Biochimica et Biophysica Acta, 611 (1980) 358—362

BBA 68903

INACTIVATION OF ADENYLATE CYCLASE BY PHENYLGLYOXAL AND OTHER DICARBONYLS

EVIDENCE FOR EXISTENCE OF ESSENTIAL ARGINYL RESIDUES

DOUGLAS J. FRANKS [a], GODFREY TUNNICLIFF [b] and THAT T. NGO [a]

[a] *Clinical Research Institute of Montreal and l'Université de Montréal, 110 Pine Avenue West, Montreal, Quebec, H2W 1R7 (Canada), and* [b] *Indiana University School of Medicine, Evansville Center for Medical Education, 8600 University Boulevard, Evansville, IN 47712 (U.S.A.)*

(Received May 15th, 1979)

Key words: Adenylate cyclase; Phenylglyoxal; Dicarbamyl reagent; Arginyl residue

Summary

Rat brain adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) activity was markedly reduced when the enzyme was preincubated at 23°C for periods up to 30 min with phenylglyoxyal (2.5—20 mM), an agent that binds specifically to arginyl residues. The kinetics indicated the interaction of phenylgloxal with one arginyl residue was responsible for the inactivation. Protection of inactivation of the enzyme by phenylglyoxal was attained in the presence of ATP and to a lesser extent by ADP and 5′-AMP but not by cyclic-AMP or Mg^{2+}. In addition, 2,3-butanedione and 1,2-hexanedione, compounds that also react with arginyl residues, each inactivated adenylate cyclase to varying degrees. Furthermore, this inactivation was enhanced in the presence of borate ions. These observations strongly suggest that brain adenylate cyclase possesses essential arginyl residues.

Introduction

The chemical modification of amino acids at the active site of an enzyme can provide much information on the role of these amino acid residues in the enzyme structure and function. The use of phenylgloxal [1] and α-dicarbonyl compounds [2—4] as specific probes for arginyl residues has increased our awareness of the participation of such residues in the anionic substrate binding site of enzymes [5]. Among the enzymes studied are hydrolytic enzymes [6—8], dehydrogenases [9—12], transaminases [13,14], transferases [15—17],

Abbreviation: Tes, *N*-tris[hydroxymethyl]methyl-2-amino-ethanesulfonic acid.

polymerases [18,19], kinases [20,21], a mutase [22] and a number of ATP-dependent enzymes [23—25].

Adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) catalyses the hydrolysis of ATP and cyclization of AMP to form cyclic 3′,5′-adenosine monophosphate. Though this enzyme is implicated in the mechanism of action of many hormones, little is known of its catalytic mechanism. Some studies have been performed to determine the physical structure of the mammalian enzyme [26], and partial purification has been achieved [27]. Few studies have been directed toward elucidating the structure of the active site though it has been demonstrated that sulfhydryl or dithiol groups are essential for activity [28,29]. To investigate the role of arginyl residues in the catalytic mechanism of adenylate cyclase and the possibility that such residues may serve as ionic recognition sites for the binding of ATP, we have studied the effects of phenylglyoxal, glyoxal, 2,3-butanedione and 1,2-cyclohexanedione on the activity of brain adenylate cyclase. Each of the arginine-modifying agents were shown to irreversibly inhibit the enzyme activity and evidence supporting an essential role of arginyl residues in the mechanism of action of adenylate cyclase is presented.

Experimental

Preparation of adenylate cyclase. Male Wistar rats (Charles River) were killed by cervical dislocation and the brain rapidly removed. Brains were homogenized immediately in ice-cold 0.25 M sucrose containing 10 mM Tes, and 1 mM dithiothreitol, pH 7.5, using a motor-driven teflon-glass homogenizer. The homogenate was centrifuged at 600 × *g* for 10 min, the supernatant decanted and centrifuged again at 20 000 × *g* for 10 min. The pellet thus obtained was made up in a small volume of the same medium and dispersed in a Dounce homogenizer. The protein concentration was determined by the method of Lowry et al. [30] and the volume adjusted to give a protein concentration of 4—6 mg/ml.

Preincubation with phenylglyoxal. 40 μl of this enzyme preparation were added to an equal volume of a phenylglyoxal solution (5—40 mM) at 23°C. The inactivation reaction was allowed to proceed for an appropriate time interval and was terminated by dilution of the samples to 1 ml with 10 mM Tes, 1 mM dithiothreitol, pH 7.5. The adenylate cyclase was recovered by centrifugation at 20 000 × *g* for 5 min, washed with a further 1 ml of Tes/dithiothreitol, centrifuged again and the pellet dispersed in 80 μl of the above buffer by vigorous mixing. Adenylate cyclase activity was determined immediately in triplicate 20-μl samples of this preparation.

Adenylate cyclase assay. The method used has been described in detail elsewhere [31]. The reaction mixture contained 25 mM Tes (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM cyclic AMP + 0.01 μCi cyclic [^{3}H]AMP, 0.5 mM MgATP + 0.5 μCi [α-^{32}P]ATP and 40—60 μg of enzyme protein in a total volume of 0.1 ml. The reaction was initiated by addition of enzyme and allowed to proceed at 37°C for 4 min. The incubation was terminated by the addition of 0.1 ml of 10 mM ATP at 0°C. Under these conditions the reaction was linear with respect to incubation time and enzyme protein concentration.

Cyclic AMP was isolated from the incubates by a procedure modified [32] from that of Salomon et al. [33]. After dilution of the sample to 0.6 ml with H_2O the sample was applied to a 5 × 0.5 cm column of Dowex AG50-WX4 resin and the column washed with 2.5 ml H_2O. The column was washed with a further 5 ml of water and this eluate added directly to a 3.0 × 0.5 cm column of neutral alumina. Cyclic AMP was then eluted from the alumina with 4 ml of 50 mM Tris (pH 7.5) and the eluate collected directly into a scintillation vial containing 10 ml of Aquasol (New England Nuclear). The ^{32}P cpm and ^{3}H cpm were determined in a Beckman liquid scintillation spectrometer. The assay blank was 15—20 cpm and 75—85% of the applied cyclic AMP was recovered.

Results and Discussion

Phenylglyoxal is reasonably specific for arginyl residues [1], but reaction will occur over long-time periods with the ϵ-amino group of lysine and with sulfhydryl residues; however, this latter interaction is reversible [35].

Adenylate cyclase is rapidly and irreversibly inactivated by incubation with phenylglyoxal at 23°C (Fig. 1). The inactivation rate obeys pseudo first-order kinetics and is dependent on the phenylglyoxal concentration. A plot of log $[1/t_{0.5}]$ versus log [I] in which $t_{0.5}$ is the half time of inactivation and [I] is the inhibitor concentration has been used to determine the number of reactive amino acid residues at the active site of an enzyme [34]. Such a plot should be a straight line of slope n where n is the number of inhibitor molecules reacting with each active unit of enzyme to form an enzyme-inhibitor complex. For adenylate cyclase the plot of $[1/t_{0.5}]$ versus log [phenylglyoxal concentration] (not shown) was a straight line (correlation coefficient 0.99) having a slope of 0.83. This strongly suggests that inactivation is the result of the reaction of a single arginyl residue.

In addition to the effects of phenylglyoxal, brain adenylate cyclase is also

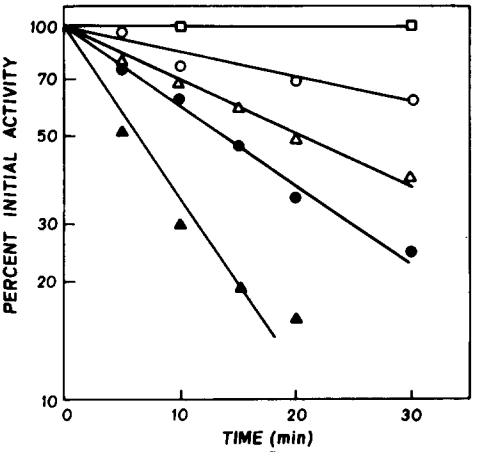

Fig. 1. Time course of inactivation of brain adenylate cyclase by increasing concentrations of phenylglyoxal. Adenylate cyclase was preincubated with phenylglyoxal for the time shown. After washing, adenylate cyclase activity was assayed as described in Experimental. □, no phenylglyoxal; ○, 2.5 mM phenylglyoxal; △, 5 mM phenylglyoxal; ●, 10 mM phenylglyoxal; and ▲, 20 mM phenylglyoxal.

TABLE I

EFFECT OF ARGININE-SPECIFIC REAGENTS ON THE ACTIVITY OF BRAIN ADENYLATE CYCLASE

The adenylate cyclase preparation was preincubated for 5 min at 23°C with the agents indicated. The concentration of all the agents used was 10 mM. After washing, adenylate cyclase activity was then assayed as described in Experimental. The percent inactivation was calculated by taking the activity of non-treated enzyme after 5 min at 23°C as 0% inhibition.

Agent	50 mM borate	Inactivation of adenylate cyclase (%)
None	—	0
	+	0
Phenylglyoxal	—	64
	+	65
Glyoxal	—	25
	+	26
2,3-Butanedione	—	18
	+	33
1,2-Cyclohexanedione	—	51
	+	68

inactivated by glyoxal, 2,3-butanedione and 1,2-cyclohexanedione (Table I). When borate ions were added to the incubation mixture an enhancement of the inactivation brought about by 2,3-butanedione and 1,2-cyclohexanedione was observed. However, borate ion has no effect on the inactivation of the enzyme by phenylglyoxal and glyoxal. The effect of borate are consistent with the proposals of Riordan et al. [5] that borate stabilizes the arginine-dicarbonyl complex.

To demonstrate whether or not these arginyl residues are essential for the interaction of adenylate cyclase with its substrate, Mg ATP, a series of protection experiments were performed. As shown in Table II, 10 mM ATP was able to partially protect against inactivation by 10 mM phenylglyoxal. ADP and 5′-AMP were less effective while Mg^{2+}, a cofactor of adenylate cyclase, and the reaction product cyclic AMP were unable to protect against inactivation. This suggests that the effects of the inactivator are occurring at the catalytic site or induce a conformational change that modifies this site.

TABLE II

PROTECTION OF INACTIVATION OF ADENYLATE CYCLASE BY DIFFERENT AGENTS

The enzyme was first preincubated for 10 min at 23°C in the presence of protecting agent alone. After 10 min phenylglyoxal was added and the incubation was continued for a further 20 min. After washing adenylate cyclase activity was assayed as described in Experimental. Final concentration of all agents was 10 mM.

Agent	Adenylate cyclase (% initial activity)
None	100
Phenylglyoxal	18
Phenylglyoxal + 5′-ATP	50
Phenylglyoxal + $MgCl_2$	15
Phenylglyoxal + 5′-ADP	42
Phenylglyoxal + 5′-AMP	35
Phenylglyoxal + cyclic 3′,5′-AMP	24

The results presented thus provide evidence that brain adenylate cyclase possesses essential arginyl residues.

Acknowledgements

This work was supported by a grant from the Medical Research Council of Canada (MA 5718) to D.J.F. The authors wish to thank Mrs. M. Dupuis and Mrs. H. Leung for excellent technical assistance and Dr. P. Hamet and Dr. A. Barbeau for helpful advice and encouragement.

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