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ORIGINAL ARTICLE

Mechanism of cysteine-dependent inactivation of aspartate/glutamate/cysteine sulfinic acid α-decarboxylases

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Abstract Animal aspartate decarboxylase (ADC), glutamate decarboxylase (GDC) and cysteine sulfinic acid decarboxylase (CSADC) catalyze the decarboxylation of aspartate, glutamate and cysteine sulfinic acid to β-alanine, γ-aminobutyric acid and hypotaurine, respectively. Each enzymatic product has been implicated in different physiological functions. These decarboxylases use pyridoxal 5-phosphate (PLP) as cofactor and share high sequence homology. Analysis of the activity of ADC in the presence of different amino determined that beta-alanine production from aspartate was diminished in the presence of cysteine. Comparative analysis established that cysteine also inhibited GDC and CSADC in a concentration-dependent manner. Spectral comparisons of free PLP and cysteine, together with ADC and cysteine, result in comparable spectral shifts. Such spectral shifts indicate that cysteine is able to enter the active site of the enzyme, interact with the PLP-lysine internal aldimine, form a cysteine-PLP aldimine and undergo intramolecular nucleophilic cyclization through its sulfhydryl group, leading to irreversible ADC inactivation. Cysteine is the building block for protein synthesis and a precursor of cysteine sulfinic acid that is the substrate of CSADC and therefore is present in many cells, but the presence of cysteine (at comparable concentrations to their natural substrates) apparently could severely inhibit ADC, CSADC and GDC activity. This raises an essential question as to how animal species prevent these enzymes from cysteine-mediated inactivation. Disorders of cysteine metabolism have been implicated in several neurodegenerative diseases. The results of our study should promote research in terms of mechanism by which animals maintain their cysteine homeostasis and possible relationship of cysteine-mediated GDC and CSADC inhibition in neurodegenerative disease development.

Keywords Cysteine · Inactivation · PLP-containing decarboxylase · Hypotaurine · β-Alanine · γ-Aminobutyric acid

Introduction

Animal glutamate decarboxylase (GDC), aspartate decarboxylase (ADC, also called aspartate α-decarboxylase or aspartate 1-decarboxylase) and cysteine sulfinic acid decarboxylase (CSADC) catalyze the decarboxylation of α-carboxyl group of glutamate, aspartate and cysteine sulfinic acid to produce γ-aminobutyric acid (GABA), β-alanine and hypotaurine, respectively; these amine products play important role in living organisms. For example, GABA is a chief inhibitory neurotransmitter and a regulator of neuronal excitability in animals. Hypotaurine is the direct precursor of taurine, a chemical that plays several physiological functions in mammals (Holmes et al. 1992; Foos and Wu 2002; Hayes and Sturman 1981). β-alanine is one of the two precursors for the formation of carnosine (β-alanyl-L-histidine), a peptide that plays an important role as an intracellular buffer and antioxidant (Hipkiss

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2009; Hama et al. 1971; Bellia et al. 2011). Carnosine has also been demonstrated to enhance muscular performance (Smith et al. 2009; Artioli et al. 2010; Smith et al. 2011). As a result, β-alanine has been recommended as a beneficial dietary supplement (Smith et al. 2009; Artioli et al. 2010).

Although β-alanine plays several physiological roles in mammals, ADC has not been identified in any animal species except insects. In insects, β-alanine is used to synthesize *N*-β-alanyldopamine (NBAD) and *N*-β-alanylhistamine. NBAD is oxidized easily by insect phenoloxidase and laccase, and its oxidized form can react with nucleophilic groups on proteins, leading to protein cross-linking. This has been considered as one of the primary mechanisms involved in insect cuticle hardening or sclerotization (Arakane et al. 2009; Moussian 2010; Morgan et al. 1987; Hopkins et al. 1984; Kramer et al. 1984). β-alanine has also been shown to inactivate the neurotransmitter histamine within the synaptic clefts of insect photoreceptor cells (Hardie 1987; Stuart et al. 2007). Therefore, β-alanine plays unique physiological functions in insects.

Despite playing different physiological roles in animal and insects, ADC, CSADC and GDC all use pyridoxal 5-phosphate (PLP) as cofactor and share fairly high sequence identity (>40 %). Among these enzymes, GDC is ubiquitous and therefore is considered the prototype. As a result, the ADC and CSADC sequences in some sequenced genomes often have been named GDC or GDC-like protein without further differentiation. Our previous study determined that insect ADC and GDC have no overlap substrate specificity (Richardson et al. 2010). Insects do not have a specific CSADC and there has been no report regarding the ADC in mammals. In our recent study, however, we demonstrated that insect ADC could use cysteine sulfinic acid and cysteic acid as its substrates (Fig. 1). Accordingly, insect ADC has the typical CSADC activity (Liu et al. 2012). The function of insect ADC in taurine production may be as important (if not more) as its function in β-alanine production, and the enzyme could have been named CSADC if cysteine sulfinic acid were the first tested substrate. Overlap in substrate specificity between insect ADC and mammalian CSADC indicates that it is necessary to critically compare the substrate specificity and biochemical properties of these GDC-like enzymes. It has also been reported that the activity of human GDC is negatively affected by the presence of aspartate (Porter and Martin 1987). Aspartate-induced GDC inhibition suggests that non-substrate amino acids might interfere with the catalytic efficiency of GDC-like. Therefore, to more practically comprehend the regulation of these enzymes, it is necessary to understand conditions that affect the functions of GDC-like protein family.

In this study, we expressed several GDC-like enzymes, including human CSADC, mosquito GDC, mosquito ADC and *Drosophila* ADC, and assessed the activities of these enzymes to their natural substrates in the presence of other proteogenic amino acids. We initially observed that in the presence of similar concentrations of aspartate and cysteine sulfinic acid, the β-alanine production (ADC activity) was diminished and the reaction was driven primarily toward hypotaurine production (CSADC activity). Both aspartate

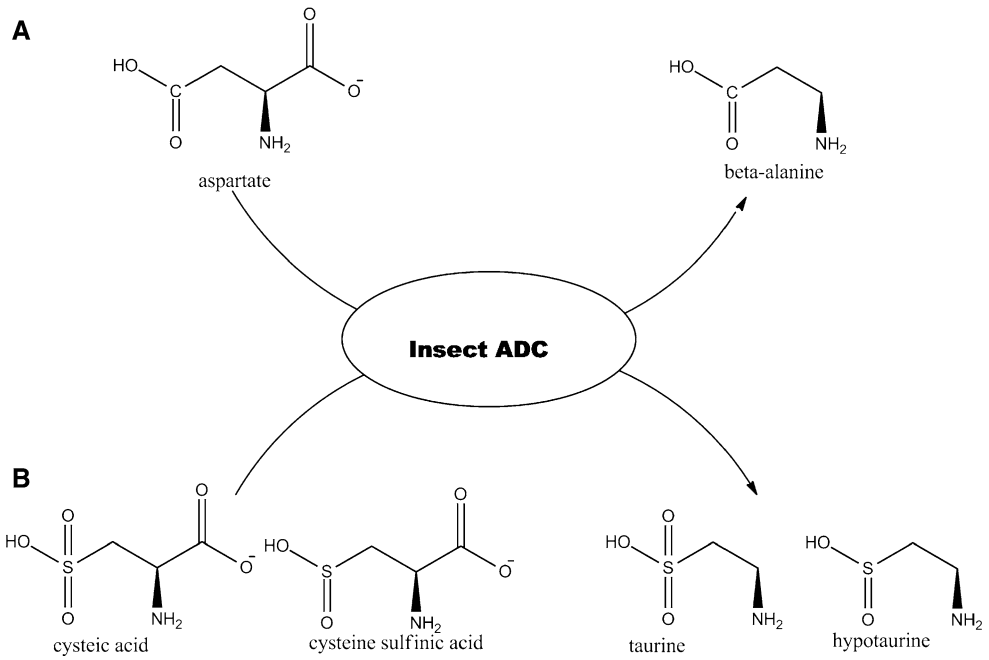

Fig. 1 Reactions catalyzed by insect ADC. **a** Aspartate 1-decarboxylation. **b** Cysteine sulfinic acid decarboxylation and cysteic acid decarboxylation

and cysteine sulfinic acid likely are present in many tissues and cells. Subsequent screening of insect ADC with either aspartate or cysteine sulfinic acid in the presence of each of the other proteogenic amino acids revealed that cysteine effectively inactivated the activity of insect ADC. Further analysis determined that the activities of mosquito GDC and human CSADC were also inactivated by cysteine in a concentration-dependent manner.

In this communication, we provide data that describe the effect of cysteine on the activities of GDC-like proteins. Specifically, we use insect ADC as a model enzyme to discuss the mechanism of cysteine-dependent inactivation of GDC-like enzymes.

Materials and methods

Chemicals

All chemicals used in this report were from Sigma-Aldrich, unless specified otherwise.

*Ae*ADC expression and purification

*Ae*ADC was expressed in BL21 and purified to homogeneity as described in a previous study (Richardson et al. 2010). A Bio-Rad protein assay kit was used to determine protein concentration with bovine serum albumin as a standard. *Ae*ADC was concentrated to 20 μg/μl in 20 mM phosphate buffer (pH 7.0).

Assays of ADC and CSADC activities of *Ae*ADC in the presence of different amino acids

Prior to enzyme activity assays, concentrated enzyme preparations were diluted with phosphate buffer containing 1 mg of bovine serum albumin (for enzyme stabilization). Amino acid solutions in this manuscript were adjusted to pH 7.0 before use. The typical reaction mixtures of 50 μl, containing 2 μg of purified *Ae*ADC, and 5 mM of aspartate or 5 mM of cysteine sulfinic acid, were prepared in 200 mM phosphate buffer (pH 7.0) in the absence or presence of 5 mM each of the proteogenic amino acids (the final concentration for tyrosine was 2 mM due to its low solubility). The reaction mixtures were incubated at 25 °C and the reaction was stopped by addition of two volumes of 100 % ethanol 10 min after incubation. The mixture was then treated with an equal volume of *o*-phthaldialdehyde (OPT) reagent as described in a previous method (Richardson et al. 2010). Product formation was based on the detection of *β*-alanine-OPT derivative by reverse-phase liquid chromatography with electrochemical detection (HPLC-ED). The mobile phase consisted of 50 mM phosphate buffer (pH 3.5) containing 25 % acetonitrile at a flow rate of 0.5 ml per minute. The oxidation potential of the working was maintained at +750 mV with an Ag/AgCl reference electrode.

Time and dose dependence of cysteine inhibition on *Ae*ADC

ADC activity assays in the presence of different amino acids identified cysteine as an effective inactivator of *Ae*ADC activity. To assess the type of ADC inhibition by cysteine, a series of solutions of 400 μl containing 6.25 mM aspartate and a varying concentration of cysteine were freshly prepared in 100 mM phosphate buffer (pH 7.0) and mixed with 100 μl of *Ae*ADC solution to determine the effect of cysteine concentration on ADC activity. The final concentration of the reaction contained 5 mM of aspartate and a series of cysteine concentrations (0.05, 0.1, 0.2, 1, 2 and 5 mM). The enzyme preparation contained 0.2 mg of *Ae*ADC and 1 mg of bovine serum albumin (for enzyme stabilization) per milliliter and was prepared in the same buffer. The reaction was initiated by mixing 100 μl enzyme preparation into 400 μl aspartate and cysteine solution. At 2-, 4-, 6-, and 8-min intervals, 50 μl of the reaction mixture was withdrawn and mixed with 100 μl of 100 % ethanol to stop reaction, followed by the described product derivatization and quantification procedures.

Spectral changes of *Ae*ADC or PLP in the presence of cysteine

Initial data indicated that inhibition of *Ae*ADC by cysteine seemed to be due to its interaction with PLP cofactor, which likely led to the noticeable spectral changes of the enzyme. To evaluate the possible interaction of aspartate, cysteine sulfinic acid, or cysteine and *Ae*ADC, 10 μl of 5 mM aspartate, cysteine sulfinic acid or cysteine was first mixed with 80 μl of 200 mM phosphate buffer (pH 7.0), followed by rapid addition of 10 μl of 20 μg/μl of *Ae*ADC and spectral analysis. The spectral changes of the *Ae*ADC and amino acid mixtures from 300 to 480 nm were monitored at each 45-s interval for a 10-min period using an Aligent 8453 UV/visible spectrophotometer. Data were processed using the Aligent UV/visible ChemStation software. To determine the possible interaction of free PLP with aspartate, cysteine sulfinic acid or cysteine, free PLP was prepared in phosphate buffer and mixed with each of the compounds (with a final concentration of PLP and

cysteine at 0.2 and 0.5 mM) and the possible spectral changes of the PLP and cysteine mixture were monitored from 300 to 480 nm as those described for *Ae*ADC and amino acid mixtures.

Effect of cysteine on *Anopheles gambiae* GDC (*An*GDC), *Hu*CSADC and *Ae*ADC Q377L activities

The inhibition of *Ae*ADC by cysteine was verified. This questions if cysteine has similar effect on other GDC-like proteins. To assess the general effect of cysteine on GDC-like enzymes, *An*GDC, *Hu*CSADC and *Ae*ADC Q377L (a mutation showing different substrate selectivity) (Liu et al. 2012) were expressed as *Ae*ADC and the effect of cysteine on their activity was analyzed similarly as described for *Ae*ADC. Typically, reaction mixtures of 50 µl, containing 2 µg of purified *An*GDC or *Hu*CASDC and 5 mM of glutamate or 5 mM of cysteine sulfinic acid, were prepared in 200 mM phosphate buffer (pH 7.0) in the absence or presence of 5 mM of cysteine. The other conditions, including incubation time, product derivatization and product quantitation, were identical to those described for *Ae*ADC activity assays, except for the increase of acetonitrile (50 %) in the mobile phase during detection GABA.

Computer modeling

Enzyme activity assays and spectral changes of *Ae*ADC in the presence of cysteine indicated that cysteine could enter into the active site of *Ae*ADC and interact with the internal aldimine, leading to the formation of the PLP–cysteine complex. To elucidate the structural basis of cysteine inhibition, *Ae*ADC structural model was generated using the atomic coordinates of *Hu*CSADC (PDB: 2JIS) as templates. *Ae*ADC shares 52 % sequence identity with *Hu*CSADC overall and 56 % identity between their PLP-binding domains. The predicted *Ae*ADC structural model was then used to assess the structural basis of cysteine binding through molecular docking (Trott and Olson 2010). AutoDockTools and Autodock Vina were used to evaluate the interaction of the substrate and the enzyme (Trott and Olson 2010). The structure of cysteine for docking was prepared using PRODRG (Schuttelkopf and van Aalten 2004). Cysteine is one of the preferred substrates for animal kynurenine aminotransferases and these enzymes are not inactivated by cysteine. To further understand and clarify the mechanism of cysteine inhibition to GDC-like proteins, the predicted *Ae*ADC structural model was compared with an *Aedes aegypti* kynurenine aminotransferase structural model with cysteinyl aldimine (PDB: 2R5C) (Han et al. 2008). The active site for cysteine is found in one of the biological dimers (Han et al. 2008).

Results

The presence of cysteine sulfinic acid on the activity of *Ae*ADC to form beta-alanine

When *Ae*ADC was mixed with aspartate, accumulation of *β*-alanine was observed in the reaction mixture, but when the same concentration of cysteine sulfinic acid was also incorporated into the reaction mixture, production of *β*-alanine was diminished and instead a high level of hypotaurine was accumulated (Fig. 2a, b). When the concentration of aspartate was increased to fourfold of cysteine sulfinic acid in the reaction mixture, the ADC activity was still inhibited (Fig. 2c) suggesting that *Ae*ADC favored cysteine sulfinic acid as its substrate, or cysteine sulfinic acid could much better compete with aspartate for binding/

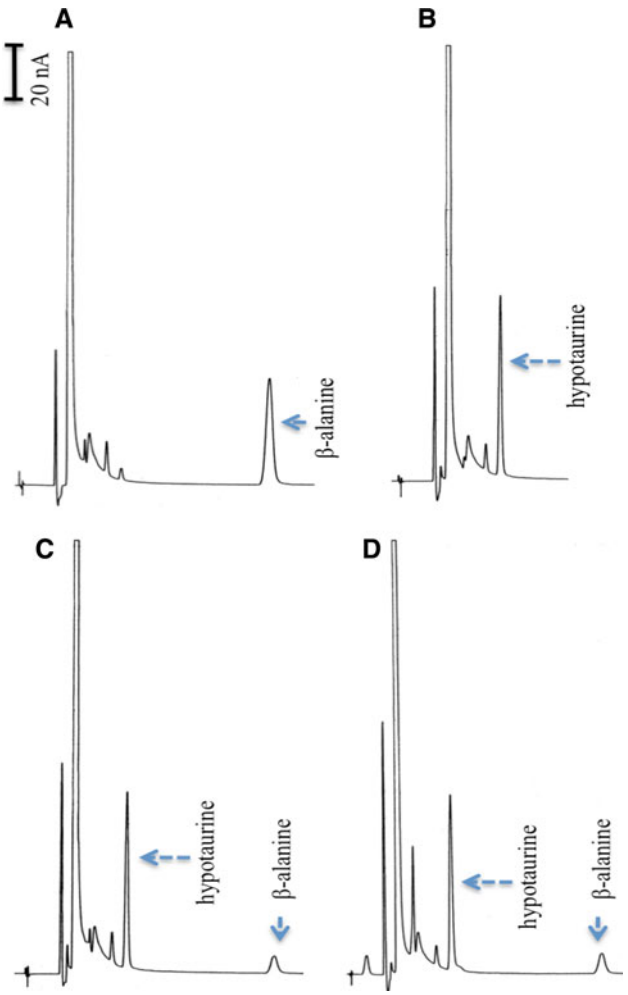

Fig. 2 The ADC or CSADC activity of *Ae*ADC in the presence of cysteine sulfinic acid or aspartate. **a** The ADC activity of *Ae*ADC with 5 mM of aspartate. **b** The CSADC activity of *Ae*ADC with 5 mM of cysteine sulfinic acid. **c** The ADC activity of *Ae*ADC with 5 mM of aspartate in the presence of 5 mM of cysteine sulfinic acid. **d** The ADC activity of *Ae*ADC with 20 mM of aspartate in the presence of 5 mM of cysteine sulfinic acid

catalysis. The same results were obtained when *Dm*ADC was analyzed in the same manner (not shown).

Effects of amino acids on *Ae*ADC activities

When *Ae*ADC was incubated with cysteine sulfinic acid in the presence of each of 20 proteogenic acids, its CSADC activity was not significantly affected by any of the tested amino acids (Fig. 3a). When *Ae*ADC was incubated with aspartate in the presence of each of 19 remaining proteogenic amino acids, its ADC activity was diminished in the presence of cysteine. No other amino acids showed any significant effect on either increase or decrease of its ADC activity (Fig. 3b). The selective cysteine-dependent

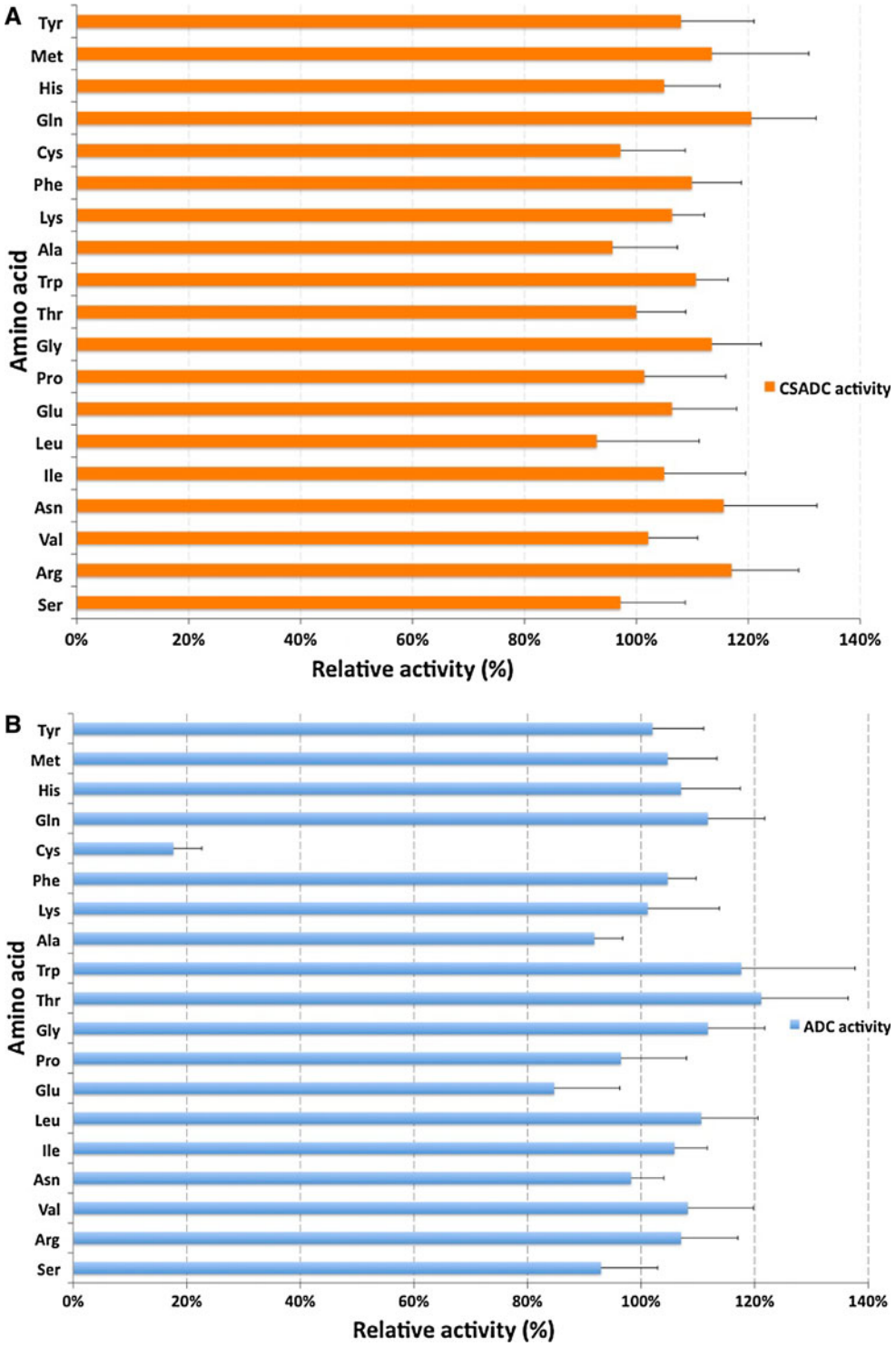

Fig. 3 The effect of amino acids on the activities of *Ae*ADC. **a** The CSADC activity of *Ae*ADC with the addition of different amino acid. **b** The ADC activity of *Ae*ADC with the addition of different amino acid. Each 50 µl reaction mixture contained 5 mM of substrate (either aspartate or cysteine sulfinic acid), 200 mM phosphate buffer (pH, 7.0), 0.4 µM of PLP and 5 mM of amino acid addition. The reaction was triggered by adding 2 µg *Ae*ADC, incubated at 25 °C for 10 min and stopped by adding two volumes of 100 % ethanol

inhibition of the ADC activity, but not the CSADC activity of *Ae*ADC, raises an essential question regarding the chemical mechanism of such selective inhibition.

Time and concentration dependence of cysteine inhibition

When *Ae*ADC was mixed with solutions containing 5 mM of aspartate and a varying concentration of cysteine, the level of ADC activity inhibition was approximately proportional to the concentrations of cysteine in the reaction mixtures (Fig. 4a), suggesting somewhat a competitive nature of the inhibition. Based on the specific activity calculated during a 10-min incubation period, the presence of 2 mM of cysteine or above resulted in 80 % inhibition of ADC activity (Fig. 4a). However, a progressive decrease in the specific activity was observed when the reaction mixtures containing *Ae*ADC, 5 mM of aspartate and 1 mM of cysteine were stopped at 2, 4, 6 and 8 min after incubation (Fig. 4b). This suggests that the interaction of cysteine with *Ae*ADC actually led to progressive and irreversible inactivation of the enzyme. The kinetics of *Ae*ADC inactivation by cysteine was calculated to be $K_{obs} = -0.364$/min. The inactivation efficiency can reach almost 100 % beyond 10 min of incubation (not shown). Prolonged pre-incubation of cysteine with *Ae*ADC will completely kill the ADC activity of the enzyme (please see supplemental figure 1).

The time and concentration dependency of cysteine inactivation of the *Ae*ADC activity differed from the cysteine inactivation of CSADC activity. When *Ae*ADC was first incubated in 5 mM of cysteine for 5 or 10 min and then mixed with cysteine sulfinic acid, the CSADC activity of the *Ae*ADC was greatly decreased compared to a

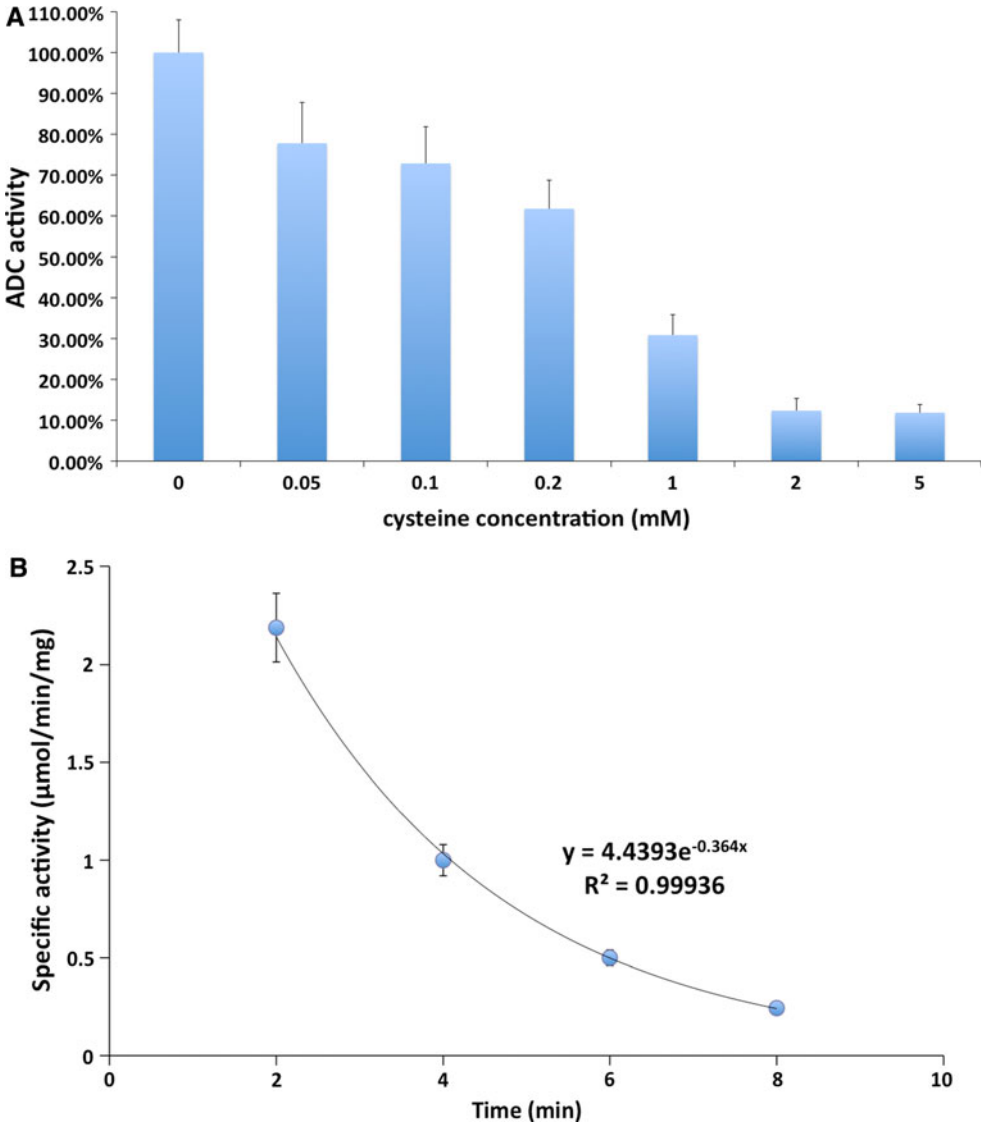

Fig. 4 The dose and time dependence of cysteine inhibition on ADC activity. **a** Different concentrations of cysteine were tested on the inhibition of ADC activity. **b** The velocity of ADC activity was measured at different time point when the reaction mixture contained 5 mM of aspartate and 1 mM of cysteine

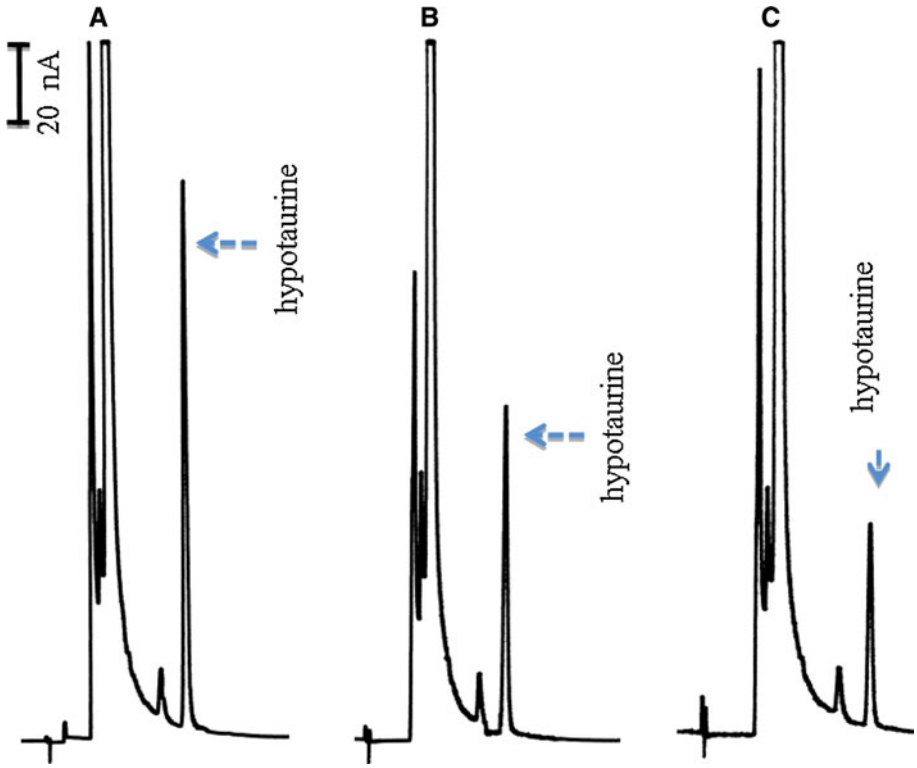

Fig. 5 The effect of cysteine pre-incubation on the CSADC activity of *Ae*ADC; 10 µl of 25 mM cysteine was added into each 50 µl reaction mixture containing 2 µg *Ae*ADC, 200 mM phosphate buffer (pH 7.0) and 0.4 µM of PLP for 0 min (**a**), 5 min (**b**) or 10 min (**c**) at 25 °C. The reaction was triggered by adding 10 µl of 25 mM of cysteine sulfinic acid and incubated at 25 °C for 10 min before it was stopped

cysteine-free control reaction. The level of cysteine-*Ae*ADC inactivation increased proportionally to cysteine pre-incubation time (Fig. 5). Under the applied conditions, the specific activity was 2.2 µmol/min/mg for the 10-min cysteine-pre-incubated ADC as compared to 5.5 µmol/min/mg for the enzyme without cysteine pre-incubation. These results indicate that cysteine sulfinic acid competes effectively with cysteine to bind to *Ae*ADC, which prevents or greatly slows down the interaction of cysteine with the enzyme so that the rate of product formation was not noticeably affected during a 10-min incubation period (see Fig. 3a). In contrast, cysteine competed more effectively than aspartate to react with *Ae*ADC when mixed with the enzyme at the same time, leading to a progressive inactivation of the enzyme and diminished product formation (see Fig. 3b).

Interaction of aspartate, cysteine sulfinic acid or cysteine with *Ae*ADC

*Ae*ADC contains a PLP cofactor that is covalently linked with a conserved lysine residue as an internal aldimine. This internal aldimine produces absorbance peaks with λ_{max} around 335 and 405 nm. The *Ae*ADC spectrum remains unchanged after 2 h of incubation at 25 °C. This indicates that the *Ae*ADC is stable at 25 °C. When either aspartate or cysteine sulfinic acid (each at 0.5 mM final concentration) was mixed with *Ae*ADC in phosphate buffer at pH 7.0, there were no obvious changes of the spectrum of the enzyme (Fig. 6a, b). When 0.5 mM of cysteine was mixed with *Ae*ADC solution, progressive increase and decrease of the 335-nm peak and the 405-nm peak, respectively, were observed (Fig. 6c). Increasing the concentration of cysteine in the mixture increased the rate of spectral change (not shown). When aspartate and cysteine (each at 0.5 mM final concentration) was mixed with the enzyme, a similar increase of the 335-nm peak and decrease of the 405-nm peak were observed (Fig. 6d). However, the addition of 0.5 mM of cysteine and cysteine sulfinic acid did not result in significant change in the spectra of the enzyme within a 10-min period (Fig. 6e).

Effects of different amino acids on the UV/visible spectrum of free PLP

Free PLP has a peak absorbance around 390 nm and a small peak (or a shoulder) at 330 nm at pH 7.0. When cysteine was mixed with free PLP, a progressive decrease of the 390-nm peak and increase of the 335-nm peak were observed (Fig. 7a). These spectral changes are similar to those observed in *Ae*ADC and cysteine mixture except that the decrease was at 405 nm for the enzyme as opposed to the decrease at 390 nm for free PLP (Fig. 6c). In contrast, no apparent spectral changes of the PLP were observed when aspartate or cysteine sulfinic acid was mixed with free PLP (Fig. 7b). These results indicate that cysteine forms complex with PLP under physiological pH conditions.

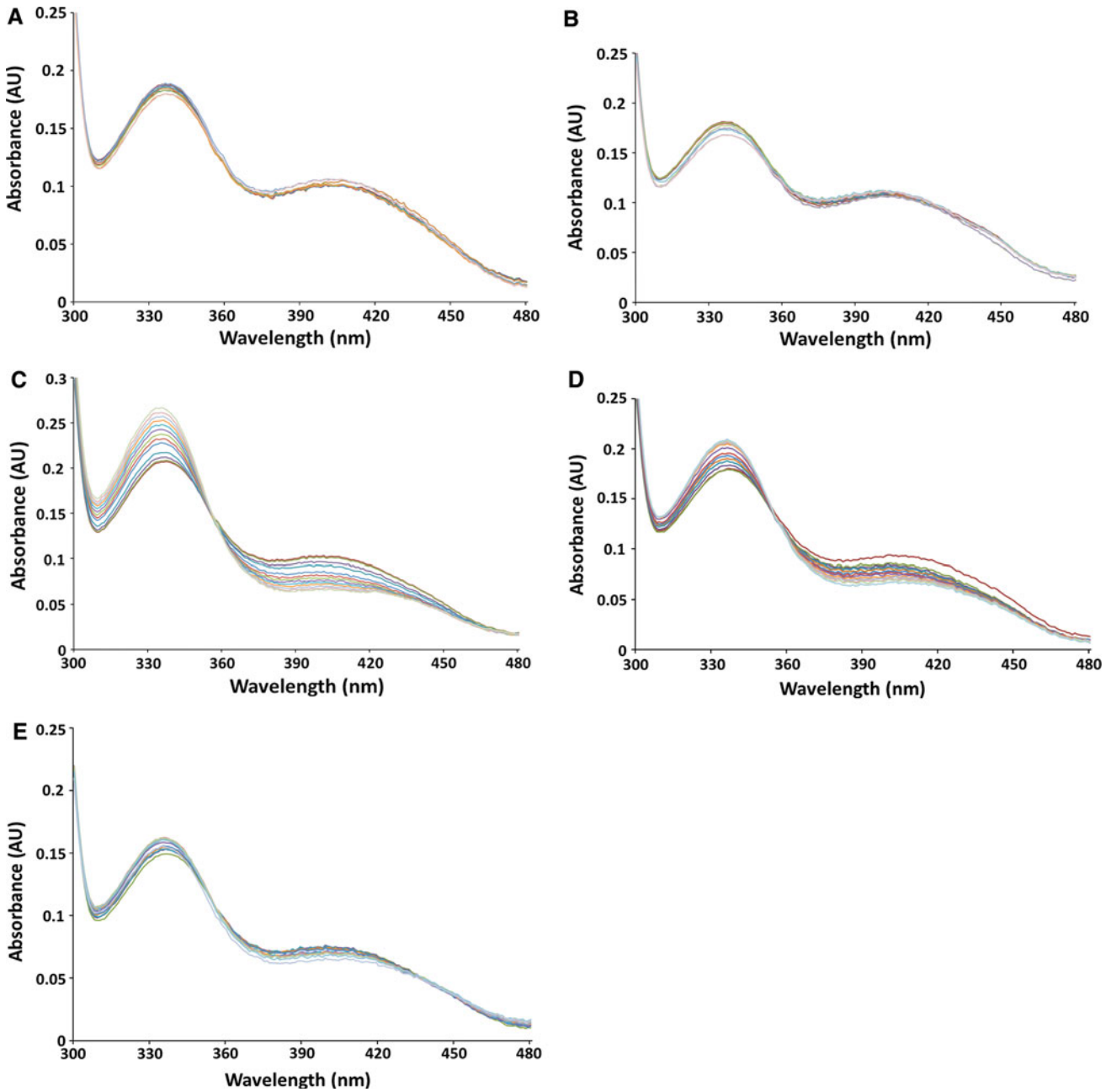

Fig. 6 The UV/visible spectra of *Ae*ADC under different conditions. **a** The spectra of *Ae*ADC with the addition of aspartate (0.5 mM final concentration). **b** The spectra of *Ae*ADC with the addition of cysteine sulfinic acid (0.5 mM final concentration). **c** The spectra of *Ae*ADC with the addition of cysteine (0.5 mM final concentration). **d** The spectra of *Ae*ADC with the addition of aspartate and cysteine (each 0.5 mM final concentration). **e** The spectra of *Ae*ADC with the addition of cysteine sulfinic acid and cysteine (each 0.5 mM final concentration). The total reaction volume is 100 µl; 10 µl of 20 µg/µl *Ae*ADC was incubated with 0.5 mM of different compounds at 25 °C for 10 min in 200 mM phosphate buffer (pH 7.0). The change of spectra was monitored in a period of 10 min and each recoding was taken at an interval of 45 s

Reaction of cysteine with the internal aldimine in *An*GDC, *Hu*CSADC, and *Ae*ADC Q377L

Cysteine is chemically similar to glutamate, aspartate and cysteine sulfinic acid. It was predicted that cysteine is likely able to enter the active sites of GDC and CSADC, react with the internal aldimine, form cysteine–PLP complex and inhibit their activities in the same manner. When *An*GDC was mixed with glutamate in the presence of cysteine, the inhibition of GDC was clearly observed (Table 1) and such inhibition was approximately proportional to the concentration of cysteine in the reaction

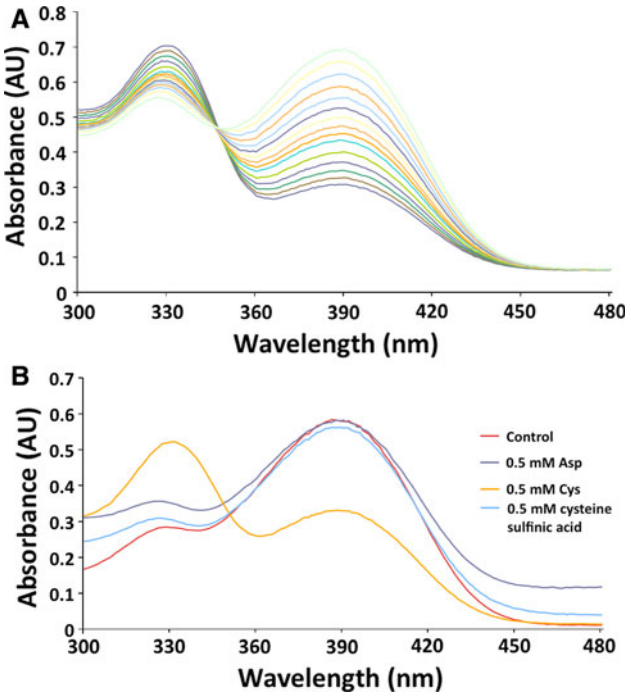

Fig. 7 The UV/visible spectra of free PLP under different conditions. **a** The spectra of free PLP with the addition of cysteine. The total reaction volume is 100 µl; 0.2 mM of PLP was incubated with 0.5 mM of cysteine at 25 °C for ten min in 200 mM phosphate buffer (pH 7.0). Each spectrum was taken at an interval of 45 s. **b** The spectra of free PLP under different conditions. The total reaction volume is 100 µl. *Red line* shows the spectrum of 0.2 mM of PLP in 200 mM phosphate buffer (pH 7.0) at 10 min. *Dark blue line* shows the spectrum of 0.2 mM of free PLP and 0.5 mM of aspartate in 200 mM phosphate buffer (pH 7.0) at 10 min. *Light blue line* shows the spectrum of 0.2 mM of free PLP and 0.5 mM of cysteine sulfinic acid in 200 mM phosphate buffer (pH 7.0) at 10 min. *Orange line* shows the spectrum of 0.2 mM of free PLP and 0.5 mM of cysteine in 200 mM phosphate buffer (pH 7.0) at 10 min (color figure online)

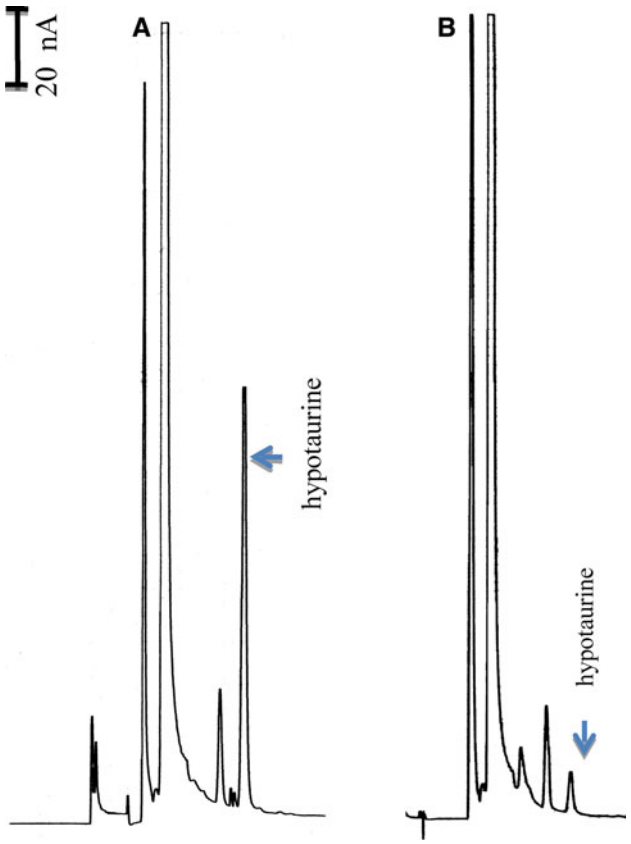

Fig. 8 The effect of cysteine on the *Ae*ADC Q377L. 10 µl of 25 mM of cysteine was added into each 50 µl reaction mixture containing 2 µg *Ae*ADC Q377L, 5 mM of cysteine sulfinic acid, 200 mM phosphate buffer (pH 7.0) and 0.4 µM of PLP. The reaction was stopped and derivatized after 10 min of incubation at 25 °C. The production of hypotaurine for control group (**a**) and cysteine-addition group (**b**) was tested and compared. The electrochemical detection was the same as previously

Table 1 The activities of wild-type *Ae*ADC, *Ae*ADC Q377L, *An*GDC and *Hu*CSADC with or without cysteine addition

Enzyme	Substrate	Specific activity (µmol/min/mg)	
		Control	With cysteine (5 mM)
*Ae*ADC wild-type	Aspartate	7.39 ± 0.6	1.59 ± 0.2
	Cysteine sulfinic acid	6.70 ± 0.8	6.37 ± 0.5
*Ae*ADC Q377L	Aspartate	2.14 ± 0.2	Not detectable
	Cysteine sulfinic acid	5.82 ± 0.4	0.6
*An*GDC	Glutamate	1.07 ± 0.2	Not detectable
*Hu*CSADC	Cysteine sulfinic acid	4.89 ± 0.5	3.61 ± 0.2

The assaying conditions are described in "Materials and methods"

mixtures (not shown). Both human CSADC and *Ae*ADC use cysteine sulfinic acid as substrate, but human CSADC has no activity on aspartate. When human CSADC was mixed with both cysteine sulfinic acid and cysteine at the same time, the activity of the enzyme was decreased, which is different from that observed from *Ae*ADC (Table 1).

We recently proposed that an active site glutamine residue (Q377) in *Ae*ADC plays a major role in its ability to use aspartate as substrate, because mutation of the active site Q377 to leucine (L377) diminished its ADC activity with no apparent effect on its CSADC activity. When the mutant *Ae*ADC was mixed with both cysteine and cysteine sulfinic acid at an equal molar concentration, the CSADC activity of the mutant *Ae*ADC was considerably reduced (Fig. 8; Table 1).

Analysis of the interaction between cysteine and PLP-dependent enzymes through molecular docking

To assess the mechanism of cysteine-dependent inactivation against GDC-like proteins, cysteine was used as the

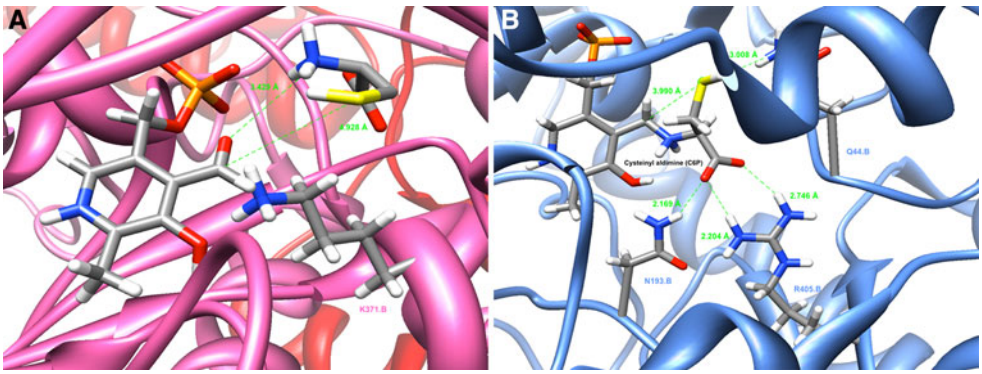

Fig. 9 Computational analysis of interactions between cysteine and *Ae*ADC or *Hu*CSADC. **a** The relative position of cysteine and the PLP at the active site of predicted *Ae*ADC structure. **b** A model showing the relative position of cysteinyl aldimine and the active site residues of *Ae*KAT1. The homology model of *Ae*ADC are shown as *red* and *pink ribbons*. The structural model was generated with Swiss model using the atomic coordinates of *Hu*CSADC (PDB: 2JIS) as templates. The crystal structural model of *Ae*KAT1 is shown as *blue* and *cornflower blue ribbons*. The distances are indicated with *green dashed lines* (color figure online)

ligand to perform the docking experiment in *Ae*ADC. The orientation of cysteine within *Ae*ADC active site and its interaction with the active site residues were compared with those of cysteinyl aldimine in a kynurenine aminotransferase that uses cysteine as a substrate (Han et al. 2008; Han et al. 2009). The optimum docking position (predicted by Autodock Vina) was based on chemical potential that takes into account both the bound conformation preference and the free energy of binding (i.e., the ligand in the position has the lowest energy) (Trott and Olson 2010). The orientation of cysteine was slightly different at the active site between the two enzymes (Fig. 9a, b). Distances between the α-N atom or sulfhydryl group of the substrates and the C4′ atom of the lysine–PLP or PLP were labeled. The results showed that the sulfhydryl group was cycled back, making it within 5 Å to the C4′ atom of the PLP in *Ae*ADC (Fig. 9a). Notice that the cysteine does not form Schiff base with PLP in the predicted model of *Ae*ADC, making the distance between the sulfhydryl group and the imine carbon slightly longer than it should have been. In contrast, the cysteine was stretched at the active site of the kynurenine aminotransferase; the sulfhydryl group and carboxyl group of cysteinyl aldimine interact with the surrounding residues (Q44, R405, and N193), physically restraining the sulfhydryl group from interacting with the external aldimine (Fig. 9b).

Discussion

This is the first detailed report concerning the potential effect of cysteine on the regulation of animal ADC, CSADC and GDC. Our study determined that (1) cysteine can enter into the active site of the above enzymes and undergo transaldimination (i.e., the replacement of ε-amino group of internal aldimine with the amino group of an incoming substrate to form external aldimine), resulting in the formation of cysteine-PLP aldimine and 2) the aldimine intermediate then undergoes intramolecular cyclization (nucleophilic addition) through the sulfhydryl group to form a stable covalently linked complex (Fig. 10), leading to the irreversible inactivation of the enzyme. In addition to the above findings, we demonstrate that the cysteine inactivation of GDC-like proteins is dependent on the relative affinities of cysteine and natural substrates of the GDC-like proteins. This is clearly illustrated by the varied efficiency of cysteine-dependent inactivation of ADC, CSADC and ADC Q377L (due presumably to enhanced inactivator binding). Our results lead to an intriguing question as to how living species prevent the inactivation of GDC-like proteins by cysteine. This manuscript should serve as a useful reference toward comprehensive understanding of the regulation of GDC-like proteins in vivo. This work also highlights a possible research direction regarding the effect of the disorder of cysteine regulation on the physiological functions of GDC-like proteins. The toxicity of excess cysteine has been demonstrated in rabbits and rats (Andine et al. 1991; Lehmann 1987; Lehmann et al. 1993).

Cysteine is indispensible in animals as a building block for protein synthesis and a precursor for the production of glutathione and taurine. This is particularly true for young individuals as growth is always accompanied with active protein synthesis, which requires adequate supplies of cysteine and which may explain in part the relatively high levels of cysteine in young individuals. The plasma cysteine concentration ranges from 80 to 200 μmol/l in healthy individuals (Stipanuk et al. 2006) and cysteine tends to decrease along the aging process (Hildebrandt et al. 2002; Droge 2005). Cysteine is reactive and potentially toxic because oxidation of cysteine in the presence of oxygen may produce reactive oxygen species, but under

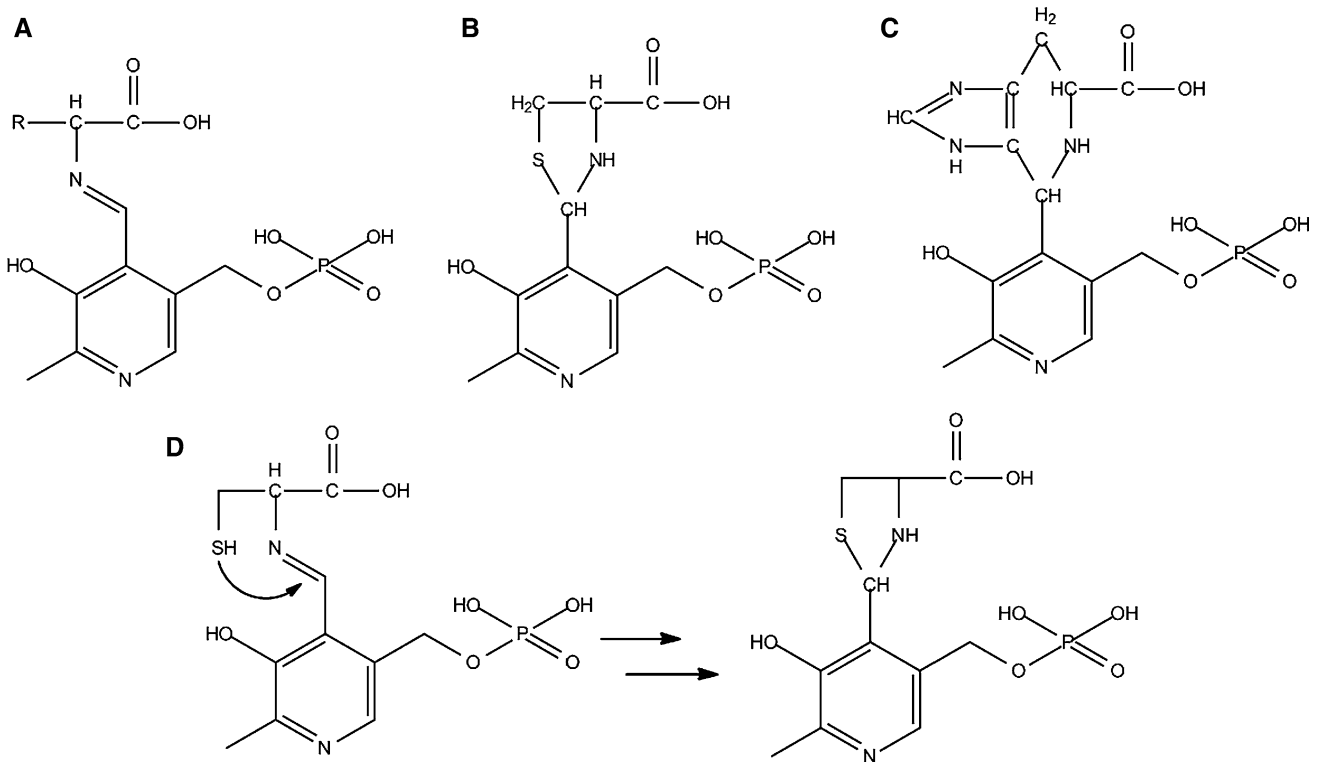

Fig. 10 The proposed interaction between free PLP and amino acids. **a** The Schiff base between amino acid and PLP; **b** 4-thiazolidine-carboxylic acid derivative; **c** the cyclic compound between histidine and PLP; **d** the intramolecular cyclization to form the 4-thiazolidin-ecarboxylic acid derivative

other conditions it may help scavenge radical species if the reactive compounds are already formed (Bourdon et al. 2005; Miki and Funato 2012). Disorders of cysteine regulation often result in disease conditions (Olney et al. 1990). Relative to the control children, children with autism had lower plasma cysteine concentration (James et al. 2004). In addition, cysteine, together with its metabolic derivative homocysteine, is defined as exotoxin (Coyle and Puttfarcken 1993). For example, malfunction of cysteine dioxygenase (the primary cysteine catabolism enzyme) can lead to elevated cysteine levels that have been associated with several major neurodegenerative diseases (Heafield et al. 1990; Coyle and Puttfarcken 1993; McCaddon et al. 1998; Olanow 1993; Diaz-Arrastia 2000). The association of cysteine disorders with neurodegenerative diseases is more related to the aging process (Zecca et al. 2004) and the precise role of cysteine disorder in neurodegenerative diseases remains to be established.

In addition, the hepatic CDO activity increases while CSADC activity decreases as the intake of sulfur-containing amino acids elevates (Stipanuk et al. 2002). Cysteine dioxygenase catalyzes the oxidation of cysteine to cysteine sulfinic acid that is then decarboxylated by CSADC in the taurine biosynthesis pathway (McCoy et al. 2006). Cysteine dioxygenase has important medical implications and is generally considered as an important enzyme regulating cysteine (Stipanuk 2004). Accordingly, it seems reasonable to suggest that a deficiency of the enzyme could potentially lead to the cysteine accumulation that in turn may affect the biosynthesis of GABA and taurine due to the cysteine-dependent inactivation of GDC and CSADC, respectively. The inactivation of decarboxylase by cysteine may explain in part the tight regulation of cysteine in mammals and other species.

Many decarboxylases contain PLP as a cofactor that forms an internal aldimine with a conserved lysine residue. The internal aldimine is essential to all PLP-dependent decarboxylation (Oliveira et al. 2011). Formation of internal aldimine between active site lysine and PLP involves the formation of a carbinolamine intermediate and subsequent dehydration to form imine (internal aldimine). Recent studies determined that a conserved active site cysteine residue (C446 in *Hu*GDC67; C455 in *Hu*GDC66) that promotes the dehydration of the carbinolamine intermediate is essential for the formation of the internal aldimine in human GDC (Oliveira et al. 2011). We initially speculated that free cysteine might form disulfide bond with this active site cysteine, resulting in a progressive inactivation of the enzyme. However, the relative rapid rate of *Ae*ADC inactivation by cysteine excludes such possibility, because formation of disulfide bond between free cysteine and residue cysteine requires time-consuming oxidation.

When *Ae*ADC was incubated with its substrate or non-substrate amino acids, there was no noticeable change of the spectrum of *Ae*ADC. However, the apparent spectral changes of *Ae*ADC in the presence of cysteine clearly indicate the interaction of cysteine with enzyme-bond PLP. Amino acids react with PLP and the interaction of cysteine and PLP results in the formation of imine and subsequent intramolecular cyclization of the site chain to form a stable complex (Fig. 8) (Schonbeck et al. 1975; Buell and Hansen 1960). The incorporation of cysteine to PLP led to progressive 390-nm peak decrease and 335-nm peak increase that corresponded to the changes observed from *Ae*ADC with cysteine, except that the peak for *Ae*ADC was at 405 nm instead of 390 nm. The formation of thiazolidinic compound between PLP and cysteine was demonstrated by nuclear magnetic resonance (Abbott and Martell 1970). The spectral characteristics of the cysteine–PLP derivative, isolated from cysteine-treated *Ae*ADC (see supplemental figure 2), is also consistent with that of thiazolidinic species reported in literature.

It has generally been considered that PLP can react with the amino group of amino acids, leading to imine production, but there has been no detailed discussion about the pathway in vivo. Although the reaction likely proceeds under physiological conditions, the equilibrium unlikely favors the production of their imine complex between free PLP and any given amino acids. Nucleophilic addition to the carbonyl carbon is an acid-promoted reaction. To undergo nucleophilic addition to the carbonyl carbon of PLP, however, the weak nucleophilic amino group needs to be unprotonated. This may explain some increase in imine formation between PLP and amino acids at relative basic conditions in vitro (although the carbonyl carbon of PLP should be more reactive at relatively acidic condition) (Heyl et al. 1948). Because the amino group of any given amino acids is in general positively charged, imine formation between PLP and amino acids should be a minor pathway in vivo (no PLP would have been available for decarboxylases and aminotransferase otherwise). Indeed, if aldehydes could react very easily with the amino group of amino acids and other biological amines, it would be a disaster as numerous potentially toxic imine complexes would have circulated in the body of living species (including humans). This leads to a question why cysteine readily reacts with PLP.

Although the pKa of the amino group is around 9 or above, there should be a very small fraction of any given amino acid with its amino group unprotonated at physiological pH. It is reasonable to predict that, when PLP is mixed with different amino acids, an insignificant level of Schiff base might be formed and the reaction is in dynamic process (association–dissociation) in the solution under physiological pH (this explains why studies regarding the Schiff base formed between amino acids and PLP were done in either basic or organic solutions (Heyl et al. 1948). Like any other amino acid, the formation of imine between cysteine and PLP should be a minor pathway, but the subsequent intramolecular nucleophilic addition of the sulfhydryl group toward the imine (which apparently proceeds easily) helps to eliminate the imine complex, which likely breaks the equilibrium and drives the reaction toward imine formation. This may explain the rather progressive accumulation of the cyclized cysteine–PLP complex, because intramolecular cyclization to form the thiazolidine ring through the sulfhydryl group should be fairly rapid if it were not due to the rate-limiting step of imine formation.

Insect ADC shares high sequence identity with mammalian CSADC and both can use cysteine sulfinic acid as a substrate. Aspartate could inactivate GDC by converting the holoenzyme to apoenzyme as a result of the transamination of aspartate (Porter and Martin 1987), but glutamate could not affect insect ADC (Fig. 3). When *An*GDC was mixed with an equal molar concentration of cysteine and glutamate, its GDC activity was diminished (Table 1). The CSADC activity of insect ADC was not significantly affected by cysteine, unless cysteine was pre-incubated with the enzyme. In contrast, when *Hu*CSADC was added to a mixture of an equal molar concentration of cysteine and cysteine sulfinic acid, the production of hypotaurine was diminished (Table 1). These results indicate that cysteine is an inactivator of decarboxylases that use aspartate, glutamate and cysteine sulfinic acid as their substrates. There has been no report discussing cysteine as an inactivator of PLP-dependent decarboxylases in literature, although cysteine was reported to inhibit the activity of crayfish GDC (Grossfeld 1985). Therefore, our data regarding the potential regulation of these enzymes by cysteine fill some knowledge gap in this area.

Our study provides solid evidence indicating that cysteine could inactivate GDC-like proteins through its reaction with the enzyme-bound PLP. PLP serves as the cofactor for many aminotransferases and decarboxylases; ~4 % of all classified enzymatic activities are PLP dependent (Percudani and Peracchi 2003). Based on the reaction mechanisms, however, one may argue that if cysteine can inactivate GDC-like proteins, why many other PLP-containing enzymes do not seem to be affected by this amino acid. Moreover, some aminotransferases actually use cysteine as one of their preferred substrates (Han et al. 2008, 2009). In reality, a number of factors likely limit the ability of cysteine to form cyclized complex with protein-linked PLP. First, cysteine may not be able to enter the active site of many PLP-containing proteins. Second, cysteine may enter into the active site, but its amino group may not be able to be positioned at close proximity to the imine bond of the internal aldimine to initiate

transaldimination. Third, even the amino group of cysteine can interact with the internal aldimine and replace the amino group of ε-amino group of active site lysine to form external aldimine, and the active site residues may restrict the flexibility of the sulfhydryl group of cysteine from undergoing nucleophilic addition to form a complex with PLP. For example, kynurenine aminotransferases can use cysteine as a substrate (Han et al. 2009, 2008), but the interactions of several active site residues with the carboxyl group and sulfhydryl group restrict the flexibility of the thiol group, thereby physically obstructing it from participating in nucleophilic addition to PLP (Fig. 10).

In addition to those mentioned above, the affinity of GDC-like proteins to their nature substrate also seems to affect the efficiency on their inactivation by cysteine. Cysteine is not quite effective in inactivating the CSADC activity of *Ae*ADC. The binding affinity of *Ae*ADC to cysteine sulfinic acid is higher than that to aspartate; this could partly explain why cysteine sulfinic acid competes more effectively than aspartate to react with the internal aldimine when cysteine is present. We noticed that *Ae*ADC Q377L showed diminished CSADC activity when it was mixed with the substrate and cysteine at the same time, suggesting that the conformation and/or hydrophobicity of the active site environment of *Ae*ADC may also affect cysteine inactivation efficiency (Fig. 9; Table 1). Analysis of cysteine on mutant *Ae*ADC suggests that the active site glutamine in wild-type *Ae*ADC may impart negative effect on cysteine-mediated *Ae*ADC inhibition. Glutamine is more hydrophilic than leucine and also can form hydrogen bonds with aspartate and cysteine sulfinic acid (see supplemental figure 3A and 3B). Cysteine is much more hydrophobic than cysteine sulfinic acid and aspartate. The increase in hydrophobicity at position 377 may favor the binding of cysteine in the *Ae*ADC mutant. However, the exact structural and chemical basis remains to be clarified.

Conclusion

In this study, cysteine was identified as an inactivator of aspartate/glutamate/cysteine sulfinic acid α-decarboxylases. The inactivation was demonstrated to be due to the interaction between cysteine and bound PLP from those enzymes. The possibility of such cysteine-dependent inactivation was dependent on the relative position of cysteine and the bound PLP at the active site. The efficiency of such inactivation is likely affected by the hydrophobicity of certain active site residues. The discovery of cysteine-dependent inactivation of aspartate/glutamate/cysteine sulfinic acid α-decarboxylases questions if pressure from the cysteine inhibition of GDC-like enzymes could has been one of the driving forces for a tight regulation of cysteine in vivo and if inactivation of GDC-like proteins due to disorders of cysteine metabolism could eventually lead to neurological symptoms. These apparently deserve further investigations.

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