EVIDENCE FOR ESSENTIAL ARGININE IN YEAST ADENYLATE CYCLASE

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1. Introduction

Adenylate cyclase, catalyzing 3′,5′-cyclic AMP formation from ATP, is a Mn-dependent, membrane-bound enzyme in baker's yeast with a slightly acidic pH optimum [1]. Little is known about the binding of substrate to the active site of adenylate cyclases of different origin. Numerous other enzymes with negatively charged substrates or cofactors have been shown to contain essential arginine as a positively charged recognition site in their active centres [2]. In this paper we describe the rapid inactivation of yeast adenylate cyclase by reagents highly specific for arginine, 2,3-butanedione [2,3], 1,2-cyclohexanedione [4] and phenylglyoxal [5]. As often found in the reaction of butanedione with arginine [2,3], borate accelerates the inactivation. Instead of ATP, its analogue, AMP-PNP, was used to demonstrate a protective effect of substrate against the inactivation. ATP could not be used because of the large amount of ATPase present in the enzyme preparation. Even though only a crude, Lubrol-dispersed enzyme was available, the results strongly suggest that arginine is involved in the binding of substrate to yeast adenylate cyclase.

2. Materials and methods

Commercial baker's yeast from our Rajamäki factories was broken with a MiniMill (Gifford Wood Co., Hudson NY) as in [6], but in 25 mM Hepes/NaOH buffer (pH 7.2) containing 1% Lubrol-PX, 0.3 mM EDTA and 0.3 M KCl. Nonsedimentable Lubrol-dispersed enzyme in the 60 min 100 000 × *g* supernatant was concentrated with solid polyethyleneglycol in a dialysis tube to 1/3rd of the original volume and stored at −20°C. Its specific activity was 0.24–0.32 nmol .min^{-1} .mg protein^{-1}.

Adenylate cyclase was assayed at 2 mM ATP (pH 6.2) as in [6]. Treatments with arginine-specific reagents were performed at 30°C in borate or Hepes (see text and legends). The reagents were diluted in the appropriate buffer and any resulting pH change corrected by addition of 1 M NaOH. The reaction was initiated by adding freshly diluted reagent to the enzyme solution so that the final protein concentration was 30 mg/ml. The course of inactivation was followed by taking 10 μl samples from the incubation mixture to 100 μl adenylate cyclase assay mixtures. When incubations with borate were done, 25 mM borate/HCl (pH 6.2) was included in the adenylate cyclase assay mixture to avoid the partial reactivation of the enzyme which otherwise occurred. The final pH of the incubation mixtures was determined directly.

The [8-^{14}C]ATP (NEN Chem.) (at a specific radioactivity of 3000 cpm/nmol) and AMP-PNP (Sigma) were purified by ion-exchange chromatography by the general method [7], then stored as dried tetrasodium salts at −20°C.

Phenylglyoxal from Sigma, 1,2-cyclohexanedione (dimer of half hydrates) and 1,3-cyclohexanedione obtained from Merck, and 2,3-butanedione from Fluka were used without further purification. The sources of other chemicals have been described [6].

Protein was assayed with Biuret reagent [8].

Abbreviations: AMP-PNP, adenylylimidodiphosphate; ATP, adenosinetriphosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulphonic acid

3. Results

3.1. *Inactivation by 2,3-butanedione, 1,2-cyclohexanedione and phenylglyoxal*

Incubation of yeast adenylate cyclase with 2,3-butanedione resulted in a rapid, and eventually complete, loss of enzyme activity, which was first order over at least 80% of the reaction (fig.1). The rate of inactivation depended on the pH (fig.2), and the borate concentration. A maximum rate was achieved in 25 mM borate. Possibly the effective concentration of 2,3-butanedione was decreased at higher borate concentrations [3]. Inactivation by 20 mM 2,3-butanedione in 25 mM Hepes buffer was slower than in 25 mM borate (fig.2), and stopped after loss of ~72% of the enzyme activity (not shown). Similar partial inactivation of enzymes by reaction of 2,3-butanedione with arginine residues in the absence of borate has been observed before (e.g., [9]).

Inactivation of adenylate cyclase by 20 mM 1,2-cyclohexanedione in 100 mM borate (pH 7.6) was first order up to at least 90% loss of activity, with a half-inactivation time of 18 min (not shown). Incubation with 1,3-cyclohexanedione did not cause inactivation in the same conditions.

Inactivation of adenylate cyclase by 20 mM phenylglyoxal in 25 mM Hepes (pH 7.6) reached 50% in 10 min and a maximum of 73% in ~1 h (not shown).

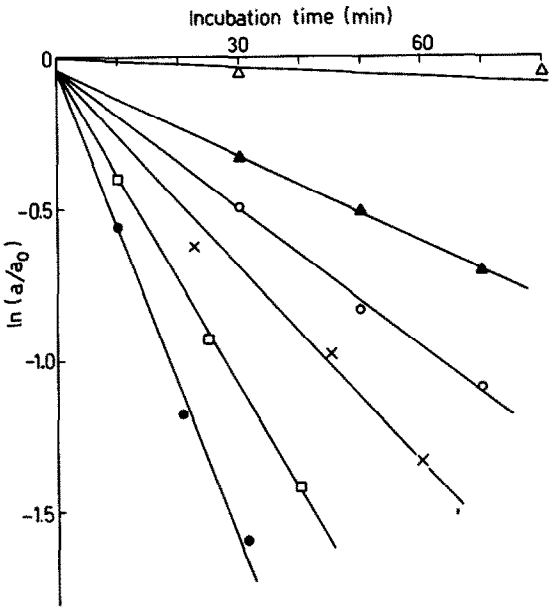

Fig.1. Inactivation of adenylate cyclase at 30°C by 2,3-butanedione in 25 mM borate (pH 7.2). Butanedione concentrations are none (△), 5 mM (▲), 6.7 mM (○), 10 mM (x), 15 mM (□) and 20 mM (●). The initial enzyme activity in the absence of 2,3-butanedione is a_0 and the activity observed after incubation is a. Protein concentration of the incubation mixture was 30 mg/ml.

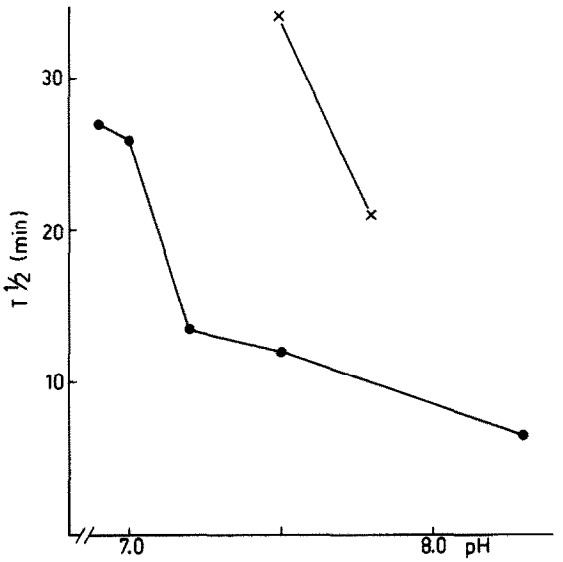

Fig.2. Half-inactivation times for adenylate cyclase at 30°C with 20 mM 2,3-butanedione in 25 mM borate (•) and 25 mM Hepes (x) as a function of pH. Protein concentration during the incubation was 30 mg/ml.

3.2. *Order of reaction with respect to 2,3-butanedione*

The increase with the concentration of 2,3-butanedione (BD) of the pseudo first-order rate constant of inactivation, k_{app} (calculated from fig.1) fitted the equation: $k_{app} = k\,[BD]^n$, with values for n and k of 1.12 and 1.8 $min^{-1}.M^{-1.12}$, respectively (fig.3, inset). The difference of n from unity appeared to be outside experimental error, and may reflect the fact [3] that 2,3-butanedione reacts with the borate buffer. Correspondingly, the plot of k_{app}^{-1} against $[BD]^{-1}$ was linear within experimental error, but had a positive intercept on the $[BD]^{-1}$ axis. The plot of k_{app}^{-1} against $[BD]^{-1.12}$ passed through the origin. The inactivation by 2,3-butanedione of some enzymes

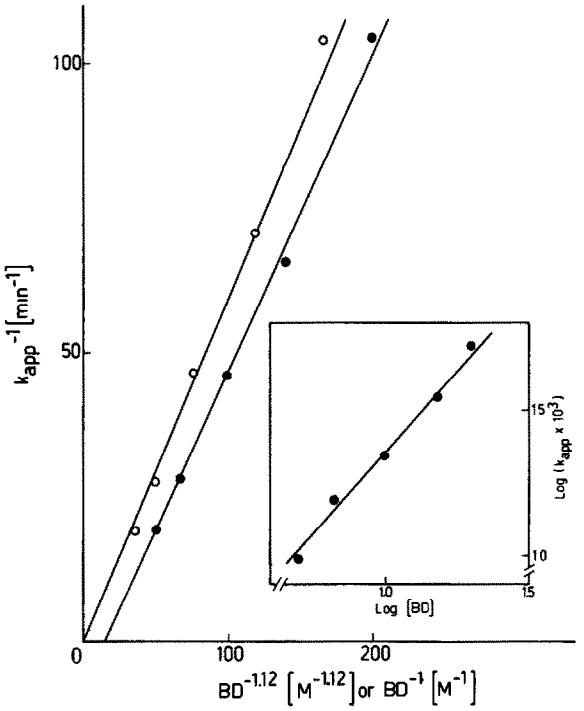

Fig.3. Dependence on the concentration of 2,3-butanedione (BD) of the pseudo first-order rate constant (k_{app}) for the inactivation in 25 mM borate (pH 7.2). Values of k_{app} were calculated from fig.1. Main figure: k_{app}^{-1} is plotted against $[BD]^{-1}$ (•) or $[BD]^{-1.12}$ (○). Inset: log (k_{app} × 10^3) is plotted against Log [BD].

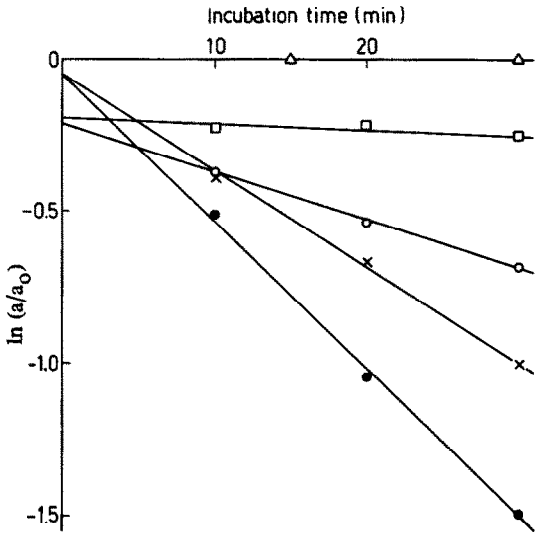

Fig.4. Effects of 4.6 mM adenylylimidodiphosphate (AMP-PNP) on the inactivation of adenylate cyclase by 20 mM butanedione in the presence of $MnCl_2$ (□), $MgCl_2$ (○) and EDTA (x), each 4.5 mM. Controls with 20 mM BD, no AMP-PNP (•) and no BD, no AMP-PNP (△) are also shown. Other conditions are the same as in fig.1.

(e.g., pyruvate kinase [10]) exhibits saturation kinetics with respect to 2,3-butanedione indicating the formation of a potentially active enzyme–2,3-butanedione complex which rearranges to an inactive complex. The results of fig.3 suggest that this is not the case during the modification of adenylate cyclase by 2,3-butanedione.

3.3. *Substrate protection against inactivation*

The synthesis of 3′,5′-cyclic AMP from 0.5 mM ATP by crude preparations of Lubrol-dispersed yeast adenylate cyclase is inhibited by > 95% by 3.4 mM AMP-PNP. However, at pH 6.2 and 5 mM $MnCl_2$, such enzyme preparations catalyse 3′,5′-cyclic AMP formation from 2 mM AMP-PNP alone at ~2.5% of the rate with 2 mM ATP alone (J. L., unpublished work). Thus, AMP-PNP appears to be a substrate for yeast adenylate cyclase, as it also is for some other adenylate cyclases [11]. The effect of 4.6 mM AMP-PNP on the inactivation of yeast adenylate cyclase by 20 mM 2,3-butanedione is shown in fig.4. The rate of inactivation was decreased by 96%, 67% or 34%, respectively, in the presence of 4.5 mM $MnCl_2$, $MgCl_2$, or EDTA. Adenosine (10 mM) did not alter the inactivation rate under these conditions. The inhibitory effect (15%) of the 0.46 mM AMP-PNP introduced to the assay mixtures has been allowed for, so that the initial small (14%) drop in activity observed in the presence of AMP-PNP and metal ions is unexplained.

4. Discussion

Yeast adenylate cyclase is rapidly inactivated by three reagents under conditions (pH 6.8–8.3) where they are highly specific for arginine. With 2,3-butanedione the inactivation is complete and, typically, accelerated by borate. Comparison of 1,2- and 1,3-cyclohexanedione showed that the dicarbonyl grouping must be vicinal for rapid inactivation to result. These results strongly suggest that yeast

adenylate cyclase contains an essential arginine residue. The powerful protection afforded by Mn-AMP-PNP, but not by adenosine, is consistent with the idea that an arginine modified by vicinal dicarbonyl compounds participates in binding the negatively charged substrate at the active centre. To our knowledge, this is the first essential amino acid residue to be identified in the active site of an adenylate cyclase, although several adenylate cyclases are inhibited by sulfhydryl blocking reagents. In addition, Mg-AMP-PNP and even AMP-PNP in the presence of EDTA protect in a less degree. Possibly they can bind to the active site more loosely and so form a steric hindrance to the approach of butanedione.

Proof that the inactivations reported here are caused by modification of arginine residues cannot be obtained until highly purified enzyme is available. Similarly, possible protection by Mn-ATP remains to be elucidated after purification of detergent-solubilized adenylate cyclase free from ATPase.

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