ADENYLATE CYCLASE ACTIVITY IN PERMEABILISED YEAST

Kaija VARIMO and John LONDESBOROUGH
Research Laboratories of the State Alcohol Monopoly (Alko), POB 350, SF-00101 Helsinki 10, Finland

Received 15 April 1982

1. Introduction

The plasma membrane of yeast can be disrupted by treatment with basic proteins [1] or organic solvents [2] to give permeabilised cells in which the concentrations of macromolecules are much higher than is possible with kinetic studies in vitro. These in situ preparations [3] are in many ways closer to physiological conditions than broken-cell preparations. We report here a study of adenylate cyclase (AC) in permeabilised *Saccharomyces cerevisiae*. The main catalytic properties of the enzyme were the same as reported in vitro [4,5]; glucose did not specifically inhibit the activity in situ as it does that of *Escherichia coli* AC [6]; and two essential groups with p*K*-values of 5.5 and 6.1 in the free enzyme were found.

2. Materials and methods

Two stages in the commercial production of bakers' yeast at our Rajamäki factories were used, semi-aerobic A3 yeast grown on molasses, and strongly aerobic A5 yeast grown on molasses plus ethanol. Yeast was disintegrated in buffer HM (25 mM Hepes/KOH/0.1 M KCl/0.55 M mannitol/0.3 mM EDTA, pH 7.0) with a Mini Mill [5], centrifuged successively 30 min at 34 000 × *g* and 60 min at 105 000 × *g*, and the 105 000 × *g* pellet suspended in buffer HM and frozen at −20°C. Portions of this 'cell-free particulate fraction' were taken for kinetic studies as required. Yeast was permeabilised at 30°C by shaking at 2 mg fresh wt/ml in one of the following solutions: 1% (v/v) toluene/4% (v/v) ethanol in buffer HM; 0.5 mg protamine/ml water or buffer HM; 0.2 mg cyt. *c*/ml water. After the times indicated, the cells were sedimented at 5°C (10 min at 2000 × *g*), washed, suspended in buffer HM and used at once or after storage overnight.

AC was assayed at 30°C by isolating [5] the c[^{14}C]-AMP formed in reaction mixtures containing 5 mM $MnCl_2$, 1.6 mM $MgCl_2$, 0.15–3.0 mM 8-[^{14}C]ATP (~3000 cpm/nmol), 0.4 mM cAMP, 10 mM phosphoenolpyruvate and 0.25 mg pyruvate kinase/ml buffered with 0.1 M Na acetate/acetic acid (pH < 5.1) 0.1 M MES/KOH (pH 5.2–5.7), 0.1 M Pipes/KOH (pH 5.8–7.0) or 0.1 M Hepes/KOH (pH > 7.0). The pH of spent reaction mixtures was measured at 30°C with a glass electrode. Median values of V_m and V_m/K_m were computed [7] from 5–7 datum points between 0.15 and 3.0 mM ATP. The ranges of the middle thirds of the sets of estimates [7] for V_m and V_m/K_m are shown by error bars in fig.2. K_m was calculated as the ratio of V_m and V_m/K_m. Reagents not described in [5] were of analytical grade.

Abbreviations: AC, adenylate cyclase; cAMP, 3′,5′-cyclic adenosine monophosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid

3. Results

3.1. *General properties of AC in permeabilised yeast*

AC activity was exposed during permeabilisation of yeast (fig.1). Progress curves for AC assays were linear up to a greater percentage conversion of ATP into cAMP with permeabilised than with broken-cell preparations, either because AC is more stable when retained within the yeast cell wall, or possibly because ATPase activity is relatively smaller in permeabilised yeast. AC activity was exposed by toluene/ethanol more slowly in A5 than A3 yeast, and only after a distinct lag, probably because A5 yeast was grown in the presence of ethanol [2]. Treatment with toluene/

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00145793/82/0000–0000/$02.75

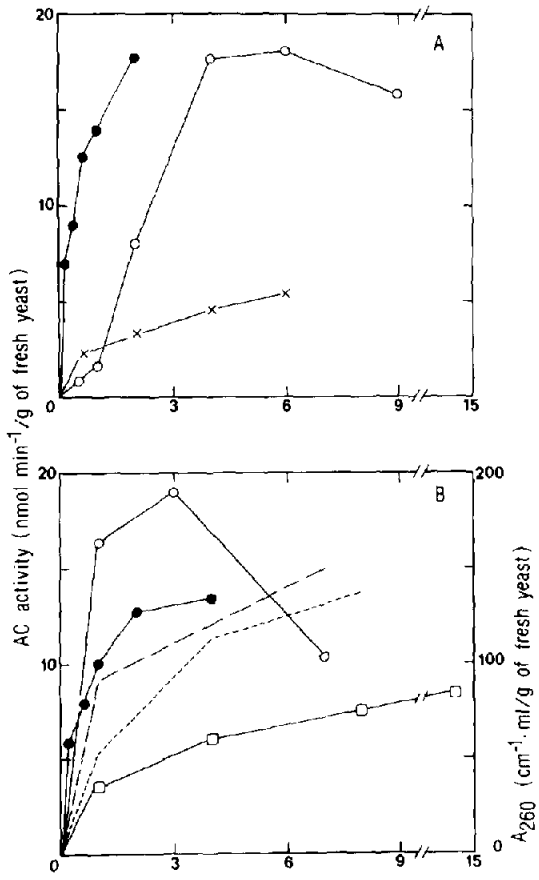


Fig.1. Exposure of AC activity during permeabilisation of yeast. Yeast were permeabilised as in section 2 and their AC activity measured at 2 mM ATP (pH 6.2). The activity in mechanical disintegrates of the yeast used was ~12 nmol . min^{-1} . g fresh $yeast^{-1}$. Results in (A) are for A3 (•) and A5 yeast (○) treated with toluene/ethanol and for A5 yeast treated with cytochrome *c* (×). Results in (B) are for A3 (•) and A5 (○) yeast treated with protamine in water and for A5 yeast treated with protamine in buffer HM (□). Release of UV-absorbing material (A_{260}) into the supernatants is shown for A5 yeast treated with protamine in water (——) or buffer HM (- - -).

ethanol or with protamine in water both caused the appearance of 18–20 nmol AC . min^{-1} . g fresh $yeast^{-1}$, compared to 12 nmol . min^{-1} in broken-cell preparations. Protamine in buffer HM or cytochrome *c* in water caused slower appearances of AC activity. However, the increase in A_{260} of the cell supernatants during treatment with protamine in either water or buffer HM reached the same value (150 cm^{-1}, ml/g yeast) as the A_{260} of perchloric acid-treated supernatants of mechanically disintegrated yeast, indicating that both treatments permeabilised essentially all the yeast.

After mechanical disintegration of protamine-permeabilised cells, ~70% of their AC activity was sedimentable at 105 000 × *g*, compared to 80% in disintegrates of untreated yeast. No activity was detected in protamine-permeabilised cells with Mg^{2+} but no Mn^{2+}. The K_m for ATP in these cells was 1.7 mM at pH 6.2.

Both protamine-permeabilised and toluene/ethanol-permeabilised cells were tested for inhibition of AC by glucose. Preincubation in up to 100 mM glucose followed by assay in up to 10 mM glucose at 2 mM ATP (pH 6.2) caused only 5–15% inhibition compared to controls without glucose. At smaller ATP concentrations, larger inhibitions by glucose occurred. However, these inhibitions increased when the capacity of the ATP-regenerating system was decreased, and progress curves in the presence of glucose were

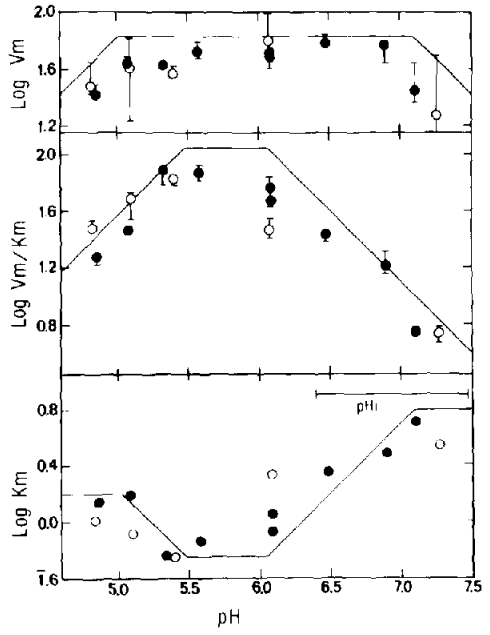


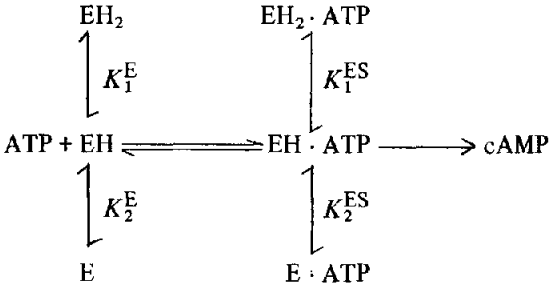
Fig.2. Dixon plots for adenylate cyclase activity. Results for toluene-permeabilised cells (•) and the cell-free particulate fraction (○) are shown. Values for V_m (nmol . min^{-1} . g fresh $yeast^{-1}$; for the isolated particulate fraction log V_m at pH 6.08 was arbitrarily set a 1.80) and V_m/K_m (nmol . min^{-1} . mM^{-1} . g^{-1}) are median estimates, and the error bars are explained in section 2. Lines of slope 0 and ∓1 were fitted using values for the dissociation constants and the pH-independent quantities derived from fig.3. The horizontal bar labelled pH_i indicates the range of physiological pH in the yeast cell [9].

markedly non-linear. These features suggested that the apparent inhibition by glucose was due to a glucose-dependent consumption of ATP by the powerful hexokinase (~150 μmol . min^{-1} . g^{-1}) present in these yeast.

3.2. *The pH-dependence of AC in toluene/ethanol-permeabilised cells*

The pH-dependency of AC was studied in yeast permeabilised with toluene/ethanol for 4 h because they contained reproducible high activities. Dixon plots [8] are shown in fig.2. Reliable data could not be obtained at pH > 7.2 because of increasing disturbance by ATPase. The data for toluene/ethanol-permeabilised cells are of higher quality than data for the cell-free particulate fraction (compare the error bars in fig.2), probably because the progress curves were linear over a greater extent. However, the behaviour of AC in the particulate fraction broadly agreed with that in situ. Experimental values for V_m varied by <30% between pH 5.0–7.0, and decreased on either side of this range. V_m/K_m showed a narrow maximum between pH 5.4–6.1 and decreased sharply at higher and lower pH values. K_m had a minimum of 0.57 mM at pH 5.3, and within the physiological pH range (6.4–7.5, [9]) varied between 2–5 mM.

This behaviour was interpreted by the simplest possible mechanism, shown in scheme 1:



Scheme 1

where, for the moment, the nature of the ATP substrate species is not defined. The values of pK_1^{ES} and pK_2^{ES} could only be approximated, because they are close to the limits of the pH range within which kinetic measurements were reliable. From plots (upper panel of fig.3) of $1/V_m$ against H^+ (at low pH) or $1/H^+$ (at high pH) according to the equations:

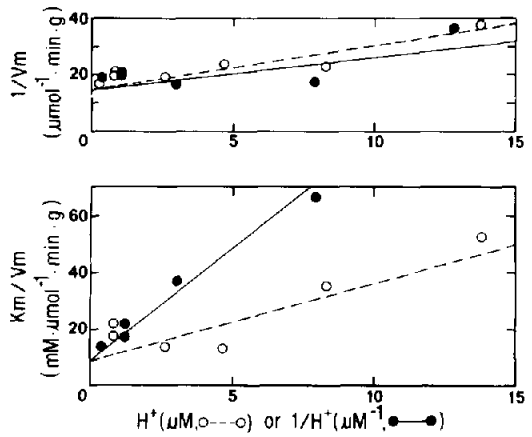


Fig.3. Dependence of V_m and K_m/V_m on H^+ activity: (upper) $1/V_m$ is plotted against H^+ between pH 4.8–6.5 (o——o) and against $1/H^+$ between pH 5.6 and 7.1 (●——●); (lower) K_m/V_m is plotted against H^+ between pH 4.8–6.1 (o---o) and against $1/H^+$ between pH 5.6–6.9 (●——●). All data are from toluene/ethanol-permeabilised cells.

$$1/V_m = 1/V_{m'} + (1/V_{m'} \cdot K_1^{ES}) \cdot H^+$$

and

$$1/V_m = 1/V_{m'} + (K_2^{ES}/V_{m'}) \cdot 1/H^+$$

values for pK_1^{ES} and pK_2^{ES} of ~5.0 and ~7.1, respectively, and for the pH-independent maximum velocity, $V_{m'}$, of 68 nmol . min^{-1} . g $yeast^{-1}$ were obtained. From analogous plots of K_m/V_m (lower panel of fig.3) values for pK_1^E and pK_2^E of 5.5 and 6.1, respectively, and for $(K_m/V_m)'$ of 9.0 mM . $nmol^{-1}$. min . g yeast were obtained. The dissociation constants of pK_1^E and pK_2^E probably belong to groups in the free enzyme because their values are well removed from the acid dissociation constants of possible substrate species (ATP^{4-} 6.7, ATP^{3-} 4.0, $MgATP^{2-}$ 4.8 [10]; $MnATP^{2-}$ ~4.4 [11,12]).

4. Discussion

Treatment of yeast with cytochrome *c* in water or with protamine in buffer HM exposed rather less AC activity than found in normal broken-cell preparations (12 nmol . min^{-1} . g fresh $yeast^{-1}$, equivalent to 43 nmol . min^{-1} . g yeast dry wt^{-1}; cf [5]). Treatment with protamine in water alone or with toluene/ethanol

exposed about twice this amount of activity, or roughly the same as found in yeast broken in the presence of Lubrol [5].

The main properties of AC in situ (apparent absolute dependence on Mn^{2+}, pH optimum 6.2, K_m 1.7 mM at pH 6.2) are the same as in vitro [5]. The high K_m cAMP phosphodiesterase of yeast also exhibits very similar behaviour in situ as in vitro, whilst ~70% of the low K_m cAMP phosphodiesterase exhibits a K_m in situ an order of magnitude greater than the value in vitro (J. L., unpublished).

Yeast AC is expected to be inhibited by glucose or one of its early metabolites, because levels of cAMP in yeast are depressed by glucose [13]. However, glucose did not directly inhibit AC in situ under conditions similar to those used [6] to demonstrate the control of *Escherichia coli* AC by glucose. The apparent absolute dependence of yeast AC on Mn^{2+} both in situ (see above) and in vitro (where rates with 5 mM $MgCl_2$ alone are <3% of those with Mn^{2+} [5]) is a puzzle, because it is unlikely that significant amounts of Mn^{2+} are available to AC in yeast cells, although the vacuole can accumulate Mn^{2+} [14]. Mn^{2+} may short-circuit a yet unknown regulatory mechanism for yeast AC, as they appear to do for mammalian ACs [15]. This may explain why convincing effectors of yeast AC have not yet been found (several glucose metabolites have been screened; K. V., J. L., unpublished), though very high concentrations (0.1–0.25 M) of several sugars inhibit yeast AC by up to 35% in the presence of Mn^{2+} [16].

The effects of pH on AC activity in the presence of Mn^{2+} (fig.2,3) probably reflect, therefore, the properties of AC catalytic subunit, uncomplicated by interactions with any regulatory subunit(s). Because the stability constant of $MnATP^{2-}$ ($2–7 \times 10^5$ M^{-1} [11,12]) is nearly 10-times that (7×10^4 M^{-1} [10]) of $MgATP^{2-}$, $MnATP^{2-}$ plus $MnATP^-$ account for at least 85% (pH 7.0)–75% (pH 5.0) of the total ATP at 3 mM ATP under our assay conditions, and for more at the smaller ATP concentrations. Presumably, $MnATP^{2-}$ is a substrate, but the accuracy of the results is too small to decide whether $MnATP^-$ (~15% of the total ATP at pH 4.8) is also a substrate.

The pK-values in free enzyme of the essential basic group ($pK_1^E = 5.5$) and acidic group ($pK_2^E = 6.1$) are close to those typical to protein imidazoles [17]. Identification cannot be made from pK data alone, but if, indeed, both groups are imidazoles, then it is of note that the separation of their practical pK-values by 0.6 corresponds to the statistical factor (log 4) for groups of identical intrinsic pK. Binding of substrate apparently assists somewhat the ionisation of group 1 and strongly hinders that of group 2, because the pK-values shift to ~5.0 and 7.1, respectively, in the enzyme–substrate complex (fig.2). Virtually nothing is known about the essential amino acid residues at the active sites of adenylate cyclases, although yeast AC contains an essential arginine residue [18], which would be expected to have $pK > 11$.

References

[1] Schlenk, F. and Zydek-Cwick, C. R. (1970) Arch. Biochem Biophys. 138, 220–225.
[2] Serrano, R., Gancedo, J. M. and Gancedo, C. (1973) Eur. J. Biochem. 34, 479–482.
[3] Reeves, R. E. and Sols, A. (1973) Biochem. Biophys. Res. Commun. 50, 459–466.
[4] Londesborough, J. and Nurminen, T. (1972) Acta Chem. Scand. 26, 3396–3398.
[5] Varimo, K. and Londesborough, J. (1976) Biochem. J. 159, 363–370.
[6] Harwood, J. P. and Peterkofsky, A. (1975) J. Biol. Chem. 250, 4656–4662.
[7] Eisenthal, R. and Cornish-Bowden, A. (1974) Biochem. J. 139, 715–720.
[8] Dixon, M. (1953) Biochem. J. 55, 161–171.
[9] Kotyk, A. (1963) Folia Microbiol. (Prague) 8, 27–31.
[10] Phillips, R. (1966) Chem. Rev. 66, 501–527.
[11] Perrin, D. D. and Sharma, V. S. (1966) Biochim. Biophys. Acta 127, 35–41.
[12] Ragot, M., Sari. J. C. and Belaich, J. P. (1977) Biochim. Biophys. Acta 499, 411–420.
[13] Schlanderer, G. and Dellweg, H. (1974) Eur. J. Biochem. 49, 305–316.
[14] Okorokov, L. A., Lichko, L. P., Kadomtseva, V. M., Kholodenko, V. P., Titovsky, V. T. and Kulaev, I. S. (1977) Eur. J. Biochem. 75, 373–377.
[15] Ross, E. M. and Gilman, A. G. (1980) Annu. Rev. Biochem. 49, 533–564.
[16] Wheeler, G. E., Schibeci, A., Epand, R. M., Rattray, J. B. M. and Kidby, D. K. (1974) Biochim. Biophys. Acta 372, 15–22.
[17] Tanford, C. (1962) Adv. Prot. Chem. 17, 69–161.
[18] Varimo, K. and Londesborough, J. (1979) FEBS Lett. 106, 153–156.