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SHORT COMMUNICATION

Ketimine reductase/CRYM catalyzes reductive alkylamination of α-keto acids, confirming its function as an imine reductase

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Abstract Recently, crystalized mouse ketimine reductase/CRYM complexed with NADPH was found to have pyruvate bound in its active site. We demonstrate that the enzyme binds α-keto acids, such as pyruvate, in solution, and catalyzes the formation of *N*-alkyl-amino acids from alkylamines and α-keto acids (via reduction of imine intermediates), but at concentrations of these compounds not expected to be encountered in vivo. These findings confirm that, mechanistically, ketimine reductase/CRYM acts as a classical imine reductase and may explain the finding of bound pyruvate in the crystallized protein.

Keywords CRYM · Ketimine reductase · Alkylamination · *N*-methyl amino acids

List of Abbreviations

CRYM	μ-Crystallin
DTT	Dithiothreitol
LCMS	Liquid chromatography-mass spectrometry
P2C	Δ^1-Piperideine-2-carboxylate
T_3	3,5,3′-Triiodothyronine
P2CR	Δ^1-Piperideine-2-carboxylate reductase
TLC	Thin layer chromatography

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Introduction

μ-Crystallin (CRYM) was first purified and identified on the basis of its strong binding to 3,5,3′-triiodothyronine (T_3), the active form of thyroxine (Vie et al. 1997). Indeed, CRYM has been described as the main cytosolic thyroid hormone binding protein (Suzuki et al. 2007). CRYM has also been shown to be the most abundant protein in lens tissue obtained from diurnal marsupials, where it plays a structural role (Segovia et al. 1997). Its enzyme function was first determined in 2011 to be a ketimine reductase, catalyzing the reduction of the imine double bonds of cyclic ketimines such as *S*-2-aminoethylcysteine ketimine and Δ^1-piperideine-2-carboxylate (P2C) (Hallen et al. 2011). Hallen et al. (2011) suggested that ketimine reductase/CRYM substrate levels and T_3 bioavailability are reciprocally linked. In the case of insufficient enzyme substrate concentrations, T_3 will remain bound to the enzyme and therefore be unable to translocate to the nucleus where its binding would normally result in gene expression. This novel interaction between amino acid metabolism and T_3 bioavailability is suggested to be of fundamental importance, linking endocrinology and core metabolic pathways (Hallen et al. 2011). A recent report has indicated that pyruvate is bound in the active site of the solved crystal structure of mouse ketimine reductase/CRYM (Borel et al. 2014). The authors suggested that the enzyme may act as an alanine dehydrogenase and/or as a deaminase in addition to its function as a ketimine reductase. In this brief communication, we offer a rational explanation for the findings of Borel et al. (2014), which further confirms that ketimine reductase/CRYM catalyzes reduction reactions similar to those of classical imine reductases such as glutamate dehydrogenase, which progress through imine intermediates (Hochreiter and Schellenberg 1969).

Methods

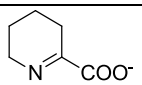
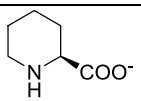
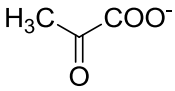
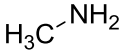
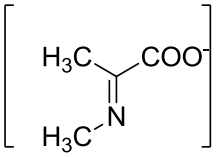
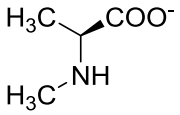
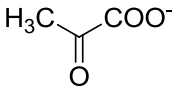
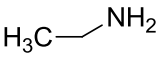
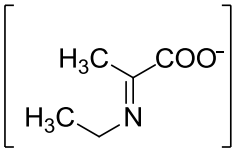
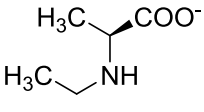
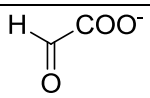
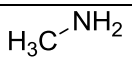
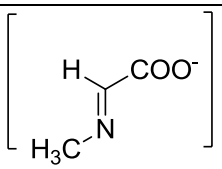
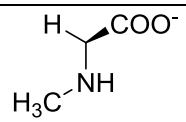
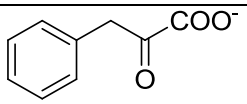
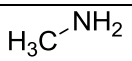
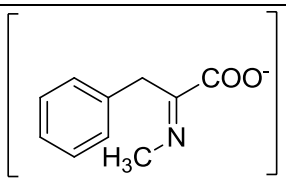
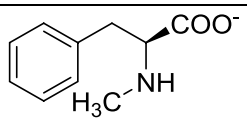
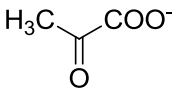
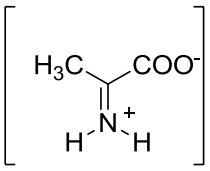
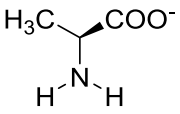
All chemicals and reagents were purchased from Sigma-Aldrich (Sydney, Australia). P2C was synthesized as previously reported (Meister 1954). Human ketimine reductase/CRYM was recombinantly expressed in *Escherichia coli*, and purified as previously reported (Hallen et al. 2011). Ketimine reductase assays were performed at 37 °C in microtiter plates with fluorescence detection (excitation 355 nm, emission 460 nm). The assay mixture contained 200 mM potassium phosphate buffer (pH 7.2), 20 % glycerol and 1 mM dithiothreitol (DTT). For the NADPH-dependent formation of *N*-methyl-L-amino acids, a modification of the assay previously reported was used (Mihara et al. 2005). This assay was adapted for use in microtiter plates and made use of fluorescence measurements (as opposed to UV absorbance measurements). The reaction mixture included 8 μM NADPH, 30 mM alkylamine, 20 % glycerol and 1 mM DTT. A BMG Novostar (Offenburg, Germany) was used for fluorescence detection. The identity of the enzyme products formed in a reaction mixture containing alkylamine and α-keto acid was confirmed using thin layer chromatography (TLC), and also by using liquid chromatography/mass spectrometry (LCMS). TLC was performed in a similar manner to that previously described using Merck silica gel 60 plates, ethyl acetate/ethanol/water/acetic acid (5:2:1:1, by volume) as solvent (Mihara et al. 2005), and *N*-alkylated amino acids were visualized with ninhydrin reagent. LCMS analysis was performed using an Agilent 1260 HPLC coupled to an Agilent 6130 single quadrupole mass spectrometer (Agilent Technologies, USA). A Phenomenex Synergi Hydro-RP C18 HPLC column (250 mm × 4.6 mm) was used and analytes were eluted isocratically (1.5 ml/min) using water/formic acid (0.05 %) except for *N*-methyl-L-phenylalanine. In this case, the eluant additionally contained 5 % acetonitrile. Kinetic data were analyzed using nonlinear regression with GraphPad Prism 5 software (GraphPad software Inc., CA, USA). Data are presented as the mean ± SEM.

Results and discussion

We demonstrate that human ketimine reductase/CRYM can utilize alkylamines (such as methylamine and ethylamine) and α-keto acids (such as pyruvate and phenylpyruvate) as enzyme substrates (Table 1). Under physiologically neutral conditions (pH 7.2), an apparent K_m of 2.9 ± 0.3 mM and apparent V_{max} of 2 ± 0.3 μmol/min/mg were obtained for pyruvate at fixed concentrations of NADPH (8 μM) and methylamine (30 mM). Even at a concentration of 30 mM methylamine, the enzyme is not saturated with this alkylamine (Fig. 1). In comparison, under the same conditions, the cyclic ketimine substrate P2C was previously found to have an apparent K_m of 13 ± 1 μM and apparent V_{max} of 7.4 ± 0.2 μmol/min/mg (Hallen et al. 2015). The reaction products (Table 1) were identified by TLC and LCMS (sarcosine, [TLC] R_f 0.10, [LCMS] retention time 1.82 min/*m/z* $(M + H)^+$ 90.1; *N*-methyl-L-alanine, [TLC] R_f 0.15, [LCMS] retention time 1.92 min/*m/z* $(M + H)^+$ 104.1; *N*-ethyl-L-alanine, [TLC] R_f 0.17, [LCMS] retention time 2.16 min/*m/z* $(M + H)^+$ 118.1; *N*-methyl-L-phenylalanine, [TLC] R_f 0.40, [LCMS] retention time 4.50 min/*m/z* $(M + H)^+$ 180.1). All *N*-alkyl amino acid reaction products were validated by comparison of R_f values on TLC plates and LCMS R_f/$(M + H)^+$ values of authentic compounds, except for *N*-ethyl-L-alanine, which is not commercially available. However, the LCMS result obtained with reaction mixture containing enzyme, NADPH, ethylamine and pyruvate is consistent with the mass of the expected product *N*-ethyl-L-alanine. We were able to detect very low ketimine reductase activity using ammonia as a substrate in the presence of pyruvate, but only at <1 % of the rate observed with the cyclic ketimine substrate P2C (Table 1). However, due to technical difficulties, we could not validate the formation of L-alanine by TLC or by LCMS.

Rates of ketimine reductase/CRYM-catalyzed reduction of imine intermediates formed from methylamine and ethylamine are relatively low compared to that observed with the cyclic ketimine substrate P2C (Table 1). Moreover, concentrations of alkylamines (methylamine, ethylamine) or ammonia required to promote CRYM-catalyzed imine reduction are very high and not encountered in vivo (Table 1). For example, concentrations of ammonia in the normal brain are ~180 μM (Cooper and Plum 1987), phenylpyruvate concentrations in the blood are ~0.5 μM (Turchany et al. 1993), and rat brain methylamine concentration is reported to be ~547 ng/g (Zhang et al. 2012), which equates to approximately 22 μM (assuming a brain water content of 80 %). There are many reports in the literature on the concentration of pyruvate in rat brain. Most reports indicate a concentration of ~e.g., 40–200 μM in normal mammalian brain (e.g., Bergmeyer 1974). Addition of an excess of 1 mM or 5 mM pyruvate to a reaction mixture, which also included 32 μM or 125 μM P2C, respectively, did not alter the observed rate at which P2C is reduced. P2C concentrations in vivo are unknown. However, the enzyme product of P2C reduction, namely pipecolate, in adult rat brain is 5.5 nmol/g or ~6.4 μM (Kim and Giacobini 1985). Though pyruvate undoubtedly binds in the active site of ketimine reductase/CRYM, our results suggest that the binding is not likely to appreciably affect enzyme catalysis under normal conditions.

Table 1 Substrate specificity of CRYM/ketimine reductase toward Δ^1-piperideine-2-carboxylate (P2CR) and various non-cyclized imine intermediates

Reactant	Reactant	Imine intermediate	Proposed Product	Reaction Rate[1]
 Δ^1-Piperideine-2-carboxylate			 L-Pipecolate	[100][2]
 Pyruvate	 Methylamine		 *N*-Methyl-L-alanine[3]	25
 Pyruvate	 Ethylamine		 *N*-Ethyl-L-alanine[4]	24
 Glyoxylate	 Methylamine		 Sarcosine[3]	16
 Phenylpyruvate	 Methylamine		 *N*-Methyl-L-phenylalanine[3]	12
 Pyruvate	NH_4^+ Ammonia		 L-Alanine[5]	<1

The reaction mixture contained 5 mM α-keto acid, 30 mM alkylamine or ammonia, 8 μM NADPH, 200 mM potassium phosphate buffer (pH 7.2), 20 % glycerol, 1 mM DTT and varying amounts of enzyme. In the case of Δ^1 -piperideine-2-carboxylate (P2C), the reaction mix contained 125 μM P2C and 8 μM NADPH and 2.61 nM enzyme (17.6 ng enzyme in a 200 μL volume in a microplate well), in an identical buffer. All readings were carried out in quadruplet

[1] Per cent activity relative to the rate observed with NADPH and the cyclic ketimine substrate P2C

[2] Addition of 5 mM pyruvate to the P2C reaction mixture did not change the reduction reaction rate

[3] TLC and LCMS identical to authentic compounds

[4] LCMS confirms expected mass of product—no available commercial compound

[5] Unable to validate due to technical difficulties

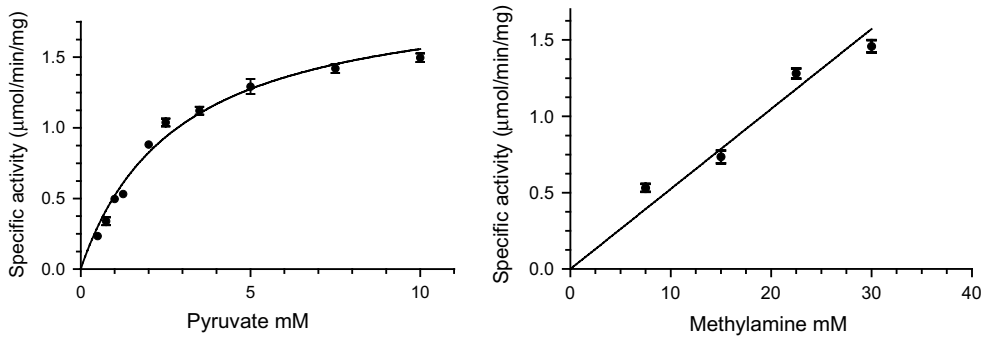


Fig. 1 *Left* plot of specific activity in the presence of 8 µM NADPH, 30 mM methylamine, 10.4 nM enzyme (70.4 ng enzyme in a 200 µL volume), and varying concentrations of pyruvate ($K_{m\ app}$ 2.9 ± 0.3 mM, $V_{max\ app}$ 2 ± 0.3 µmol/min/mg); *right* plot of the specific activity in the presence of 8 µM NADPH, 10 mM pyruvate and varying concentrations of methylamine. It should be noted that even at 30 mM methylamine the enzyme is not saturated. The concentration of reactants exceeds that which would be expected in vivo (~50—200 µM pyruvate, 22 µM methylamine) and thus this reductive alkylamination of pyruvate is not likely to proceed in vivo to any measureable extent (see the text). All readings were conducted in quadruplet

Ketimine reductase/CRYM is homologous with a number of prokaryote and archaea enzymes involved in amino acid metabolism, namely ornithine cyclodeaminase (Goodman et al. 2004) and lysine cyclodeaminase (Gatto et al. 2006), which have been shown to catalyze reactions that proceed via a cyclic ketimine intermediate. This phylogenetic relationship would explain how CRYM has evolved as a cyclic ketimine reductase, but does not explain the role of CRYM in reductive alkylamination of α-keto acids. CRYM is, however, also homologous with bacterial/archaeal alanine dehydrogenase, which is responsible for the reversible reductive amination of pyruvate to L-alanine (Gallagher et al. 2004). Ketimine reductase/CRYM has been shown previously not to act as either an ornithine cyclodeaminase (Kim et al. 1992) or as an alanine dehydrogenase (Schroder et al. 2004). Bacterial Δ^1-piperideine-2-carboxylate reductase (P2CR) was previously found to catalyze the reduction of imines formed from alkylamines and α-keto acids. However, it was concluded that the ability of bacterial P2CR to utilize alkylamines as substrates is not likely to be of significance in vivo and that the enzyme's preferred substrates are cyclic imines such as P2C (Mihara et al. 2005; Muramatsu et al. 2005, 2006). The present work shows that mammalian ketimine reductase/CRYM catalyzes similar reactions to those catalyzed by bacterial P2CR even though the mammalian and bacterial enzymes are not homologous. The current research, together with prior research on bacterial P2CR, suggests that alkylamines and pyruvate first condense to form an imine intermediate, which is then subsequently reduced using NADPH as a cofactor. Imine formation from a reaction mixture containing carbonyls and alkylamines is a well characterized reaction that occurs spontaneously in aqueous solutions and is generally freely reversible (Jenks 1969). No measurable activity at neutral pH was detected in the reverse reaction with $NADP^+$ (0.1 mM), *N*-methyl-L-alanine (5 mM), and enzyme (26.1 nM). This is consistent with previous research on mammalian ketimine reductases which demonstrated that under physiological conditions the enzyme only catalyzes the forward reaction (Meister and Buckley 1957; Nardini et al. 1988). This brief communication also provides an explanation for the prior finding of pyruvate in the active site of the enzyme crystal structure by Borel et al. (2014).

Mammalian ketimine reductase reaction is known to be enantiospecific and only the L-enantiomer product is formed in vivo (Chang 1976; Meister and Buckley 1957; Nardini et al. 1988). In this short communication, we do not address the stereochemistry of the product obtained by reduction of the imine generated from α-keto acid/alkylamine. However, it is reasonable to suggest that only the L-enantiomer is produced (except in the case of sarcosine, which does not possess D- and L-enantiomers).

In conclusion, the present findings show that mammalian/human ketimine reductase/CRYM catalyzes reduction of non-cyclic imines in a similar fashion to that noted previously for glutamate dehydrogenase. However, a ketimine reductase/CRYM-catalyzed reaction at neutral pH in the reverse direction could not be demonstrated. Thus, ketimine reductase/CRYM-catalyzed reductive amination/alkylamination of α-keto acids (or oxidation of L-amino acids/*N*-alkyl-L-amino acids) is not likely to be of physiological importance in mammals in vivo. Nevertheless, mammalian ketimine reductase/CRYM may be of importance as a means of synthesizing *N*-L-alkylamino acids in vitro for use as chemical building blocks.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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