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A REACTIVE ARGININE IN ADENYLATE KINASE

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Summary

Adenylate kinase (ATP:AMP phosphotransferase, EV 2.7.4.3) from pig heart is inactivated by the specific arginyl reagent phenylglyoxal. During inactivation two molecules of phenylglyoxal are incorporated into the protein indicating the modification of one of the 11 arginine residues. The modification of other amino acids is ruled out. Chemical modification of this essential residue is prevented by high concentrations of the substrates AMP, ADP and $MgATP^{2-}$. The protection of the substrates is explained by the formation of a ternary abortive enzyme-substrate complex ESS. The dissociation constants $K_D^* = [ES] \cdot [S]/[ESS]$ are determined from the kinetic data of inactivation and protection.

Introduction

The atomic structure of adenylate kinase is known from X-ray data at 3 Å resolution [1]. The exact location of the substrate binding site is not yet possible, however, because crystallisation of the enzyme-substrate complex has not been achieved. In order to explore the active site of the enzyme, further chemical modification of amino acids within the active centre is necessary. Histidine [2] and cysteine [3] are assumed to be located at the catalytic site of adenylate kinase, but the position of these amino acids in relation to the substrates and their function in catalysis are not known.

For the binding of substrates in phosphokinases one or more cationic groups in the enzyme interacting with the phosphate groups of the substrates were postulated by Crane [4]. A positively charged area in the enzyme could be formed by the protonized ϵ-amino group of lysine or by the guanidinium group of arginine. The amino acid sequence of porcine adenylate kinase from skeletal muscle [5] reveals among the 194 amino acids 11 arginyl residues and 21 lysine residues. Takahashi [6] introduced the reagent phenylglyoxal which modifies arginyl residues under mild conditions. The present paper describes

the modification of arginine in adenylate kinase using this reagent. Protective effects of the natural substrates against loss of activity and the determination of the dissociation constants of the complexes between enzyme and AMP, ADP and $MgATP^{2-}$ are also shown.

Materials

AMP, ADP, ATP, NADH and phosphoenolpyruvate were purchased from Boehringer (Mannheim, Germany). Phenylglyoxal monohydrate was obtained from Fluka (Buchs, Switzerland). [*Me*-^{14}C]Acetophenone was a product of Farbwerke Hoechst AG. [^{14}C]Phenylglyoxal prepared according to Riley and Gray [7] had a specific activity of $1.05 \cdot 10^4$ cpm/μmol. Adenylate kinase with a specific activity of 1900 units/mg (ADP formation), pyruvate kinase (ATP : pyruvate phosphotransferase, EC 2.7.1.40) with a specific activity of 250 units/mg (ATP formation) and lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) with a specific activity of 460 units/mg (NADH oxidation) were isolated from porcine heart.

Methods

Enzyme assay

The rate of oxidation of NADH at 25°C was monitored at 366 nm with an Eppendorf photometer. A unit of activity was defined as 1 μmol of product formed per min. Specific activity was defined as the number of units per mg of protein.

The activity of adenylate kinase was measured in a coupled enzyme assay determining the production of ADP by means of pyruvate kinase and lactate dehydrogenase. Here a unit of activity was defined as 2 μmol of ADP formed per min. The assay contained in 1.2 ml 0.1 M triethanolamine · HCl buffer (pH 7.6) $1.4 \cdot 10^{-3}$ M $MgCl_2$, $8 \cdot 10^{-2}$ M KCl, $8 \cdot 10^{-4}$ M ATP, $2.3 \cdot 10^{-3}$ M AMP, $1.8 \cdot 10^{-4}$ M NADH, $3.5 \cdot 10^{-4}$ M phosphoenolpyruvate, 10 units lactate dehydrogenase, 10 units pyruvate kinase and rate-limiting amounts of adenylate kinase.

Analytical procedures

The protein concentration of adenylate kinase was determined from absorbance at 277 nm using $A_1^{1\%}{}_{cm} = 5.38$ [8]. Radioactivity was determined by liquid scintillation techniques using a Packard Tricarb instrument. The scintillation solution contained 120 g naphthaline, 75 mg POPOP and 4 g PPO in 1000 ml of dioxane [9].

Inactivation was accomplished at 37°C by adding the calculated amount of phenylglyoxal monohydrate, dissolved in 20 μl of water, to adenylate kinase (1 mg) in 1 ml of 0.1 M triethanolamine · HCl buffer (pH 7.6) containing $1.4 \cdot 10^{-3}$ M $MgCl_2$ and $8 \cdot 10^{-2}$ M KCl. Aliquots were removed at intervals of 2 min and after dilution assayed for enzymatic activity. In experiments examining the protective effect of the substrates, AMP, ADP and ATP were added to the enzyme solution before addition of the inactivator. During investigations with ADP the enzyme was dissolved in 0.1 M triethanolamine · HCl buffer (pH

7.6). The $MgATP^{2-}$ concentration was calculated using a stability constant of $2.09 \cdot 10^4$ M^{-1} [10].

Determination of the radioactivity incorporated into protein

1.35 mg [^{14}C] phenylglyoxal monohydrate dissolved in 0.1 ml water was added to 2 mg adenylate kinase dissolved in 2 ml 0.1 M triethanolamine · HCl buffer (pH 7.0). Enzymic activity was measured in intervals of 3 min. At the same time 0.1 ml of the solution were removed and the protein was precipitated by adding to 1 ml of ice-cold 25% trichloroacetic acid. The precipitate was collected by filtration on a glass fibre filter (Whatman GF/C) and freed from adsorbed inactivator by washing with trichloroacetic acid, water and dioxane. The filters were placed in counting vials containing a mixture of 9 ml scintillation solution and 1 ml water.

Identification of the modified amino acid

Adenylate kinase (5 mg) was incubated with 4 mg [^{14}C] phenylglyoxal monohydrate in 0.1 M triethanolamine · HCl buffer (pH 7.0). The reaction was terminated at 5% residual activity by the addition of 1 M $HClO_4$. The precipitate was collected by centrifugation, washed with 1 M $HClO_4$ and water and then suspended in 1.5 ml of 10^{-2} M HCl. The hydrolysis with pepsin and other proteinases was performed as previously described [11]. The hydrolysis was terminated by passage of the digestion mixture through a column (1.5 × 80 cm) of Sephadex G-10 equilibrated with 0.1 M acetic acid. In the ninhydrin-positive fraction exhibiting radioactivity the arginine derivative was identified as previously described [11].

Results and Discussion

As recently reported there is no difference between adenylate kinase from porcine heart and from porcine skeletal muscle [12]. In the presence of $2.6 \cdot 10^{-3}$ M phenylglyoxal the activity of adenylate kinase from porcine heart

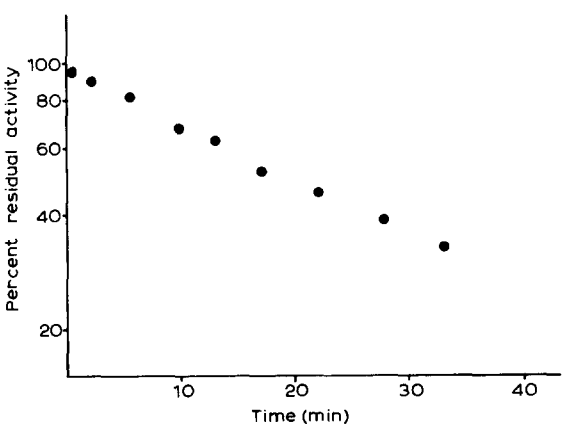

Fig. 1. Semi-log plot of changes in the activity of adenylate kinase (1 mg/ml) during inactivation by $2.6 \cdot 10^{-3}$ M phenylglyoxal in 0.1 M triethanolamine · HCl buffer (pH 7.6) containing $1.4 \cdot 10^{-3}$ M Mg^{2+} and $8 \cdot 10^{-2}$ M K^+ at 37° C versus time.

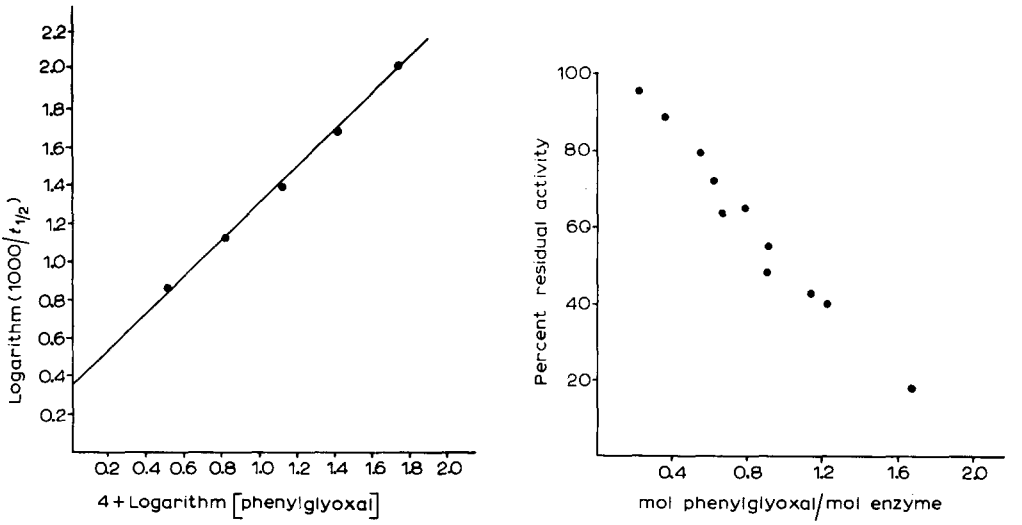

Fig. 2. Order of inactivation with respect to the concentration of phenylglyoxal. Plot of log $1/t_{1/2}$ versus log concentration of inactivator. The slope of the plot (1) equals the order of the reaction. Conditions are the same as in Fig. 1.

Fig. 3. Correlation between residual activity (in percent) and incorporation of inactivator (mol/mol) during inactivation of adenylate kinase (1 mg/ml) by $4 \cdot 10^{-3}$ M phenylglyoxal in 0.1 M triethanolamine · HCl buffer (pH 7.0).

declined within 20 min to 50% of its original activity (Fig. 1). The cations Mg^{2+} and K^+ have no effect on inactivation.

A plot of the log of adenylate kinase activity versus time of inactivation gives straight lines at different concentrations of phenylglyoxal. This indicates that the inactivation reaction approximately obeys first-order kinetics. The order of inactivation with respect to phenylglyoxal concentration was determined by the method of Levy et al. [13] from a plot of log of the reciprocal of the half-times versus log concentration of inactivator (Fig. 2). The points fit a straight line with a slope equal to 1. This value indicates the binding of one molecule of phenylglyoxal to one arginine as inactivation occurs. Because of the great number of arginine residues present in adenylate kinase it was of interest to find out whether all or only some residues react with phenylglyoxal. We therefore used radioactively labelled phenylglyoxal and determined the incorporation of radioactivity into the protein during inactivation. A linear relationship between the loss of activity of adenylate kinase and the radioactivity incorporated was found (Fig. 3). Per mol of adenylate kinase (mol. wt 21 500) 2 mol of inactivator are incorporated at complete inactivation. According to the observation of Takahashi [6] two molecules of phenylglyoxal react with one molecule of arginine. Consequently only one arginine residue of the 11 residues present in adenylate kinase reacts with phenylglyoxal during inactivation. In order to find out whether modification of amino acids other than arginine occur, we hydrolysed inactivated enzyme with a mixture of proteinases. After chromatography of the amino acid mixture on Sephadex G-10 we found one ninhydrin-positive fraction containing radioactivity incorporated into one amino acid. This amino acid derivative shows the same behaviour as a

model synthesized from arginine and phenylglyoxal. Therefore, the modification of other amino acids apart from arginine is excluded. From the kinetic data of inactivation and from the ratio of incorporation of radioactivity we can conclude that reaction of one molecule of phenylglyoxal with a single arginine leads to inactivation and that the same arginine subsequently reacts with a second molecule of phenylglyoxal. The known instability of the arginine derivative prevented the isolation of the corresponding tryptic peptide. Thus we are not able to integrate the modified arginine into the sequence and the tertiary structure of adenylate kinase.

The loss of activity is decreased in the presence of the substrates AMP, ADP and $MgATP^{2+}$. If we assume that phenylglyoxal is only reacting with the free enzyme and not with the enzyme-substrate complex, then is:

$$\begin{array}{l} E + S \overset{K_D}{\rightleftharpoons} ES \\ \downarrow k \\ E^* \end{array}$$

with $K_D = \dfrac{[E] \cdot [S]}{[ES]}$ and $k_{obs} = \dfrac{k \cdot [E]}{[E] + [ES]}$

where E means the free enzyme, ES the enzyme-substrate complex, E* the inactive enzyme, k the rate constant of the modification reaction and k_{obs} the measured pseudofirst-order rate constant. It can be shown that

$$\frac{1}{k_{obs}} = \frac{1}{k} + \frac{[S]}{k \cdot K_D} \tag{1}$$

and a plot of the experimental half-times of inactivation at a constant concentration of inactivator versus the concentration of the protecting substrates should be linear, because the half-time and the reciprocal of k_{obs} only differ by a constant multiplication factor. The dissociation constant K_D of the enzyme-substrate complex can be determined from such a plot. As shown in Fig. 4 we find a linear relation between half-time of inactivation and the concentrations of the three protecting substrates. Dissociation constants determined in this way are shown in Table I. The dissociation constant of $MgATP^{2-}$ (4.5 mM) is two orders of magnitude higher than the dissociation constant of $MnATP^{2-}$ (44 μM) measured using an other method [14]. This discrepancy can be explained by the formation of a ternary abortive enzyme-substrate complex, in which both substrate binding sides are occupied by the same substrate. For AMP such a complex is already known [15]. Phenylglyoxal is able to react with the free enzyme as well as with the enzyme-substrate complex.

$$\begin{array}{ccc} E + S \overset{K_D}{\rightleftharpoons} ES & + S \overset{K_D^*}{\rightleftharpoons} & ESS \\ \downarrow k_1 \quad\quad \downarrow k_2 & & \\ E^* \quad\quad E^* & & \end{array}$$

with $K_D^* = \dfrac{[ES] \cdot [S]}{[ESS]}$

At low concentrations of substrates at which 50% of the total enzyme and

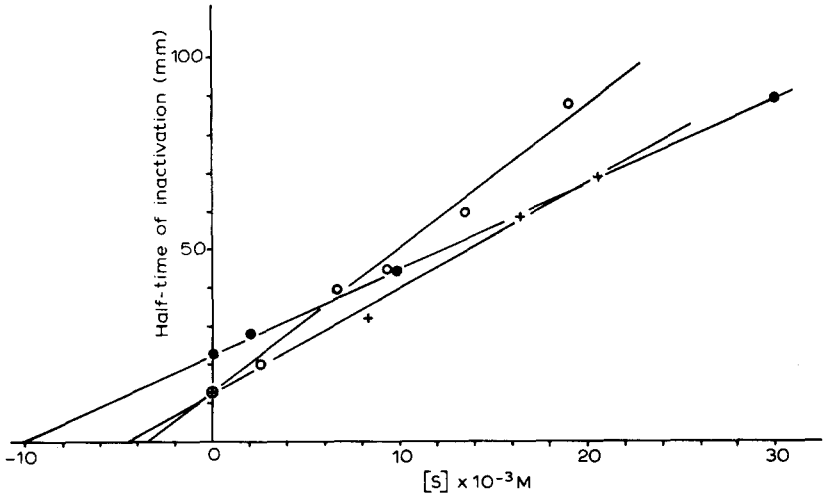

Fig. 4. Effect of substrates on the inactivation of adenylate kinase (1 mg/ml). Plot of half-time of inactivation versus concentration of AMP (○), ADP (●) and $MgATP^{2-}$ (+). The inactivation was performed in 0.1 M triethanolamine · HCl buffer (pH 7.6) with $2.4 \cdot 10^{-3}$ M phenylglyoxal in the experiments with ADP and in the same buffer containing $2.4 \cdot 10^{-2}$ M Mg^{2+} and $8 \cdot 10^{-2}$ M K^+ with $3.2 \cdot 10^{-3}$ M phenylglyoxal in the experiments with AMP or ATP.

more are complexed, if we assume K_D values about 50 μM, the rate of inactivation is not altered. Therefore, we must conclude that the enzyme-substrate complex reacts with phenylglyoxal as fast as the free enzyme. Then is

$$k_1 = k_2 = k$$

$$k_{obs} = \frac{k\left(K_D \cdot \frac{[ES]}{[S]} + [ES]\right)}{K_D\left(\frac{[ES]}{[S]} + [ES] + [ESS]\right)}$$

$$\frac{1}{k_{obs}} = \frac{1}{k} + \frac{[S]^2}{k(K_D \cdot K_D^* + K_D^* \cdot [S])} \qquad (2)$$

and for $K_D \gg [S]$

$$\frac{1}{k_{obs}} = \frac{1}{k} + \frac{[S]}{k \cdot K_D^*}$$

TABLE I

DISSOCIATION CONSTANTS OF THE TERNARY ABORTIVE COMPLEXES BETWEEN ADENYLATE KINASE AND THE SUBSTRATES AT 37°C, AS DETERMINED FROM FIG. 4

Substrate	K_D
AMP	$3.5 \cdot 10^{-3}$ M
ADP	$10 \cdot 10^{-3}$ M
$MgATP^{2-}$	$4.5 \cdot 10^{-3}$ M

which corresponds to Eqn. 1. Thus the dissociation constants determined from the kinetic data of protection describe the dissociation of a ternary enzyme-substrate complex.

It can not be decided whether the modified arginine itself participates in the binding of the substrates or if this residue is necessary for the maintenance of an active protein structure. The importance of arginine residues for the binding of substrate and coenzyme, however, was shown at lactate dehydrogenase [11] and the alcohol dehydrogenases [16]. These observations and the present results point to the important role arginine side chains play in enzymes with anionic substrates.

Acknowledgements

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