Cysteine-25 of adenylate kinase reacts with dithiothreitol to form an adduct upon aging of the enzyme

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Abstract Adenylate kinase (AK) ages in solution in the presence of DL-dithiothreitol (DTT) with a gradual activity decrease. Upon dilution with 4 M guanidine hydrochloride denatured native and aged AK, both recover to the same activity as the fresh enzyme. Mass spectroscopy and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole chloride modification kinetics studies identify that the residue cysteine-25 of the enzyme reacts with DTT to form an adduct. The formation of the unusual bridging DTT adduct of AK appears to be the result of a stable DTT–protein complex. The K_M for AMP, ADP and MgATP of the DTT-modified enzyme does not differ significantly from that of the intact enzyme, whereas the secondary and tertiary structures of the enzyme change obviously. These results indicate that cysteine-25 may not be involved directly in substrate binding, but may play an important role in maintaining secondary and tertiary structures of native AK, as well as the conformation interconversion in the catalytic cycle. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Protein aging; Multiple native form; Disulfide bridge; Dithiothreitol adduct; Conformation interconversion; Rate-limiting step

1. Introduction

Because of its low redox potential (−0.33 V at pH 7) and its rapid rate of recyclization, DL-dithiothreitol (DTT) was considered not to form stable mixed disulfide compounds and therefore was widely used as a protective reagent in redox-related protein studies [1]. However, Li et al. [2] first reported a DTT adduct of RNase A in which an intramolecular disulfide loop is formed by a bridging DTT with two intermolecular disulfide bonds.

Adenylate kinase (AK, EC 2.7.4.3) catalyzes the reaction MgATP+AMP⇔MgADP+ADP, which is essential for cell survival [3–10]. This small kinase, containing two cysteine residues and no disulfide bond, has also been considered a 'model kinase' in the study of structure–function relationship of kinases [11]. In order to protect oxidation of free thiol groups of AK, disulfide compounds such as DTT were often used as protective reagents.

It is shown in the present report that AK ages at 4°C in the presence of DTT to a conformation with marked decreasing activity. Mass spectroscopy and kinetics studies identify that the residue cysteine-25 of the enzyme reacts with DTT to form an adduct. This result demonstrates that DTT can form a stable mixed disulfide bridge with protein thiols by interacting with specific local residues. The K_M for AMP, ADP and MgATP of the DTT-modified enzyme does not differ significantly from that of the intact enzyme, whereas its activity is obviously lower than that of the intact enzyme. This indicates that the conformational interconversions might be the rate-limiting step in the catalytic mechanism of AK.

2. Materials and methods

2.1. Reagents

ATP, ADP, AMP, NADP, NADH, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, hexokinase, glucose-6-phosphate dehydrogenase, DTNB (5′,5′-dithio-bis(2-nitrobenzoic acid)), DTT, and NBD chloride (7-chloro-4-nitrobenz-2-oxa-1,3-diazole) were Sigma products. Guanidine hydrochloride (GdmHCl), ultrapure, was obtained from Life Technologies (USA). All other reagents were local products of analytical grade. GdmHCl solution, DTT solution, K^+-HEPES buffer and Tris–HCl buffer were always prepared fresh just before being used.

2.2. Preparation and activity assay of AK

The enzyme was prepared essentially according to Zhang et al. [12]. The final preparation had a specific activity greater than 2000 U/mg which showed only a single peak in SDS electrophoresis, gel filtration and reversed-phase FPLC. One unit is defined as the amount of enzyme catalyzing the formation of 1 μmol ATP generated from ADP per minute in the backward reaction or the formation of 1 μmol ADP per minute in the forward reaction.

The activity of ADP formation was measured by a coupled enzyme procedure with pyruvate kinase and lactate dehydrogenase. The decrease of the NADH concentration was followed at 340 nm in a UV-1601 spectrophotometer (Shimazu, Japan), thermostatted at 25°C. The final assay mixture was: 50 mM Tris–HCl, pH 7.5, 2 mM β-mercaptoethanol, 75 mM KCl, 4 mM phosphoenolpyruvate, 2.5 mM $MgCl_2$, 0.2 mM NADH, 20 U/ml pyruvate kinase, 40 U/ml lactate dehydrogenase, 2 mM ATP, 2 mM AMP and 0.5 mg/ml bovine serum albumin.

The activity of ATP formation was measured by a coupled enzyme procedure with hexokinase and glucose-6-phosphate dehydrogenase. The reaction mixture contained 50 mM Tris–HCl, pH 8.1, 2 mM β-mercaptoethanol, 6.7 mM glucose, 0.67 mM NADP, 0.5 mg/ml bovine serum albumin, 2.1 mM $MgCl_2$, 10 U of hexokinase, 20 U of glucose-6-phosphate dehydrogenase. The measurements were done by following the reduction of NADP at 340 nm in a UV-1601 spectrophotometer (Shimazu, Japan), thermostatted at 25°C.

The concentration of AK was determined by measuring the absorp-

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Abbreviations: AK, adenylate kinase; GdmHCl, guanidine hydrochloride; DTNB, 5′,5′-dithio-bis(2-nitrobenzoic acid); NBD, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; DTT, DL-dithiothreitol

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tion at 280 nm with $A^{1\%}_{1cm} = 5.2$; the concentration of DTNB was determined with $\varepsilon_{324} = 17.78 \times 10^3$ M^{-1} cm^{-1}.

2.3. Methods

The numbers of free thiol groups in cysteine residues were determined using Ellman's reagents for fresh and aged AK. The reaction system contained 5.0 μM AK, 0.5 mM DTNB in 50 mM Tris–HCl buffer, pH 8.1. The measurements were done at 412 nm and 25°C with a UV-1601 spectrophotometer. The sulfhydryl group modification reactions with NBD chloride were carried out by addition of a small aliquot of a solution of NBD chloride in ethanol according to Price and Cohn [13]. The final concentration of ethanol was below 1% and was shown to have no effect on the activity of the enzyme. The kinetic reactions of NBD chloride with AK are shown in the form of a semilogarithmic plot. From the later, linear portion of the plot the pseudo first order rate constant of the slow phase of the reaction can be obtained. Division by the concentration of NBD chloride gives the second order rate constant. After correction for the contribution of the slowly reacting group a semilogarithmic plot for the fast reacting group is constructed, and the rate constant for the fast reaction can be obtained from the slope, as for the slow reaction.

For detecting the global conformational change of AK, circular dichroism (CD) measurements in the far-UV region of 200–250 nm were made on a Jasco-720 spectropolarimeter with a cuvette of path length 0.1 mm, while the near-UV CD measurements ranged from 260 to 320 nm with a cuvette of path length of 3 mm. Spectra were collected with a 0.5 nm band width, response time of 1.0 s, and scan speed of 20 nm/min. Each spectrum was obtained as an average of six runs and corrected for contribution of the buffer.

The digestion process of AK by trypsin was performed in 50 mM Tris–HCl buffer, pH 8.1, 1 mM EDTA at 20°C overnight. The molar ratio of trypsin:AK as 1:100 (AK concentration is 1.0 mg/ml).

Matrix-assisted laser time of flight mass spectrometry (MALDI-TOF MS) was used for analyzing the molecular weight of AK and mixture of digestion peptides. The measurement was operated in linear mode on a BIFLEX3 type mass spectrometer made by Bruker (USA). The instruments use a fixed frequency laser (N_2 laser source at 337 nm) and ion extraction voltage is 20 kV, linear flight distance is 65 cm. A delay extraction accessory was introduced to improve the mass resolution (operating voltage is 15 000 V). XTOF software, a Windows-based program available from Bruker, was used for data processing and plotting. The sample was desalted and dissolved in a sulfosalicylic acid matrix to a concentration of 5×10^{-6} M.

3. Results

3.1. Aging of freshly prepared AK in the absence and in the presence of various concentrations of DTT

Freshly prepared AK usually had a specific activity of 2000 ± 100 U/mg for the ATP formation reaction. Upon aging of 1.0 mg/ml enzyme solution in the presence of 10 mM fresh DTT in 50 mM Tris–HCl buffer, pH 8.1, the activity decreases gradually (Fig. 1). In the range of 10–65 mM, the aging process is independent of the concentration of DTT. The aging process is not changed yet, when DTT is in its oxidation form (data not shown). In the absence of DTT, the activity remains fairly stable at 4°C and slightly decreases to about 1800 U/mg in 10 days. Substrates can protect the activity of aged enzyme from decreasing. In the presence of 10 mM DTT and 3 mM ADP or MgATP, the activity decreases at 4°C in 12 days to about 1600 U/mg; in the presence of 10 mM DTT and 3 mM AMP or MgADP to about 1200 U/mg. The concentration of enzyme has a great influence on the activity decrease. At the beginning of aging, the lower the aged enzyme concentration, the faster the activity decreases (data not shown). The activity change of the ADP formation reaction shows no significant difference from that of the ATP formation reaction (data not shown).

When the activity of aged AK in the presence of 10 mM DTT decreases to lower than 1200 U/mg, the light scattering intensity of the aged AK solution begins to increase with time, implying the formation of aggregation (inset in Fig. 1). The aged enzyme, in the presence of 10 mM DTT with activity higher than 1200 U/mg, after denaturation in GdmHCl can be refolded by dilution with activity restored near to that of the fresh enzyme. However, aged AK incubated for a longer period with activity lower than 1200 U/mg is no longer fully reactivable. Hereafter, unless specially indicated, aged AK refers to the enzyme aged in the presence of 10 mM DTT with a specific activity more than 1200 U/mg.

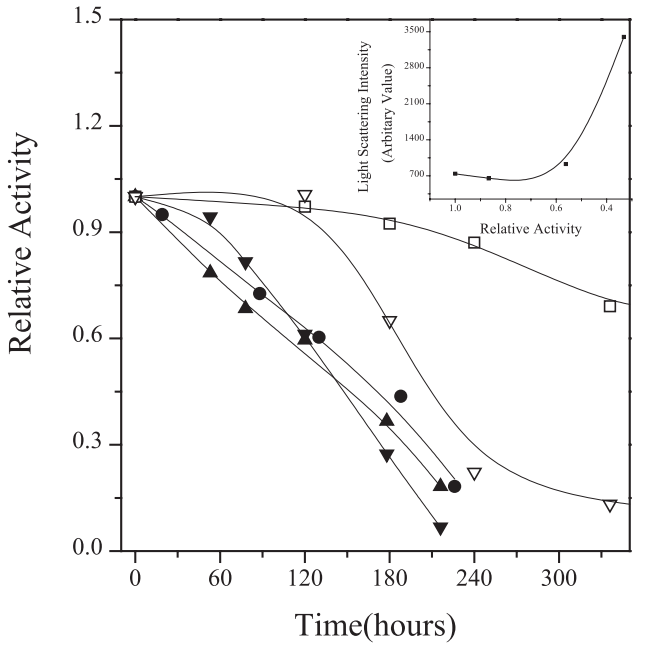

Fig. 1. Aging of freshly prepared AK at different temperatures or different concentrations of DTT. Freshly prepared AK, 1.0 mg/ml, in 50 mM Tris–HCl buffer (pH 8.1) containing 10 mM DTT (▼), 20 mM DTT (●), or 65 mM DTT (▲), was aged at 20°C; or aged in the same buffer containing 0 mM DTT (□) or 10 mM DTT (▽) at 4°C. The activity of the ATP formation reaction was assayed at 25°C at different time intervals as indicated. Inset plot is the light scattering intensity versus relative activity of AK. The activity change of the ADP formation reaction is no different from that of the ATP formation reaction (data not shown).

Fresh AK has a molecular mass of 21 685 Da, determined by MALDI-TOF MS, with a resolving power of 10^3. Upon aging, a new peak appears in the position of molecular mass 21 837 Da, which is about the molecular mass of fresh AK (21 685 Da) plus 152 Da. The intensity of peak 21 685 decreases with aging time, while that of peak 21 837 increases. The difference of 152 in molecular mass between fresh and aged AK is exactly the molecular mass of oxidized DTT, indicating that aged species are adducts of fresh AK and oxidized DTT through intermolecular (mixed) disulfide bonds, namely a bridging oxidized DTT.

In order to determine the binding site of DTT, aged and fresh AK was digested by trypsin. TOF MS was used for analyzing the molecular weight of the digested fragments. Two new peaks appeared in the aged AK sample. One is in the position of molecular mass 1331 Da, which is about the molecular mass of a fragment of residues 22–31 plus the molecular mass of oxidized DTT, the other is in the position of molecular mass of 1963 Da, which is about the molecular

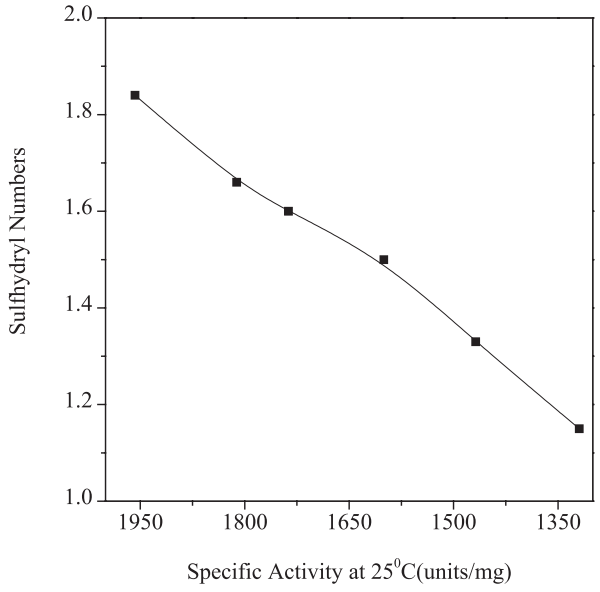

Fig. 2. Sulfhydryl numbers of aged AK with different activity remaining. 5.0 μM AK with different activity reacts with 0.5 mM DTNB in 50 mM Tris–HCl buffer, pH 8.1 at 25°C.

mass of a fragment of residues 10–27 plus the molecular mass of oxidized DTT. No new peak in the aged AK sample was found in the molecular mass range of a fragment containing cysteine-187 plus DTT (about 2344 Da). This result indicates that the binding site of DTT is at cysteine-25.

For fresh AK, the reaction of the free sulfhydryl groups with DTNB proceeds to completion in several minutes (data not shown), and about 1.9 eq of DTNB complex formed per mol of enzyme molecular consumed. This agrees closely with the reported value of two sulfhydryl groups in AK [14]. Upon aging, the free sulfhydryl groups decrease linearly with remaining activity of the aged enzyme in the native state (Fig. 2). Only one free sulfhydryl group can be modified by DTNB for aged AK with 1200 U/mg activity remaining.

It was reported that the thiol group of cysteine-25 in porcine muscle AK reacts with NBD chloride approximately 40-fold faster than the other one of cysteine-187 because of their different locations on the interior or in the surface of the enzyme molecular [13]. Here the reaction of aged or fresh rabbit muscle AK with NBD chloride was also carried out under pseudo first order conditions to facilitate the kinetic analysis. The rate constants for reactions between fresh or aged AK and NBD chloride can be obtained from the slopes of the semilogarithmic plot (Fig. 3). The second order rate constants with NBD chloride for aged AK with 1400 U/mg activity were approximately 1330 M^{-1} min^{-1} for the fast phase and 108 M^{-1} min^{-1} for the slow phase. For aged AK with 1200 U/mg activity, the second order rate constants were approximately 320 M^{-1} min^{-1} for the fast phase and 102 M^{-1} min^{-1} for the slow phase; and for fresh AK, 2300 M^{-1} min^{-1} and 115 M^{-1} min^{-1}. The burst phase amplitude and the reactivity of the fast reacting SH was reduced, while the reactivity of the slow reacting SH was basically unchanged, and there is hardly any burst phase existing in modifying kinetics of aged AK with 1200 U/mg remaining activity. The above results implied that it was mainly the thiol group of cysteine-25 that was involved in the reaction with oxidized DTT during the AK aging process.

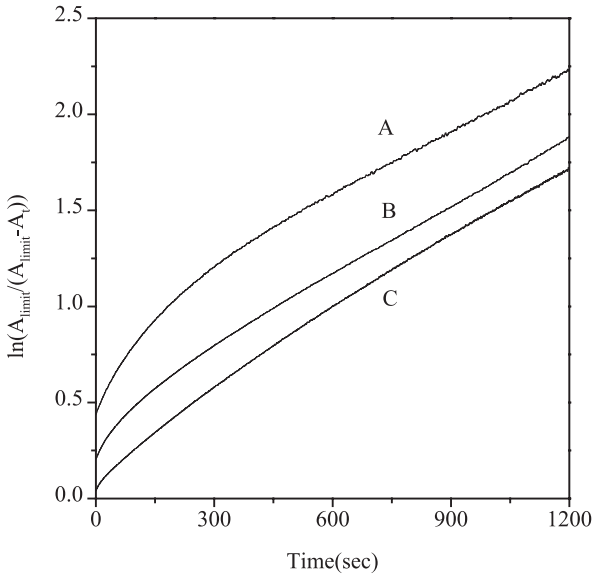

Fig. 3. The kinetic reaction of NBD chloride with fresh or aged AK in the form of a semilogarithmic plot. 375 μM NBD chloride reacts with AK (line A: 3.2 μM for fresh AK with 1900 U/mg activity; line B: 5.1 μM for aged AK with 1400 U/mg activity; line C: 5.1 μM for aged AK with 1200 U/mg activity) in 50 mM K^+–HEPES, pH 7.9, at 25°C. A_t represents the absorbance change at 420 nm after time t, and A_∞ is the limiting change in absorbance at 420 nm.

3.2. The effects of fresh DTT on aged AK

Aged AK, with 1200 U/mg remaining activity and about 1.0 eq free thiol group determined by Ellman's reagent, can recover to 1800 U/mg activity and about 1.9 eq free thiol groups when incubated with 10 mM fresh DTT for 20 h (Fig. 4). Moreover, aged AK with a molecular mass of 21 835 Da

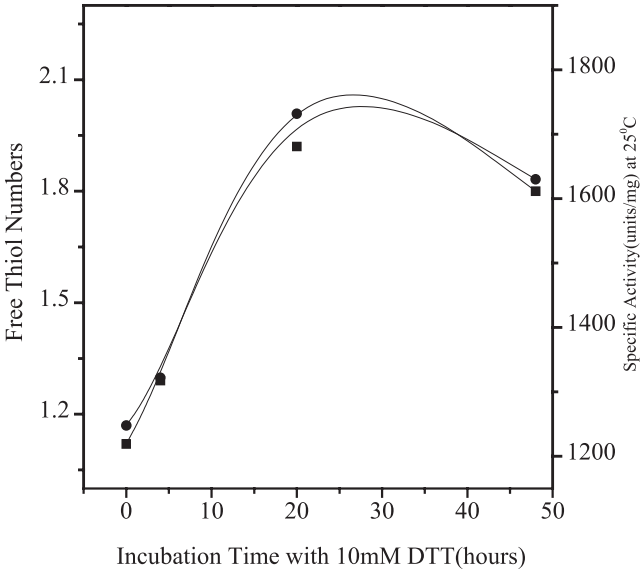

Fig. 4. The effects of 10 mM fresh DTT on sulfhydryl group numbers and specific activity of aged AK. 1.0 mg/ml aged AK with 1200 U/mg remaining activity and about 1.0 eq free thiol group was incubated with 10 mM fresh DTT at 20°C, then at different intervals as indicated. Catalytic activity or free thiol group numbers (free DTT molecules were removed through a desalting column) were measured. Circles: specific activity of AK; squares: free thiol group numbers determined by Ellman's reagent.

can recover to a molecular mass of 21 691 Da by fresh DTT determined by MALDI-TOF MS.

3.3. Conformational difference of aged from fresh AK

Like the fully active enzyme, aged AK with decreased activity shows a single peak with similar retention time on size exclusion chromatography indicating no aggregated dimer species of low activity existing. However, upon aging, it shows a significant decrease in the amplitude of negative ellipticity in CD spectra in both the far- and near-UV regions (Fig. 5) suggesting obvious changes in secondary and tertiary structures.

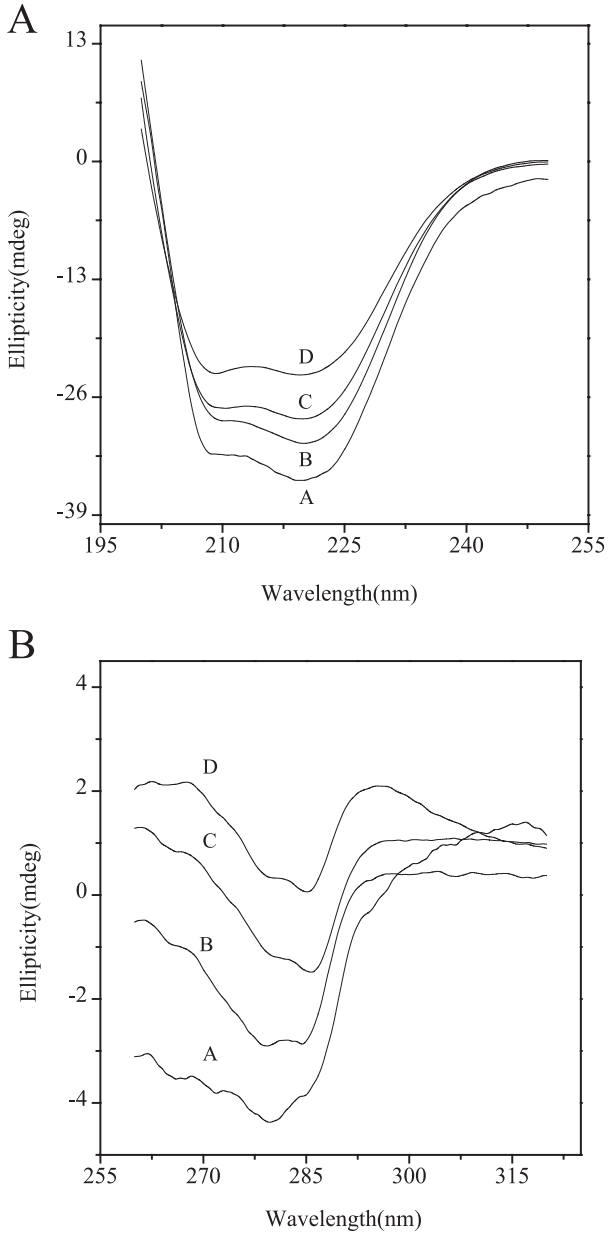

Fig. 5. CD spectra of AK with different activity. The concentration of enzyme was 100 μM in 50 mM Tris–HCl buffer containing 2 mM EDTA, pH 8.1. CD studies in the far-UV region of 200–250 nm or in the near-UV region of 260–320 nm were done at 20°C with a 0.1 mm or a 3 mm cuvette, respectively. A: Far-UV CD spectra, curves A–D represent enzymes with activity 1820, 1690, 1400 and 1090 U/mg, respectively. B: Near-UV CD spectra, curves A–D represent enzymes of activity 1820, 1690, 1400 and 1090 U/mg, respectively.

Table 1
Kinetic parameters of fresh and aged AK

	Activity 1800 U/mg (mM)	Activity 1200 U/mg (mM)
K_M(ADP)	0.015 ± 0.001	0.015 ± 0.001
K_M(MgADP)	0.026 ± 0.002	0.052 ± 0.003
K_M(MgATP)	0.060 ± 0.010	0.073 ± 0.015
K_M(AMP)	0.120 ± 0.01	0.105 ± 0.02

The K_M values of aged and fresh AK are summarized in Table 1. Comparing with fresh AK, the K_M for AMP, ADP, and MgATP of aged AK does not change greatly, while the K_M for MgADP of aged AK is two-fold higher than that of fresh AK. However, the activity change of the ADP formation reaction shows no difference from that of the ATP formation reaction, indicating that the substrate binding should not be the rate-limiting step in the catalytic cycle of AK.

4. Discussion

4.1. Activity decrease of aged AK might result from the formation of an adduct between oxidized DTT and the thiol group of cysteine-25

Aging of proteins in solution may result from certain covalent changes. It was reported that aging of α-B-crystallin leads to deamidation of asparagine and peptide cleavage [15]. The results here have provided some evidence for intermolecular disulfide bridge formation between oxidized DTT and the thiol group in cysteine-25 of AK.

X-ray crystallographic structure shows that the environments of the cysteine-25 and cysteine-187 residues of AK are markedly different. Cysteine-25 is located in the interior of the enzyme, whereas cysteine-187 is on the surface of the structure [16]. The rate of the bimolecular reaction of DTT with cysteine-187 should be much slower than that of oxidized DTT recyclization, so DTT cannot modify cysteine-187. Cysteine-25 is located in the interior and close to the catalytic center of the enzyme, and as a result, first DTT could approach the enzyme close to the location of cysteine-25 via a cleft, then DTT reacts with cysteine-25 to form an adduct. The same reaction properties of cysteine-25 and cysteine-187 were also observed in NBD chloride modification. Cysteine-25 reacts with NBD chloride approximately 40-fold faster than cysteine-187 [13]. Moreover, that the concentration of DTT has no effect on the aging process also suggests that the reaction of the enzyme with DTT is under a two-step mechanism. Firstly, the enzyme binds rapidly with DTT to form a stable complex:

$$E + DTT \Leftrightarrow E{\cdot}DTT$$

Then, the enzyme–DTT complex should take a mono-molecular reaction to form the intermolecular disulfide bond:

$$E{\cdot}DTT \xrightarrow{slow} E{-}DTT$$

This mono-molecular process is independent of the DTT concentration.

4.2. The structural and functional role of cysteine-25

According to the X-ray structure, cysteine-25 is located in

the interior of the molecule, close to the catalytic center [16], but this Cys-25-Ser mutant was found to have relatively little perturbed kinetic constants: $K_{cat} = 113\ s^{-1}$; $K_m(MgATP) = 0.073$ mM (as for wild-type AK, $K_{cat} = 610\ s^{-1}$; $K_m(MgATP) = 0.042$ mM) [17]. Combined the kinetic analysis of the mutant C25S with the interaction studies for histidine-36 and cysteine-25, these results suggest that the sulfhydryl groups are not directly involved in the catalytic mechanism or in substrate binding. From our results, since the K_M values for AMP, ADP and MgATP of DTT-modified AK do not differ significantly from that of intact enzyme while the specific activity decreases, one is led to conclude that the sulfhydryl groups might not play a direct role in substrate binding. The same conclusion was also obtained in a very early work by Kress et al. [18].

4.3. *Conformation interconversion are the rate-limiting step in the catalytic mechanism of AK*

Although the catalytic kinetics of AK have been studied extensively [19–23], a full understanding of the mechanism is still lacking. The basic kinetic pattern is random Bi–Bi [20], but whether the chemical step or the physical step(s) is rate-limiting is again controversial. Furthermore, it was reported by Kuby et al. [21] that the nature of the AP_5A inhibition changes qualitatively from competitive inhibition with respect to either substrate in the forward reaction (MgATP or AMP) to non-competitive in the backward reaction with either substrate (MgADP or ADP). In order to give a convincing explanation for the inhibitory nature of AP_5A, an iso-random Bi–Bi catalytic mechanism based on the multiple native forms of AK was proposed, one form of AK can bind with substrates MgATP and AMP, while the other can bind with MgADP and ADP. In a catalytic cycle, the conformation interconversion of the free enzyme and the enzyme–substrate ternary complex were presumed to be the rate-limiting steps [24].

The K_M values for AMP, ADP and MgATP of DTT-modified AK do not change greatly, indicating that the conformational changes of the DTT-modified AK have no great effects on its substrate binding ability. The activity decrease of DTT-modified AK should result from the decrease of the conformation interconversion rates, suggesting that the conformation interconversion is the rate-limiting step in the catalytic cycle of AK.

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