and the molar ratio of water to surfactant concentration is shown in Fig. 2 at constant enzyme concentration for two CTAB concentrations (0.2 and 0.3 M). The value of V_{max} increased with θ (or ω_0) at 0.2 M CTAB (Fig. 2A). At this CTAB concentration, it was impossible to form reversed micelles above values of 7.4% v/v and

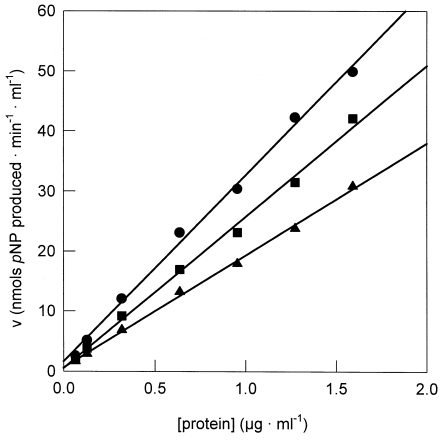

Fig. 1. Initial velocity of hydrolysis of *p*NPP in reversed micelles [0.1 M (▲), 0.2 M (■), and 0.3 M CTAB (●)] as a funtion of *p*NPPase concentration.

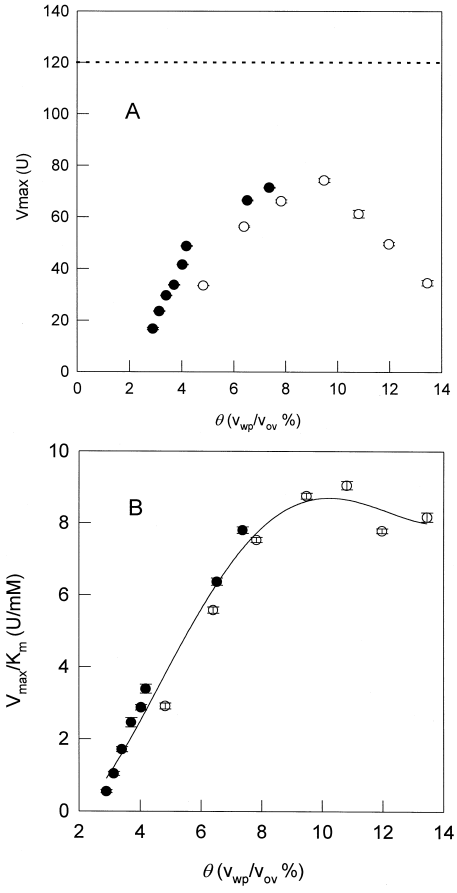

Fig. 2. (A) Dependence of the maximum activity of *p*NPPase of *H. salinarum* (V_{max}) in reversed micelles (●, 0.2 M CTAB; ○, 0.3 M CTAB) vs. θ (percentage of water, v/v). (B) Dependence of the V_{max}/K_m of *p*NPPase of *H. salinarum* in reversed micelles (●, 0.2 M CTAB; ○ 0.3 M CTAB) vs. θ (percentage of water, v/v). The straight line represents the value of V_{max} in aqueous buffer.

20.5 for θ and ω_0, respectively, because the reversed micellar system was not thermodynamically stable. At 0.3 M CTAB, the value of V_{max} showed a bell-shaped activity profile (Fig. 2A) with an optimum θ value around 9.5% v/v (or ω_0 value around 16.65). The value of K_m decreased with θ value while V_{max}/K_m increased (Fig. 2). The decreasing of specific constant (V_{max}/K_m) and increasing of K_m in reversed micelles implied a weakened binding between enzyme and substrate. This change of binding affinity could be due to interactions between enzyme molecule and surfactant since by decreasing the θ value (or ω_0 value), the micellar dimensions decrease, but the number of micelles is constant and the possibility of contact between enzyme and the surfactant increases [9].

A bell-shaped curve of enzyme activity as a function of θ (or ω_0 value) has been observed for many enzymes in reversed micellar systems with different types of surfactants, and it is, in fact, a quite general feature of reversed micellar enzymology [1]. The reason for this dependency has not been made fully clear yet, but numerous factors have been suggested to be the cause [16].

When the CTAB concentration was increased, the ω_0 value dependency on the maximum *p*NPPase activity changed; $\omega_0 = 20.5$ (or higher) for 0.2 M CTAB and $\omega_0 = 16.65$ for 0.3 M CTAB (Fig. 2A). The mentioned experimental results may suggest that the maximum enzyme activities, at different ω_0 values, were due to CTAB concentration and θ value, and not only to the ω_0 value. It was found that the average micellar diameter depended on AOT concentration for ω_0 values between 5 and 25 [17]. They also concluded that the reaction rates catalysed by enzymes solubilised in reversed micelles depended on both the water and surfactant concentrations, and not on the degree of hydration or micellar diameter [17]. Later, Chen and Chang [18] found similar results with the lipase-catalysed hydrolysis of milk fat in reversed micelles formed by soybean lecithin in isooctane.

When θ value (or ω_0 value) increased, the size of aqueous core also increased together with free water (water not linked at the polar head of the surfactant) [17]. The microenvironment around the *p*NPPase could be more similar to the aqueous media; when the value of ω_0 was high enough, both kinetic parameters (V_{max}/K_m and K_m) approached those found in the aqueous media. When the θ value was higher than 9.5, the activity of *p*NPPase decreased (Fig. 2A). Probably, this feature could

be due to the inhibition by CTAB. When the θ value (or ω_0 value) was increased, the stability of the reversed micellar system decreased and the possibility of contact between enzyme and surfactant (and subsequently the organic solvent) increased.

These previous results were obtained using a fixed overall enzyme concentration ($[E]_{ov}$) in the reaction medium. Fig. 3 shows the effect of varying the overall enzyme concentration ($[E]_{ov}$ from 0.097 to 0.175 μg/ml) on the *p*NPPase activity. The reason for increasing the $[E]_{ov}$ was to keep the water pool enzyme concentration constant ($[E]_{wp} = 1.6$ μg/ml) when the ω_0 values were increased with 0.3 M CTAB/1 M 1-butanol/cyclohexane as reversed micellar system. When $[E]_{wp}$ was kept constant at 1.6 μg/ml, the $V_{max,wp}$($V_{max}/[E]_{wp}$) increased hyperbolically with increasing θ values (Fig. 3A, curve a). The enzymatic activity showed the typical bell-shaped curve when the $V_{max,ov}$-($V_{max}/[E]_{ov}$) was considered; the value of ω_0 of maximal activity did not change: $\omega_0 = 16.65$ (Fig. 3B, curve b). Above $\theta \approx 10$, when the reversed micelles were formed, there was foam in the medium implying that the enzyme denatured. In this experience, the units for V_{max} were nanomoles of *p*NP formed per minute per milliliter.

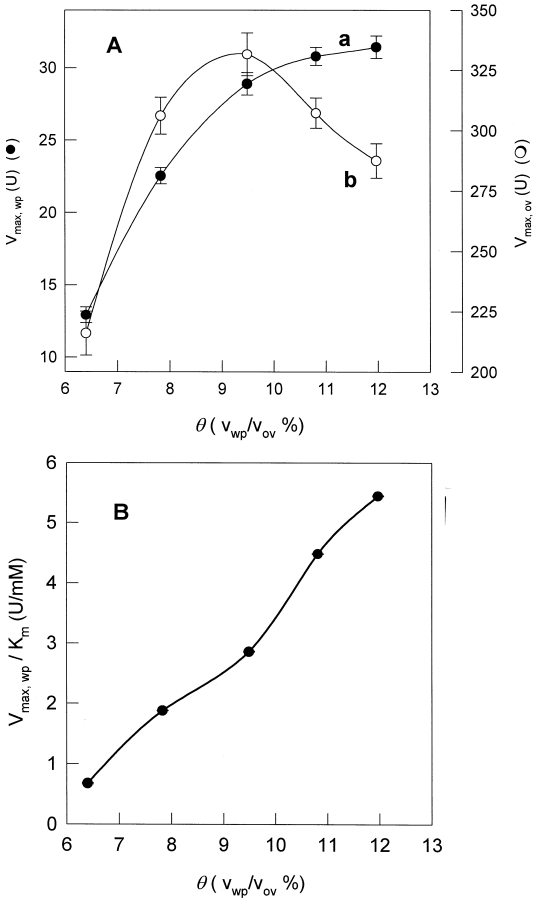

Fig. 3. (A) Effect of the water content on the maximum activity of *p*NPPase from *H. salinarum* (V_{max}) measured at constant enzyme concentration in the water pool ($[E]_{wp} = 1.6$ μg/ml) in reversed micelles (0.3 M CTAB/1 M 1-butanol/cyclohexane) at all ω_0 values. (●) $V_{max,wp}$ (U); (○) $V_{max,ov}$ (U). (B) Dependence of the $V_{max,wp}/K_m$ of *p*NPPase of *H. salinarum* in reversed micelles vs. θ (percentage of water, v/v).

The behaviour shown in Fig. 3A was not expected in a bulk aqueous reaction medium with a water-soluble enzyme acting on a water-soluble substrate. When using reversed micelles, the emphasis is placed on the homogeneous nature of the microenvironment of each molecule as reversed micelles have an enzyme excess, and they can, thus, accommodate all enzyme molecules in an equivalent microenvironment [21]. The reduction in specific activity may well be related to the distribution of reversed micelles, or a partial denaturalisation would be caused by contact between the enzyme and the surfactant or organic solvent [19,20]. It is also possible that, at higher enzyme concentration, enzyme aggregates may form, limiting the specific activity [21].

3.3. Effect of CTAB concentration

The effect of surfactant concentration on the activity of *p*NPPase from *H. salinarum* was studied at $\omega_0 = 10.27$, at a constant enzyme concentration and varying corresponding surfactant concentrations from 0.05 to 0.3 M CTAB (obviously, the water content (θ) was also increased from 0.93% to 5.55% v/v). The results are shown in Fig. 4. When water and surfactant are modified simultaneously so that ω_0 remains

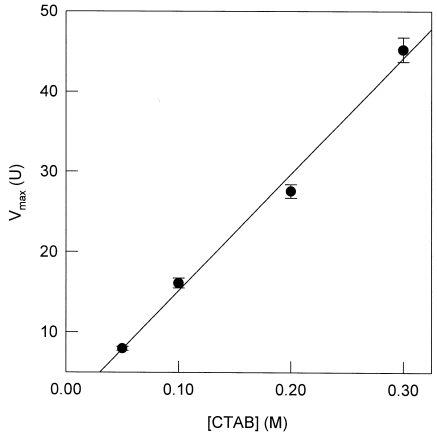

Fig. 4. Effect of the surfactant concentration on V_{max} of the activity of *p*NPPase from *H. salinarum* in the reversed micellar system. The value of ω_0 was constant ($\omega_0 = 10.27$).

constant, reversed micelles change in concentration but not in size. The increase of the micellar population increases the rate of collisions and exchanges of micellar contents could be the cause of the increase of V_{max} when the reversed micellar population is increased (Fig. 4).

On the other hand, the effect of surfactant concentration on *p*NPPase from *H. salinarum* was also studied at $\theta = 5\%$ v/v and at a constant enzyme concentration by varying ω_0 from 13.89 to 6.94 and the corresponding surfactant concentration from 0.2 to 0.4 M CTAB (Fig. 5). When the surfactant concentration increases, the size of the water pool in the reversed micelles decreases, keeping the water content constant and increasing the possibility of contact between the enzyme and the surfactant. This contact could explain the decrease of enzymatic activity when the CTAB concentration was increased (Fig. 5). This behaviour was also found within the human placental alkaline phosphatase in AOT/isooctane [9].

It is generally recognised that by increasing the surfactant concentration (at least for AOT and some other surfactants [22] at a constant value of ω_0), the number of reversed micelles will increase, but their physical properties will be similar since they will have the same aggregation number and size [18]. The Stokes radii of the micelles were found to be independent of AOT concentration when ω_0 was below 20 [23]. Similar results have been reported in an independent study for ω_0 up to 45 [18]. Accordingly, increasing the surfactant concentration should only result in more empty micelles without significantly changing the enzyme activity. This was shown to be true for the water-soluble enzymes, α-chymotrypsin and trypsin [22]. On the other hand, the effect of increasing surfactant concentration of reversed micelles on membrane active enzymes, such as peroxidase, acid phosphatase, γ-glutamyltransferase, and different lipases [24], was, in all cases, a reduction in catalytic activity. It was suggested that the decrease of enzyme activity under such conditions was characteristic of membrane association [24]. The molecular mechanism of this phenomenon has not yet been established.

In aqueous medium, CTAB exhibited non-competitive inhibition of the catalytic hydrolysis of *p*NPP (Fig. 6) with $K_i = 28 \pm 4$ mM. Enzymatic activity was determined according to Ref. [14], with different concentrations of CTAB ranging from 5.0 to 45.9 mM. Different surfactants such as Aerosol-OT, sodium cholate, and CTAB are non-competitive inhibitors in this enzymatic hydrolysis in aqueous medium and

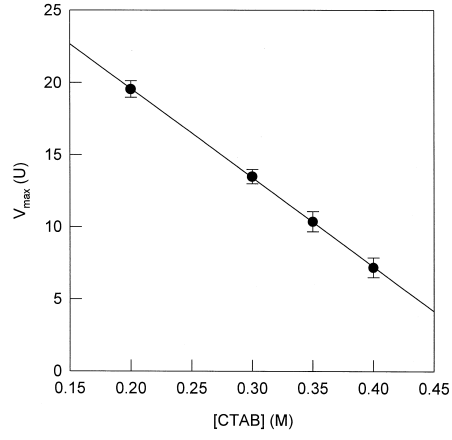

Fig. 5. Effect of the surfactant concentration on V_{max} of the activity of *p*NPPase of *H. salinarum* in the reversed micellar system. The value of θ was constant ($\theta = 5\%$ v/v).

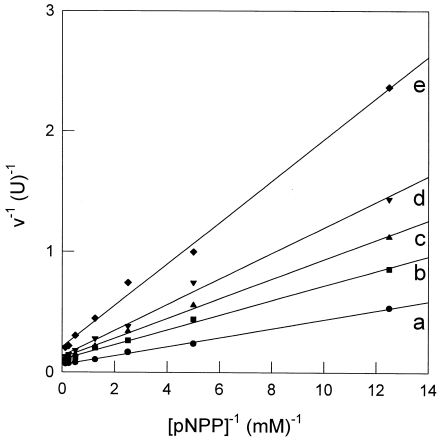

Fig. 6. Lineweaver–Burk plots in presence of CTAB in an aqueous buffer (100 mM Tris–HCl pH 8.5, 0.85 M NaCl, 20 mM 2-mercaptoethanol, and 10 mM $MnCl_2$). (a) 5.0 mM CTAB, (b) 12.5 mM CTAB, (c) 20.9 mM CTAB, and (e) 45.9 mM CTAB.

reversed micelles [11]. If a surfactant in an aqueous medium shows an inhibitory effect on the catalytic process, the results in a reversed micelles system using the same surfactant should show such inhibitory effect. Enzyme kinetics in a reversed micelles system may very often contain elements of inhibition [11] because binding of surfactants to proteins and enzymes is a general physico-chemical feature.

3.4. Effect of cosurfactant concentration

CTAB requires the presence of a cosurfactant in the formation of reversed micelles. The influence upon enzyme activity of the varying concentrations of cosurfactant, 1-butanol, relative to that of the surfactant, for *p*NPP hydrolysis is shown in Fig. 7. This variation was represented as a ratio of concentration of 1-butanol relative to that of CTAB.

It is generally believed that the cosurfactant influences the size of reversed micelles and the fluidity of the interface and, therefore, the structural relationship between enzyme and reversed micelles. Increasing the alcohol concentration in a microemulsion of cationic surfactant leads to an increase in the interface elasticity and micelles dimension [25], which can explain the slight increase in V_{max} found for ratios around 4. However, when the interface elasticity is too high, a decrease in the micelle dimension is observed [25]. This may explain the reduction in V_{max} at higher ratio values. The profiles observed may result from a combination of effects related to micelle structure and influence upon the kinetics of the catalyst.

3.5. Effect of temperature

In aqueous media, *p*NPPase was stable up to 40°C but its activity was completely lost at 47.5°C at 0.85 M NaCl [14]. This contrasts with the marked thermal stability of many halophilic enzymes and many alkaline phosphatases of bacterial and fungal origin. In reversed micelles, V_{max} was maximum around 50°C, for $\omega_0 =$ 12.50 and $\omega_0 = 19.43$ (Fig. 8); and the enzyme was found to be more stable in reversed micelles than in aqueous media.

Solvation is necessary for protein function; however, excessive solvation may lead to the loss of the native protein structure. This is more patent at relatively high temperatures in which many of the weak bonds that maintain the native structure are destabilised and solvated. Therefore, it was rationalised in a low water

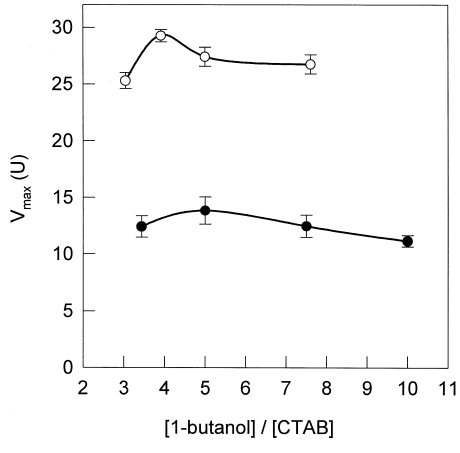

Fig. 7. Effect of the molar ratio [1-butanol]/[CTAB] on the V_{max} of *p*NPPase solubilised in reversed micelles of CTAB (0.2 M)/1-butanol/cyclohexane with $\omega_0 = 7.44$ (●), and $\omega_0 = 10.27$ (○).

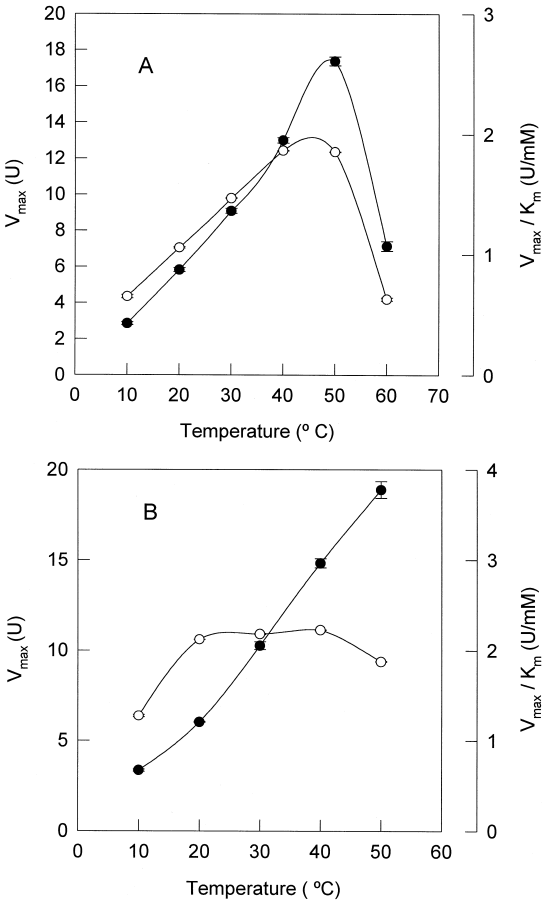

Fig. 8. Effect of temperature on the maximum activity, V_{max} (●) and V_{max}/K_m (○) of *p*NPPase of *H. salinarum* in reversed micelles at $\omega_0 = 12.5$ (A), and $\omega_0 = 19.43$ (B).

environment (reversed micelles) that enzymes should exhibit a high resistance to thermal denaturation. The *p*NPPase dispersed in organic solvent employing the reversed micellar system with low water concentrations was found to exhibit a higher thermostability than in an aqueous medium, as can be observed in a number of different enzymes in reversed micelles [4].

From de Arrhenius plot (log V_{mas} vs. $1/T$), where T is the absolute temperature, it found for the enzyme reaction an activation energy of 39.5 kJ mol^{-1} in aqueous buffer [14], and this value decreased in reversed micelles: 33.7 and 33.2 kJ mol^{-1} for $\omega_0 = 12.50$ and $\omega_0 = 19.43$, respectively. These results imply that, once entrapped into the reversed micelles, the enzyme reaction has lower activation energy irrespective of ω_0.

4. Conclusions

The *p*NPPase was more stable in reversed micelles than in aqueous medium. The *p*NPPase activity in the system of reversed micelles was determined, principally, by three factors: number of micelles, percentage of free water, and the enzymatic inhibition by CTAB. The latter factor is the most complex, since it would determine the CTAB concentration in the water pool ($[CTAB]_{wp}$) and the inhibition of the *p*NPPase activity. The magnitude of this inhibition was determined by the system variables (ω_0, [CTAB], [1-butanol], and temperature).

These results open the possibility of working with halophilic enzymes in organic solvents using apolar substrates, which has been impossible up to the present date.

Acknowledgements

We thank Dr. A.A. Carbonell-Barrachina for the useful discussion. This work was supported by funds from CICYT-BT 87-0033 and EC Generic Project Biotechnology of Extremophiles contract BIO-CT93-02734.

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