BioMetals **15:** 421–427, 2002.

Isolation of GIF from porcine brain and studies of its zinc transfer kinetics with apo-carbonic anhydrase

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Received 28 January 2002; accepted 2 March 2002

Key words: carbonic anhydrase (CA), GIF (Cu_4Zn_3MT-3), zinc transfer, kinetic

Abstract

Neuronal growth inhibitory factor (GIF) of porcine brain, was isolated and purified by a similar procedure which was used on the isolation of human and bovine GIF. The native porcine protein with stoichiometry of $4Cu^{+}$, $3Zn^{2+}$ was obtained for the first time. The kinetics of zinc transfer from Cu_4Zn_3MT-3 to apo-carbonic anhydrase were studied, and zinc transfer rate constants and thermodynamic parameters were obtained. It is found that like other MTs, porcine Cu_4Zn_3MT-3 can also transfer its zinc atom to apoCA, even much easier than other MTs. A possible association mechanism has been proposed, the formation of Cu_4Zn_3MT3-apoCA complex may be the rate-determining step. The obtained data indicate besides its neuronal growth inhibitory function, GIF might play a role in cellular Zn homeostasis in brain.

Introduction

Metallothioneins (MTs) are a group of low-molecular weight, cysteine and metal rich metalloproteins (Margoshes *et al.* 1957). Although metallothioneins have been known for over 40 years, their special structures and important functions still provoke the interest of many scientists (Kägi *et al.* 1998; Vašák *et al.* 2000). It was reported that MTs (Cd_5Zn_2MT-1/-2 and Zn_7MT-1/-2) were involved in heavy metal detoxification and essential metal homeostasis (Sadhu *et al.* 1989). Cu_4Zn_3MT-3, also called neuronal growth inhibitory factor (GIF), is a metalloprotein associated with Alzheimer's disease (Uchida *et al.* 1991). Some excellent studies have been conducted on GIF of human, bovine and rat brain using UV, CD, MCD, luminescence, EXAFS, ^{113}Cd NMR and ^{1}H NMR spectroscopies (Bogumil *et al.* 1996, 1997, 1998; Faller *et al.* 1999; Vašák *et al.* 2000). Nevertheless, only a few studies were carried out on the isolation and characterization of MT3 from porcine (Chen *et al.* 1996), and until now, the stoichiometry of Cu, Zn in native porcine GIF is not ascertained. In this work we report the detailed isolation and characterization of porcine MT3.

Although native GIF (68 amino acids) exhibits approximately 70% amino acid sequence identity to the family of mammalian metallothioneins (MT-1 and MT-2 isoforms, 61 amino acids), including the preserved array of 20 Cys residues, only GIF exhibits growth inhibitory activity in neuronal cell culture assays (Uchida *et al.* 1991). When compared with mammalian MTs, the changes in the primary structure of MT3 result in an obviously increased structural flexibility, which may be described for its biological function (Faller *et al.* 1999). It is generally believed that MT1 and MT2 play a role in the regulation of zinc distribution in cells and organisms, such as it could serve as a reservoir for zinc while preventing metal toxicity and also involved in zinc transfer to apo-metalloproteins (Nielson *et al.* 1983; Stillman *et al.* 1987). Accordingly, MT3 is proposed to exhibit the same function as MT1 and MT2 in zinc transfer reaction. Thus, we studied zinc transfer kinetic

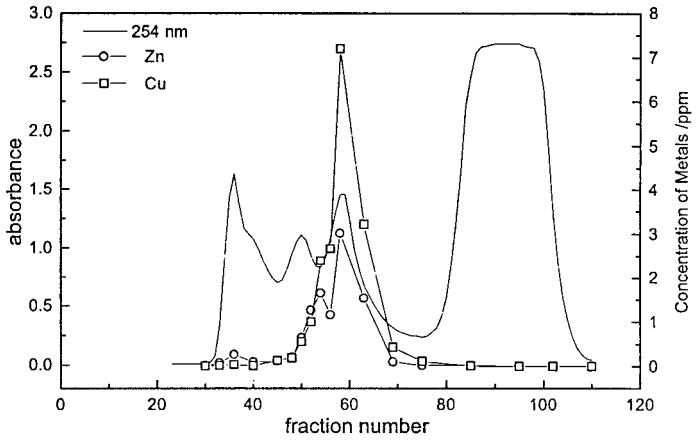

Fig. 1. Gel-filtration elution profiles of the resultant solution of porcine brain tissues. Elution was performed on a Sephadex-G75 column, 2.6 × 90 cm, equilibrated with Tris-HCl, 0.01 M, pH 7.6, 5.0 ml aliquots of fractions were collected and monitored by the absorbance at 254 nm and the content of Cu and Zn in each fraction was determined by ICP method.

from Cu_4Zn_3MT3 to apoCA. It might be helpful in understanding the native GIF functions.

Experimental

Materials

Sephadex G-75, G-50, G-25 were purchased from Parmacia, DEAE-Cellulose DE-52 from Watman, Tris base, 1,4-Dithithreitol (DTT), bovine carbonic anhydrase (BCA) and p-Nitrophenyl acetate were purchased from Sigma. Other reagents are from Reagent. grade.

Isolation of identification of porcine GIF

Isolation and purification were performed by combination methods used for isolation of human brain (Uchida *et al.* 1991) and bovine brain (Bogumil *et al.* 1996). All buffers were nitrogen saturated or degassed prior to use to minimize oxidation. 160 g porcine brain from health porcine was homogenized with 500 ml mixture of 0.01 M Tris-HCl pH 7.60 buffer solution, anhydrous ethanol, chloroform (v/v 1.00:1.00:0.03) and 1 mmol DTT. The homogenate was centrifuged at 43, 000 × *g* for 30 min at 4 °C to remove the precipitate. Three volumes of anhydrous ethanol (−20 °C) were slowly added to the supernatant with stirring. After incubation at −20 °C overnight. The precipitate was collected by centrifuged at 28, 000 × *g* for 30 min, the pellet was dissolved in 15 ml of buffer (0.01 M Tris-HCl, pH 7.6) by stirring for 30 min, The suspension was centrifuged again at 30, 000 × *g* for 30 min and dried at room temperature. The dried precipitate was dissolved in 10 mM Tris/HCl buffer solution, pH 7.60, then centrifuged at 28, 000 × *g* for 30 min. The resultant supernatant was applied to a Sephadex G-75 column, 2.6 × 90 cm, preequilibrated with 10 mM Tris/HCl buffer solution, pH 7.60, and eluted at a flow rate of 1 ml/min with the same buffer. 5 ml aliquot fractions were collected, the absorption was monitored at 254 nm. The metal contents in each fraction were determined by ICP. The Cu,Zn-rich fractions were pooled, ultrafiltrated and lyophilized. The sample was dissolved in 5 ml 20 mM Tris/HCl, pH 7.60 buffer solution and further purified on a DEAE-Cellulose DE-52 column, 1.0 × 20 cm, preequilibrated with 20 mM Tris/HCl buffer solution, pH 7.60. A continuous linear gradient of 5–300 mM NaCl in the above buffer solution was used to separate proteins and the absorption was monitored at 254 nm. Meanwhile, the metal contents in each fraction were again determined by ICP. The metal-rich fractions were pooled, and desalted on a Sephadex G-25 column, 1.6 × 50 cm, preequilibrated with water, using water as eluant. The fractions of GIF was pooled and lyophilized. The content of Cu, Zn and S per mol protein, were determined on JOBIN YVON JY38S inductively coupled ploasma (ICP) spectrophotometer according to the previous literature (Bongers *et al.* 1988); the scanned emission lines were, Cu 324.754 nm, Zn 213.856 nm, S 181.987 nm, respectively. Amino acid composition was analyzed with amino acid analyzer after hydrolysis with 6 M HCl at 110 °C for 24 h.

HPLC analysis and SDS-PAGE

The purified sample solution was injected onto a C_{18} reverse-phase column. (Hewlett Packapd 300SB 0.46 × 25 cm) equilibrated in 5 mM ammonium acetate, pH 6.9. Elution was performed with a linear gradient from 0 to 80% acetonitrile in the same buffer solution at a flow rate of 1 ml/min (30 min) with detection at 254 nm. The SDS-PAGE was used to confirmed the purity of GIF. The gel was cast in an electrophoretic apparatus (Mini-protean II, Bio-Rad). The separation gel contains 16.5% total acrylamide (6% bis-acrylamide), 1 M Tris/HCl, pH 8.45, 0.1% SDS, 0.08% ammonium persulfate and 5 μM of TEMED. The stacking gel consists of 5% total acrylamide, 750 mM Tris/HCl, pH 8.45, and other components as separation gel. Sample with equal volume of sample buffer (50 mM Tris/HCl, pH 6.8, 1 mM DTT, 2% SDS, 0.1% Bromophenol Blue, 10% glycerol) were mixed and boiled for 3 min before loading into the well. After electrophoresis, the gel was stained by Coomassie Blue (G-250, 0.025%). The molecular weight of the marker were 43, 29, 18.4, 14.3, 6.2, 3.4, 2.3 kDa, respectively.

Preparation of other metallothionein isoforms

Rabbit liver Cd_5Zn_2MT and Zn_7MT were prepared as described (Comeau, *et al.* 1992). All of them were characterized by amino acid analysis and metal content in them were determined by ICP. The protein concentration of MT1 and MT2 were determined by the absorbance at 220 nm of apo-MT at pH 2 ($\varepsilon_{220} = 47,300\ \text{mol}^{-1}\ \text{l}^{-1}\text{cm}^{-1}$) (Buhler *et al.* 1979). The concentration of MT3 was determined by the absorbance at 255 nm. ($\varepsilon_{255} = 48,000\ \text{mol}^{-1}\ \text{l}^{-1}\ \text{cm}^{-1}$) (Bogumil *et al.* 1996).

Reaction of metallothionein with apo-CA

Zinc transfer reactions from Cu_4Zn_3MT-3 to apo-CA were studied according to the literature method (Armstrong *et al.* 1966). Briefly, Cu_4Zn_3MT3 was incubated with apoCA in 0.01 M Tris/HCl buffer solution (pH 7.60) at certain temperature and monitor the enzyme activity of CA in a certain time interval within 1 hour. Since the hydrolytic rate of p-nitrophenyl acetate is proportional to the concentration of reconstituted enzyme, the formation of p-nitrophenolate can be monitored at 348 nm. A linear best fit was used to calculate the reactive rate, activity is expressed as percent of that measured for native CA. The concentration of native CA was determined by ultraviolet absorption ($\varepsilon_{280} = 57,000\ \text{mol}^{-1}\ \text{l}^{-1}\ \text{cm}^{-1}$) (Armstrong *et al.* 1966).

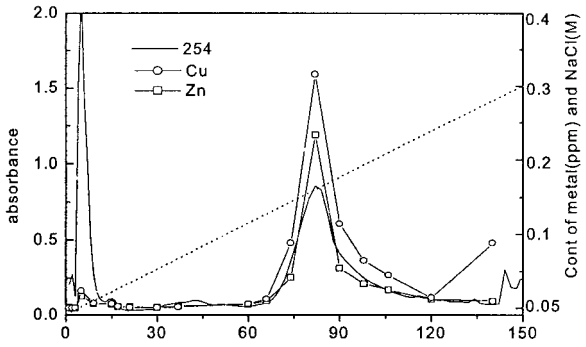

Fig. 2. Ion exchange chromatography of Cu,Zn-MT sample on DEAE-Cellulose DE-52 column, 1.0 × 20 cm, eluted with a continuous linear gradient of 5–300 mM NaCl in 0.02 M, pH 7.6, Tris-HCl buffer solution, the elution was monitored by absorbance at 254 nm and the content of Cu and Zn in each fraction was determined by ICP method.

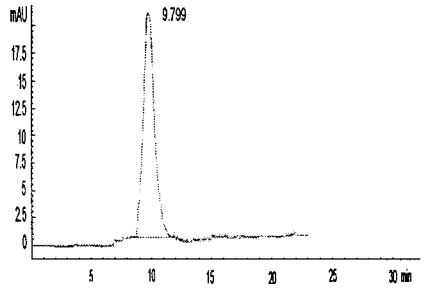

Fig. 3. Chromatographic property of purified porcine GIF as determined by HPLC. C_{18} reverse-phase HPLC column (Hewlett Packapd 300 SB 0.46 × 25 cm). A linear gradient from 0∼80% acetonitrile in 5 mM ammonium acetate, pH 6.9, flow rate of 1 ml/min, detection with 254 nm.

Results and discussion

Isolation and identification of GIF

GIF was isolated from porcine brain by means of a procedure as described in former with a yield of 1.5–2.0 mg GIF/Kg porcine brain tissue. A typical elution profile monitored at 254 nm is shown in Figure 1, in which there are a high molecular weight component between 30 and 50 fractions, and a small molecule component from 70 to 110 fractions. Both of them almost have no metal of Cu and Zn. The fractions from 50 to 70 rich of Cu, Zn were collected, desalted and concentrated, then subjected to DEAE-Cellulose DE-52 column (Figure 2). The metal rich fractions from

70 to 90 appeared as a peak at the NaCl concentration of approximately 0.2 M on the elusion profile. These fractions were pooled, desalted and lyophilized. The purity of isolated porcine brain protein was determined by HPLC and SDS-PAGE. The elution profile of GIF on a C_{18} reverse-phase HPLC column is shown in Figure 3. The protein was eluted as a single peak at 25% acetonitrile. In addition, as shown in Figure 4, SDS-PAGE indicates a single protein band with a molecular weight of approximately 6–7 kDa. These evidences suggest that the obtained protein is pure. The content of Cu, Zn and S determined by ICP showed that the protein contains 3.7 Cu^{+}, 2.6 Zn^{2+} and 21S/mol protein. This result is consistent with the Cu, Zn, S stoichiometry in GIF of human and bovine (Bogumil *et al.* 1996; Uchida *et al.* 1991). In order to confirm that the obtained protein is really MT3 rather than MT1 and MT2, the amino acid composition of the protein was further analyzed, and the results are listed in Table 1. According to literatures, a significant difference was found between MT3 and other mammalian MTs isoforms. There are 8∼9 Glu residues in MT3 of human, bovine and horse, while 1∼2 Glu residues in their MT1 and MT2. (Kobayashi *et al.* 1993; Pountney *et al.* 1994; Uchida *et al.* 1991). The content of Glu residue in protein can be regarded as a characteristic of MT3 which distinguished itself from other mammalian MT1 and MT2 isoforms. In our case, near 8 Glu residues was found, which is consistent with the content of Glu residues in GIFs reported previously. All above obtained results show that a native porcine brain MT3 with stoichiometry of $4Cu^{+}$ and $3Zn^{2+}$ was obtained for the first time.

Spectral characteristics of Cu_4Zn_3MT3

Since aromatic amino acids and histidine are absent in mammalian MTs (Margoshes *et al.* 1957), the optical properties of MT will mainly contribute from the metal-thiolate cluster and the polypeptide chain. Figure 5a shows a typical absorption spectra of Cu_4Zn_3MT3. There is a characteristic absorption shoulder near 260 nm originate predominately from Cys-Cu (I) LMCT transitions (Hasler *et al.* 1998). Due to the high stability of the Cu(I)-GIF complex (Uchida *et al.* 1991), the apo-GIF was obtained by exposure the native GIF to approximately 1 M HCl. Then applied on gel filtration to remove the metal ions. By comparison with the absorption of native GIF, the absorption near 260 nm disappeared in Figure 5b. It suggested that the Cu^{+} has been separated from the native GIF.

Zinc transfer from Cu_4Zn_3MT3 to apo-CA

The curves of the restoration of apo-CA activated by Cu_4Zn_3MT3 versus incubation time for different molar ratio of [Cu_4Zn_3MT3]/[apoCA] at pH 7.6 in 10 mM Tris/HCl buffer, 25 °C were illustrated in Figure 6. It shows that when the ratio of [Cu_4Zn_3MT3]/[apo-CA] = 2.22, Cu_4Zn_3MT3 activates apo-CA and restores CA activity to about 75% after 1 h. When the molar ratio are 1.00, 0.44 and 0.33, the acticity restoration of apoCA are 49%, 37% and 20%, respectively. It is obvious that the restoration of apoCA depends on the molar ratio of [Cu_4Zn_3MT3]/[apoCA]. On the other hand, it can be seen from the result, although the apparent concentration of zinc ion in the experiments is much higher than the concentration of apo-CA, the enzyme activity of apo-CA is restored partly. That is to say, no more than one zinc ion per Cu_4Zn_3MT-3 molecular is involved in the zinc transfer process. As a comparison, we have also investigated the activity restorations of apoCA activated by other metallothionein isoforms (Zn_7MT-1/-2 and Cd_5Zn_2MT-1/-2). Under the same condition as described above, when the molar ratio of [MT]/[apoCA] ≈ 1, ([apoCA] = 2.67 μm, [MT] = 2.86 μm), after incubated at 25 °C for 1 h, the activity restorations of apoCA activated by Cu_4Zn_3MT3, Zn_7MT-1, Zn_7MT-2, Cd_5Zn_2MT-1 and Cd_5Zn_2MT-2 are 49.5%, 44.1%, 37.6%, 36.2%, and 22.3%, respectively. These results show that the ability to donate its zinc ion to apoCA for Cu_4Zn_3MT3 is even more than those for MT1 and MT2. The reason for the phenomenon is not known clearly, it may be related to the detailed folding of Cu_4Zn_3MT3. A CPCP tetrapeptide in the N-terminus of Cu_4Zn_3MT3 probably leads to relaxation of the β-domain. Zinc ion can be released from Cu_4Zn_3MT3 molecular more easily than from other metallothionein isoforms, since the solution structure of α-domain for MT3 determined by NMR method is similar to that for MT1 and MT2, besides adding a loop in the C-terminus (Öz *et al.* 2001). The curves of the restoration of apo-CA activated by Cu_4Zn_3MT3 versus incubation time at various temperatures in 10 mM Tris/HCl buffer pH 7.6 were illustrated in Figure 7. It shows that in the molar ration of [Cu_4Zn_3MT3]:[apoCA] = 1, when the temperature are 15 °C, 25 °C, 30 °C and 37 °C, the activity restorations of apoCA are 39.6%, 49.5%, 62.1% and, 70.2% respectively.

In previous studies, Li *et al.* have studied the reaction of apo CA with horse kidney Zn-MT and

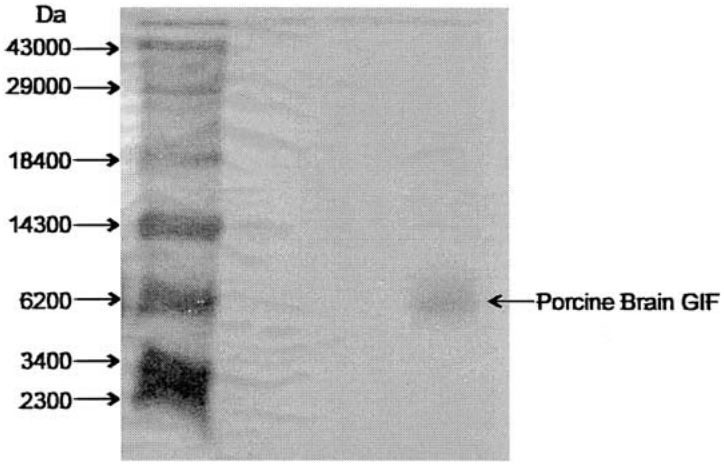

Fig. 4. SDS-PAGE of porcine brain GIF. Left: molecular weight marker, the molecular weight of the marker (from top to bottom) is: 43, 29, 18.4, 14.3, 6.2, 3.4 and 2.3 kDa, respectively. Right: porcine brain GIF.

Table 1. Amino acid composition of Cu_4Zn_3MT-III purified from porcine brain.

	Cys	Asp	Glu	Ser	His	Gly	Thy	Arg	Ala
Calc[a]	20	3	8	6	0	6	4	0	5
Found	18.41	3.66	7.59	6.03	0.14	6.21	3.50	0.06	3.60
	Tyr	Val	Met	Phe	Ilu	Leu	Lys	Pro	Gln
Calc[a]	0	1	1	0	0	0	8	5	1
Found	0.09	1.00	0.43	0.16	0.24	0.28	6.92	3.35	1.00

[a]From the porcine brain MT-3 in literature (Chen *et al.* 1996).

$Zn(NO_3)_2$. As a consequence, a single reaction occurred and that it was first-order in Zn-MT and apoCA, respectively. Then kinetics were plotted for a second-order reaction. Furthermore, the activation parameters obtained by them from kinetic run between 0 °C and 38 °C were positive $\Delta H^{\neq}$ and negative $\Delta S^{\neq}$ for Zn-MT, and positive $\Delta H^{\neq}$, $\Delta S^{\neq}$ for $Zn(NO_3)_2$. These distinctly different values indicated that the two reactions proceed by different mechanisms. The second-order kinetics and negative $\Delta S^{\neq}$ in the Zn-MT and apoCA reaction suggested an association mechanism, and the reaction was described as following: Zn-MT + apoCA $\rightarrow$ [Zn-MT-apoCA]* $\rightarrow$ CA + products.

Li *et al.* proposed that a direct interaction, possibly binding of the two proteins in the rate-determining transition state of this reaction. On the other hand, the positive $\Delta S^{\neq}$ in the reaction between Zn^{2+} and apoCA was considered due to the stripping off of water molecules from Zn^{2+} in the transition state for the reaction (Li *et al.* 1980).

To gain a further insight into the mechanism of the zinc transfer reaction between Cu_4Zn_3MT3 and apoCA, it is essential to carry out the kinetic studies on the reaction. Then kinetic analysis was performed in the same way of zinc transfer from Zn-MT to apoCA. A second-order reaction took place, and the overall expression for the reaction was found to be: $\ln([Cu_4Zn_3MT3]/[\text{apo-CA}]) = ([Cu_4Zn_3MT3]_0 - [\text{apo-CA}]_0)\, k_2 t - \ln([Cu_4Zn_3MT3]_0/[\text{apo-CA}]_0)$

By plotting $\ln([Cu_4Zn_3MT3]/[\text{apo-CA}])$ *vs* time (min) at different temperatures, a series of straight lines were obtained. The zinc transfer rate constants can be deduced from the slops and were marked in Figure 8. The results indicated the capacity of MT3 to reconstitute CA increased with the temperature increasing from 15 °C to 37 °C, the rate constant at 37 °C (2.79×10^3 M^{-1} min^{-1}) is more than 4-fold of that obtained at 15 °C (1.19×10^4 M^{-1} min^{-1}). The temperature dependence of reconstitution of apoCA restored by Cu_4Zn_3MT3 is shown in Figure 9. The active energy for the reaction E_a was obtained according the Arrhenius equation by least-square fitting. The $\Delta_r^{\neq}H$ and the $\Delta_r^{\neq}S$ at 25 °C can be also sobtained and listed as following:

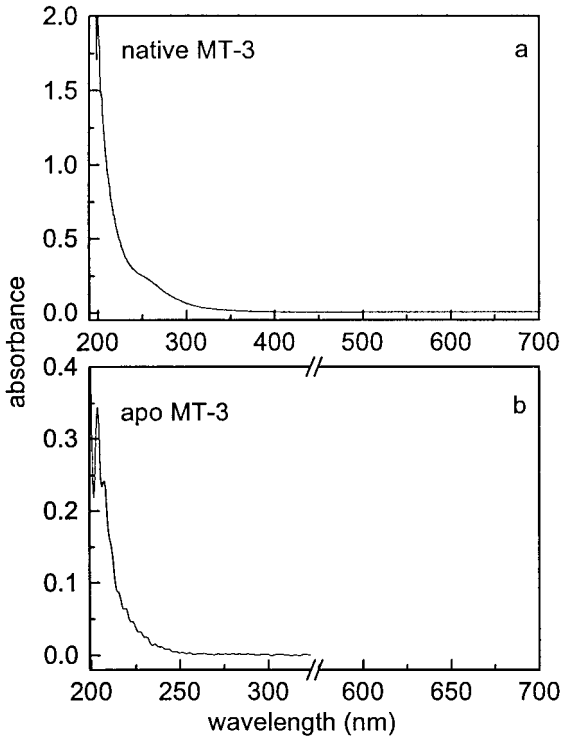

Fig. 5. Absorption spectrum of native porcine GIF (a) and apo-GIF (b). Protein concentration: 1×10^{-5} M in 0.01 M Tris-HCl (pH 7.0). Temperature: 25 °C.

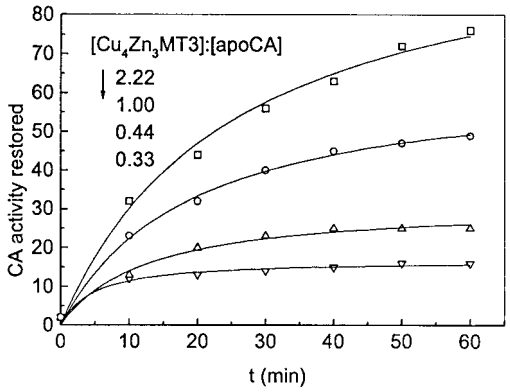

Fig. 6. Restoration of apoCA activity by porcine Cu_4Zn_3MT3 with different concentration in 0.01 M Tris/HCl, pH 7.6, 25 °C.

$E_a = 38.759$ KJ mol^{-1}
$\Delta_r^{\neq}H = 36.281$ KJ mol^{-1}
$\Delta_r^{\neq}S = -84.681$ J mol^{-1} K^{-1}

The positive value of $\Delta_r^{\neq}H$ and the negative value of $\Delta_r^{\neq}S$ for the zinc reaction from Cu_4Zn_3MT3 to apoCA are in agreement with the activation parameters for zinc transfer from Zn-MT to apoCA reaction. It indicates that the two reactions proceed

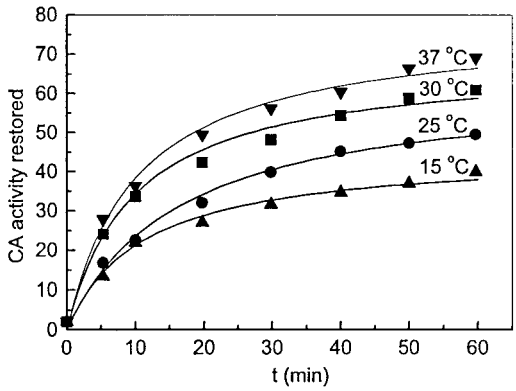

Fig. 7. Time dependence of restoration of apoCA activity by porcine Cu_4Zn_3MT3 at various temperatures with the molar ratio of [Cu_4Zn_3MT3]/ [apoCA] = 1.

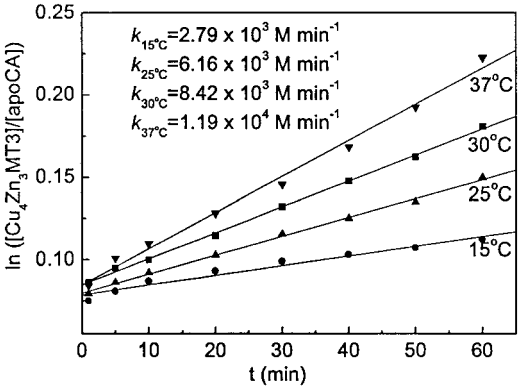

Fig. 8. Plot ln([Cu_4Zn_3MT3]/[apoCA]) versus time at different temperature from 15 °C ~ 37 °C. $[Cu_4Zn_3MT3]_0 = 2.86$ μM and $[apoCA]_0 = 2.67$ μM.

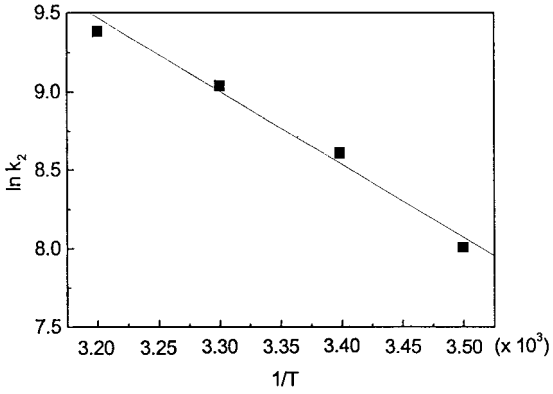

Fig. 9. Temperature dependence of the reconstitution of apoCA by porcine brain Cu_4Zn_3MT3. Reaction mixtures were incubated at temperatures between 15–37 °C. Assays for recovery of activity was run at 25 °C.

by the similar mechanism. Based on these facts, an association mechanism for the reaction of zinc transfer from Cu_4Zn_3MT3 to apoCA may be suggested, Cu_4Zn_3MT3 bind firstly to apoCA, forming a protein-protein complex $[Cu_4Zn_3MT3\text{-}apoCA]^*$, a binding step takes place according to the following scheme: $Cu_4Zn_3MT3 + apoCA \rightarrow [Cu_4Zn_3MT3\text{-}apoCA]^* \rightarrow CA + products$

The overall reaction exhibits a second-order kinetics and a first-order reaction takes place in each reactant. A direct zinc transfer takes place within the intermediate complex. Then the complex transfer into CA and products rapidly, and the formation of the intermediate complex is the rate determining transition state of this reaction. In conclusion, the present kinetic study of zinc transfer from Cu_4Zn_3MT3 to apoCA show that GIF behaves as a transient reservoir of zinc ion as well as other MTs isoforms. Actually, native GIF acts as the zinc ion donor in biological system, indicating GIF might play a role in cellular Zn homeostasis, despite its neuronal growth inhibitory function.

Acknowledgement

This work was supported by the National Natural Science Foundation of China, the Natural Science Foundation of Jiangsu Province and the Doctoral Foundation of the National Education Ministry of China.

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