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Chemical modification studies on arginine kinase: Essential cysteine and arginine residues at the active site

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Abstract

Chemical modification was used to elucidate the essential amino acids in the catalytic activity of arginine kinase (AK) from *Migratoria manilensis*. Among six cysteine (Cys) residues only one Cys residue was determined to be essential in the active site by Tsou's method. Furthermore, the AK modified by DTNB can be fully reactivated by dithiothreitol (DTT) in a monophasic kinetic course. At the same time, this reactivation can be slowed down in the presence of ATP, suggesting that the essential Cys is located near the ATP binding site. The ionizing groups at the AK active site were studied and the standard dissociation enthalpy (ΔH°) was 12.38 kcal/mol, showing that the dissociation group may be the guanidino of arginine (Arg). Using the specific chemical modifier phenylglyoxal (PG) demonstrated that only one Arg, located near the ATP binding site, is essential for the activity of AK.

Keywords: Arginine kinase; *Migratoria manilensis*; Chemical modification; Essential amino acid

1. Introduction

As a member of the phosphagen kinase family, arginine kinase (AK; ATP; Arg *N*-phosphotransferase, EC 2.7.3.3) plays a key role in the interconnection of energy production and utilization in invertebrates, which is analogous to the creatine kinase reaction in vertebrates [1]. It catalyzes the reversible phosphorylation of Arg by MgATP to form phosphoArg and MgADP [2]:

$$\text{Arginine} + \text{ATPMg}^{2+} \overset{\text{AK}}{\rightleftarrows} \text{Phosphoarginine} + \text{ADPMg}^{2+}$$

Cys residues exist in all dimeric or monomeric AK sequences and the role of Cys has been reported by chemical modification with different reagents [3,4]. The modification of dimeric AK with *o*-phthalaldhyde revealed that only one Cys was essential for the enzyme activity, existing in the region between two subunits [5]. Several hypotheses have pointed out that the Cys may not be catalytically important, but may be involved in a hinge movement required for the enzyme to become active [6,7]. Modification of AK by DTNB suggested that the Cys was near the ATP binding site and may play an important role in the conformational changes caused by the transition-state analog not in the binding of the transition-state analog [8].

With the exception of Cys [9], other important amino acid residues Trp [10], Asp [11], Gly [12] and Val [12] have also been reported by chemical modification and site mutagenesis in either binding of substrates or in the catalytic activity of the enzyme. However, whether Arg residues involve directly in catalysis has not been reported to date. Recently, a crystal structure for monomeric AK from the horseshoe crab *Limulus* suggested that the catalytic center, where reversible transfer of the phosphate occurs, had Arg124, Arg126, Arg229, and Arg280 that attract ADP/ATP to the active site [13]. Thus, we choose monomeric AK from *Migratoria manilensis* to study the roles of Cys and Arg in further detail.

We have purified AK from locust and investigated its characteristics [14,15]. This report focuses on the essential Cys and Arg residues, which are located close to the active site of AK. The Tsou's plot analysis indicated the presence of only one essential

Abbreviations: AK, arginine kinase; DTNB, 5, 5-dithiobis-(2-nitrobenzoic acid); PG, phenylglyoxal; DTT, dithiothreitol; Arg, arginine; Cys, cysteine; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis

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Cys in the active center of locust AK. In addition, the reactivation course of substrate kinetics of DTNB-modified AK was used to demonstrate the function of reactive Cys. While the ΔH° of AK is closer to the value of the ionization constant for guanidyl, a further study by a specific chemical modifier PG demonstrated that Arg is involved in the enzymatic activity. The Lery's plot showed that only one Arg is essential for the catalytic activity of *M. manilensis* AK.

2. Materials and methods

2.1. Materials and chemicals

AK was prepared from the leg muscle of locust according to the procedure of Li et al. [14]. The purified enzyme was judged to be homogeneous by PAGE and SDS-PAGE, and was stored at −20 °C. To ensure the active AK, the thawed enzyme within 1–2 days was used in the experiment. The protein concentration was determined from the absorbance at 280 nm by the coefficient 0.67 mM^{-1} cm^{-1} [16]. Chemicals ATP, Arg, DTT, DTNB and PG were purchased from Sigma (USA).

2.2. Modification of AK by DTNB

To determine the number of essential Cys residues, Tsou's plot method was used [17]. In this method, the modification was carried out by incubating the AK in 50 mM Tris–HCl buffer, pH 8.0, with different concentrations of DTNB for 20 min. The concentrations of molar ratios of DTNB and AK ranged from 4 to 0.025. The extents of modification were measured spectrophotometrically at 412 nm by the coefficient 13.6 mM^{-1} cm^{-1} and the residual enzyme activity for different modifications were measured at the same time. AK activity was determined by the pH-continuous method [18].

2.3. Reactivation of DTNB-modified AK by DTT

AK was first completely modified by DTNB as described above and reactivated by different concentrations of DTT (0.01–0.15 mM) for 20 min at 30 °C. The excess DTNB was removed by gel filtration through a Sephadex G-75 column, and AK-TNB was obtained. For each concentration, the residual enzyme activity and the absorption spectrum were recorded by a spectrophotometer.

Tsou's kinetic theory of the substrate reaction in the presence of an activator could be applied to study the reactivation kinetics of AK by DTT. In this method, 3.5 nM DTNB-modified AK was added to the AK assay system, including 2 mM ATP, 10 mM Arg, 50 mM $MgAC_2$ and 50mM Tris–HCl buffer, pH 8.0 in the presence of different concentrations of DTT. The course of reactivation was followed by the direct continuous pH-spectrophotometric assay at 575 nm on an Ultrospec 4300 pro UV/Visible spectrophotometer (Pharmacia) [18]. Different concentrations of substrates were added to further determine the role of Cys residues.

All the reactions were carried out at 30 °C except where mentioned otherwise.

2.4. Determination of the standard dissociation enthalpy (ΔH°) of ionizing groups

The ionizing group at the enzyme active site was determined by measuring the pK_e value of groups. In this method, the pH (pH range from 8.06 to 9.42) effects were first assayed using a Lineweaver–Burk plot. The pK_e value was determined by the second plot of the apparent pK_m versus pH with the intersection of the two lines with slopes of zero and negative 1. The different pK_e values of the ionizing groups at different temperatures were then measured at temperatures from 20 to 35 °C. A plot of pK_e versus $1/T$ gives a straight line with slope which is used to determine the ΔH°.

2.5. Inactivation of AK by PG

The specific chemical modifier PG was used to study the role of Arg in AK. Reactions of AK with different concentrations of PG were carried out in 50 mM Tris–HCl buffer, pH 8.0. After 90 min of modification, the residual enzyme activity was measured [19].

The number of essential Arg residues was examined by a semi-logarithmic plot which was easier than Tsou's, when the total Arg residues were unknown. The enzyme was treated with PG (3– 15 μM) in 50 mM Tris–HCl buffer, pH 8.0. At different time intervals, 10 μM enzymes were taken out for activity determination [19]. Different concentrations of substrates (ATP and Arg) were used in activity assays to further determine the positions of the essential Arg residues in the active site. After 90 min, the residual activity was estimated as described above. The enzyme was incubated without modifier as control.

3. Results

3.1. The number of essential Cys residues determined by Tsou's method

Fig. 1 suggests that there are two phases during the modification by DTNB. In the first phase, the activity of AK didn't reduce when modified by low concentrations of DTNB, suggesting that there are some unessential Cys residues with the fastest reaction rates. When the fraction of DTNB was increased, the activity of AK was decreased (the second phase). So the model is fitted to Eq. (1) [20]:

$$a^{1/i} = \frac{[nx - (n - s - p)]}{p} \quad (1)$$

where a being the fractional activity remaining, x being the fractional reactive Cys residues remaining, i being the number of Cys residues essential for AK, n the total number of Cys residues ($n = 6$ here), s being the number of unessential Cys residues with the fastest reaction rates, p being the number of Cys residues with slow reaction rates, n-s-p being the number of Cys residues with the slowest reaction rates.

According to Tsou's method, when the plot of $a^{1/i}$ versus x obtained a straight line, the value of i stands for the number of essential residues. In Fig. 1 when $i = 1$, a linear relationship