Amino acid metabolism in mammalian brain

Discovery and characterisation of mammalian ketimine reductase

A Thesis Submitted in Partial Fulfilment of
The Degree of Doctor of Philosophy from Macquarie University
by

André Hallen, BSc (Hons.)

40317463

Graduation Date: 15th September, 2015

Primary Supervisor: Prof. Peter Karuso

Associate Supervisor: Prof. Paul Haynes

Adjunct Supervisor: Prof. Arthur Cooper, New York Medical College, USA

Chemistry and Biomolecular Sciences Department
Macquarie University
Sydney 2109
Australia

Higher Degree Research Office
Macquarie University
NSW 2109 Australia
T: +61 (2) 9850 7345
F: +61 (2) 9850 6198
hdrexam@mq.edu.au
ABN 90 952 801 237
CRICOS Provider No 00002J

MACQUARIE
University
SYDNEY·AUSTRALIA

14 July 2015

Ref: 40317463

Andre Hallen
10/14-18 Jones St
Ryde NSW 2112

Dear Mr Hallen,

I am pleased to be able to advise that the Dean of Higher Degree Research on behalf of the Higher Degree Research Committee considered your corrections report and determined that your thesis entitled "Amino Acid Metabolism in Mammalian Brain: Discovery and Characterisation of Mammalian Ketimine Reductase"[1] be accepted as satisfying the requirements for the award of PhD. May I offer you my sincere congratulations.

Please find enclosed a copy of the examiners' reports. Your supervisor(s) has also been sent a copy of the reports, the reserved copy of your thesis and a copy returned by one of your examiners. You are given the opportunity to amend any typographical or editing errors. Amendments need to be completed by no later than 14 Aug 2015.

Australian Higher Education Graduation Statement: The Australian government requires that the Statement be provided to all candidates on completion of the requirements for a particular higher education award. It provides a description on the nature, level, context and status of studies that were pursued. In order for the Statement to be issued, if you have not done so, you are required to submit an abstract of your thesis to hdrexam@mq.edu.au with the subject of "Australian Higher Education Graduation Statement" within 1 month of the date of this letter at the very latest. The submitted abstract must strictly be no longer than 100 words.

Digital Thesis: Completing higher degree research candidates are required to submit a digital copy of their thesis so that it may be added to the Australian Digital Theses Program. Information regarding this program and the Digital Thesis Submission Policy and Forms are available from the Higher Degree Research Office (HDRO) website. Completed forms and digital documentation should initially be forwarded to the Higher Degree Research Office.

Hardbound Thesis: Three Copies of your thesis should be permanently bound and lodged at the **Faculty Higher Degree Research Office** in your Faculty by no later than 14 Aug 2015 together with a copy of your Certification of Completion Form:
http://www.hdr.mq.edu.au/information_for/thesis_examiners/documents/Certification_of_Completion_Form_-_June_09.pdf. One copy of your thesis will be housed in the University Library, one copy will be sent to your supervisor and one copy will be sent to the Executive Dean of Faculty.

Important: Please note that if you required and were given an ethics clearance to conduct your research, you must include a copy of your final ethics approval letter in your permanently bound corrected thesis as an appendix.

Graduation Ceremony: For details regarding graduation ceremony procedures please refer to the University website – http://www.mq.edu.au/graduation/.

On behalf of the Higher Degree Research Committee, please accept my congratulations on your fine achievement and my best wishes for your future career.

Should you have any queries regarding this matter, please feel free to contact me.

Yours sincerely,

Dr Ren Yi
Director, Research Training and International Research Training Partnerships
(for and on behalf of)
Professor Sakkie Pretorius
Deputy Vice-Chancellor (Research)

---

[1] **Please review your thesis title. If your thesis title is incorrect, please contact the Higher Degree Research Office via hdrexam@mq.edu.au immediately**

MACQUARIE UNIVERSITY

PART II:

REPORT OF EXAMINER TO THE CANDIDATE ON A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Please state concisely the grounds on which the recommendation is based, indicating the strengths and weaknesses of the thesis. This should be in sufficient detail for candidates to gain clear understanding of your opinion of their work and the means by which your conclusions were reached.

Where further work is required, please indicate clearly those revisions and/or corrections which you wish to see made. Supplementary sheets may be attached.

Name of Candidate: HALLEN, Andre

Name of Examiner: Dr Eduard A. Struys

With interest and appreciation, I have reviewed the thesis of the candidate Andre Hallen on the subject of the discovery and characterization of mammalian ketamine reductase.

Although biochemical pathway of lysine has been intensively studied for decades, there are still many questions and debates on the actual biochemical catabolic route of this essential amino acid. Studies performed in the 70's of the previous century, postulated that lysine can be degraded via two distinct routes i.e. the pipecolic acid branch and the saccharopine branch.

The current thesis by Andre Hallen provided evidence that P2C can be converted by ketamine reductase/CRYM into l-pipecolic acid, substantiating the claim that lysine can be degraded via the l-pipecolic acid branch.

This thesis contains several complicated biochemical techniques and procedures, combined with impressive organic chemistry to prepare the not commercially available (and labile) substrates like for instance P2C. In this sense, the current work integrates several disciplines to further unravel the complexity of metabolic pathways and routes.

For future work, I would strongly advise to incorporate Mass Spectrometry into this research. It is of vital importance to measure the actual formed product of ketamine reductase/CRYM, instead of measuring the conversion of the cofactor involved. Only this approach, would yield unambiguous results, and verifies that the presumed reaction has indeed taken place.

Overall, this thesis is well written, and papers are published in peer-reviewed journals. The presented figures are clear and concise.

I strongly appreciate this biochemical work, illustrating that our current knowledge about the actual function of proteins/enzymes is still not complete, and that with a technical integrative approach we can further unravel novel functions of known enzymes, and their actual role in mammalian biochemistry.

Signature of Examiner: ....

Date: May 15th 2015.

MACQUARIE UNIVERSITY

PART II: **REPORT OF EXAMINER TO THE CANDIDATE**

ON A THESIS SUBMITTED FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

Please state concisely the grounds on which the recommendation is based, indicating the strengths and weaknesses of the thesis. This should be in sufficient detail for candidates to gain clear understanding of your opinion of their work and the means by which your conclusions were reached.

Where further work is required, please indicate clearly those revisions and/or corrections which you wish to see made. Supplementary sheets may be attached.

Name of Candidate: HALLEN, Andre

Name of Examiner: Professor Philip Board

See attached.

Signature of Examiner: ....

Date: 25/5/15

A report on a PhD thesis submitted by Andre Hallen

The thesis submitted by Andre Hallen consists of five manuscripts that have either been published or submitted for publication in recognized international peer reviewed journals. The candidate is the first author on all these works and has clearly made the major contribution to the conception and execution of the studies. In addition, the candidate has included three other papers to which he has made more modest contributions. The combined works involve a multi-disciplinary approach indicating that the candidate has mastered a wide range of techniques. The experimental work appears to have been undertaken with skill and the interpretation of the results is consistently well reasoned reflecting a sound understanding of the field and the issues involved. The manuscripts are well written and easy to read. It was very good to see classical enzymology coupled with bioinformatics and structural biology. In all, the thesis represents an excellent and extensive contribution to knowledge very worthy of the award of the degree.

Chapters 2,5and 6 contain the major experimental contributions and chapters 3 and 4 are reviews. While the reviews are more extensive than would occur in a classical thesis they are scholarly and authoritative and integrate the novel findings of the candidate's research into the broad context of the field and will be of great value to others working in this area.
The experimental chapters report the laborious purification and characterization of a mammalian ketimine reductase and the proteomics analysis that lead to the novel discovery that the protein had previously been characterized as a lens crystalin in some marsupials and importantly that the enzyme is a thyroid hormone binding protein in the brain. The inhibitory interactions with T3 and T4 are of physiological significance and have been well characterized in chapter 5. This discovery and the related discussion of the interdependent roles of ketimine reductase, cyclic ketimines and thyroid hormone bioavailability is particularly interesting and constitutes a significant original contribution.

I only have one issue that may have been investigated further by the candidate but has not been discussed in any detail. Experience from other enzyme families suggests that distantly related genes with related or variant functions can be identified by low stringency alignments. There are several references in the thesis to the possibility of another ketamine reductase in mammals. Given the candidate undertook some sequence alignments to identify the closely homologous ketimine reductases in other species I would have expected that he may have undertaken searches of the human or mouse transcriptomes looking for low identity variants that may represent the other suspected gene. In addition he could have searched the human, mouse or other transcriptomes with the sequence of the PC2 reductase from P. syringae, or the ornithine cyclodeaminase or alanine dehydrogenase sequences mentioned in the bioinformatics section on page 27. A comment on the results of such searches could be included in the final thesis if they have been undertaken.

There is a very pleasing absence of typographical errors. Two that I have noted :
P7 L7, "reductase" is superfluous.
P39, bottom of left column. Garweg et. al. should be ref [62] not [60].

MACQUARIE UNIVERSITY

PART II: **REPORT OF EXAMINER TO THE CANDIDATE**

ON A THESIS SUBMITTED FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

Please state concisely the grounds on which the recommendation is based, indicating the strengths and weaknesses of the thesis. This should be in sufficient detail for candidates to gain clear understanding of your opinion of their work and the means by which your conclusions were reached.

Where further work is required, please indicate clearly those revisions and/or corrections which you wish to see made. Supplementary sheets may be attached.

Name of Candidate: HALLEN, Andre

Name of Examiner: Professor Glenn King

SEE ATTACHED REPORT

Signature of Examiner: .... **Date:** 05/07/15

Report on PhD thesis by André Hallen

This is an outstanding thesis that was a pleasure to read. It is certainly one of the best theses that I have had the pleasure of reviewing in the past decade. In addition to clearly describing the excellent and important data obtained by the candidate, the thesis was almost entirely free of grammatical and typographical errors.

The thesis describes the purification and identification of ketimine reductase from lamb forebrain followed by recombinant production and characterization of the human homolog. This is an important breakthrough as even thought this enzyme activity was described in bovine cerebellum as early as the 1980s, the identity of the enzyme responsible had until now proved elusive. Surprisingly, the responsible enzyme proved to be identical with mu-crystallin, which plays a structural role in the lens of diurnal marsupials and which is the major cytosolic thyroid hormone binding protein in mammalian brain. Thus, this research provides the first evidence implicating thyroid hormones in regulation of mammalian amino acid metabolism. Thesis chapters 2 and 5 are published primary research articles in high-quality journals, while chapters 3 and 4 are published review articles, also in high-quality journals. The work described in chapter 6 has also been submitted for publication. Thus, this thesis has produced an impressive output of three primary research articles and two reviews. The candidate was also a coauthor on three other publications during the period of this candidature. Thus, there is clearly no doubt that the thesis "makes a distinct contribution to knowledge in the area in which it deals" and that it "contains a substantial amount of material suitable for publication". Thus, I enthusiastically recommend award of the degree. The errors listed below are extremely minor and have no bearing on the scientific content; I have listed them in case the candidate and supervisor wish to make these amendments, but I consider correction of these minor errors to be optional.

Optional corrections

- Page 7, line 11: The end of this sentence does not make grammatical sense ("....and may be regulate enzyme activity *in vivo*")
- Page 13, line 8: Should be "...well known protein".
- Replace Chapter 5 with published paper.
- Page 124, 4th line in Section 7.2.1: Replace "30 mins" with "30 min".
- Page 124, 5th line from bottom: Add a space between number and units in "0.946 g".
- Page 124, 4th line from bottom: Add a space between number and units in "1 g".
- Page 125, line 7: Add a space between number and units in "7 g".
- Page 124, line 13: Replace "15 mins" with "15 min".
- Page 124, line 21: Change "...in a similar manner as described" to "...in a similar manner to that described".
- Page 124, line 23: Add a space between number and units in "0.1 M NaOH".
- Page 125, line 3: Add a space between number and units in "1 M NaOH".
- Page 125, line 8: Add a space between number and units in "1 M HCl".

TABLE OF CONTENTS

Acknowledgements **1**
Thesis declaration **3**
Summary **7**
Chapter 1: Introduction **9**
Chapter 2: The discovery of a mammalian brain ketimine reductase and identification of its gene **13**
  Overview 13
  Published paper 15
    Abstract 15
    Introduction 15
    Materials and methods 17
      Materials and assays 17
      Purification protocol 17
      Preparation of lamb brains 17
      Anion exchange chromatography 17
      Hydrophobic interaction chromatography 17
      Flow-through protocol 18
      Sodium dodecyl sulphate- polyacrylamide gel electrophoresis 18
      Protein identification by MS/MS 18
      Expression and purification of recombinant proteins in *E.coli* 18
      Gel filtration 18
    Results 18
      Identification of recombinant human CRYM as ketimine reductase 19
      Michaelis-menten kinetics 19
      Inhibition studies 20
    Discussion 21
    References 22
    2.3 Supplementary material not included in publication 24
      2.3.1 Protein purification chromatograms 24
      2.3.2 Gel filtration 25
      2.3.3 Protein isoforms μ-crystallin/ketimine reductase 26
      2.3.4 Bioinformatics 27
      2.3.5 pH optimum 28
      2.3.6 Gene expression 28
      2.3.7 Erratum 29
Chapter 3: Review: Imine reductases and their mechanisms **31**
  Overview 31
  Published paper 32
    Abstract 32
    Introduction 32
      Metabolic importance of the GDH reaction 33
      Glutamate dehydrogenase catalysis proceeds via an imine intermediate 35
      The central importance of cyclic imines in the metabolism of L-lysine 35
      The central importance of cyclic ketimines in the inter-related metabolism of 5-carbon amino acids 37
      The discovery of specific imine reductases 38
      Sulphur-containing cyclic ketimines: Novel metabolic intermediates with potentially important neurochemical properties 39
      μ-Crystallin: the first definitively identified mammalian ketimine reductase 41
      Neurochemical importance of ketimine reductase/CRYM/T3 binding 42
    Summary 43
    References 43

Chapter 4: Review Lysine metabolism in mammalian brain **47**
- Overview 47
- Published paper 48
  - Abstract 48
  - Introduction 49
    - The saccharopine pathway: a major degradative pathway for lysine in extracerebral tissues and fetal brain but a minor pathway in adult mammalian brain 49
    - The nature of the first step of the pipecolate pathway in brain remains unresolved 51
    - The discovery of specific cytosolic P2C reductases 54
    - μ-Crystallin: the first P2C reductase identified in mammalian genome databases 55
    - The significance of CRYM/KR in psychiatric and neurological disease 58
    - The peroxisomal connection: oxidation of L-pipecolate 59
    - The convergence of pathways and the mystery of B_6-dependent epilepsy solved 61
    - Interconnection between lysine and tryptophan pathways: developmental and regulatory aspects 62
  - Summary 66
  - References 66

Chapter 5: Enzyme catalysis, mechanisms, and regulation under physiological neutral conditions **71**
- Overview 71
- Submitted paper 72
  - Abstract 72
  - Introduction 73
  - Methods 75
    - Activity assays 75
    - Substrate and inhibitor *in silico* docking 77
  - Results 77
    - Substrate specificity 77
    - Enzyme inhibition 79
    - *In silico* docking of substrates and inhibitors 80
  - Discussion 82
  - References 84
- **Supplementary material** 87
  - A. Enzyme assays 88
  - B. Inhibitor assays 89
  - C. Multiple sequence alignment 91
  - D. In Silico docking 92
  - E. Organic synthesis- Δ^2-thiazoline-2-carboxlate 93
  - References 98

Chapter 6: Synthesis of *N*-alkylamino acids by ketimine reductase/CRYM 99
- Overview 99
- Submitted paper 100
  - Abstract 100
  - Introduction 100
  - Methods 101
  - Results and discussion 101
  - References 104
  - **Supplementary materials** (LC-MS data) 105

Chapter 7: Experimental 107
7.1 Enzyme assays 108
  7.1.1 Standardisation of NAD(P)H concentrations 108
  7.1.2 Fluorescence standard curve 108
  7.1.3 Protein concentration 109
  7.1.4 Catalytic rate 109
  7.1.5 Specific activity 109
7.2 Organic synthesis 110
  7.2.1 *S*-2-Aminoethylcysteine ketimine (AECK) 110
  7.2.2 Lanthionine ketimine (LK) 115
  7.2.3 Cystathionine ketimine (CysK) 117
  7.2.4 Δ^1-Pyrroline-2-carboxylate (Pyr2C) 118
  7.2.5 Δ^1-piperideine-2-carboxylate (P2C) 121

Conclusions and future directions 125

Appendices

A1. Published paper 128
New insights into the metabolism of organomercury compounds: Mercury-containing cysteine S-conjugates are substrates of human glutamine transaminase k and potent inactivators of cystathionine χ-lyase

A2.Published book chapter 138
Chapter 3: Role of glutamine transaminases in nitrogen, sulfur, selenium, and 1-carbon metabolism

A3.Published letter to the editor 156
µ-Crystallin/CRYM functions as a ketimine-reducing enzyme and plays a role in thyroid hormone bioavailability due to strong inhibition/regulation by thyroid hormones

LIST OF FIGURES AND TABLES

Chapter 1: Introduction

Fig.1.1 Reactions catalysed by ketimine reductase/CRYM 10
Fig.1.2 Central role of ketimine reductase/CRYM in biochemical pathways 11
Fig.1.3 Synthesis of *N*-alkylamino acids 12

Chapter 2: The discovery of a mammalian brain ketimine reductase and identification of its gene

Fig.A Flowchart depicting the protocols used in enzyme purification 14
Fig.1 A selection of NADH/NADPH-dependent enzyme reactions catalysed by ketimine reductases 16
Fig.2 Metabolic pathways leading to the sulphur-containing cyclic ketimines 16
Fig.3 Purification of forebrain ketimine reductase 19
Fig.4 Identification of forebrain ketimine reductase as CRYM by MS/MS 20
Fig.5 Michaelis-Menten non-linear regression fit of kinetic data 21
Fig.6 Inhibition of ketimine reductase activity by T3 at pH 5 21
Fig.7 Inhibition of ketimine reductase activity by T3 at pH 7 21
Table 1. Relative reaction rates with different ketimine substrates catalysed by various ketimine reductases 20

Supplementary material not included in publication

Fig.S1 Chromatograms for lamb forebrain purification protocols 24
Fig.S2 Gel filtration of human recombinant ketimine reductase/CRYM 25
Fig.S3 Plot of K_{av} vs log MW of the protein standards 25
Fig.S4 Gel filtration of partially purified ketimine reductase 27
Fig.S5 μ-Crystallin homologues superimposed 27
Fig.S6 Sequence alignment of μ-crystallin, alanine dehydrogenase and ornithine cyclodeaminase 28
Fig.S7 pH optimum for ketimine reductase/CRYM 29
Fig.S8 Gene expression of ketimine reductase/CRYM in rat brain 30

Chapter 3: Review: Imine reductases and their mechanisms

Fig. 1 Enzymatic pathways for the formation of cyclic ketimines and their reduction by glutamate dehydrogenase 37
Fig. 2 Lysine catabolism in mammalian tissues 37
Fig. 3 Metabolic inter-conversions of 5-C amino acids in mammalian tissues showing the central importance of the cyclic ketimines 38
Fig. 4 Metabolic pathways leading to the formation of sulfur-containing cyclic ketimines ... 40
Fig. 5 Reduction of sulphur-containing cyclic ketimines 40

Chapter 4: Review Lysine metabolism in mammalian brain

Fig. 1 The saccharopine pathway for the metabolism of L-lysine in mammals 50
Fig. 2 The pipecolate pathway for the metabolism of L-lysine in mammals 52
Fig. 3 Structures of several known CRYM/KR substrates 58
Fig. 4 The interaction of L-lysine and L-tryptophan pathways in mammals 64
Fig. 5 The remarkable amino acid substrate versatility of α-aminoadipate aminotransferase/kynurenine aminotransferase II 64

Chapter 5: Enzyme catalysis, mechanisms, and regulation under physiological neutral conditions

Fig. 1 Metabolic pathways involving sulphur-containing cyclic ketimines 74
Fig. 2 Lysine/ornithine catabolism and inter-connected pathways 75
Fig. 3 Substrate inhibition of human ketimine reductase/CRYM 76
Fig. 4 *In silico* docking of substrates to ketimine reductase/CRYM 80
Fig. 5 Proposed mechanism for the reaction catalyzed by ketimine reductase 81

Fig. 6 Thyroid hormones docked into the crystal structure of ketimine reductase 82
Table 1. Substrate specificity of ketimine reductase/CRYM 78
Table 2. Docking scores and relative reaction rates exhibited by ketimine reductase/CRYM toward various substrates at two pH values.. 78
Table 3. Inhibition of ketimine reductase/CRYM by thyroid hormones 83
Table 4. Inhibition off ketimine reductase/CRYM by aromatic heterocyclic analogues 84

Supplementary material

Fig. S.1 Michaelis-Menten kinetic plots for cyclic ketimine reductase/CRYM.......................... 88
Fig. S.2 Inhibition plots obtained with thyroid hormones and analogues.............................. .89
Fig. S.3 Inhibition plots with cyclic substrate analogues of ketimine reductase/CRYM.......... 90
Fig. S. 4 Multiple sequence alignment of eukaryote ketimine reductase/CRYM 91
Fig. S. 5 Docked ligands in the active site of human ketimine reductase/CRYM...................... 92
Fig. S. 6 ^{1}H NMR and ^{13}C NMR for Δ^2-thiazoline-2-carboxylate ethyl ester 94
Fig. S. 7 ^{1}H MNR and ^{13}C NMR for Δ^2-thiazoline-2-carboxylate 95
Fig. S. 8 IR spectra for Δ^2-thiazoline-2-carboxylate and its ethyl ester.................................... 96
Fig. S. 9 UV spectra for Δ^2-thiazoline-2-carboxylate and its ethyl ester 97

Chapter 6: Synthesis of *N*-alkylamino acids by ketimine reductase/CRYM

Fig. 1 Kinetic plots of methylamine and pyruvate as co-substrates for ketimine reductase/CRYM 103
Table 1. Substrate specificity of CRYM/ketimine reductase toward Δ^1-piperideine-2-carboxylate and various non-cyclised imine intermediates.. 102

Supplementary materials (LC-MS data)

Fig. S. 1 LC-MS data for sarcosine 105
Fig. S. 2 LC-MS data for *N*-methyl-L-alanine 105
Fig. S. 3 LC-MS data for *N*-ethyl-L-alanine... 106
Fig. S. 4 LC-MS data for *N*-methyl-L-phenylanine 106

Chapter 7: Experimental

Fig. 7.1 NADPH standard curve 108
Fig. 7.2 Bradford protein concentrations.. 109
Fig. 7.3 Enzyme catalytic progress curve 110
Fig. 7.4a 1D ^{1}HNMR spectrum of AECK ethyl ester... 111
Fig. 7.4b 1D ^{13}CNMR spectrum of AECK ethyl ester curve 112
Fig. 7.4c 2D HSQC spectrum of AECK ethyl ester... 112
Fig. 7.4d 1D ^{1}HNMR spectrum of AECK sodium salt 113
Fig. 7.4e 1D ^{13}CNMR spectrum of AECK sodium salt... 114
Fig. 7.4f 2D HSQC spectrum of AECK sodium salt 114
Fig. 7.5a 1D ^{1}HNMR spectrum of lanthionine ketimine... 116
Fig. 7.5b 1D ^{13}CNMR spectrum of lanthionine ketimine 116
Fig. 7.5c 2D HSQC spectrum of lanthionine ketimine 117
Fig. 7.6a 1D ^{1}HNMR spectrum of Pyr2C ethyl ester 119
Fig. 7.6b 1D ^{13}CNMR spectrum of Pyr2C ethyl ester 119
Fig. 7.6c 1D ^{1}HNMR spectrum of Pyr2C sodium salt... 120
Fig. 7.6d 1D ^{13}CNMR spectrum of Pyr2C sodium salt.. 120
Fig. 7.7a 1D ^{1}HNMR spectrum of P2C sodium salt 121
Fig. 7.7b 1D ^{13}CNMR spectrum of P2C sodium salt 122
Fig. 7.7c 2D HCOSY of P2C sodium salt 122
Fig. 7.7d 2D HSQC of P2C sodium salt 123

ACKNOWLEDGEMENTS

My journey towards a science degree began from a hospital ward. I am fortunate to have recovered from a serious chronic illness which took away my opportunities for decades. I know many who were never as fortunate as me and I am forever grateful for the opportunities I have had. No matter how hard life can be I always have a little happiness inside me. I am most grateful that my parents and family instilled in me the virtues of honesty and decency which have carried me through many difficult times and hopefully will carry me further in my quest to understand the biochemistry of the brain. They took care of me when I was ill and gave me the support I needed to rebuild my life. I could not have wished for a better family and support.

This research was self-initiated and I am grateful to Macquarie University for allowing me to do my own research. My undergraduate degree in Biological Chemistry at Macquarie University gave me a solid foundation in all the knowledge I required to do this research. My supervisor, Professor Peter Karuso, has been a rock-solid support and a seemingly endless source of knowledge about practically everything. I am very grateful for his support and for taking me on in my PhD under very difficult circumstances and for pushing me along. He is always one to insist on doing things the “correct way” and never accepting shoddy reasoning. Thanks also go to my associate supervisor, Professor Paul Haynes, for introducing me to Proteomics which was crucial in elucidating the identity of ketimine reductase, and he has always had an interest in my research. My adjunct supervisor, Professor Arthur Cooper is also one of the most decent and honest persons I have ever known. A true gentleman who has helped me in an area of biochemistry where few are more noted and more experienced and much thanks goes for his support and assistance. Without his support and input my research would not have evolved the way it has. I would also like to thank my Honour’s supervisor Associate Professor Joanne Jamie who still to this day contributes to my research with her broad knowledge of organic chemistry, medicinal chemistry, and biochemistry. Many thanks to Dr. Andrew Piggott for his assistance in running NMR spectroscopy and Jason Smith for his assistance with LC-MS. Thanks also to my initial PhD supervisor Professor Robert Willows who initiated me into this career of being a biochemist.

I have been blessed with numerous friends who over the years have supported me in every way. At times my research would have died without their support. Too many to even mention all of them. Thanks to Adam, John, Andrew, Neilma, William, David, Laurelle, Louise, Brian, Stefan, Soo, Robyn and so many others. Most noted of all is Professor Philip Kuchel who gave me a start in this scientific pursuit before I even started university. I am forever thankful for that start in a new life and he will always be remembered for everything he has done for me over the years. He has always been one to encourage me when times were difficult and to push me along when I started to falter. It has always been an ambition of mine to prove to him that his faith in me has been warranted. My little dog, Jack, also has been a constant companion and support for almost 15 years and we have been through many difficult times together - small in size but a giant in personality. Most thanks of all goes to my best friend Jenny who has ensured that every day has a little happiness in it. A true little happy spirit and the nicest person I have ever known.

During my PhD candidature I have learnt a lot about life and people, not only about research. Those lessons have only strengthened me and made me more determined to pursue my research further. I was too naïve and trusting previously but I have now matured into a more discerning individual, more suited to a life in research. As Winston Churchill once said “This is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning”. So it is with my research and I hope to be able to carry on in the future with this journey of discovery in such an important field such as neurobiochemistry. I have still much to do in life as I have only finished the beginning.

THESIS DECLARATION

This thesis is composed of my own original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis. I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. This research was self-initiated and was not pre-existing research at Macquarie University. This research emanated from my own interest in an area of brain biochemistry, which was relatively unexplored, and which appeared to be potentially of immense importance. The ground work for my research during my PhD candidature was laid by me as an Honours student at Macquarie University, however I was unable to purify a brain ketimine reductase during that time for gene identification. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution.

I acknowledge that an electronic copy of my thesis must be lodged with Macquarie University Library and, immediately made available for research and study. I acknowledge that copyright of all material contained in my thesis resides with the copyright holder(s) of that material. My PhD candidature was supported by a MQ-RES scholarship and research costs were provided by the university to facilitate my research.

Statement of contributions to jointly authored works contained in the thesis

Corresponding author underlined and submitting PhD candidate in bold.

Chapter 2:

[1]**Hallen, A.**, [2]Cooper A.J., [3]Jamie J.F., [4]Haynes P.A., [5]Willows R.D., *Mammalian forebrain ketimine reductase identified as μ-crystallin; potential regulation by thyroid hormones*, J Neurochem. 2011 Aug; 118(3): 379-87.

[1]Responsible for conception and experimental design, all experiments, and first draft of paper. [1,2,3,4,5]Responsible for editing and writing of final paper. [3]Responsible for advice on

organic synthesis. [4]Responsible for advice on proteomic analysis. [5]Responsible for advice and assistance on biochemical procedures and analysis of data.

Chapter 3:

[1]**Hallen, A.**, [2]Jamie, J.F., and [3]Cooper, A.J.L., *Imine reductases: A comparison of glutamate dehydrogenase and ketimine reductases in the brain*, Neurochemical research, 2014; 39(3): 527-41.

[1,2]Responsible for conception and first draft of paper. [1,2,3]Responsible for editing and writing of final paper.

Chapter 4:

[1]**Hallen, A.**, [2]Jamie, J.F., and [3]Cooper, A.J.L., *Lysine metabolism in mammalian brain: an update on the importance of recent discoveries*, Amino Acids. 2013 Dec; 45(6): 1249-72.

[1,2]Responsible for conception and first draft of paper. [1,2,3]Responsible for editing and writing of final paper.

Chapter 5:

[1]**Hallen, A.**, [2]Cooper, A.J.L., [3]Jamie, J.F., and [4]Karuso, P., *Insights into enzyme catalysis and thyroid hormone regulation of cerebral ketimine reductase/μ-crystallin under physiological conditions*, Neurochem Res. 2015 May; 40:1252–1266.

[1]Responsible for conception and experimental design, all experiments, and first draft of paper. [1,2,3,4] Responsible for editing and writing of final paper. [2]Responsible for advice on biochemistry. [3,4]Responsible for advice on organic synthesis.

Chapter 6:

[1]**Hallen, A.**, [2]Cooper, A.J.L., [5]Smith J.R., [3]Jamie, J.F., and [4]Karuso, P., *Ketimine reductase/CRYM catalyses reductive alkylamination of α-keto acids, confirming its function as an imine reductase,* Amino Acids. 2015 July, DOI 10.1007/s00726-015-2044-8.

[1]Responsible for conception and experimental design, all experiments, and first draft of paper. [1,2,3,4]Responsible for editing and writing of final paper. [2]Responsible for advice on biochemistry. [3,4]Responsible for advice on organic synthesis. [5]Responsible for LC-MS spectrometry.

Appendices: Other jointly authored works published during my PhD candidature

Bridges, C.C., Krasnikov, B.F., Joshee, L., Pinto, J.T., [1]**Hallen, A.**, Li J., Zalups, R.K., Cooper, A.J., *New insights into the metabolism of organomercury compounds: mercury-containing cysteine S-conjugates are substrates of human glutamine transaminase K and potent inactivators of cystathionine γ-lyase.* Arch Biochem Biophys. 2012 Jan 1; 517(1): 20-9.

[1] Responsible for HPLC enzyme assays, preparation of the chemical figures, editing final draft.

[1]**Hallen A.**, [2]Cooper A.J. *Letter to the editor: μ-crystallin/CRYM functions as a ketimine-reducing enzyme and plays a role in thyroid hormone bioavailability due to strong inhibition/regulation by thyroid hormones.* J Clin Endocrinol Metab. 2015 Jan; 100(1):L15-6.

[1,2] Responsible for writing and editing.

Arthur J. L. Cooper, Thambi Dorai, Bhuvaneswari Dorai, Boris F. Krasnikov, Jianyong Li, **André Hallen**[1], John T. Pinto.: *Glutamine in clinical nutrition. Chapter 3: Role of glutamine transaminases in nitrogen, sulfur, selenium, and 1-carbon metabolism*, R. Rajendram et al (editors), Nutrition and Health, New York 2015.

[1] Writing section on sulfur-containing cyclic ketimines, preparation of the chemical drawings, and editing of final draft.

I declare that throughout my PhD candidature I have acted in an ethical and honest manner as regards my research and my undertakings with others. I declare that I have not attempted to gain advantage from other researchers' work without their knowledge or permission and that to the best of my knowledge I have correctly referenced, acknowledged, and cited the original authors in the publications included in my thesis. I also declare that I have not willfully misinterpreted, fabricated or falsified any data in my thesis. I undertake the responsibility that any violations of the above will result in disciplinary action by Macquarie University and may also result in penal action from any sources who have not been correctly cited or appropriately acknowledged or whose permissions have not been granted where required.

André Hallen (student number 40317463) Date: Aug 10, 2015

SUMMARY

There are subtle differences in amino acid metabolism in mammalian brain, especially as regards lysine degradation. In lysine degradation the pipecolate pathway predominates in adult mammalian brain whereas the saccharopine pathway predominates in the rest of the body. This suggests that this pathway may play an important role in brain development and functioning. A key enzyme involved in the pipecolate pathway is a ketimine reductase which is involved in the reduction of Δ^1-piperideine-2-carboxylate to form L-pipecolate. This enzyme is also involved in proline metabolism in the reduction of Δ^1-pyrroline-2-carboxylate to form L-proline. In addition the enzyme is involved in the reduction of cyclic ketimines originating from sulfur-containing amino acid metabolism. Prior to this research no mammalian ketimine reductases had been identified in mammalian genomes. The enzyme was identified as the protein μ-crystallin (CRYM). It was found to be strongly inhibited regulated by thyroid hormones and may regulate enzyme activity *in vivo*. A novel catalytic mechanism is also suggested based on *in silico* docking where an active site arginine residue acts as a proton donor. Prior to this research it was not known as an enzyme, however it was known as an important thyroid hormone binding protein and a structural protein in diurnal marsupial lens.

This multi-disciplinary research project involved organic synthesis of enzyme substrates, classical biochemical enzyme purification, proteomic identification, *in silico* docking, bioinformatics, and kinetic analysis of activity and inhibition. This research was self-initiated and this thesis consists of three research papers and two reviews which have been published or submitted during the course of my candidature with myself as primary author. Publications which I have also been involved in as a minor co-author and a letter to the editor are included in the appendices.

This research is an important contribution to our understanding of amino acid metabolism in the mammalian brain, and also its relationship with thyroid hormones.

CHAPTER 1

INTRODUCTION

This thesis documents the discovery and characterisation of an important cytosolic enzyme in brain amino acid metabolism – namely ketimine reductase (E.C. 1.5.1.25). This research was motivated by my interest in this enzyme and its substrates, which were relatively under-researched in spite of the enzyme being a major enzyme documented in every major biochemistry textbook. This thesis is presented as a series of published and submitted papers. Each publication has its own introduction, abbreviations, and references. In addition the two reviews (included in Chapter 3 and 4) provide a detailed overview of this research. Therefore this introduction is a very brief overview of the chapters of the thesis as to reduce repetition.

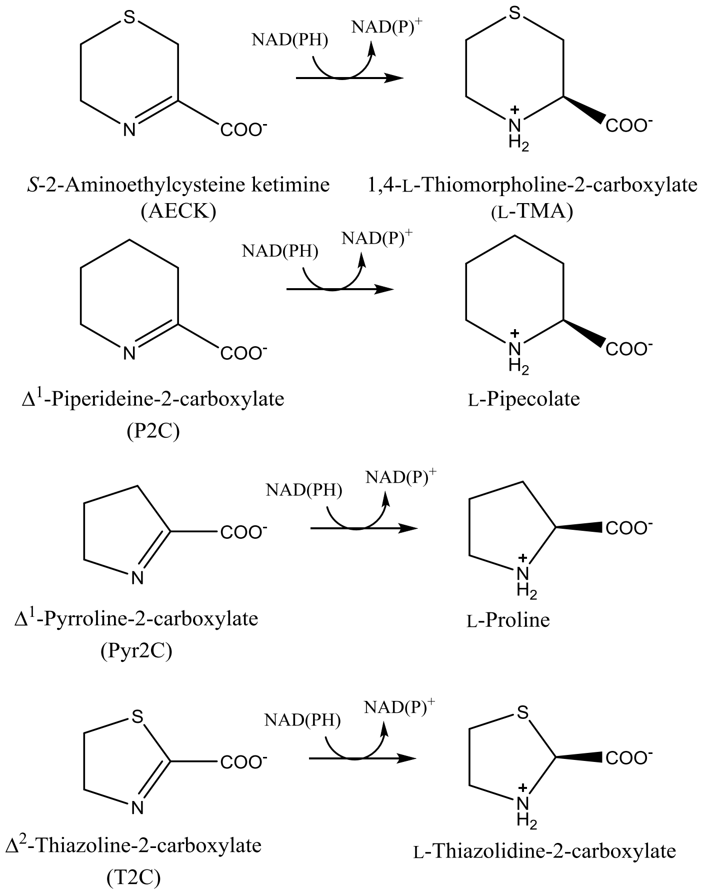

NAD(PH)
NAD(P)+
S-2-Aminoethylcysteine ketimine (AECK)
1,4-L-Thiomorpholine-2-carboxylate (L-TMA)
Δ1-Piperideine-2-carboxylate (P2C)
L-Pipecolate
Δ1-Pyrroline-2-carboxylate (Pyr2C)
L-Proline
Δ2-Thiazoline-2-carboxylate (T2C)
L-Thiazolidine-2-carboxylate

Fig. 1.1. Some of reactions catalysed by ketimine reductase/CRYM as documented in this research. The enzyme is a cytosolic enzyme and can utilize either NADH or NADPH as a necessary cofactor

In **chapter 2** the purification of a ketimine reductase from lamb brain using activity guided assays is described. Ketimine reductases catalyse the reduction of imine double bonds (C=N) using NADH or NADPH as cofactor. In this chapter enzyme activity and inhibition by thyroid hormones was primarily performed at an acidic pH (pH 5) where activity was found to be optimal, and used NADH primarily as a cofactor. The enzyme was purified using assay-guided protocols and entailed the use of multiple chromatography steps. The sulfur-containing cyclic ketimine *S*-2-aminoethylcysteine ketimine (AECK) was used as the substrate in enzyme purification. Another important substrate, Δ^1-piperideine-2-carboxylate (P2C), was also identified. The enzyme was identified as the protein μ-crystallin (CRYM) which prior to this research was not known to be an enzyme, but was known as one of the main cytosolic thyroid hormone binding proteins as well as a structural protein in diurnal marsupial lens.

Chapter 3 consists of a review comparing the imine reduction mechanism of glutamate dehydrogenase and mammalian ketimine reductases highlighting historical discoveries, culminating in the discovery of ketimine reductase as μ-crystallin. Mammalian ketimine reductases are involved in a number of different biochemical pathways, namely in lysine, proline, and sulfur-amino acid metabolism.

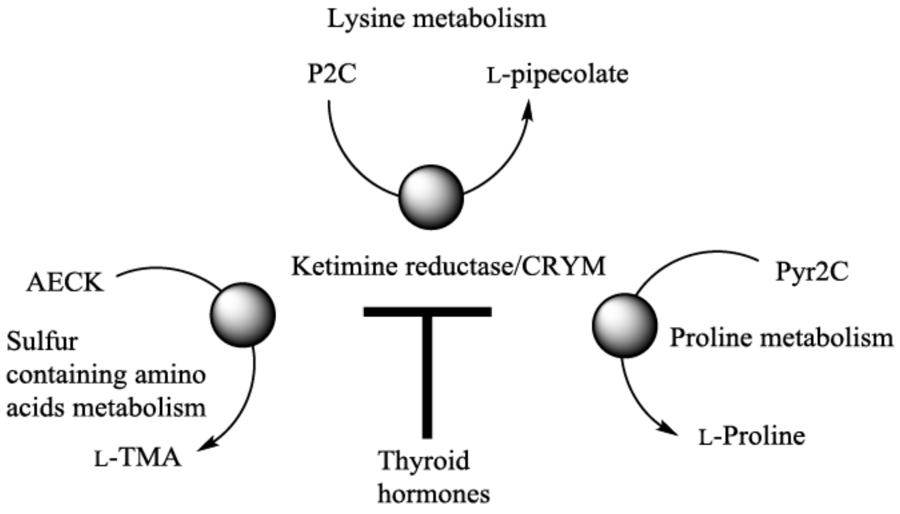


Fig. 1.2. Central role of ketimine reductase/CRYM in biochemical pathways. Ketimine reductase/CRYM is an important catalytic enzyme in a number of amino acid metabolic pathways as depicted. Its inhibition/regulation is therefore potentially also of importance in these pathways.

Chapter 4 consists of a review describing lysine metabolism in mammalian brain. There is an important distinction between the catabolism of lysine in the brain compared to the rest of the body. Lysine is broken down through two pathways, the saccharopine pathway, and the pipecolate pathway. The enzymes responsible for lysine breakdown in the brain and their potential neurochemical and neuropathological roles are discussed. Ketimine reductase/CRYM is an important enzyme in the pipecolate pathway reducing P2C to form L-pipecolate. Its role in this pathway is discussed in relation to its potential inhibition/regulation by thyroid hormones.

In **chapter 5** characterisation of ketimine reductase/CRYM was rather performed under physiological neutral conditions and new enzyme substrates are described as well as the inhibition/regulation by various thyroid hormone analogues and small substrate analogues. Δ^1-Pyrroline-2-carboxylate (Pyr2C) and Δ^2-thiazoline-2-carboxylate (T2C) were identified as enzyme substrates. NADPH was used as a reducing agent as this is the case *in vivo* as opposed to NADH. Research results also suggest that under neutral conditions the enzyme primarily acts as a P2C/Pyr2C reductase (E.C. 1.5.1.21) whose role in lysine and proline metabolism is reviewed in chapter 3 and 4. From the results of *in silico* docking an arginine active site residue is postulated to act as a proton donor in enzyme catalysis.

In **chapter 6** the ability of the enzyme to also synthesise *N*-alkylamino acids is documented from α-keto acids (such as pyruvate) and alkylamines (such as methylamine). This is not likely to occur *in vivo* however it may be of some commercial importance in the production of enantiospecific building blocks for drug synthesis. The reduction of the imine intermediate by ketimine reductase/CRYM further clarifies that the enzyme only acts as an imine reductase.

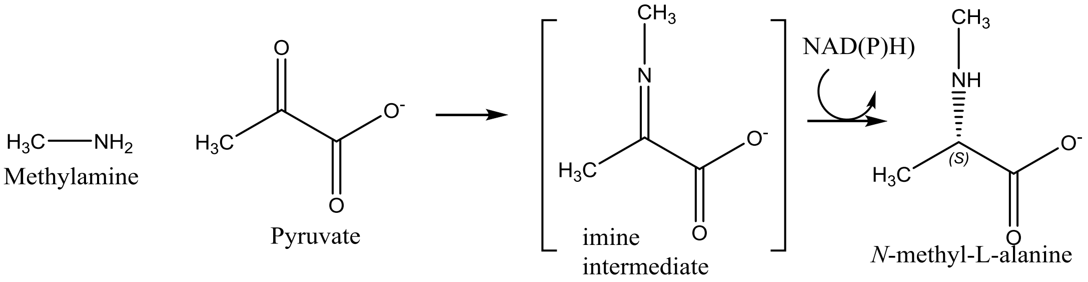


Fig. 1.3. Synthesis of *N*-alkylamino acids. Though not likely *in vivo* the reaction may nonetheless be important as a commercial synthetic reaction. The reaction further validates the ketimine reductase/CRYM mechanism as being one of imine bond reduction. The reaction of amines with aldehydes or ketones is a well characterised reaction resulting in the formation of imines.

In **chapter 7** experimental methods and data are provided in addition to those included in the publications that constitute this thesis.

The **appendices** consist of a research publication on glutamine transaminases and a book chapter on glutamine transaminase. Ketimine reductase/CRYM substrates may originate from the enzyme action of glutamine transaminases. A letter to the editor is also included stressing that ketimine reductase/CRYM is strongly inhibited/regulated by thyroid hormones.

Chapter 2

The discovery of a mammalian brain ketimine reductase and identification of its gene

2.1 Overview

Prior to this research no mammalian ketimine reductase had been successfully purified to the extent where its gene could be identified. The primary aim of the research described in this chapter was to purify a ketimine reductase from ovine forebrain using assay-guided protocols. This entailed the use of many different chromatography steps and ultimately the identification of the gene through the use of proteomic mass spectrometry analysis, thus combining traditional old school biochemistry with cutting edge contemporary proteomics (Fig. A). The gene identity was confirmed by expression of recombinant human μ-crystallin (CRYM) and characterisation of its enzyme properties. Surprisingly the ketimine reductase was a well known protein and one of the main cytosolic thyroid hormone binding proteins as well as a structural protein in mammalian marsupial lens [1]. However its enzyme function was previously unknown. This research was also the first to suggest that thyroid hormones act to regulate the enzyme activity. It was previously suggested that there exist two different mammalian ketimine reductases, one that was partially purified from porcine kidney [2] and one that was partially purified from bovine cerebellum [5]. However, only one ketimine reductase was able to be purified in this research from ovine forebrain. After exhaustive purification attempts a ketimine reductase was also purified from ovine cerebellum but it was also found to be μ-crystallin.

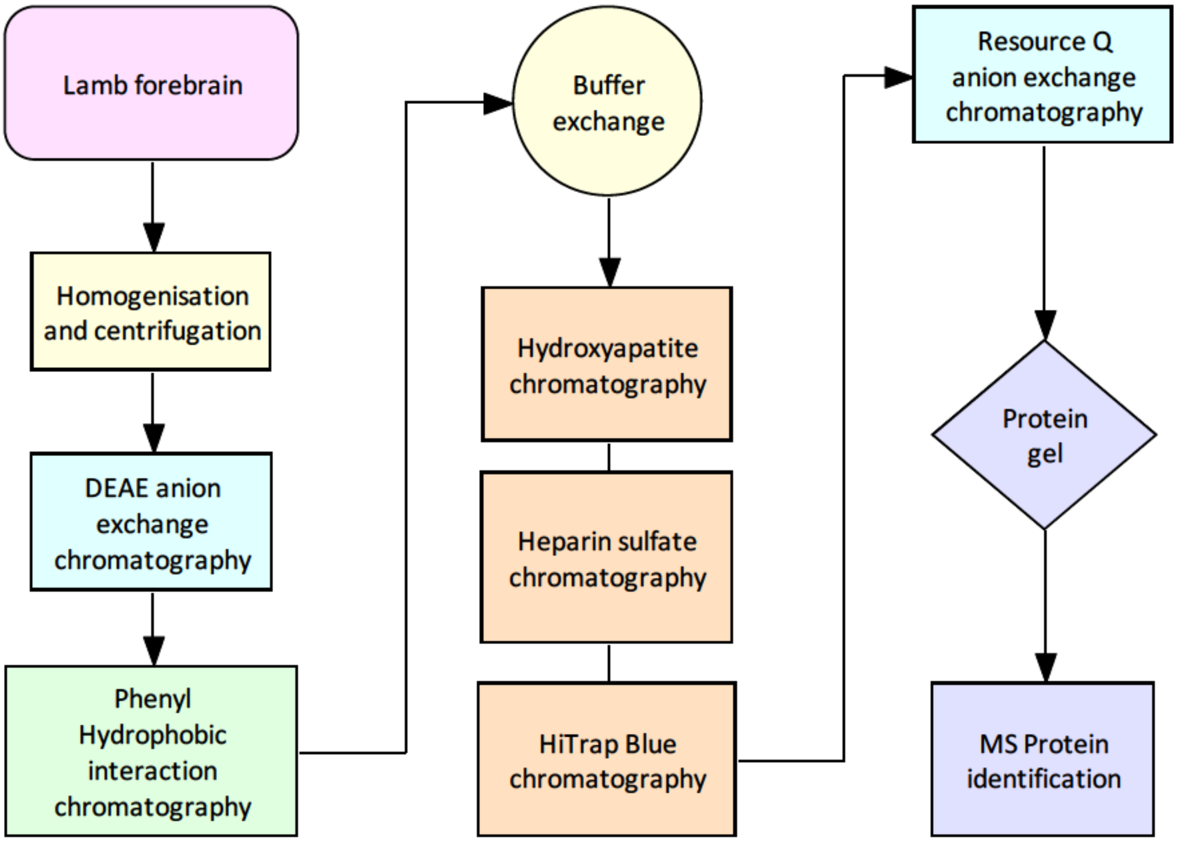

Lamb forebrain
Homogenisation and centrifugation
DEAE anion exchange chromatography
Phenyl Hydrophobic interaction chromatography
Buffer exchange
Hydroxyapatite chromatography
Heparin sulfate chromatography
HiTrap Blue chromatography
Resource Q anion exchange chromatography
Protein gel
MS Protein identification

Figure A. Flowchart depicting the protocols used in enzyme purification and identification of mammalian forebrain ketimine reductase

2.2 Published paper:

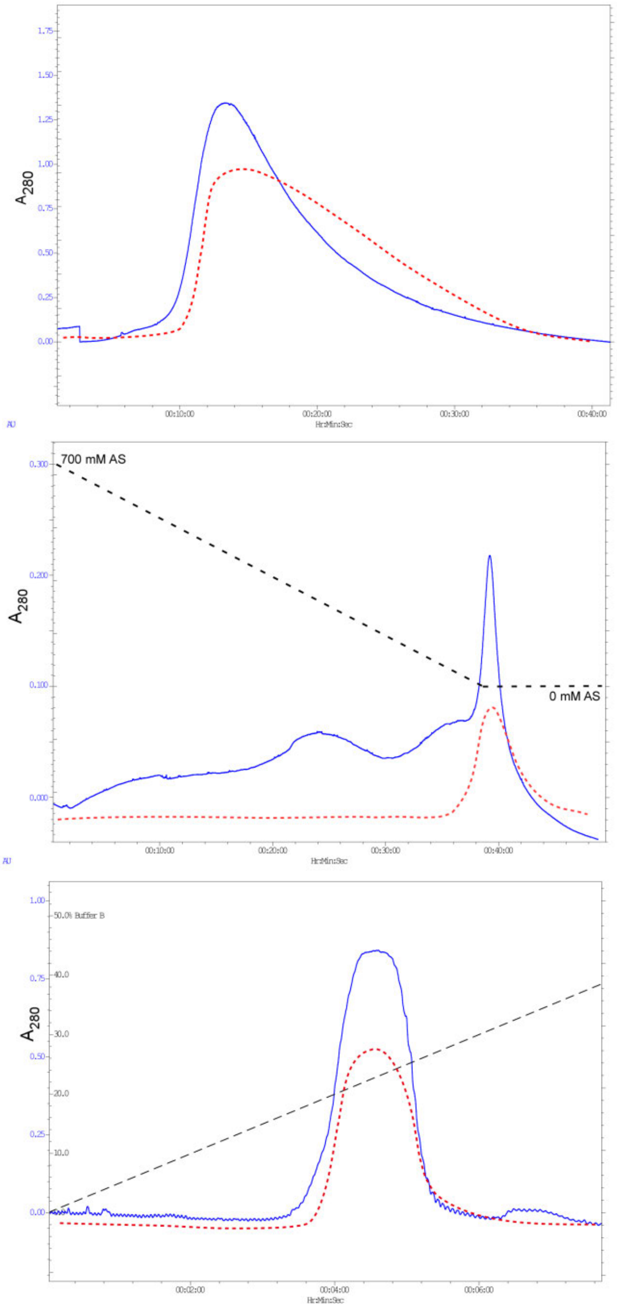
Mammalian forebrain ketimine reductase identified as μ-crystallin; potential regulation by thyroid hormones.

Pages 15-23 of this thesis have been removed as they contain published material under copyright. Removed contents published as:

Hallen, A., Cooper, A.J.L., Jamie, J.F., Haynes, P.A. and Willows, R.D. (2011), Mammalian forebrain ketimine reductase identified as μ-crystallin; potential regulation by thyroid hormones. *Journal of Neurochemistry*, 118: 379-387. doi.org/10.1111/j.1471-4159.2011.07220.x

2.3 Supplementary material not included in publication

2.3.1 Protein purification chromatograms



A. **DEAE** step: the column was washed with 95 mM KCl, and protein eluted with 165 mM KCl. Ammonium sulfate (AS) was then added to the active fractions to 700 mM concentration.

B. **Phenyl sepharose HP** step: protein was eluted with a 700-0 mM ammonium sulfate (AS) gradient elution. Active enzyme eluted with 0 mM AS.

C. **Flowthrough protocol**. After buffer exchange protein was run through a hydroxyapatite and heparin column directly onto a blue sepharose column. Protein was then eluted from the blue sepharose column onto a Reseource Q anion exchange column. The chromatogram is the final step with elution off Resource Q with a 0-500 mM KCl gradient.

Fig. S1. Chromatograms for lamb forebrain purification protocols. The blue trace represents the UV absorbance at 280 nm. The active region is highlighted by the red dotted line and the gradient by the black dotted line in each case.

2.3.2 Gel filtration

Recombinant human ketimine reductase/CRYM was eluted from a Superdex 200HR gel filtration column and the MW of its native form calculated from its elution volume. Phase distribution coefficient (K_{av}) values were calculated from the elution volumes (V_e) of reference proteins and ketimine reductase/CRYM (Equation 1, Fig. S2, and 3.). The MW of CRYM was calculated using its V_e = 14.2 mL (K_{av} 0.40) which equates to ~70 kDa. This is approximately 2× the value of the band on the denaturing SDS-PAGE gel thus implying that the full length transcript of CRYM exists as a dimer in its native form.

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

V_o = void volume as measured with Blue dextran = 7.72 mL
V_t = total column volume = 24 mL

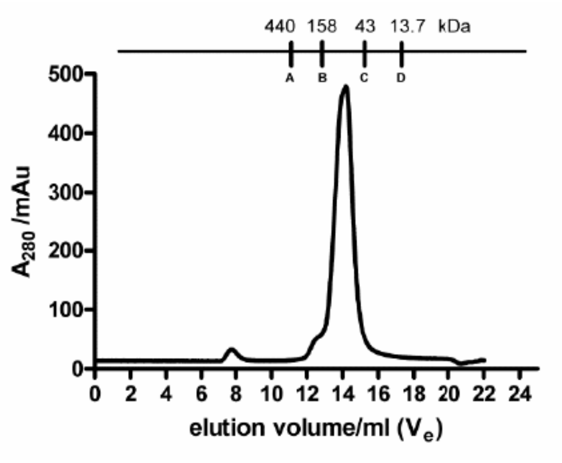


Fig. S2. **Gel filtration**: Elution of the full length recombinant human CRYM isoform 1 from a Superdex 200HR gel filtration column run at 0.5 mL/min. The column was calibrated with GE Healthcare Protein standards (A: ferritin, 440 kDa; B: aldolase, 158 kDa; C: ovalbumin, 43 kDa; and D: ribonuclease, 13.7 kDa). CRYM eluted at 14.2 mL which corresponds to an apparent M_r of 70,000, suggesting that native CRYM is a homodimer.

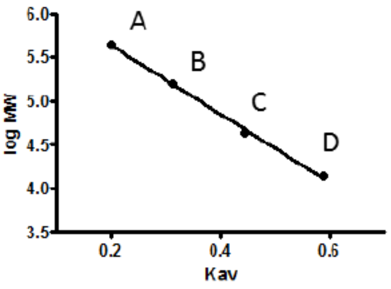


Fig. S3. Plot of K_{av} vs log MW of the protein standards in Fig. S2. The value of the log MW for CRYM is interpolated from the linear regression plot.

2.3.3 Protein isoforms μ-Crystallin/ketimine reductase (*Homo sapiens*)

The exons are represented by alternating colours.

Isoform 1: NCBI Reference Sequence: NM_001888.3   Theoretical pI/Mw: 5.06 / 33775.59

```
        10         20         30         40         50         60
MSRVPAFLSA AEVEEHLRSS SLLIPPLETA LANFSSGPEG GVMQPVRTVV PVTKHRGYLG

        70         80         90        100        110        120
VMPAYSAAED ALTTKLVTFY EDRGITSVVP SHQATVLLFE PSNGTLLAVM DGNVITAKRT

       130        140        150        160        170        180
AAVSAIATKF LKPPSSEVLC ILGAGVQAYS HYEIFTEQFS FKEVRIWNRT KENAEKFADT

       190        200        210        220        230        240
VQGEVRVCSS VQEAVAGADV IITVTLATEP ILFGEWVKPG AHINAVGASR PDWRELDDEL

       250        260        270        280        290        300
MKEAVLYVDS QEAALKESGD VLLSGAEIFA ELGEVIKGVK PAHCEKTTVF KSLGMAVEDT

       310
VAAKLIYDSW SSGK
```

Isoform 2: NCBI Reference Sequence: NM_001014444.2 Theoretical pI/Mw: 5.16 / 29425.66

```
        10         20         30         40         50         60
MQPVRTVVPV TKHRGYLGVM PAYSAAEDAL TTKLVTFYED RGITSVVPSH QATVLLFEPS

        70         80         90        100        110        120
NGTLLAVMDG NVITAKRTAA VSAIATKFLK PPSSEVLCIL GAGVQAYSHY EIFTEQFSFK

       130        140        150        160        170        180
EVRIWNRTKE NAEKFADTVQ GEVRVCSSVQ EAVAGADVII TVTLATEPIL FGEWVKPGAH

       190        200        210        220        230        240
INAVGASRPD WRELDDELMK EAVLYVDSQE AALKESGDVL LSGAEIFAEL GEVIKGVKPA

       250        260        270
HCEKTTVFKS LGMAVEDTVA AKLIYDSWSS GK
```

The longer isoform 1 was purified from ovine forebrain as noted in the publication and the human protein expressed. As noted in the overview, after exhaustive attempts ketimine reductase/CRYM was purified from ovine cerebellum with the same protocols noted in the publication and it was also found to be the longer isoform 1. As noted in the publication the ketimine reductase activity in the cerebellum was however low compared to the forebrain. Currently the validity of the presence of the shorter isoform has been questioned and its NCBI record permanently suppressed due to insufficient evidence. The shorter isoform 2 is missing much of the dimerisation domain and it would only be expected to exist as a monomer. However, a SDS-PAGE gel from kangaroo lens clearly shows two bands when detected using a CRYM antibody with the upper band corresponding to the longer isoform and the lower band to the shorter isoform [1]. During attempts of enzyme purification it was observed that there was a second region of low enzyme activity which eluted early off the DEAE anion exchange column in the wash (indicating a higher isolectric point (PI)), and when these fractions were subsequently run on a Superdex 200 HR column (Fig. S4) the active fractions eluted late corresponding to a M_r of ~ 30 kD may indicate the presence of the monomer (isoform 2). This was not pursued further and will need to be clarified in further research.

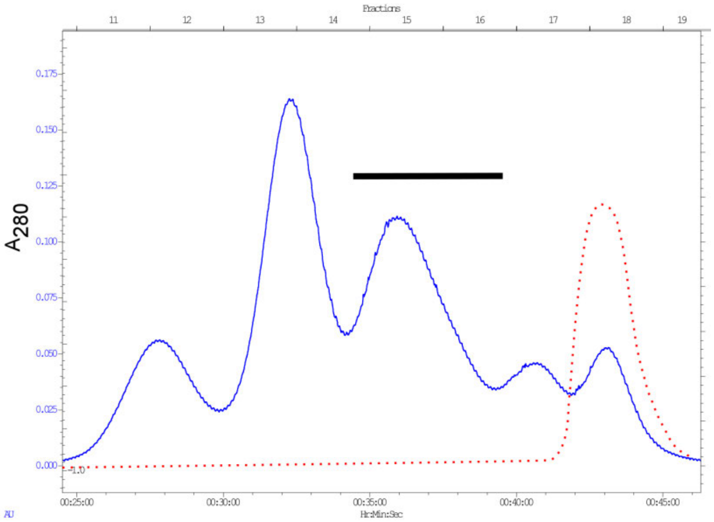


Fig. S4. **Gel filtration**: Elution of partially purified ketimine reductase from ovine forebrain on a Superdex 200HR column run at 0.4 mL/min. Enzyme activity is represented by the dotted red line and the thick black line represents the region where the homodimer of ketimine reductase/CRYM would elute. Active enzyme eluted in a region where it would have an apparent M_r of 30,000, suggesting that this partially purified enzyme may be the monomer (isoform 2).

2.3.4 Bioinformatics

Ketimine reductase/CRYM is homologous with ornithine cyclodeaminase (PDB: 1X7D, *Pseudomonas Putida*) as well as alanine dehydrogenase (*PDB: 1OMO, Archaeoglobus fulgidus*) as depicted in S5 and S6. Multiple sequence alignment of eukaryote ketimine reductase/CRYM was also performed in chapter 5 (supplementary material) highlighting catalytic residues. Ketimine reductase/CRYM has however been shown not to act as either an alanine dehydrogenase [3] or ornithine cyclodeaminase [4].

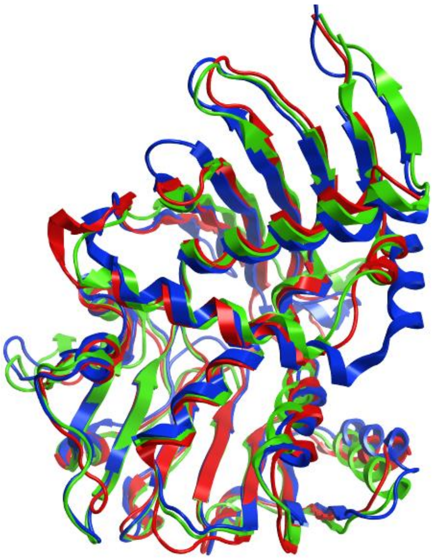


Fig.S5. μ-Crystallin homologues superimposed using MOE software illustrating the structural homology: the protein secondary structure is represented in ribbon form. Red, ketimine reductase/μ-crystallin (CRYM: PDB 2I99); green, alanine dehydrogenase (AlaDH: PDB 1OMO); blue, ornithine cyclodeaminase (OCD: PDB 1X7D).

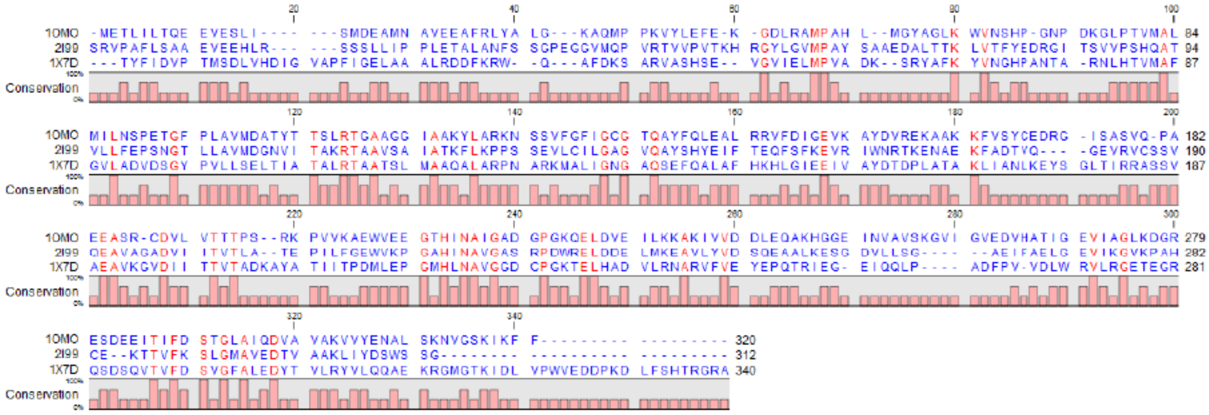

20
40
60
80
100
1OMO -METLILTQE EVESLI---- ---SMDEAMN AVEEAFRLYA LG---KAQMP PKVYLEFE-K -GDLRAMPAH L--MGYAGLK WVNSHP-GNP DKGLPTVMAL 84
2I99 SRVPAFLSAA EVEEHLR--- ---SSSLLIP PLETALANFS SGPEGGVMQP VRTVVPVTKH RGYLGVMPAY SAAEDALTTK LVTFYEDRGI TSVVPSHQAT 94
1X7D ---TYFIDVP TMSDLVHDIG VAPFIGELAA ALRDDFKRW- -Q---AFDKS ARVASHSE-- VGVIELMPVA DK--SRYAFK YVNGHPANTA -RNLHTVMAF 87
100%
Conservation
0%
120
140
160
180
200
1OMO MILNSPETGF PLAVMDATYT TSLRTGAAGG IAAKYLARKN SSVFGFIGCG TQAYFQLEAL RRVFDIGEVK AYDVREKAAK KFVSYCEDRG -ISASVQ-PA 182
2I99 VLLFEPSNGT LLAVMDGNVI TAKRTAAVSA IATKFLKPPS SEVLCILGAG VQAYSHYEIF TEQFSFKEVR IWNRTKENAE KFADTVQ--- -GEVRVCSSV 190
1X7D GVLADVDSGY PVLLSELTIA TALRTAATSL MAAQALARPN ARKMALIGNG AQSEFQALAF HKHLGIEEIV AYDTDPLATA KLIANLKEYS GLTIRRASSV 187
100%
Conservation
0%
220
240
260
280
300
1OMO EEASR-CDVL VTTTPS--RK PVVKAEWVEE GTHINAIGAD GPGKQELDVE ILKKAKIVVD DLEQAKHGGE INVAVSKGVI GVEDVHATIG EVIAGLKDGR 279
2I99 QEAVAGADVI ITVTLA--TE PILFGEWVKP GAHINAVGAS RPDWRELDDE LMKEAVLYVD SQEAALKESG DVLLSG---- --AEIFAELG EVIKGVKPAH 282
1X7D AEAVKGVDII TTVTADKAYA TIITPDMLEP GMHLNAVGGD CPGKTELHAD VLRNARVFVE YEPQTRIEG- EIQQLP---- ADFPV-VDLW RVLRGETEGR 281
100%
Conservation
0%
320
340
1OMO ESDEEITIFD STGLAIQDVA VAKVVYENAL SKNVGSKIKF F--------- --------- 320
2I99 CE--KTTVFK SLGMAVEDTV AAKLIYDSWS SG-------- ---------- --------- 312
1X7D QSDSQVTVFD SVGFALEDYT VLRYVLQQAE KRGMGTKIDL VPWVEDDPKD LFSHTRGRA 340
100%
Conservation
0%

Fig. S6. Sequence alignment of µ-crystallin (CRYM: PDB 2I99); alanine dehydrogenase (AlaDH: PDB 1OMO); ornithine cyclodeaminase (OCD: PDB 1X7D). Identical conserved residues in red. The alignment was generated using MOE software accenting structural similarities as in Fig. S5.

2.3.5 pH optimum

Ketimine reductase/CRYM was found to have a pH optimum of pH 5 using AECK as substrate and NADH as cofactor (Fig. S6) and the same as previously reported for porcine kidney ketimine reductase [2].

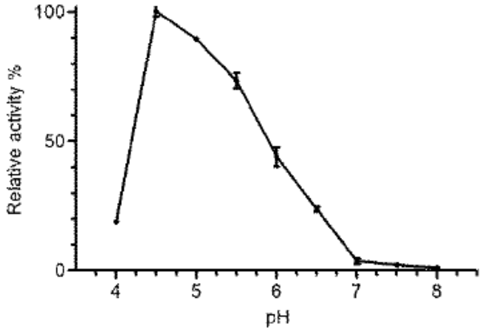


Fig. S7. pH optimum for ketimine reductase/CRYM using AECK and NADH. Sodium acetate buffer was used for the pH range 4-5.5, and potassium phosphate buffer was used for the pH range 6-8.

2.3.6 Gene expression

It was observed that ketimine reductase activity was greatest in the forebrain slices as opposed to the cerebellum. The activity is consistent with the documented gene expression (Fig. S7) [6] as well as the previously documented maximal activity of a ketimine reductase in the striatum [7]. The ketimine reductase purified from bovine cerebellum was found to have equivalent activity there compared to the cerebrum [5]. Thus it may be a separate, as yet unidentified, enzyme in the genome and deserves further research. The failure in this research to purify a second ketimine reductase may be age-related and the second enzyme may not be highly expressed in the lamb brains that were used. In future adult bovine cerebellum will be used.

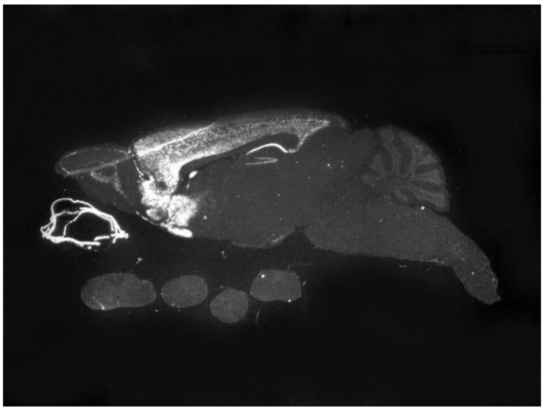


Fig. S8. Gene expression of ketimine reductase/CRYM in rat brain. The cerebellar regions have low expression compared to the regions in the forebrain which was also observed in the protein purification. (http://brainstars.org/probeset/1416776_at).

2.3.7 Erratum

In Fig. 7 of the published paper the Y axis legend is missing a factor (× 10^{-1}).

References

1. Segovia, L., et al., *Two roles for μ-crystallin: a lens structural protein in diurnal marsupials and a possible enzyme in mammalian retinas.* Mol Vis, 1997. **3**: p. 9.
2. Nardini, M., et al., *Purification and characterization of a ketimine-reducing enzyme.* Eur J Biochem, 1988. **173**(3): p. 689-94.
3. Schroder, I., et al., *A novel archaeal alanine dehydrogenase homologous to ornithine cyclodeaminase and mu-crystallin.* J Bacteriol, 2004. **186**(22): p. 7680-9.
4. Kim, R.Y., R. Gasser, and G.J. Wistow, *μ-Crystallin is a mammalian homologue of Agrobacterium ornithine cyclodeaminase and is expressed in human retina.* Proc Natl Acad Sci U S A, 1992. **89**(19): p. 9292-6.
5. Nardini, M., et al., *Bovine brain ketimine reductase.* Biochim Biophys Acta, 1988. **957**(2): p. 286-92.
6. Kasukawa, T., et al., *Quantitative expression profile of distinct functional regions in the adult mouse brain.* PLoS One, 2011. **6**(8): p. e23228.
7. Garweg, G., D. von Rehren, and U. Hintze, *L-Pipecolate formation in the mammalian brain. Regional distribution of Δ^1-pyrroline-2-carboxylate reductase activity.* J Neurochem, 1980. **35**(3): p. 616-21.

CHAPTER 3

REVIEW: IMINE REDUCTASES AND THEIR MECHANISMS

Overview

Chapter 3 is a published review highlighting historical references to the discovery of ketimine reductases and a comparison to glutamate dehydrogenases. The catalytic mechanism of glutamate dehydrogenase is known to proceed via an imine intermediate. The catalytic mechanisms are discussed in relation to imine bond reduction. The importance of ketimine reductase in the context of brain biochemistry and the potential neuro-active role of the enzyme substrates is stressed. The discovery of μ-crystallin (CRYM) as a mammalian ketimine reductase was a major discovery, however as noted in this review, research into mammalian ketimine reductases had been undertaken for decades prior to this discovery as documented in chapter 2. The novel interactions between ketimine reductase/CRYM and thyroid hormones documented in chapter 2, and later in chapter 5, are also discussed as they represent a potential regulatory mechanism for enzyme catalysis as well as potentially being of importance in the bioavailability of intracellular thyroid hormones.

NOTE: Reference: Garweg et al. [60] is incorrectly referenced and should be numbered [62]

Pages 32-46 of this thesis have been removed as they contain published material under copyright. Removed contents published as:

Hallen, A., Jamie, J.F. & Cooper, A.J.L. (2014) Imine Reductases: A Comparison of Glutamate Dehydrogenase to Ketimine Reductases in the Brain. *Neurochemical Research,* vol. 39, pp. 527–541. https://doi.org/10.1007/s11064-012-0964-1

CHAPTER 4

REVIEW: LYSINE METABOLISM IN MAMMALIAN BRAIN

OVERVIEW

Chapter 4 is a published review on lysine metabolism in mammalian brain. This review describes the important differences in mammalian lysine catabolism in the brain as compared to the rest of the body. Lysine is broken down primarily through the saccharopine pathway in the body whereas in the adult brain the pipecolate pathway predominates. This difference suggests a potential importance in brain development and functioning. The critical enzymes are described and their relationship to brain functioning and pathology highlighted. The compartmentalization of lysine catabolism is discussed with catabolism occurring in cytosol, peroxisomes, as well as mitochondria. The potential regulation of the pipecolate pathway by thyroid hormones as well as the relationship with the catabolism of tryptophan may also be of significant importance. Ketimine reductase/CRYM is a critical enzyme in the pipecolate pathway and therefore of particular importance. Furthermore research presented in chapter 5 suggests that under physiologically neutral conditions the enzyme predominantly functions as a Δ^1-piperideine-2-carboxylate (P2C)/Δ^1-pyrroline-2-carboxylate (Pyr2C) reductase.

Pages 48-70 of this thesis have been removed as they contain published material under copyright. Removed contents published as:

Hallen, A., Jamie, J.F. & Cooper, A.J.L. (2013) Lysine metabolism in mammalian brain: an update on the importance of recent discoveries. *Amino Acids* vol. 45, pp. 1249–1272.
https://doi.org/10.1007/s00726-013-1590-1

CHAPTER 5

ENZYME CATALYSIS, MECHANISMS, AND REGULATION UNDER PHYSIOLOGICAL NEUTRAL CONDITIONS

Overview

In chapter 2 “Discovery of the enzyme function of μ-crystallin (CRYM)” the emphasis had been on the isolation, purification, and identification of forebrain ketimine reductase. The purification was guided using assays performed at acidic pH (pH 5) where activity was found to be optimal using NADH as cofactor. However in this chapter emphasis is on characterization of recombinant human ketimine reductase/CRYM under physiologically neutral conditions and using NADPH as cofactor. As μ-crystallin is a cytosolic enzyme these are the catalytic conditions it would experience *in vivo*. This chapter investigates enzyme substrates not described in chapter 2 such as Δ^1-pyrroline-2-carboxylate (Pyr2C) and Δ^2-thiazoline-2-carboxylate (T2C). This research also further characterizes the catalysis of Δ^1-piperideine-2-carboxylate (P2C), *S*-2-aminoethylcysteine ketimine (AECK), and lanthionine ketimine (LK) under physiological neutral conditions. The differences in enzyme activity are discussed in relation to different tautomeric forms which predominate at different pH values. This chapter also addresses the potent inhibition/regulation of cyclic ketimine catalysis by thyroid hormones, as well as the weaker inhibition by small aromatic cyclic ketimine analogues. In this chapter an enzyme mechanism is proposed based on docking studies of substrates and inhibitors.

This chapter is a paper in Neurochemical Research journal.

Pages 72-86 of this thesis have been removed as they contain published material under copyright. Removed contents published as:

Hallen, A., Cooper, A.J.L., Jamie, J.F. et al. (2015) Insights into Enzyme Catalysis and Thyroid Hormone Regulation of Cerebral Ketimine Reductase/μ-Crystallin Under Physiological Conditions. *Neurochemical Research,* vol. 40, pp. 1252–1266.
https://doi.org/10.1007/s11064-015-1590-5

Neurochemical Research:

Insights into enzyme catalysis and thyroid hormone regulation of cerebral ketimine reductase/ μ-crystallin under physiological conditions

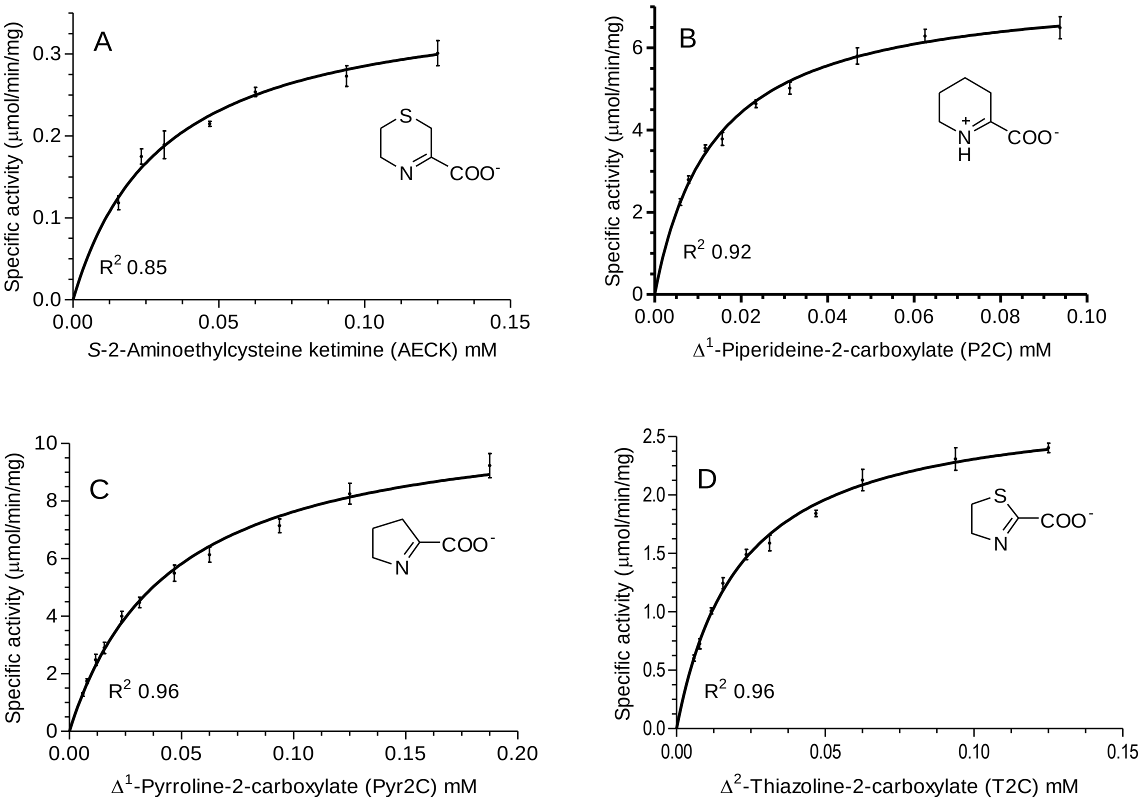
Supplementary material

André Hallen[*], Arthur J. L. Cooper[†], Joanne F. Jamie[*] and Peter Karuso[*]

[*] *Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW 2109, Australia.*

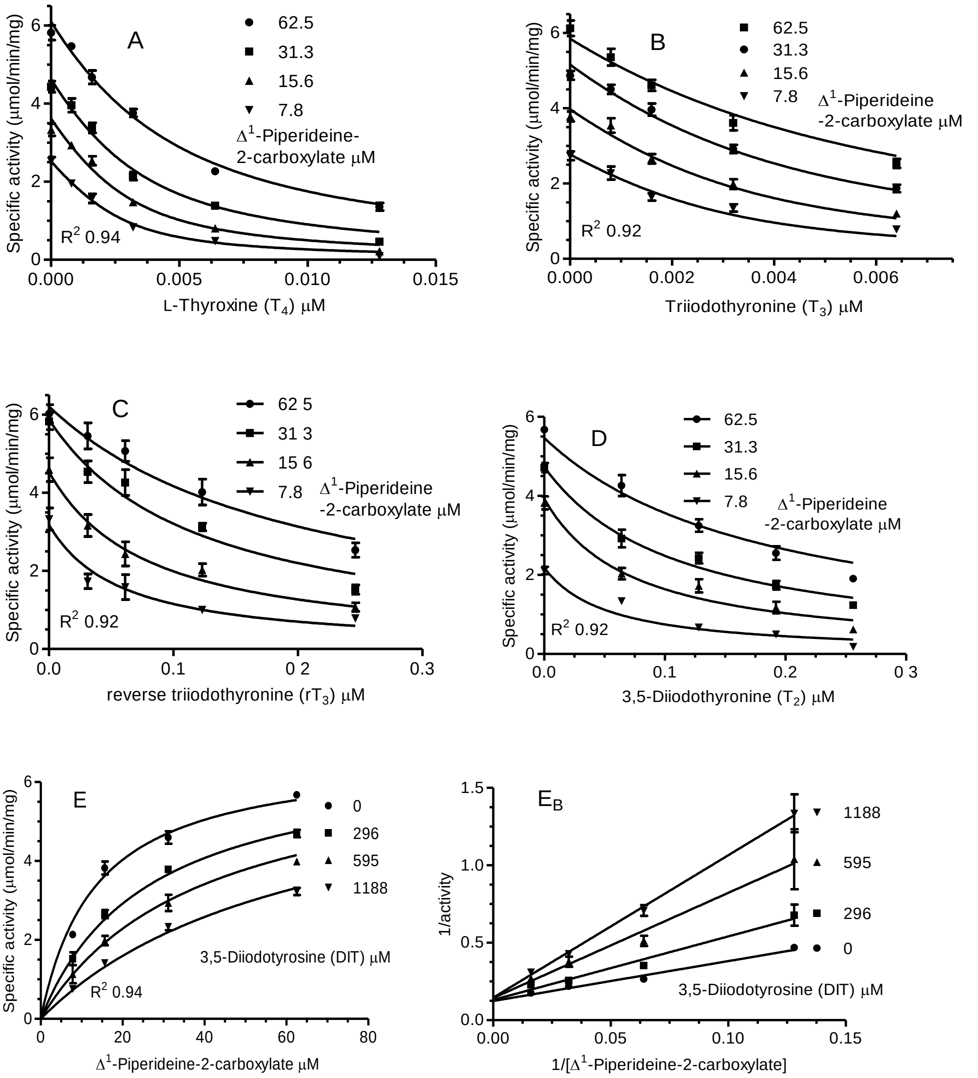
[†] *Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, New York 10595, USA.*

A. **Enzyme assays**

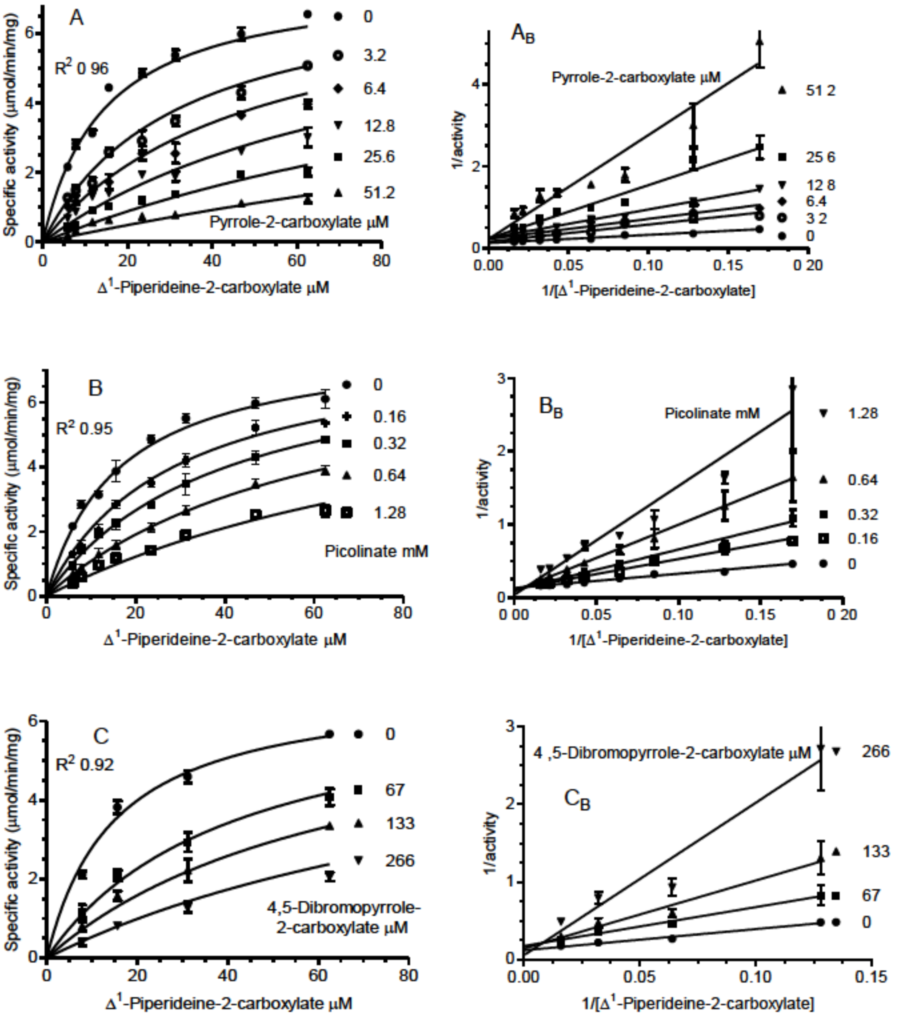


Suppl. Material Fig. 1. Michaelis-Menten kinetic plots for cyclic ketimine substrates of ketimine reductase/CRYM: (A) *S*-2-aminoethylcysteine ketimine (AECK), K_m 28 ± 4 μM, V_{max} 0.36 μmol/min/mg; (B) Δ^1-piperideine-2-carboxylate (P2C), K_m 13 ± 1 μM, V_{max} 7.36 ± 0.2 μmol/min/mg (C) Δ^1-pyrroline-2-carboxylate (Pyr2C), K_m 45 ± 2.6 μM, V_{max} 11.1 ± 0.2 μmol/min/mg, and (D) Δ^2-thiazoline-2-carboxylate (T2C), K_m 21 ± 1 μM, V_{max} 2.8 ± 0.06 μmol/min/mg. Assays were performed at 37◦ C with six replicates at each substrate concentration used. A fixed concentration of 8 μM NADPH was used for all assays and kinetic constants are therefore apparent constants. Assays were performed at pH 7.2 in a potassium phosphate buffer (200 mM), which included 20% glycerol as well as 1 mM DTT. Data are fitted using non-linear regression with GraphPad Prism 5 and results are tabulated in Table 1 of the main text. Of the four substrates assayed here only AECK is predominantly in the enzyme unfavorable enamine form at neutral pH, and this is reflected in the lower activity observed in comparison with its non-sulfur containing analogue P2C, and as such it is not likely to be a preferred substrate *in vivo* at neutral pH.

B. **Inhibitor assays**

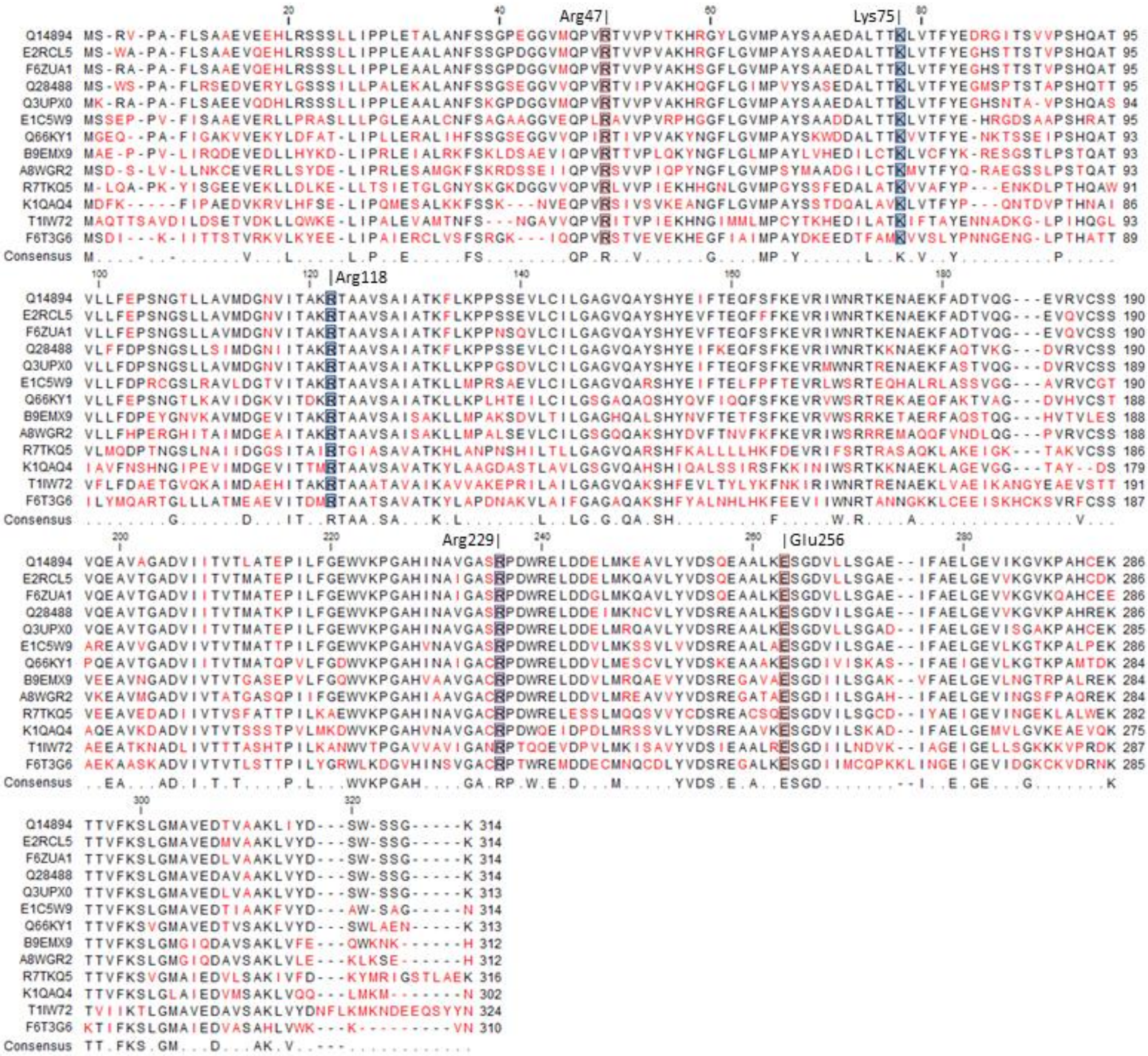


Suppl. Material Fig. 2. Inhibition plots obtained with thyroid hormones and analogues. The low apparent inhibition constants necessitated that experimental data be fitted using non-linear regression to Morrison's quadratic equation for tight binding inhibition for the thyroid hormones: A. L-thyroxine (T_4), K_i 0.60 ± 0.05 nM; B. 3,5,3'-triiodothyronine (T_3), K_i 0.75 ± 0.05 nM; C. 3,3',5'-triiodothyronine (rT_3), K_i 35 ± 3 nM; and D. 3,5-diiothyronine (T_2), K_i 32 ± 1.7 nM. Experimental data for 3,5-diiotyrosine (DIT) were fitted using non-linear regression to the equation for competitive inhibition: E. 3,5-diiotyrosine (DIT), K_i 313 ± 28 μM, E_B. Double reciprocal plot to illustrate competitive inhibition by DIT. No inhibition was observed for L-tyrosine at a concentration of 800 μM. Assays were conducted at 37° C using an enzyme concentration of 2.61 nM (17.6 ng enzyme in a 200 μL volume in a microplate well), varying concentrations of substrate (P2C), fixed NADPH concentration of 8 μM, pH 7.2 potassium phosphate buffer (200 mM) containing 20% glycerol and 1 mM DTT. Experiments were done with six replicates.



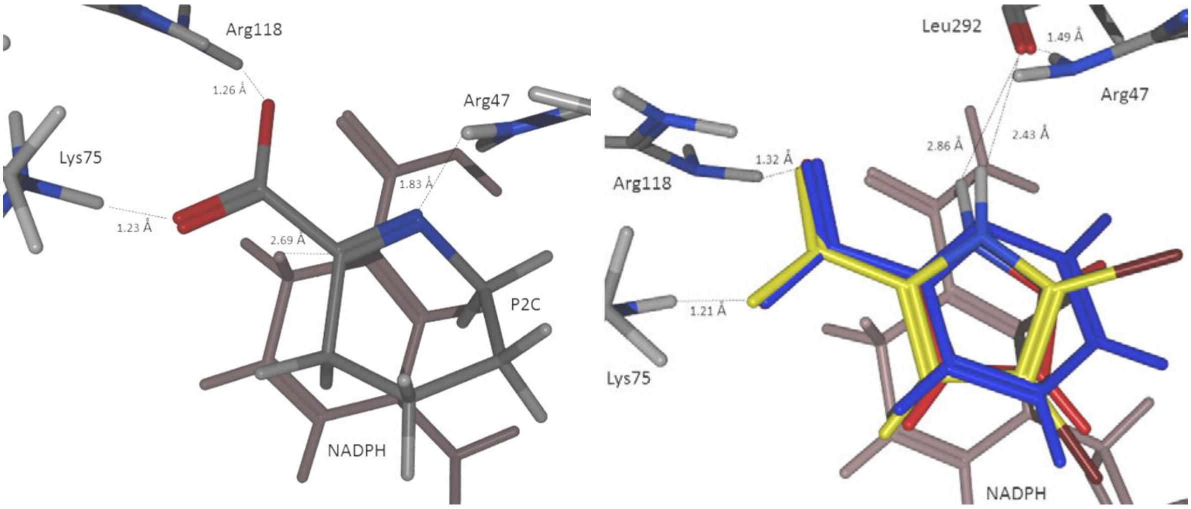
Supp. Material Fig. 3. Inhibition plots obtained with cyclic substrate analogues of ketimine reductase/CRYM. Experimental data were fit to the equation for competitive inhibition using non-linear regression: A. Pyrrole-2-carboxylate, K_i 2.7 ± 0.15 µM; B. Picolinate, K_i 220 ± 15 µM; C. 4,5-Dibromopyrrole-2-carboxylate, K_i 38 ± 4 µM. The corresponding double reciprocal plots are also depicted (A_A, B_B, C_C), which demonstrate a competitive inhibition mechanism. Assays were conducted as described above.

C. Multiple sequence alignment


Arg47
Lys75
Arg118
Arg229
Glu256
Q14894
E2RCL5
F6ZUA1
Q28488
Q3UPX0
E1C5W9
Q66KY1
B9EMX9
A8WGR2
R7TKQ5
K1QAQ4
T1IW72
F6T3G6
Consensus

Supp. Material Fig. 4. Multiple sequence alignment of eukaryote ketimine reductase/CRYM protein sequences. Non-conserved residues are shaded in red. The conserved residues Arg118 and Lys75 interact with the carboxylate of the docked substrates and bound pyruvate in the crystal structure (PDB: 4BV9). We postulate that the conserved Arg47 and Glu256 residues form a charge relay necessary for enzyme catalysis with the Arg47 residue responsible for proton donation. The Glu256 residue is solvent exposed. The conserved Arg229 residue may also from part of the charge relay via a water intermediate that it is H-bonded to. **Uniprot accession numbers**: Q14894, *Homo sapiens* (Human); E2RCL5, *Canis familiaris* (Dog); F6ZUA1, *Equus caballus* (Horse); Q28488, *Macropus fuliginosus* (Western gray kangaroo); Q3UPX0 , *Mus musculus* (Mouse); E1C5W9, *Gallus gallus* (Chicken), Q66KY1**,** *Xenopus laevis* **(**African clawed frog**),** B9EMX9*, Salmo salar (Atlantic salmon);* A8WGR2*, Danio rerio* (Zebrafish*);* R7TKQ5*, Capitella teleta (Polychaete worm)***;** K1QAQ4*, Crassostrea gigas* (Pacific oyster); T1IW72**,** *Strigamia maritima* (European centipede); F6T3G6**,** *Ciona intestinalis* (Transparent sea squirt).

D. ***In silico* docking**



Supp. Material Fig. 5. Docked ligands in the active site of human ketimine reductase/CRYM: *Left*: The top ranked docked pose of P2C into the active site (PDB: 4BV9) using the flexible docking protocols. P2C docks in the same pose as that observed using the rigid docking protocols, and is an indication that the protocols used in preparation and docking do not introduce a bias. *Right*: The highest ranked small inhibitors docked in the active site of ketimine reductase/CRYM (PDB: 4BV9) (pyrrole-2-carboxylate, red; 4,5-dibromopyrole-2-carboxylate, yellow; picolinate, blue). The docked pyrrole-2-carboxylate and its dibromo analogue can potentially form a hydrogen bond to the Leu292 residue backbone carbonyl. This hydrogen bond arrangement was not observed for the docked substrates. The docking scores do not provide information on the inhibitory strengths of the small inhibitors but do provide an indication as to how they may bind in the active site.

E. **Organic synthesis**

Δ^2-Thiazoline-2-carboxylate (T2C)

The ethyl ester of T2C was synthesized by substantial modifications of a published method [1,2]. In our hands, the method outlined in reference 2 was unusable due to possible typographical errors. Our synthetic method requires fewer steps, with comparable overall yields, and is therefore an improvement over the original synthetic method published in reference 1. Our method is therefore presented in full with accompanying analytical data.

Cysteamine hydrochloride (10 mmol/1136 mg), dissolved in absolute ethanol (10 mL), was slowly added over 3 h to a solution of ethyl cyanoformate (10 mmol /991 mg) in absolute ethanol (4 mL), with constant stirring under nitrogen for an additional 1 h at room temperature. Water (20 mL) was added and the product was extracted with dichloromethane (4 × 10 mL). The organic layer was washed with brine (5 mL), dried using magnesium sulfate, rotary evaporated, and the resulting yellow oil was distilled under vacuum (85 °C, 0.25 mPa) to yield T2C ethyl ester as a colorless oil (798 mg; 50%).

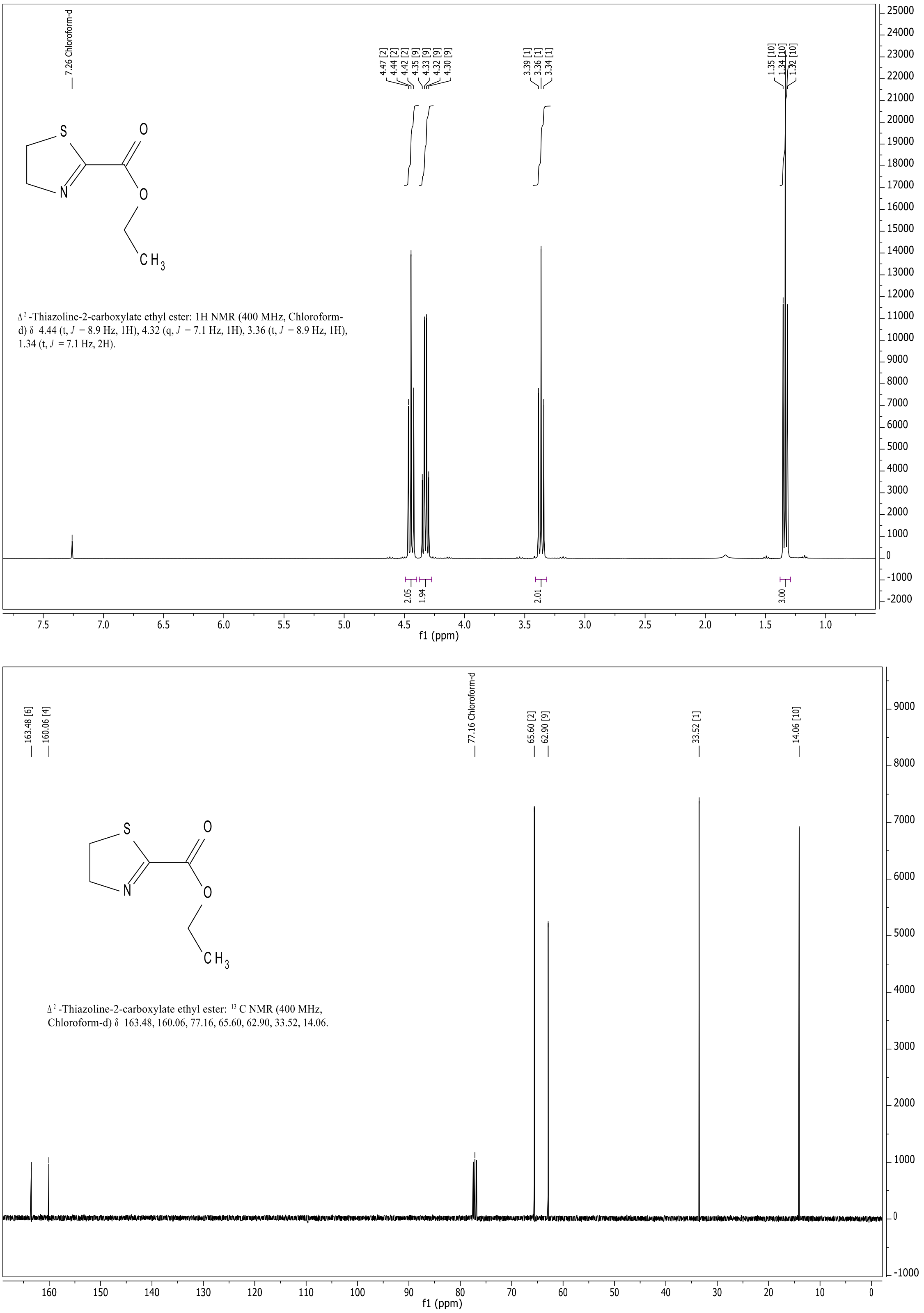
T2C ethyl ester was hydrolyzed to T2C according to a published method [3] to form the solid T2C sodium salt. Briefly T2C ethyl ester (798 mg, 5 mmol) was dissolved at room temperature in 20 mL dry dichloromethane/methanol (9:1) which included NaOH (100 mM). Under these anhydrous conditions hydrolysis was complete in 5 minutes with constant stirring. The pure sodium salt of T2C was isolated by vacuum filtration, washed with dichloromethane (10 mL), and dried under vacuum (655 mg; 86%). Analytical data for both the ethyl ester of T2C and sodium salt of T2C are similar to those previously reported by Naber *et al* [2]. No analytical data were presented for either compound by Afeefy and Hamilton [1]. Our analytical data are consistent with the proposed structures.

Δ^2-Thiazoline-2-carboxylate ethyl ester (MW 159.20): ^{1}HNMR ($CDCl_3$, 400 MHz): δ 1.35 (t, J = 7.32 *Hz*, 3H), 3.38 (t, J = 8.96 *Hz*, 2H), 4.34 (q, J = 7.32 *Hz*, 2H), 4.46 (t, J = 8.96 *Hz*, 2H). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 13.91, 33.36, 62.75, 65.45, 159.91, 163.32; MS (ESI positive mode) *m/z* 160 ($M+H^+$).

UV in PBS (pH 7.4): λ_{max} 290 (ε 1770 M^{-1}) nm. IR (ATR): ν_{max} 1746, 1722, 1606, 1246, 1060, 998 cm^{-1}.

Δ^2-Thiazoline-2-carboxylate (MW 130.14): ^{1}H NMR (D_2O, 400 MHz): δ 3.24 (t, J = 8.78 *Hz,* 2H), 4.18 (t, J = 8.78 *Hz,* 2H).^{13}C NMR* (D_2O-acetone-d_6, 400 MHz): δ 33.41, 64.44, 166.68, 171.49. MS (ESI negative ion mode) *m/z* 130 ($M–H^+$). UV in PBS (pH 7.4): λ_{max} 269 (ε 1630 M^{-1}) nm. IR (ATR): ν_{max} 1630, 1609, 1383, 1070, 1009, 797, 784 cm^{-1}.

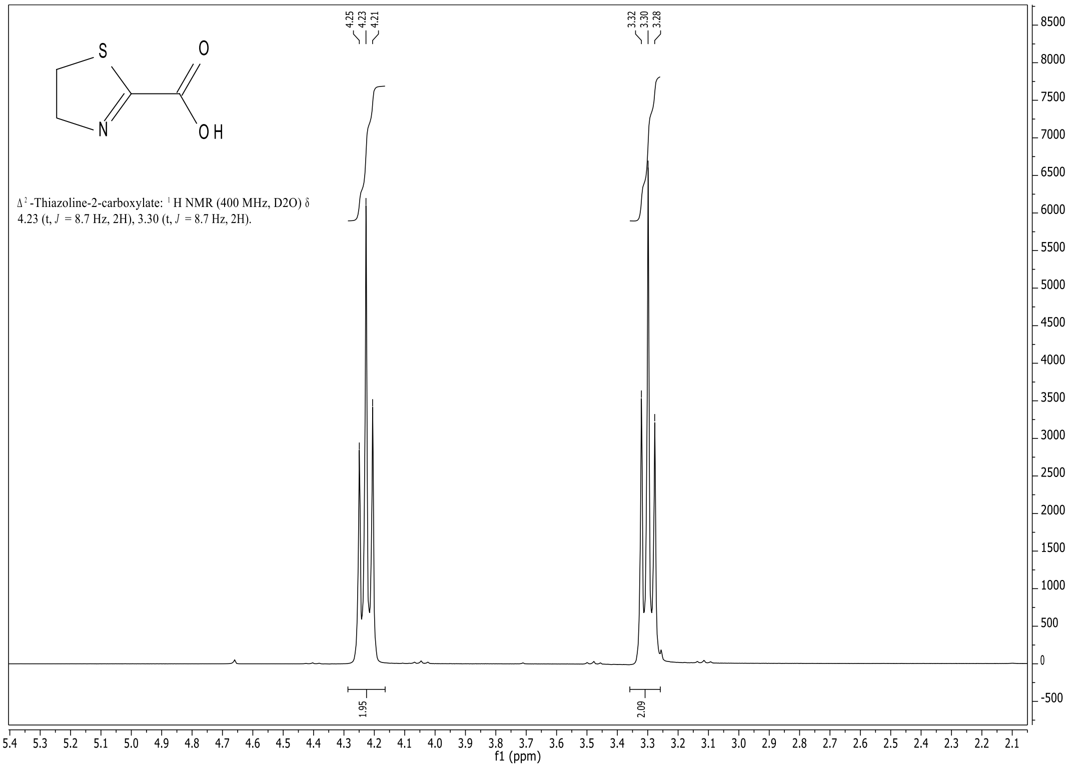
* ^{13}C NMR data as reported by Naber et al. [1] are incorrectly referenced and all the peaks in the ^{13}C NMR spectrum reported by these authors are shifted downfield by 12.61 ppm.



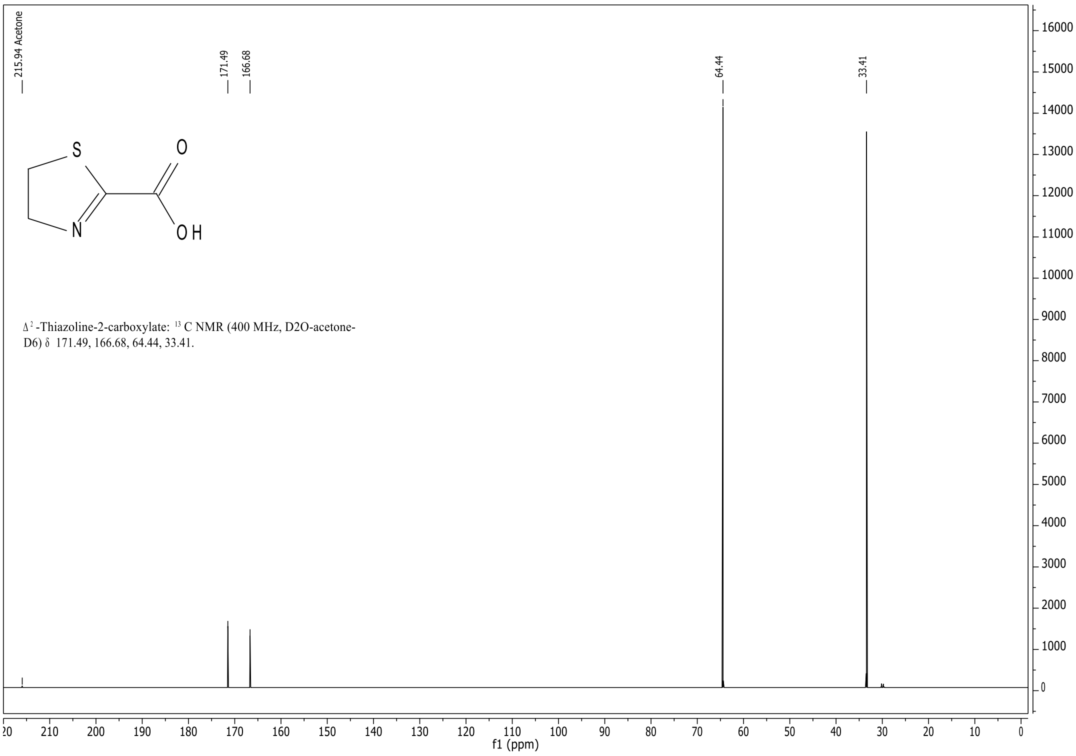
Δ² -Thiazoline-2-carboxylate ethyl ester: 1H NMR (400 MHz, Chloroform-d) δ 4.44 (t, *J* = 8.9 Hz, 1H), 4.32 (q, *J* = 7.1 Hz, 1H), 3.36 (t, *J* = 8.9 Hz, 1H), 1.34 (t, *J* = 7.1 Hz, 2H).

Δ² -Thiazoline-2-carboxylate ethyl ester: ¹³ C NMR (400 MHz, Chloroform-d) δ 163.48, 160.06, 77.16, 65.60, 62.90, 33.52, 14.06.

Supp. Material fig. 6. ^{1}H NMR (upper) and ^{13}C NMR (lower) for Δ^{2}-thiazoline-2-carboxylate ethyl ester

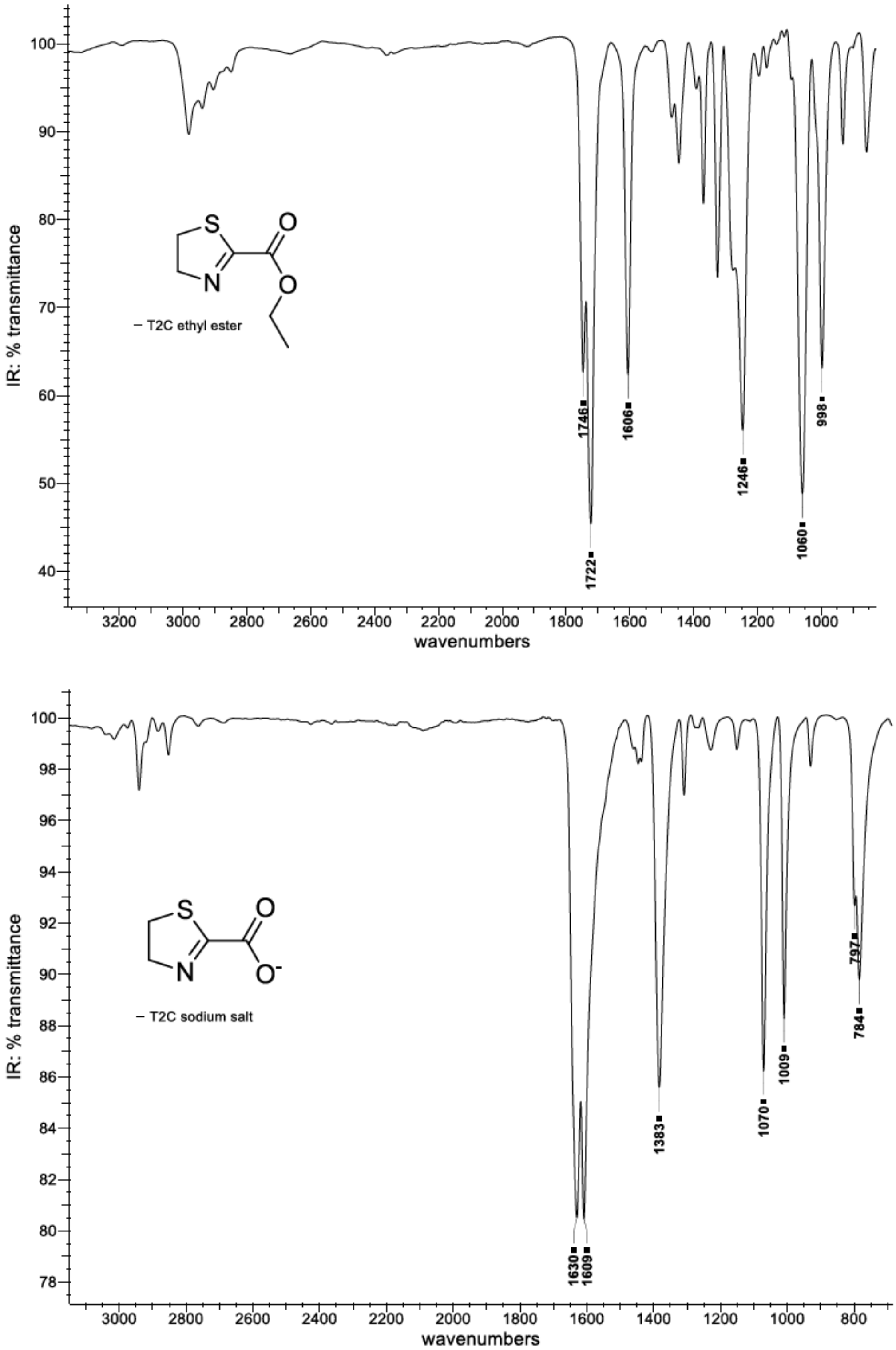


Δ^2-Thiazoline-2-carboxylate: ^{1}H NMR (400 MHz, D2O) δ 4.23 (t, *J* = 8.7 Hz, 2H), 3.30 (t, *J* = 8.7 Hz, 2H).

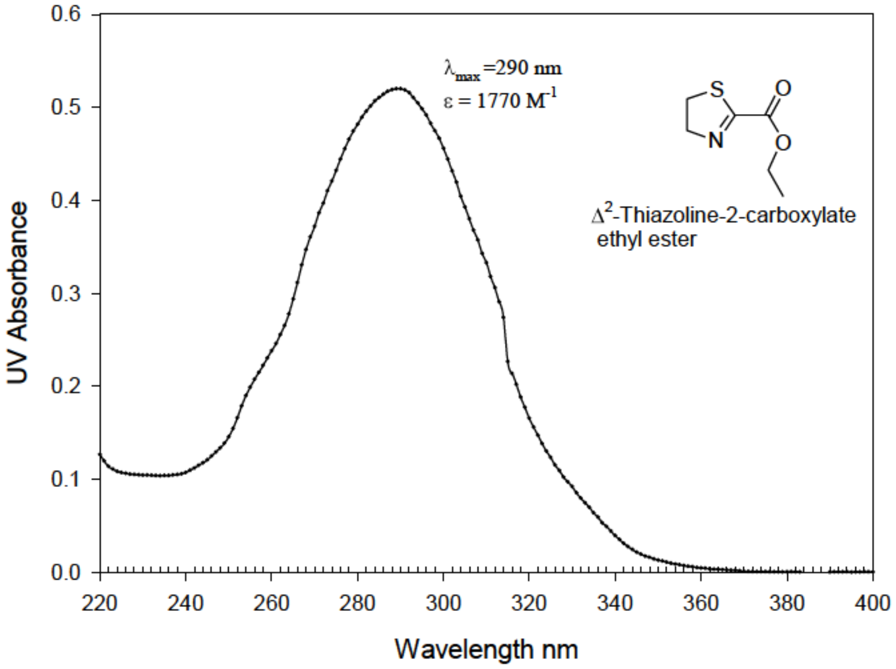


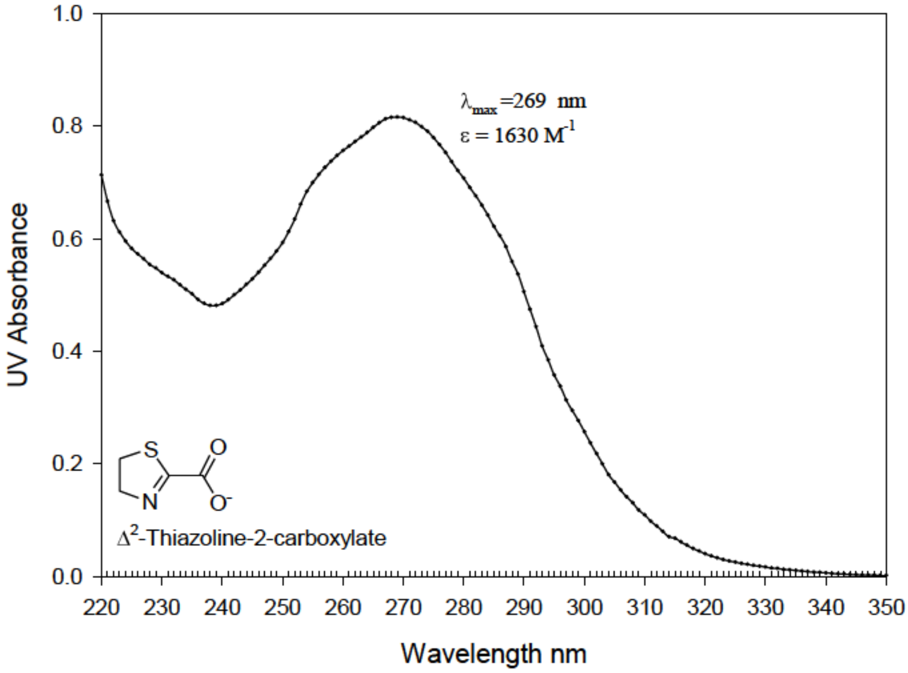
Δ^2-Thiazoline-2-carboxylate: ^{13}C NMR (400 MHz, D2O-acetone-D6) δ 171.49, 166.68, 64.44, 33.41.

Supp. Material fig. 7. ^{1}H NMR (upper) and ^{13}C NMR (lower) for Δ^2-thiazoline-2-carboxylate



Supp. Material fig. 8. IR spectra for Δ^2-thiazoline-2-carboxylate (lower) and its ethyl ester (upper)





Supp. Material fig. 9. UV spectra for Δ^2-thiazoline-2-carboxylate (lower) and its ethyl ester (upper)

References

1. Afeefy HY, Hamilton GA (1987) Acetic anhydride in aqueous solution converts Δ^2-thiazoline-2-carboxylate to an oxalyl thioester. Bioorganic Chemistry 15:262-268
2. Naber N, Venkatesan PP, Hamilton GA (1982) Inhibition of dopamine β-hydroxylase by thiazoline-2-carboxylate, a suspected physiological product of D-amino acid oxidase. Biochem Biophys Res Commun 107 (1):374-380
3. Theodorou V, Skobridis K, Tzakos AG, Ragoussis V (2007) A simple method for the alkaline hydrolysis of esters. Tetrahedron Lett 48 (46):8230-8233

CHAPTER 6

SYNTHESIS OF *N*-ALKYLAMINO ACIDS BY KETIMINE REDUCTASE/CRYM

Overview

A pyruvate molecule was observed bound in the active site of the solved x-ray structure of ketimine reductase/CRYM (Borel et al 2014). Docking studies described in chapter 3 used the monomer with bound pyruvate. Although bound in the active site it was not found to be an inhibitor or substrate. In this chapter the function of ketimine reductase/CRYM is further investigated. Its role in reductive alkylamination is described where the enzyme reduces the imine intermediate formed by the reaction of alkyl amines (e.g. methylamine) and α-keto acids (e.g. pyruvate). This research further confirms the function of ketimine reductase/CRYM as an imine reductase. Although formation of *N*-alkylamino acids is not likely to occur *in vivo* the reaction may be of some importance in the commercial synthesis of these compounds.

The following chapter is a short communication in Amino Acids journal.

Pages 100-103 of this thesis have been removed as they contain published material under copyright. Removed contents published as:

Hallen, A., Cooper, A.J.L., Smith, J.R. et al. (2015) Ketimine reductase/CRYM catalyzes reductive alkylamination of α-keto acids, confirming its function as an imine reductase. *Amino Acids*, vol. 47, pp. 2457–2461. https://doi.org/10.1007/s00726-015-2044-8

Supplementary material (LC-MS data)

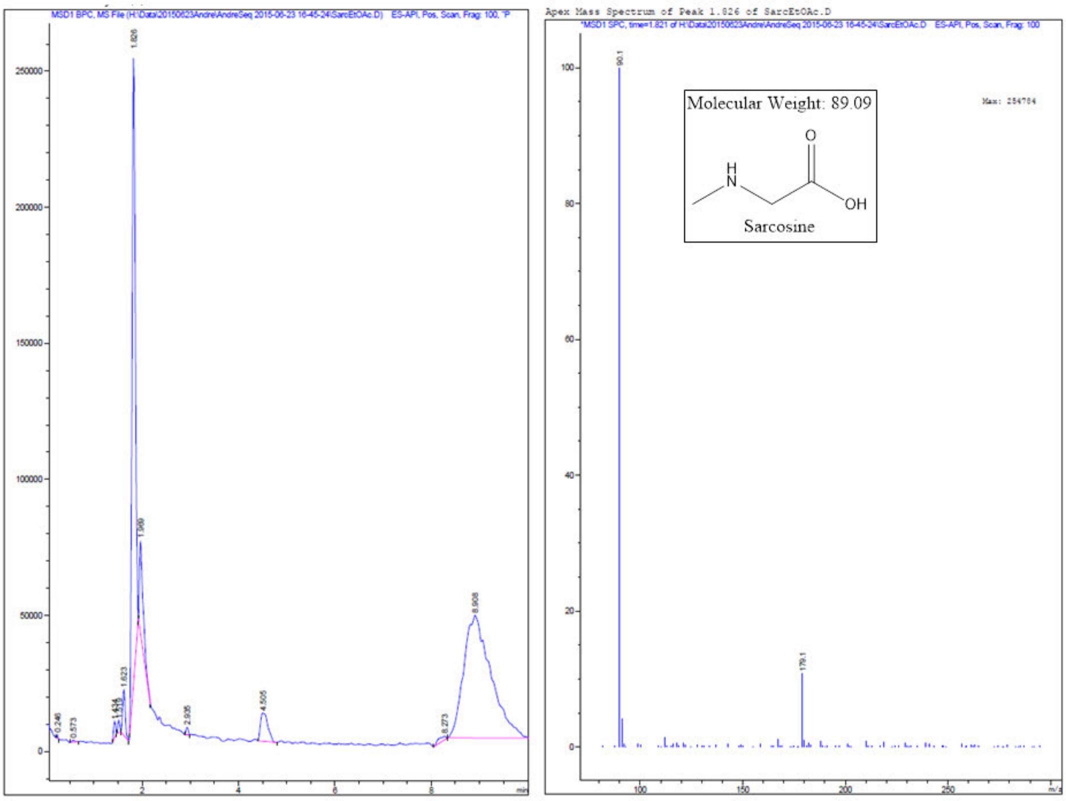


Fig. S1. LC-MS data for sarcosine. *Left*: base peak chromatogram, positive ion mode. *Right:* mass spectrum, positive ion mode.

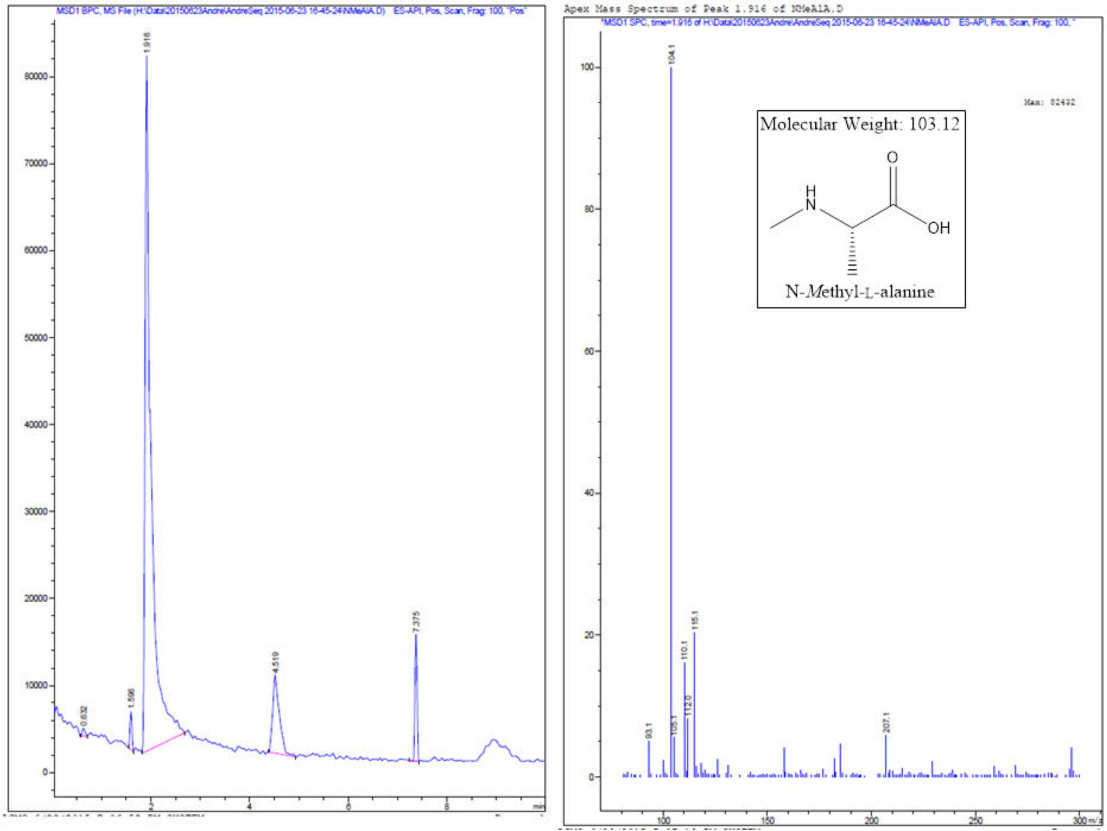


Fig. S2. LC-MS data for *N*-methyl-L-alanine. *Left:* base peak chromatogram, positive ion mode. *Right*: mass spectrum, positive ion mode.

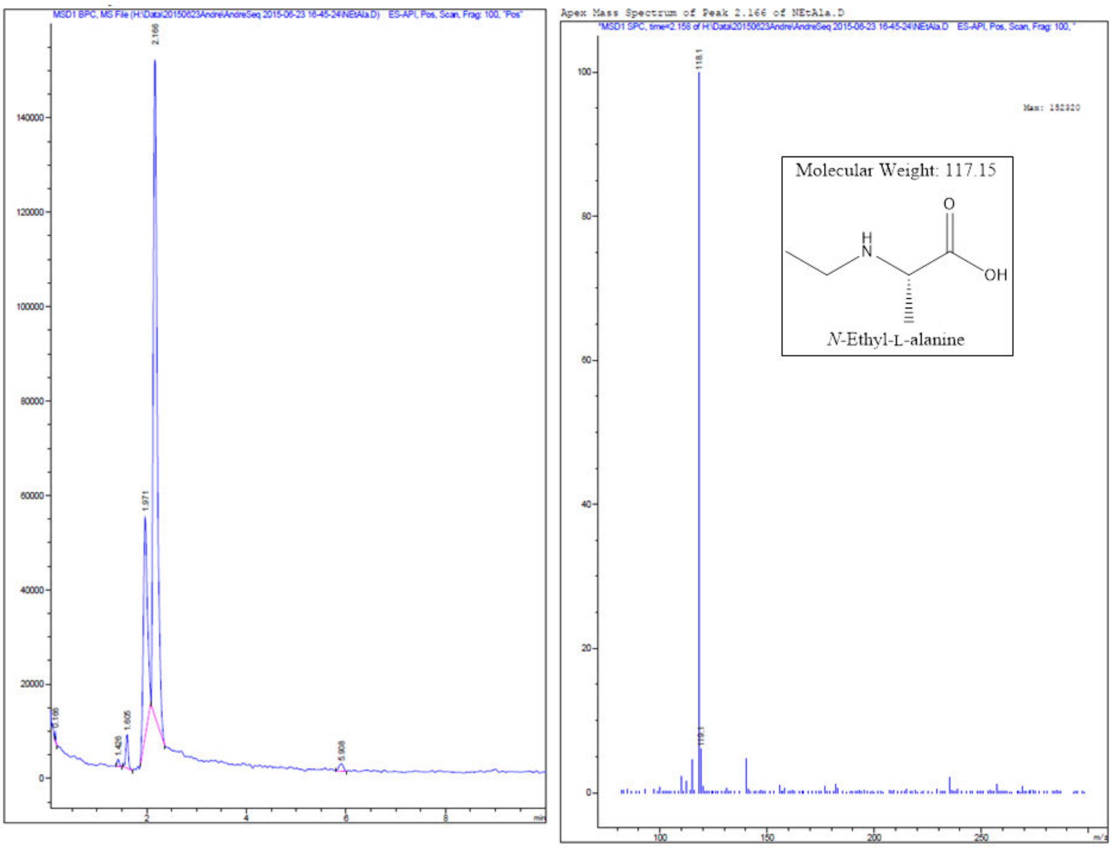


Fig. S3. LC-MS data for *N*-ethyl-L-alanine: *left* base peak chromatogram, positive ion mode. *Right:* mass spectrum positive ion mode.

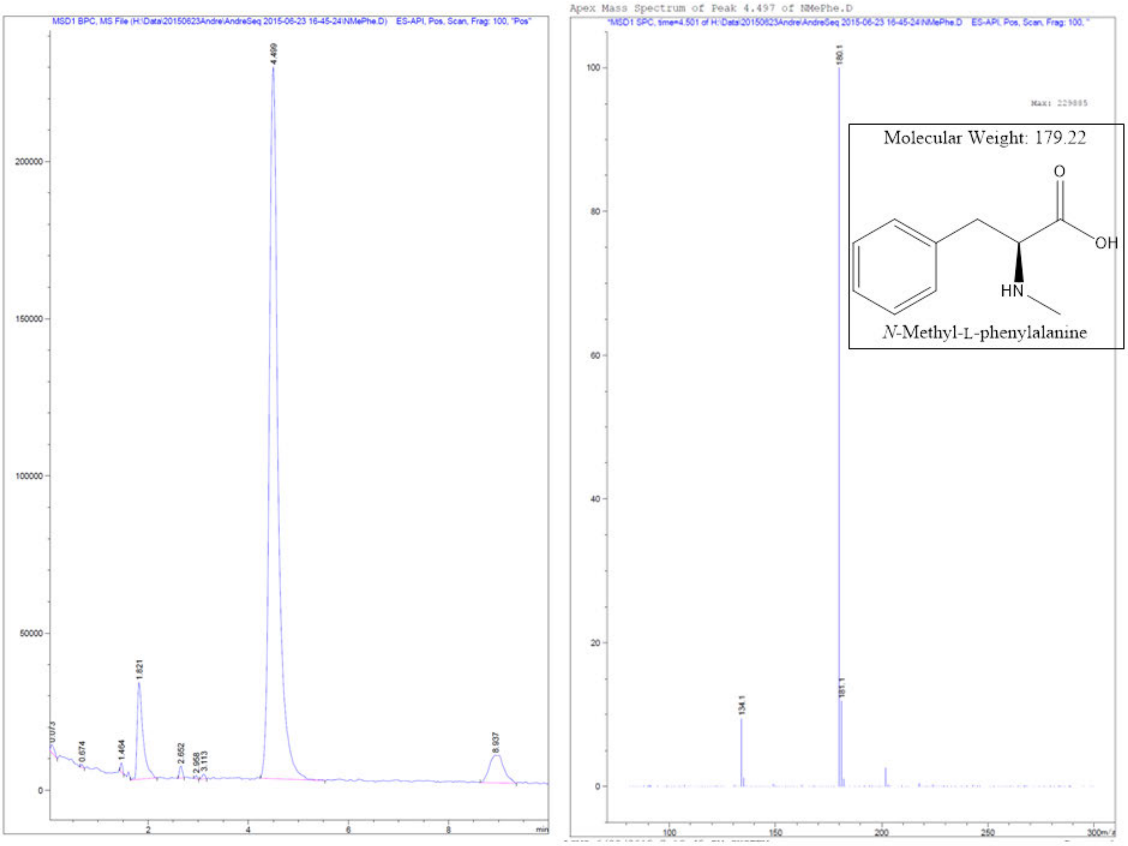


Fig. S4. LC-MS data for *N*-methyl-L-phenylalanine: *left* base peak chromatogram, positive ion mode. *Right*: mass spectrum, positive ion mode.

CHAPTER 7

EXPERIMENTAL

This chapter consists of experimental methods not included in the published papers.

7.1. Enzyme assays

A typical protocol is explained in detail.

7.1.1 Standardisation of NAD(P)H concentrations.

Standardisation of NAD(P)H concentrations was performed using the molar absorbance coefficient at 340 nm which is $\varepsilon = 6220\ M^{-1}\ cm^{-1}$. For example according to the Beer-Lambert Law ($A = \varepsilon Cl$, where A = absorbance, ε = molar absorbance coefficient, C = concentration and l = pathlength in cm) a 0.1 mM solution of NAD(P)H has an absorbance of 0.622.

7.1.2 Fluorescence standard curve:

Fluorescence assays were conducted with a Novostar microplate reader in top reading mode and the data analysed using MARS data analysis software (BMG Labtech, Germany). Using a fluorescence excitation filter of 355 nm and an emission filter of 460 nm standard curves were generated using NADPH concentrations at 2, 4, 6 and 8 μM. In order to maximize the sensitivity the gain of the photomultiplier tube was set to 90% of its maximum at 8 μM NADPH. By using linear regression analysis the relationship between concentration of NADPH and fluorescence intensity (FI) can be deduced. This can then be used in the enzyme assays. The slope of the linear regression line of fluorescence intensity (FI) vs [NADPH] represents the change in FI/μM [NADPH] (Fig. 7.1). If the slope = 5703 this equates to a 5703 change in FI for every 1 μM change in [NADPH] or 1 unit change in FI = 1/5703 μM change in [NADPH]. As there is 200 μL in the assay therefore a 1 unit change in FI equates to a change of 3.51×10^{-8} μmol [NADPH]. As the stoichiometry of the enzyme substrates: NADPH is 1:1 this also equates to an identical decrease in the concentration of enzyme substrate.

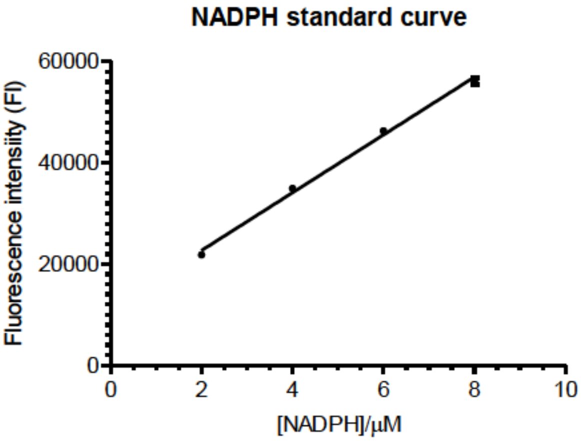


Fig. 7.1. NADPH standard curve. Standard NADP solutions were made up and 6 replicates of fluorescence readings obtained with an excitation filter (355 nm) and emission filter (460 nm). A linear regression curve was fitted with Graphpad Prism software.

7.1.3 Protein concentration

The enzyme concentration was measured by using a Bradford assay (A_{595nm}) by interpolating values using the linear regression curve of standard concentrations of bovine serum albumin (BSA). For example a stock equation solution was found to have a concentration of 0.55 mg/ml (Fig. 7.2). 2 μL of this stock enzyme solution was diluted 0.8×10^{-4} equating to 17.6 ng (1.76×10^{-5} mg) in a 200 μL assay (2.61 nM).

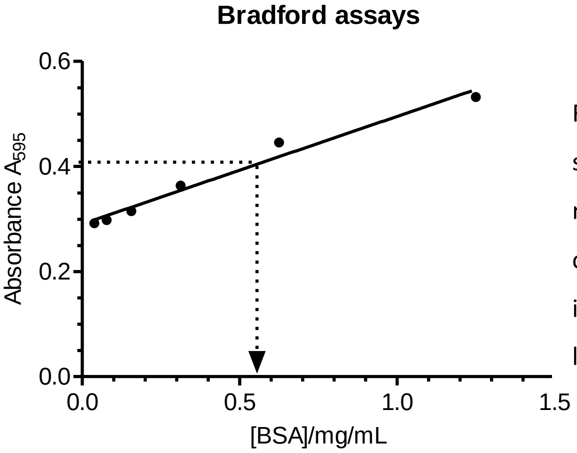


Fig. 7.2 Bradford protein concentrations. Standard solutions of BSA were prepared and absorbance readings at 595 nm obtained. The stock protein concentration was obtained by interpolation from its absorbance (0.41) as depicted by the dotted lines.

7.1.4 Catalytic rate

The rate of decrease in FI is measured from the reaction progress curve of FI against time (e.g. Fig. 7.3 using 62 μM Pyr2C as substrate at pH 5). The initial rate is determined by fitting the reaction progress curve to a 2nd order polynomial (*FI = at^2 + bt+ c, where t = time(s)*) as the initial rate is the b coefficient as:

$$\lim_{t\to 0}\frac{d}{dt}(at^2 + bt + c) = \lim_{t\to 0} 2at + b = b$$

$$\frac{dFI}{dt} = \frac{d}{dt}0.23t^2 - 100.3t + 47620 = -100.3/\text{s}\ (R^2 = 0.995).$$

Therefore the catalytic rate = 100.3 FI/s. Therefore using the conversion factor from 7.1.2 where 1 unit change in FI = 3.51×10^{-8} μmol change in substrate: 100.3 FI/s therefore equates to a change of $3.51 \times 10^{-8} \times 100.3 \times 60$ μmol/min = 2.1×10^{-4} μmol/min.

7.1.5 Specific activity

Specific activity = $\frac{catalytic\ rate}{protein\ concentration} = \frac{2.1 \times 10^{-4}}{1.76 \times 10^{-5}}$ = 11.9 μmol/mg/min

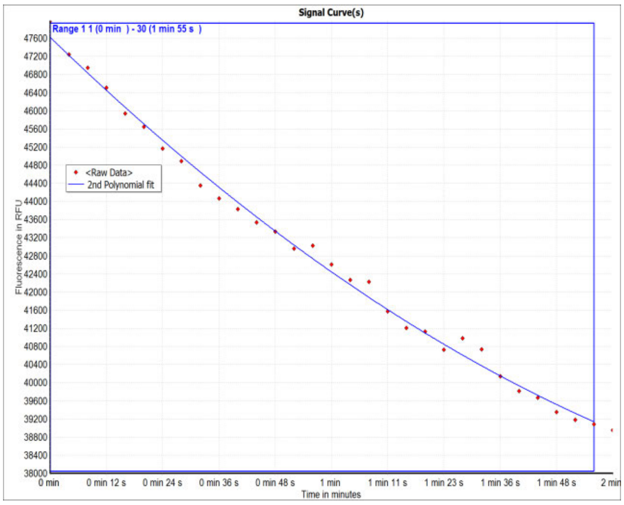


Fig. 7.3. Enzyme catalytic progress curve using Pyr2C as substrate (62 μM) and 8 μM NADPH at pH 5. The decrease in fluorescence intensity (FI) is used to measure the decrease in fluorescence of NADPH. The initial rate is determined by fitting a 2nd order polynomial to the curve and deduced as described in the text.

7.2 Organic synthesis

Chemical reagents were purchased from Sigma Aldrich (Castle Hill, Australia) and organic solvents purchased from ChemSupply (Australia).

^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance DPX400 400 MHz and AVII600 600 MHz spectrometers at 25°C and processed using MestreNova software (v. 8.1, Mestrelab Research, Santiago de Compostela, Spain).

7.2.1 *S*-2-Aminoethylcysteine ketimine (AECK)

Firstly AECK ethyl ester was synthesized by an adaption of the method of Rossi et al. (1962) [1]. Cysteamine hydrochloride (5 mmol/568 mg) was added to dry dichloromethane (40 mL) in a round bottom flask and stirred with a magnetic follower under nitrogen. Triethylamine (10 mmol/1.4 mL) in 10 mL dichloromethane and ethylbromopyruvate (5 mmol/630 μL) in 10 mL dichloromethane were simultaneously slowly added dropwise over 2 hours. The reaction was allowed to proceed to completion at room temperature (~ 4 hours). The reaction mixture was filtered to remove trimethylamine hydrobromide and hydrochloride crystals and then placed overnight in a freezer at -20°C. Remaining crystals were subsequently removed by filtration, the reaction mix dried with magnesium sulphate, and the solvent evaporated using a rotary evaporator resulting in a yellow oil of crude product (632 mg, 73%). A Biotage Isolera flash chromatography system (Biotage AB, Uppsala, Sweden) was used to purify the product. The pure product was eluted from a Biotage silica column (50g) using a 100-70% gradient (hexane/ethylacetate) over 12 column volumes and the elution of pure product was monitored by UV absorbance (300 nm). Solvent was evaporated from pooled fractions using a rotary evaporator resulting in a pure pale yellow liquid (433 mg, 50%).

AECK ethyl ester (enamine): ^{1}H NMR (600 MHz, MeOD) δ 6.17 (s, 1H), 4.21 (q, 2H), 3.53 (m, 2H), 2.97 (m, 2H), 1.29 (t, 3H). ^{13}C NMR (600 MHz, MeOD) δ 162.77, 129.05, 101.98, 61.12, 41.34, 25.94, 14.35.

The AECK ethyl ester was hydrolysed under anhydrous conditions resulting in a pale yellow solid AECK sodium salt (313 mg, mols, 38% overall yield), in a similar manner as described in chapter 3 (Supplementary material) for the synthesis of T2C sodium salt

AECK sodium salt (enamine): ^{1}H NMR (600 MHz, MeOD) δ 5.84 (s, 1H), 3.46 (m, 2H), 2.90 (m, 2H). ^{13}C NMR (600 MHz, MeOD) δ 170.33, 136.06, 96.83, 42.75, 26.37. MS (ESI) m/z: 146 (M+ H).

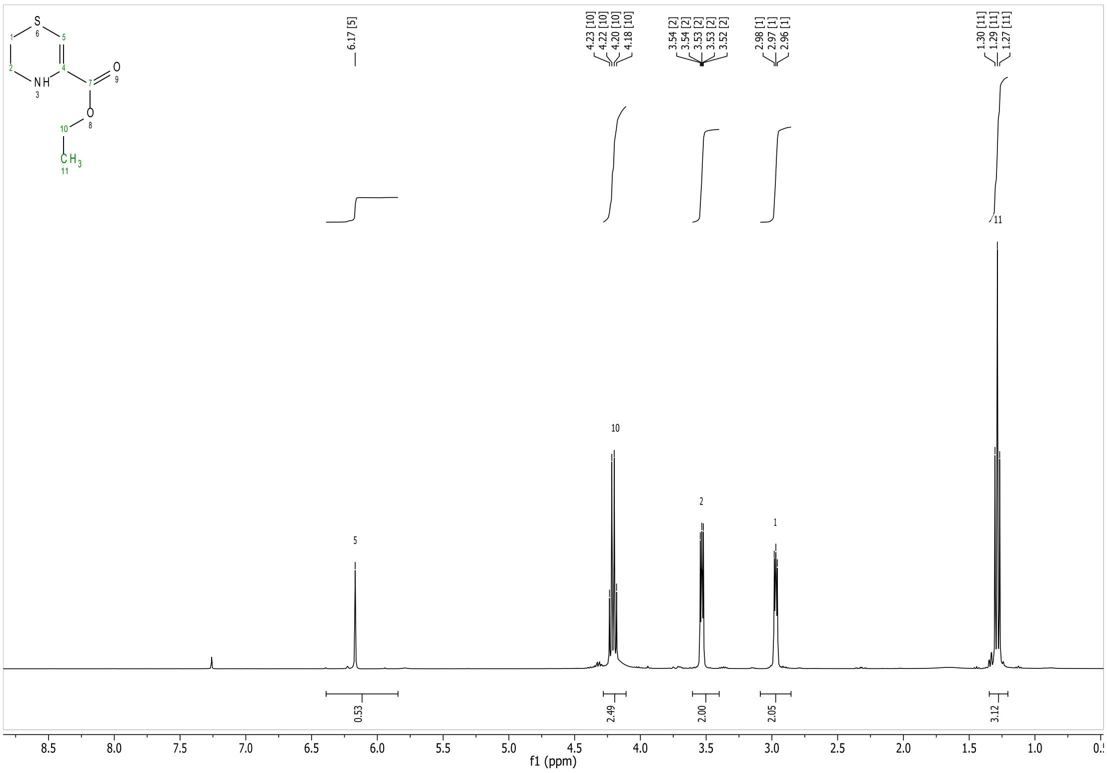


Figure 7.4a. 1D ^{1}HNMR spectrum of AECK ethyl ester (600 Mhz/MeOD).

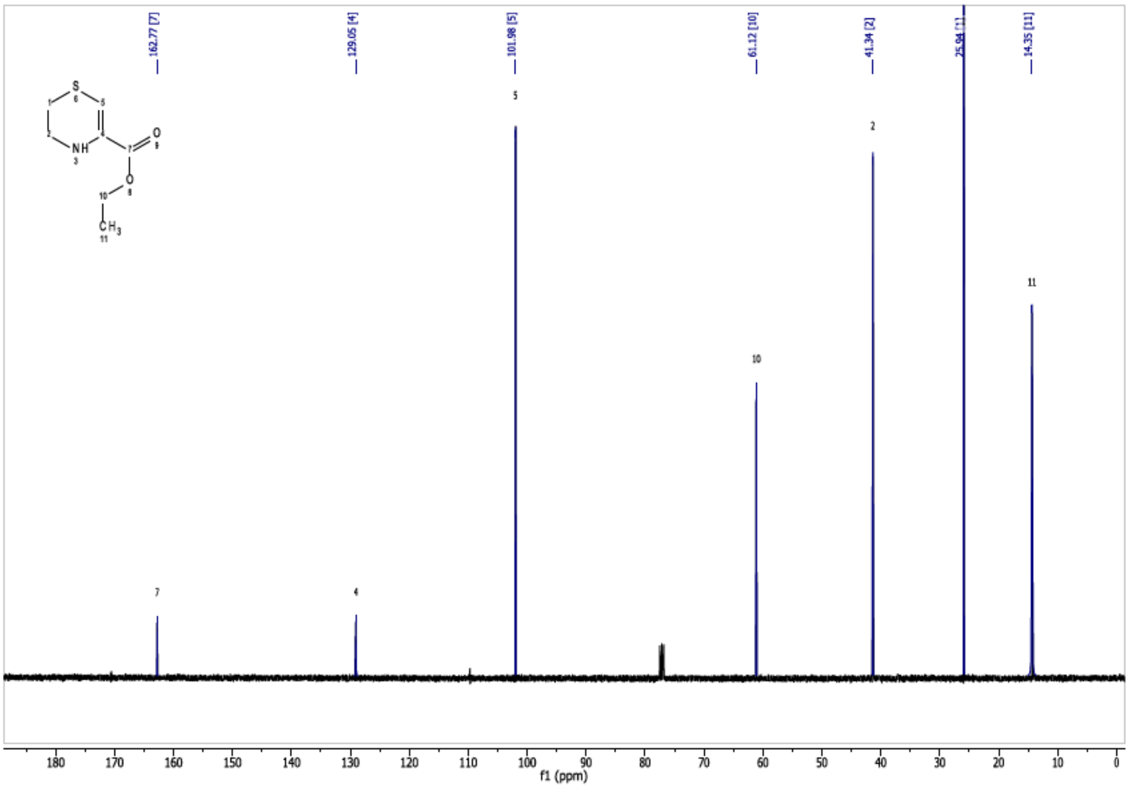


Figure 7.4b. 1D ^{13}CNMR spectrum of AECK ethyl ester. (600 Mhz/MeOD).

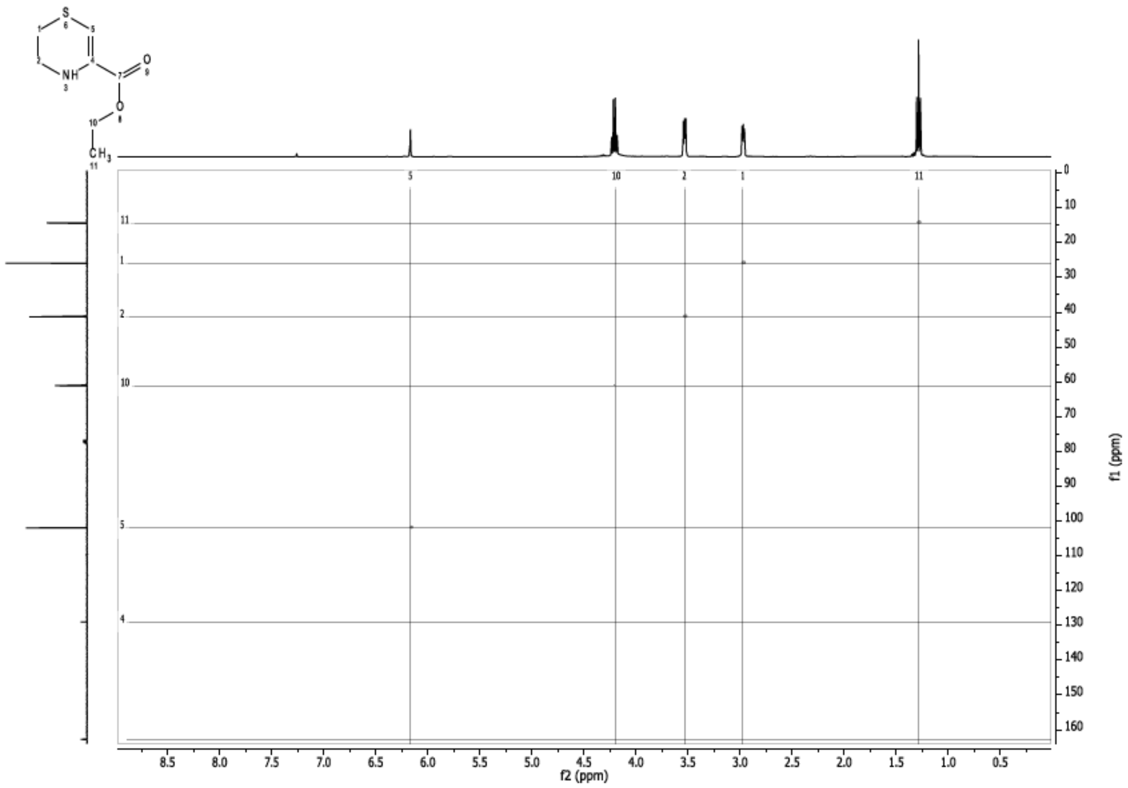
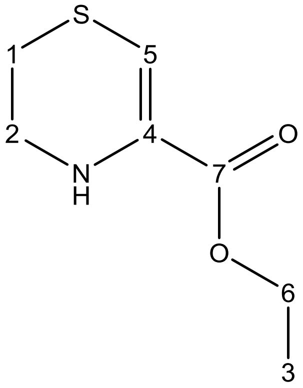


Figure 7.4c. 2D HSQC spectrum of AECK ethyl ester. (600 Mhz/MeOD).



No	^{1}H-Chemical Shift (ppm)	Multiplicity	^{13}C- Chemical Shift (ppm)
5	6.17	s	101.98
2	3.53	m	41.34
1	2.97	m	25.94
3	1.29	t	14.35
6	4.21	q	61.12
4			129.05
7			162.77

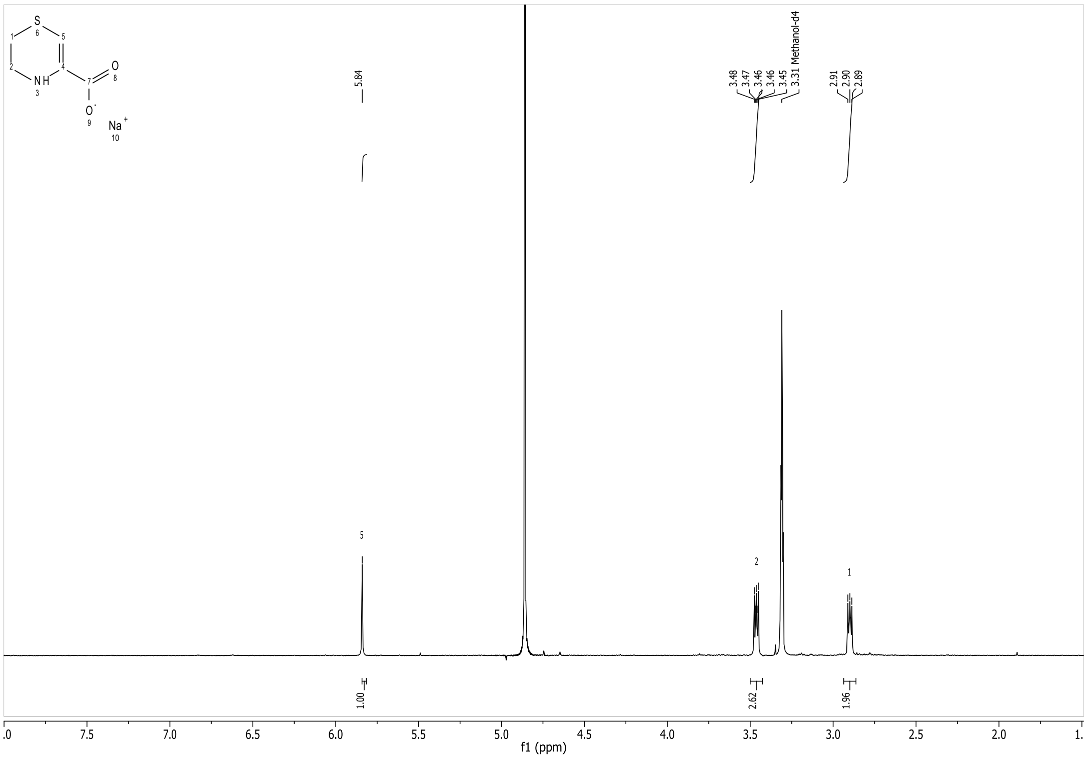


Figure 7.4d. 1D ^{1}HNMR spectrum of AECK sodium salt. (600 Mhz/MeOD).

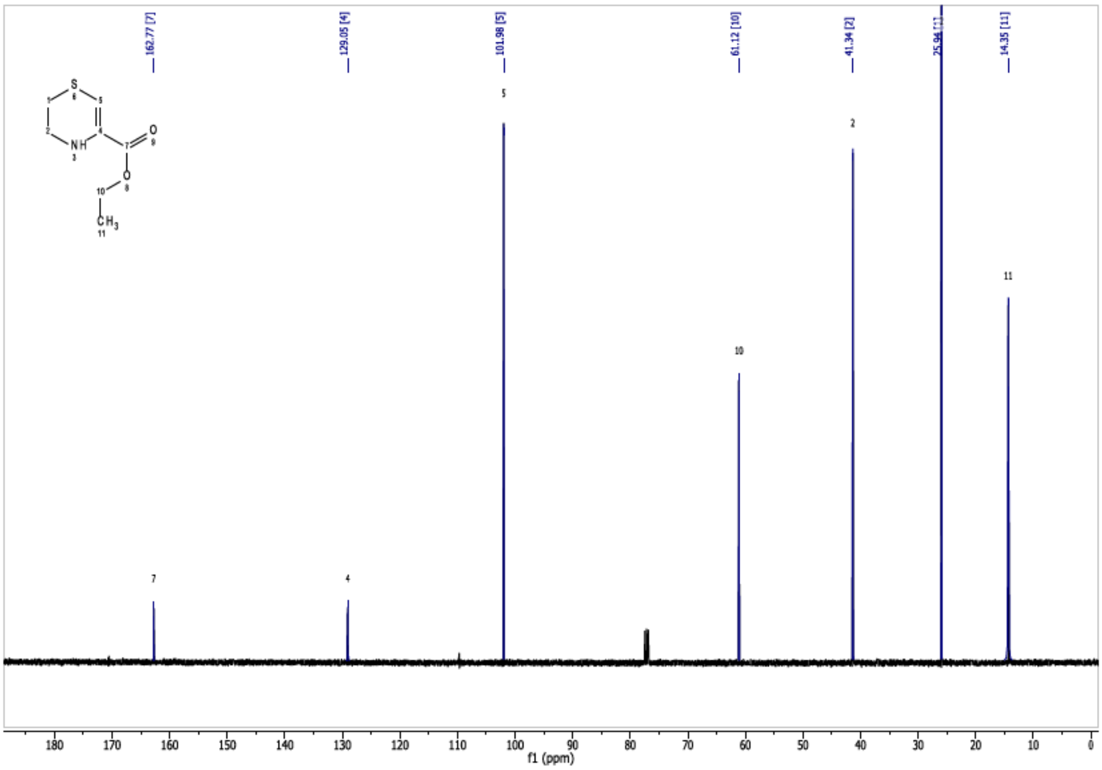


Figure 7.4e. 1D ^{13}CNMR spectrum of AECK sodium salt. (600 Mhz/MeOD).

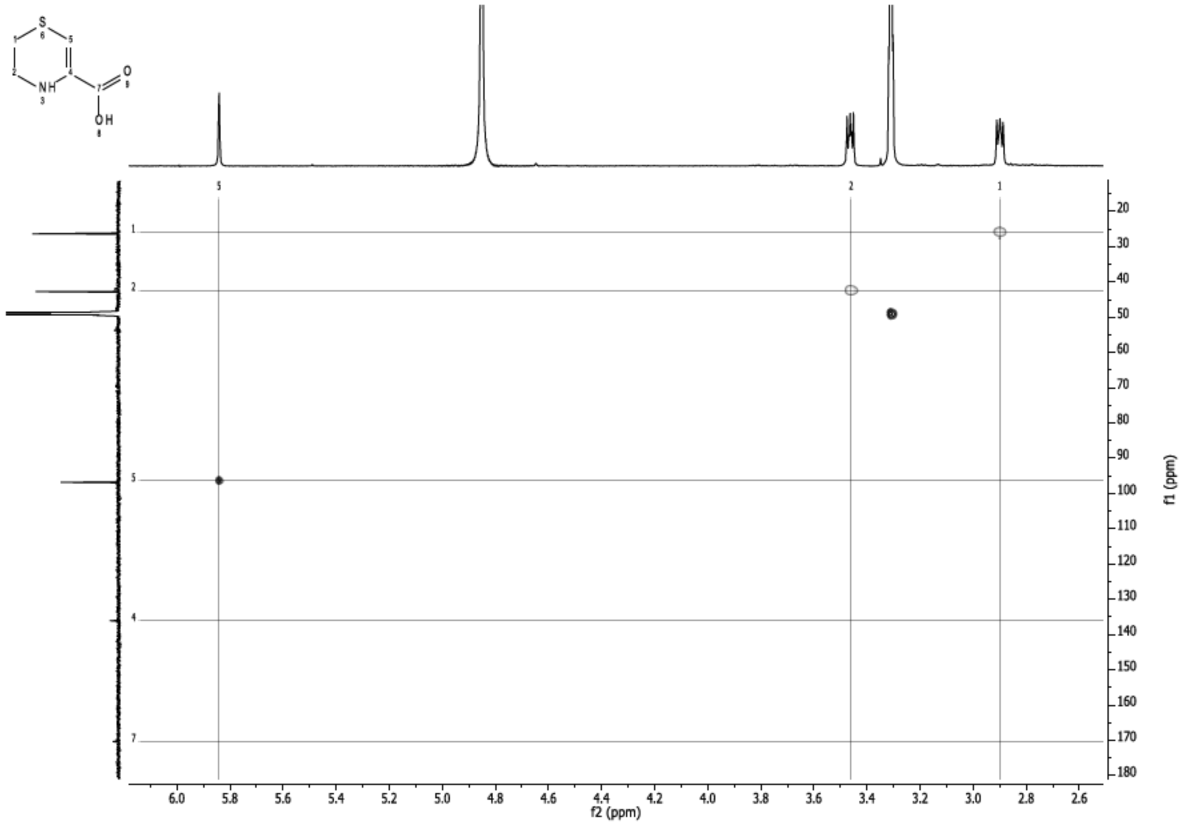
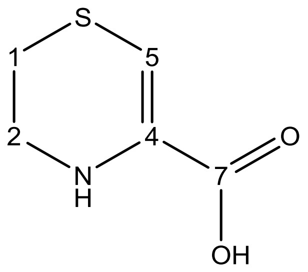


Figure 7.4f. 2D HSQC of AECK sodium salt. (600 Mhz/MeOD).



No	^{1}H-Chemical Shift (ppm)	Multiplicity	^{13}C- Chemical Shift (ppm)
5	5.84	s	96.83
2	3.46	m	42.75
1	2.90	m	26.37
4			136.06
7			170.33

AECK was also synthesized as its hydrobromide salt based on a published method by Cavallini et al. (1983) [2]. Solutions of 3-bromopyruvic acid (1 g, 5.99 mmol) and cysteamine hydrochloride (0.668 g, 6 mM) were each prepared separately in 5 mL glacial acetic acid in 15 mL falcon tubes. The cysteamine hydrochloride solution was slowly added to the bromopyruvate solution with vigorous shaking. After 30 min the resulting emulsion was broken with a glass rod, washed with glacial acetic acid (3 × 10 mL), and collected by centrifugation. The resulting AECK hydrobromide salt was flash frozen with liquid nitrogen and dried under vacuum pump over NaOH. The dried product (0.410 g, 30 %) was stored under nitrogen at -20°C. m.p. 111-113°C, (Lit. m.p. = 113-114°C [2]). The concentrations used in assays were determined by using the molar extinction coefficient at pH 8.5 (ε_{296nm} = 6200 M^{-1} cm^{-1}) [2].

7.2.2 Lanthionine ketimine (LK)

The synthesis of LK was based on a published method [2]. L-Cysteine hydrochloride (6 mmol, 0.946 g) dissolved in 2.5 mL water was added to a solution of 3-bromopyruvic acid (6 mmol, 1 g) in 2.5 mL of water, and the reaction constantly stirred. After 15 min the resulting precipitate was collected under vacuum and washed with ice-cold water (3 × 5 mL). The resulting pale yellow crystals (660 mg, 40.9%) were dried on a freeze-dryer and stored at -20°C under nitrogen. The concentrations used in assays were determined by using the molar extinction coefficient at pH 8.5 (ε_{296nm} = 6200 M^{-1} cm^{-1}) [2]

Lanthionine ketimine hydrobromide salt (enamine): ^{1}H NMR (600 MHz, MeOD) δ 6.14 (s, 1H), 4.27 (m, 1H), 3.10 (m, 2H). ^{13}C NMR (600 MHz, MeOD) δ 173.85, 165.42, 129.72, 101.84, 54.21, 27.52. MS (ESI) m/z: 190 (M + H).

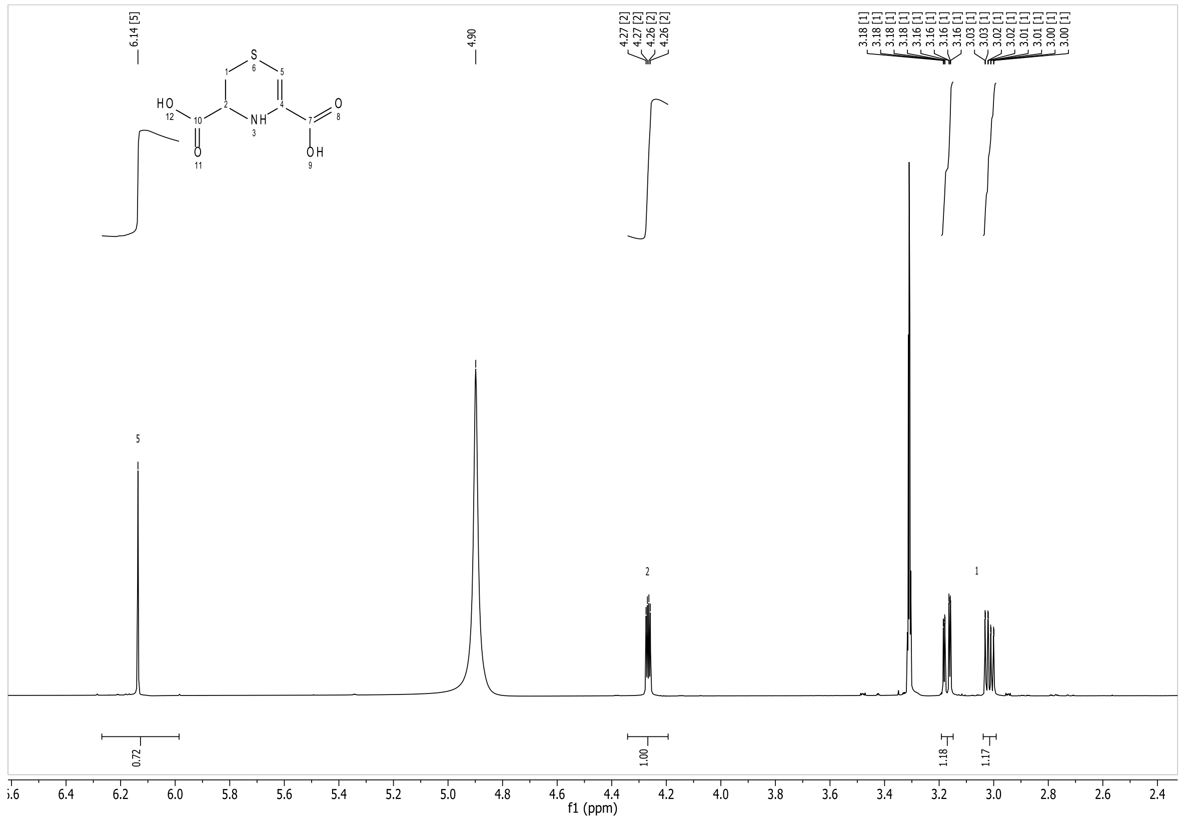


Figure 7.5a. 1D ^{1}HNMR spectrum of lanthionine ketimine (LK).

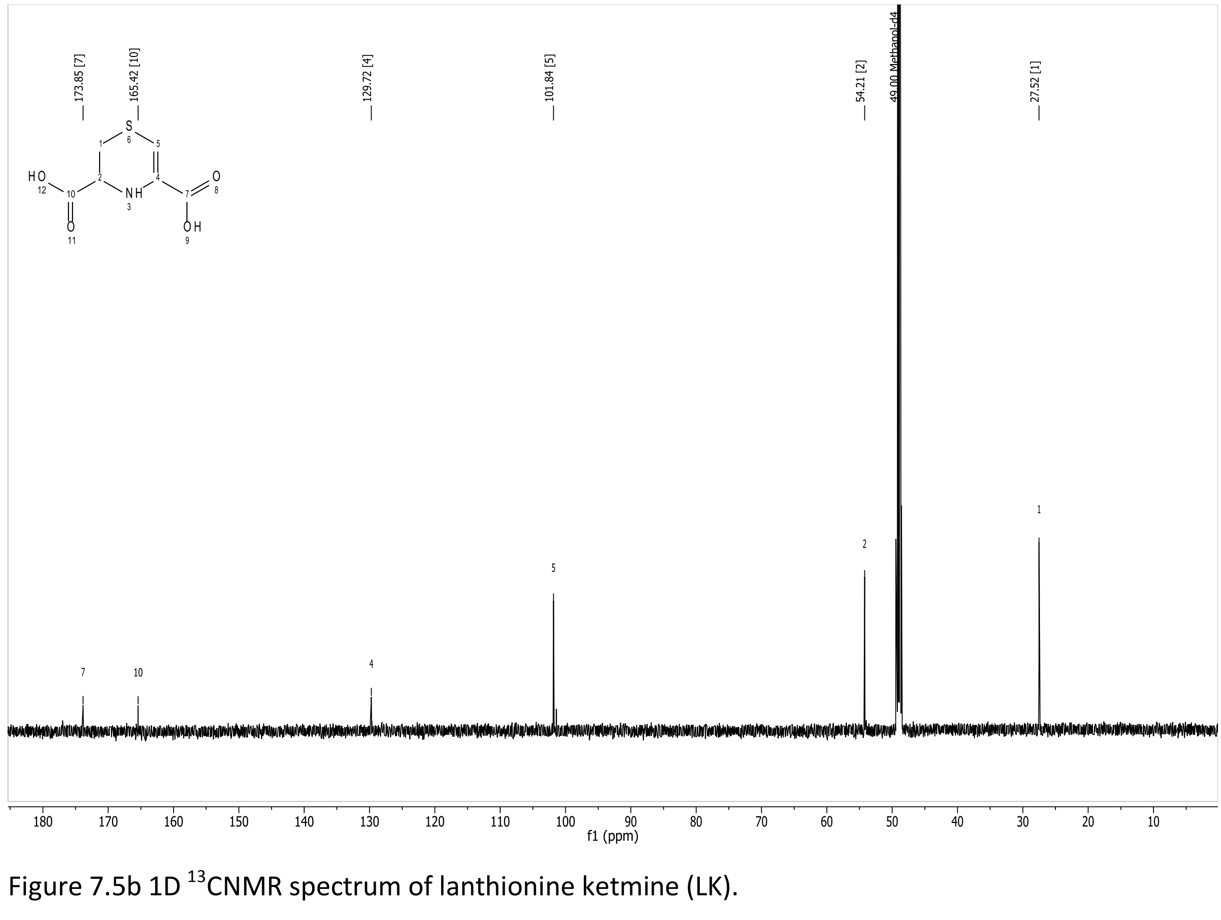


Figure 7.5b 1D ^{13}CNMR spectrum of lanthionine ketmine (LK).

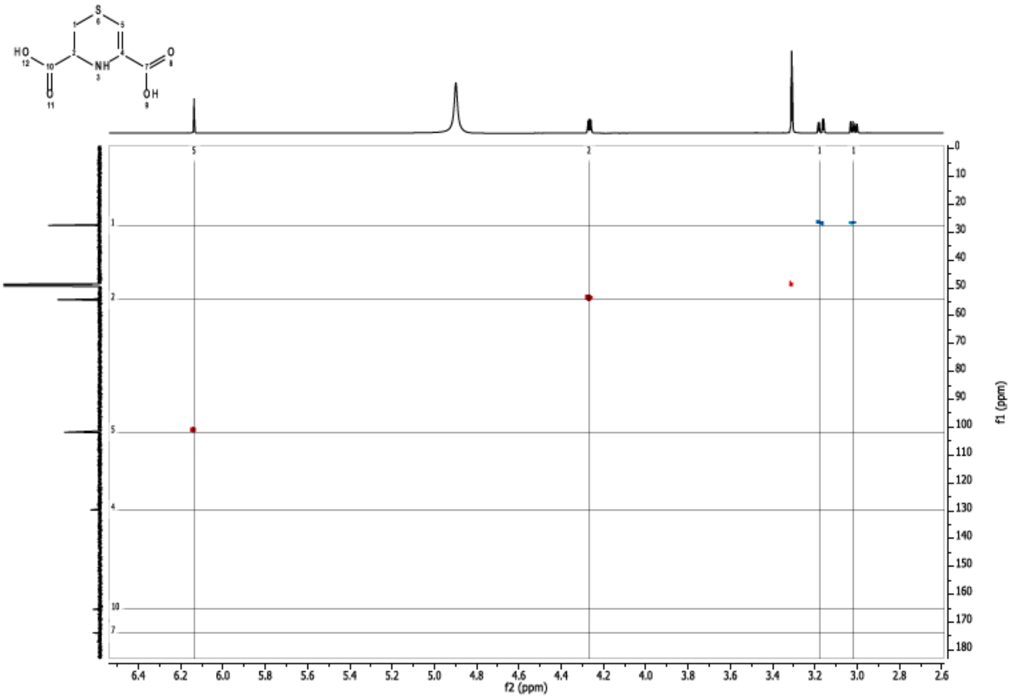


Figure 7.5c 2D HSQC of lanthionine ketimine (LK) (600 MHz, MeOD).

No	δ (ppm)	HSQC ($^{1}J_{CH}$) Correlations (ppm)
5	6.14	101.84
2	4.27	54.21
1	3.02 3.17	27.52
4		129.72
7		165.42
10		173.85

7.2.3 Cystathionine ketimine (CysK)

The synthesis of cystathionine ketimine (CysK) was based on a published method [3,4]. L-Homocysteine thiolactone hydrochloride (154 mg, 1 mmol) was dissolved in 1M NaOH (2 mL), in a 25 mL round bottom flask. The solution was then heated for 10 min in a boiling water bath to open the thiolactone ring under bubbling nitrogen. After cooling to RT 3-bromopyruvic acid (200 mg) was added and the reaction continued under bubbling nitrogen for 15 min. The reaction mix was then added to a 1 × 7 mL Dowex 50 X8 H+ form strong cation exchange column. The column had been charged with 1M HCl and then equilibrated with MilliQ water. The column was eluted with MilliQ water and 10 mL fractions collected. Fractions (70 -350 mL) were concentrated at 40°C and lyophilised to a fine pale yellow powder. The final product was dried on a freeze-dryer and stored under argon at -20°C. The final product was isolated in its open form as *S*-(2-carboxy-2-oxoethyl)-L-homocysteine (0.073 g, 33 %), m.p. 127-130°C decomposition [Lit. m.p. 130°C decomposition [4]]. The concentrations used in assays were determined by using the molar extinction coefficient in absolute ethanol (ε_{315nm} = 5000 M^{-1} cm^{-1}) [4].

7.2.4 Δ^1-Pyrroline-2-carboxylate (Pyr2C)

Pyr2C methyl ester was synthesised based on a published method by Szollosi et al. [5]. Briefly L-proline methyl ester hydrochloride (7 g, 42 mmol) was suspended in dry diethyl ether (40 mL) and trimethylamine (7 mL, 50 mmol) was added with constant stirring at room temperature. After 2.5 h the mixture was stored overnight at -20°C. The trimethylamine hydrochloride crystals were removed by filtration and the diethyl ether evaporated resulting in a yellow oil which after vacuum distillation (30 mM Hg: 85°C) resulted in a colourless liquid of L-proline methyl ester (5 mL, 85%). *tert*-Butyl hypochlorite (1 mL) and L-proline methyl ester (1 mL) were simultaneously added dropwise over 15 min to dry diethyl ether (20 mL) at -50°C. *tert*-Butyl hypochlorite was synthesized from 4% sodium hypochlorite and *tert*-butanol by a published method [6]. After stirring for 30 min the solution was warmed to room temperature and trimethylamine (2 mL) was slowly added. The solution was shielded from light and allowed to stir for a further 2 days under an atmosphere of nitrogen, after which the triethylamine hydrochloride crystals were removed by filtration. The filtrate was evaporated leaving a yellow oil of crude product which was further purified by vacuum distillation to a colourless liquid (0.5 mL, 50%). In contrast to the method used by Szollosi, who subsequently hydrolysed the esters to form sodium salts under aqueous conditions, the esters were instead hydrolysed under non-aqueous conditions in a similar manner as described in chapter 3 (Supplementary material) for the synthesis of T2C sodium salt.

Pyr2C methyl ester (imine): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.87 (m, 2H), 2.71 (m, 2H), 3.75 (s, 3H), 3.99 (m, 2H). ^{13}C NMR (400 MHz, $CDCl_3$) δ 21.95, 35.14, 52.40, 62.33, 162.99, 168.10.

Pyr2C sodium salt (imine): ^{1}H NMR (400 MHz, MeOD) δ 1.93 (m), 2.78 (m), 3.87 (m) of equal intensity. ^{13}C NMR (400 MHz, MeOD) δ 23.22, 36.93, 61.56, 171.71, 177.55. MS (ESI) m/z: 114 (M + H).

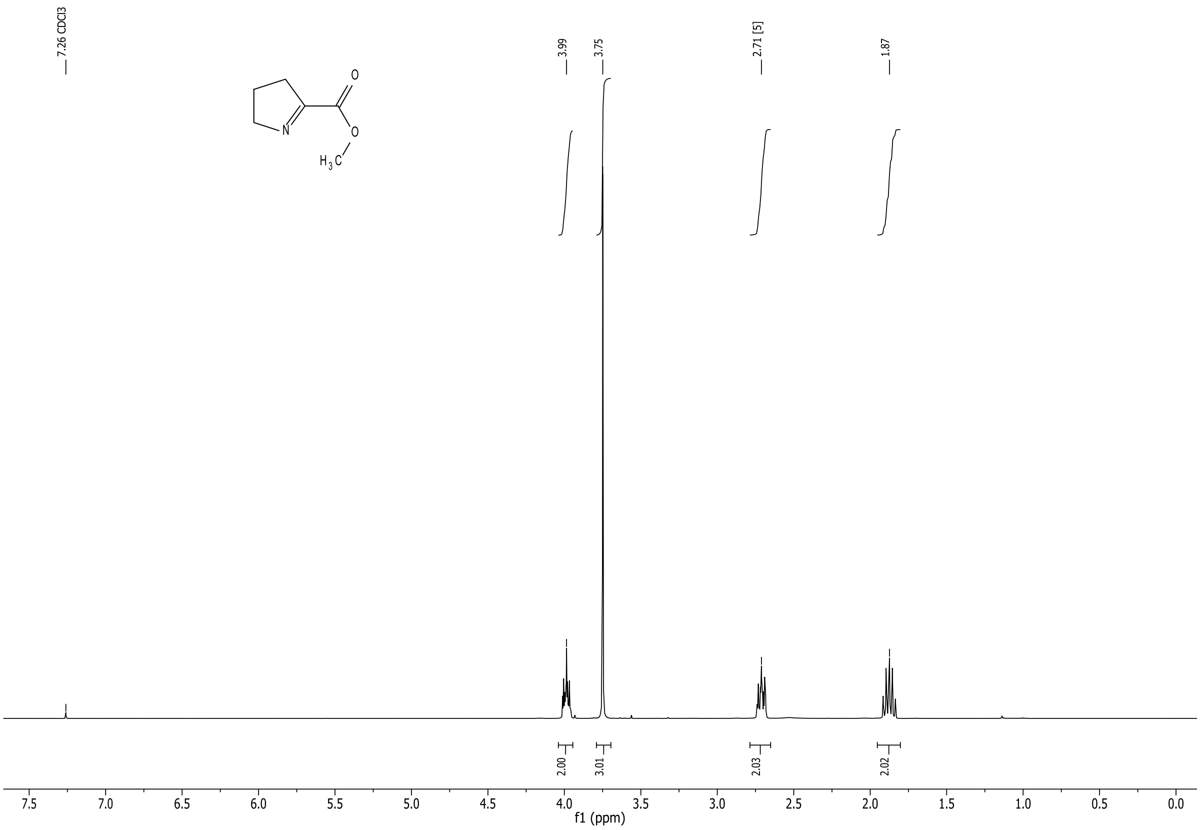


Figure 7.6a. 1D ^{1}HNMR spectrum of Pyr2C methyl ester (400 MHz, CDCl$_{3)}$.

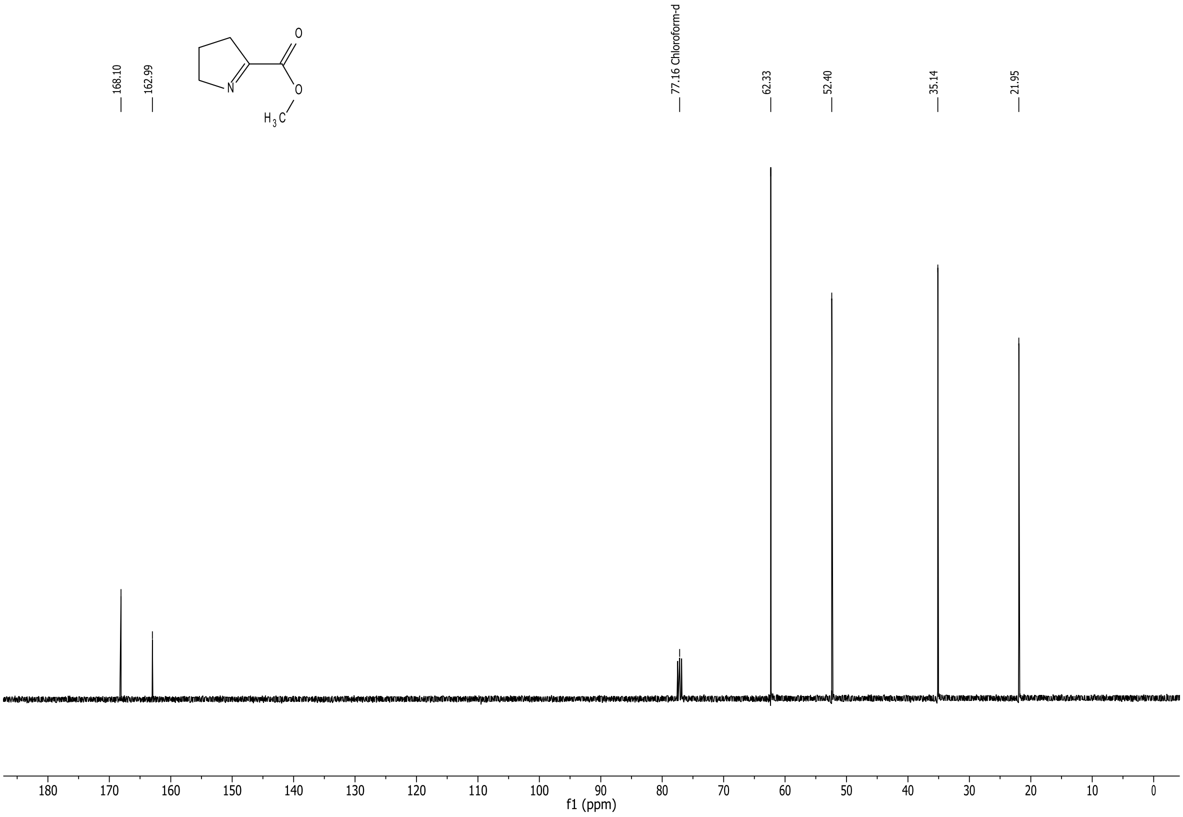


Figure 7.6b. 1D ^{13}CNMR spectrum of Pyr2C methyl ester (400 MHz, CDCl$_{3)}$.

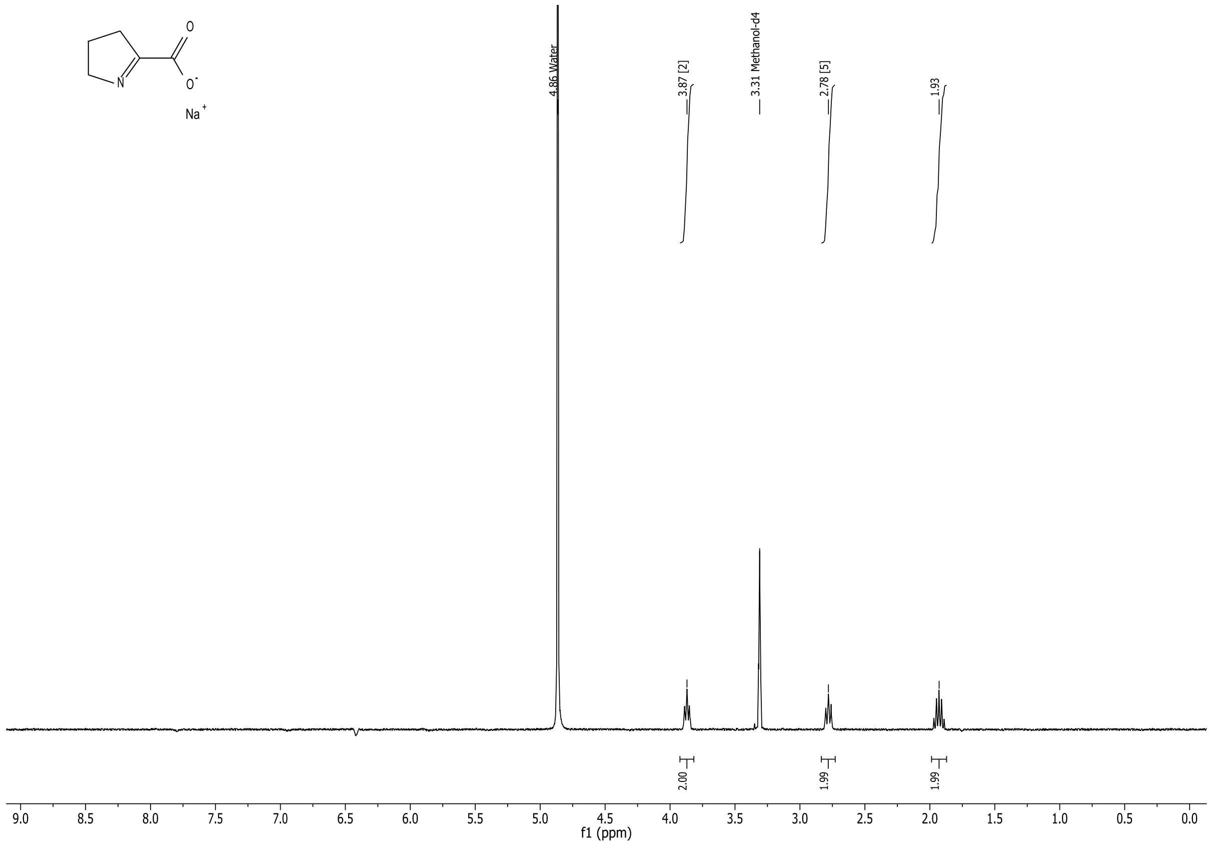


Figure 7.6c. 1D ^{1}HNMR spectrum of Pyr2C sodium salt (400 MHz, MeOD).

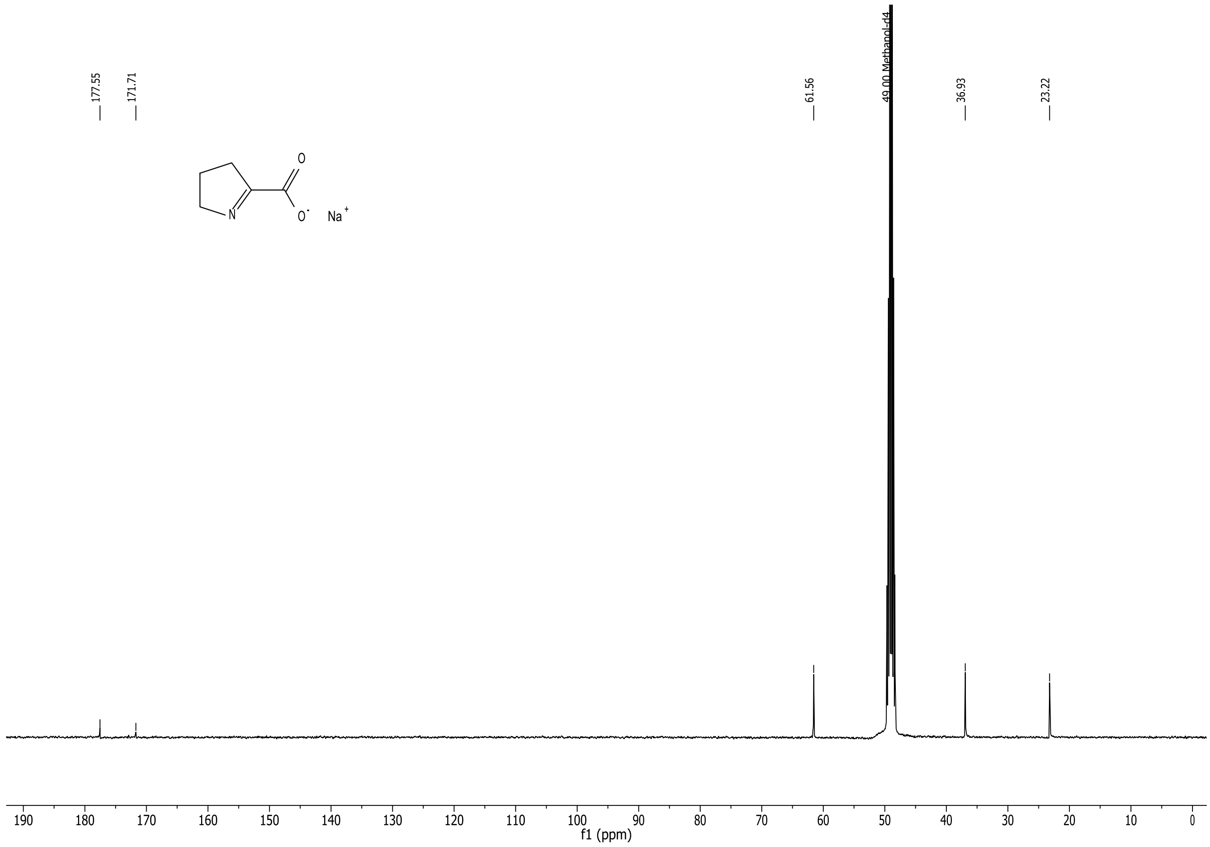


Figure 7.6d. 1D ^{13}CNMR spectrum of Pyr2C sodium salt (400 MHz, MeOD).

7.2.5 Δ^1-piperideine-2-carboxylate (P2C)

P2C methyl ester was synthesised in a similar method as Pyr2C methyl ester (7.2.4), using L-pipecolate methyl ester hydrochloride as starting material. The ester was hydrolysed under anhydrous conditions in an identical manner as previously reported, and the product recrystallized (EtOH/Hexane) to yield a fine white powder (610 mg, 52%). The P2C ester and sodium salt were found to be more unstable than their corresponding Pyr2C analogues, and consistent with literature data [7,8] were a mixture of enamine and imine tautomers in contrast to Pyr2C which was only an imine (7.2.4). The ^{13}CNMR spectrum shows evidence of some decomposition due to the lengthy acquisition time (3 hr). The ^{1}HNMR data for P2C sodium salt was consistent with the literature data [7,8]. The concentration of P2C used in the enzyme assays was standardized by using the molar extinction coefficient in 0.1 M NaOH (ε_{256nm} = 725 M^{-1} cm).

P2C sodium salt: ^{1}HNMR (600 MHz, D2O) δ (enamine) 5.67 (s, 1H, *J* = 4.1 Hz), 3.02 (m, 2H), 2.13 (m, 2H), 1.74 (m, 2H) ; (imine) 1.58 (m, 2H), 1.67 (m, 2H), 2.36 (m, 2H), 3.51(m, 2H). ^{13}CNMR (600 MHz, D2O) δ (enamine), 175.93*, 139.72, 111.30, 42.78, 23.16, 22.54; (imine) 171.59*, 173.72*, 49.37, 28.03, 22.25, 19.54. MS (ESI) m/z: 128 (M + H).

* Reference [8] notes the imine carbon resonance being downstream from the carbonyl so this is only a possible assignment. An internal standard was not used for ^{13}CNMR however the results obtained were found to be similar to those found by [8] using TSP as a standard. ^{13}CNMR was primarily used to assist to making imine/enamine assignments for comparisons with lit. values [7,8].

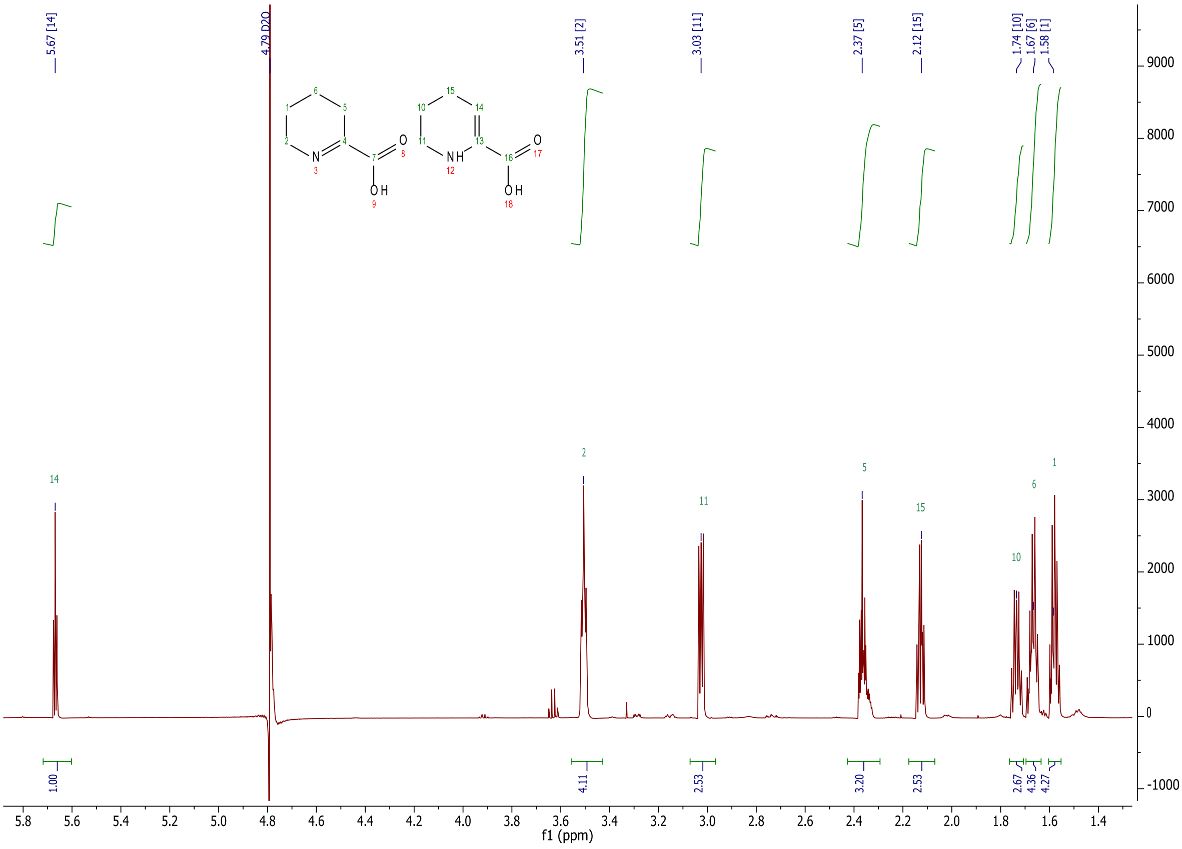


Figure 7.7a. 1D ^{1}HNMR spectrum of P2C (600 MHz, D2O).

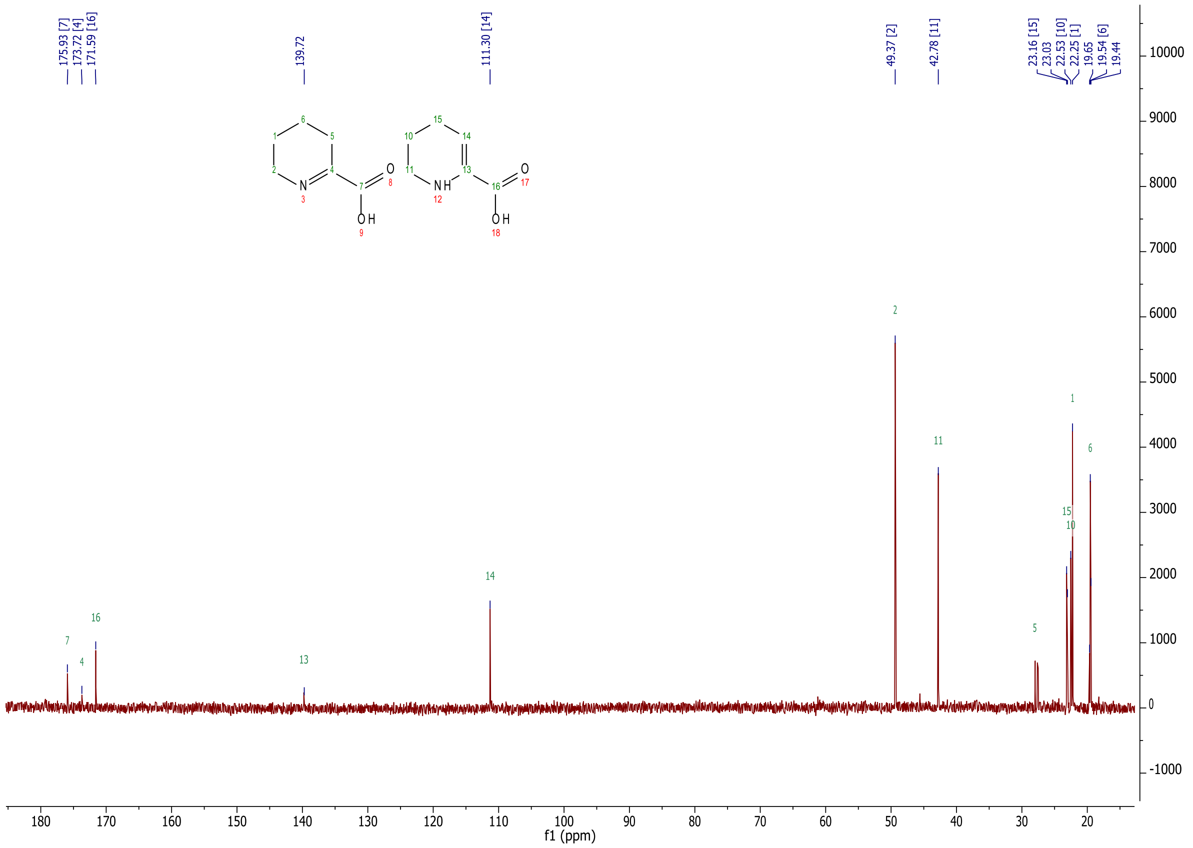


Figure 7.7b. 1D ^{13}CNMR spectrum of P2C (600 MHz, D2O). Note there is some evidence of decomposition due to the lengthy acquisition time.

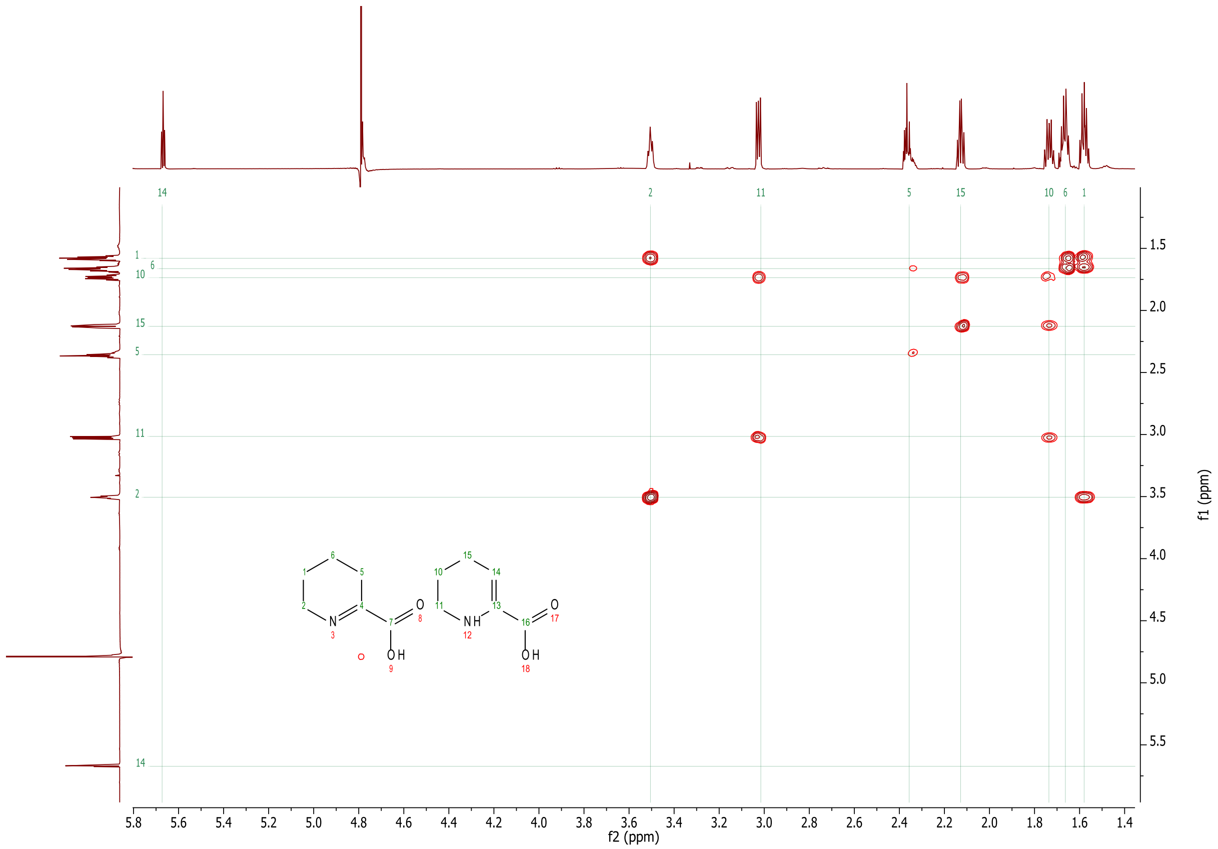


Figure 7.7c. 2D HCOSY of P2C (600 MHz, D2O).

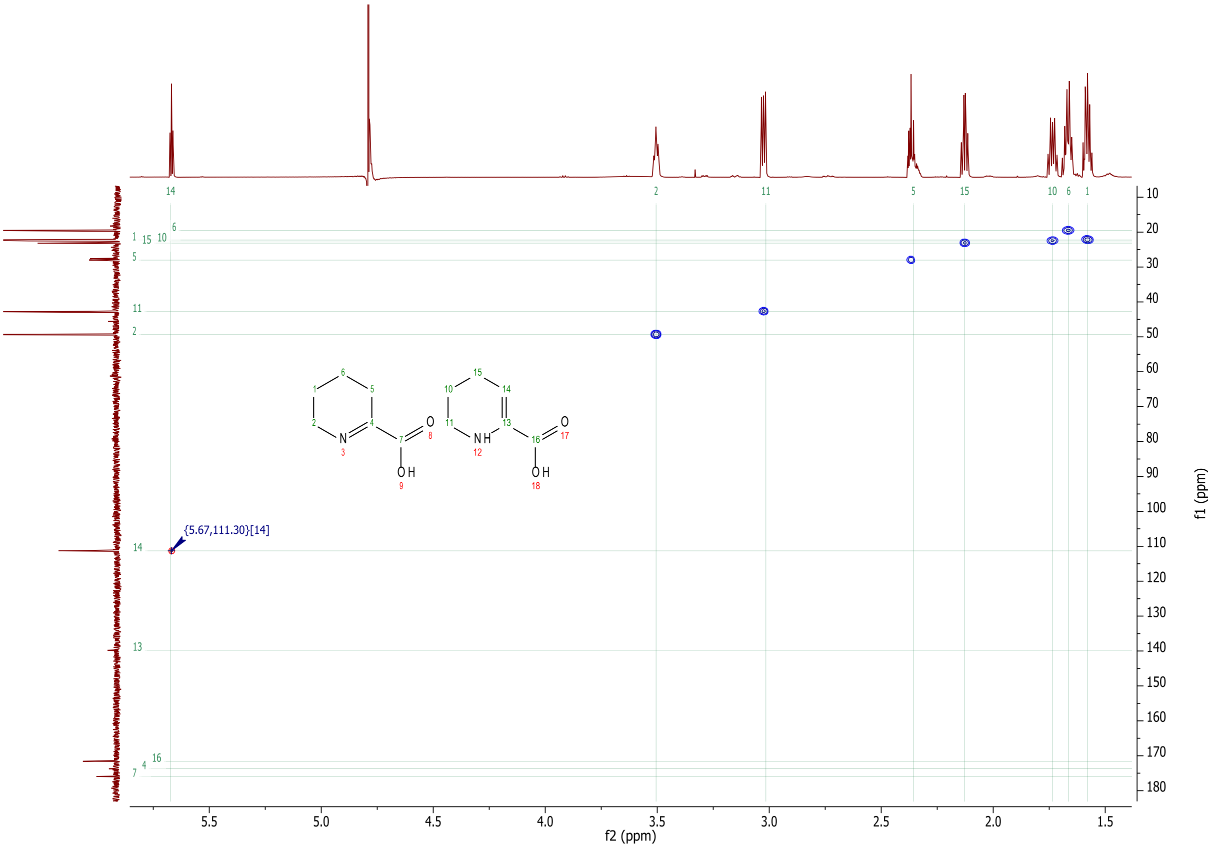


Figure 7.7d. 2D HSQC of P2C (600 MHz, D2O).

No	^{1}H-Chemical Shift (ppm)	^{13}C- Chemical Shift (ppm)
14	5.67	111.30
2	3.51	49.37
11	3.02	42.78
5	2.36	28.03
15	2.13	23.16
10	1.74	22.54
6	1.66	19.54
1	1.58	22.25
4		173.72
16*		175.93
13*		139.78
7*		171.59

*Only possible assignments as mentioned earlier.

References

1. Rossi, S., Bacehetti, T., Maiorana, S., *Dihydro-1,4-thiazines*. Gazzetta Chimica Italiana, 1962. **92**: p. 1367-78.

2. Cavallini, D., G. Ricci, and G. Federici, *The ketimine derivatives of thialysine, lanthionine, cystathionine, cystine: preparation and properties.* Prog Clin Biol Res, 1983. **125**: p. 355-63.
3. Ricci, G., et al., *Similarity of the oxidation products of L-cystathionine by L-amino acid oxidase to those excreted by cystathioninuric patients.* J Biol Chem, 1983. **258**(17): p. 10511-7.
4. Solinas, S.P., et al., *Reversible cyclization of S-(2-oxo-2-carboxyethyl)-L-homocysteine to cystathionine ketimine.* Amino Acids, 1993. **4**(1-2): p. 133-40.

5. Szollosi, G., I. Kun, and M. Bartok, *Heterogeneous asymmetric reactions. Part 24. Heterogeneous catalytic enantioselective hydrogenation of the C==N group over cinchona alkaloid modified palladium catalyst.* Chirality, 2001. **13**: p. 619-624.5.

6. Mintz, M.J. and C. Walling, *t-Butyl hypochlorite.* Org Synth, 1973. **5**: p. 184.

7. Lu, S., Lewin, A.H., Enamine/Imine tautorism in α,β-unsaturated-α-amino acids. Tetrahedron, 1998. **54**: p. 15097-15104.

8. Kamio, M., et al., The Chemistry of Escapin: Identification and Quantification of the Components in the Complex Mixture Generated by an l-Amino Acid Oxidase in the Defensive Secretion of the Sea Snail Aplysia californica, *Chem. Eur. J.* 2009, **15**, p. 1597 – 1603.

CONCLUSIONS AND FUTURE DIRECTIONS

During the course of my PhD candidature I have successfully purified and characterised an important enzyme in brain amino acid metabolism. Prior to this research μ-crystallin (CRYM) was not known to be an enzyme but rather known as an important cytosolic thyroid hormone binding protein and a structural protein in diurnal marsupial lens. I have characterised its enzyme action as a ketimine reductase responsible for the reduction of the imine bond in cyclic ketimines. I have demonstrated that the enzyme catalyses the reduction of monocarboxylate cyclic ketimines such as S-2-aminoethylcysteine ketimine (AECK), Δ^1-piperdeine-2-carboxylate (P2C), Δ^1-pyrroline-2-carboxylate (Pyr2C), and Δ^2-thiazoline-2-carboxylate (T2C) but is not very active towards the dicarboxylate substrates such as lanthionine ketimine (LK). Under physiological neutral conditions I have demonstrated that the enzyme is primarily involved in the reduction of P2C, Pyr2C and T2C. It may thus be referred to as a P2C/Pyr2C reductase which is an important enzyme in the pipecolate pathway of lysine degradation. I have also shown that the enzyme is capable of synthesising *N*-alkylamino acids from α-keto acids (e.g. pyruvate) and alkylamines (e.g. methylamine) through reduction of the intermediate imine formed by the condensation of the two co-substrates. This reaction is unlikely however under physiological conditions but does have potential commercial applications. *In silico* docking of substrates and inhibitors suggests a novel catalytic mechanism involving an arginine active site residue as a proton donor. I have also characterised the inhibition/regulation of the enzyme by thyroid hormones as well as small substrate analogues. This research suggests that only the thyroid hormones 3,5,5’-triiodothyronine (T_3) and thyroxine (T_4) can be regarded as *in vivo* regulators of enzyme activity. Under physiological neutral conditions both T_3 and T_4 have apparent sub-nanomolar inhibition constants. The interaction of thyroid hormones and ketimine reductase inhibition is an important aspect of this research and suggests that enzyme catalysis is directly related to intracellular thyroid hormone bioavailability.

Future directions:

1. Synthesis of additional potential enzyme substrates and inhibitors.
2. Purification of the monomer of ketimine reductase/CRYM from mammalian brain and its characterisation.
3. Purification of a second ketimine reductase from bovine cerebellum and its characterisation and identification.
4. Site-directed mutagenesis of ketimine reductase/CRYM to verify the *in silico* docking results.

APPENDICES

Overview

The appendices consist of two co-authored papers and a letter to the editor:

A1. A research paper on glutamine transaminase.

A2. A book chapter on glutamine transaminases.

A3. Letter to the editor re: the inhibition/regulation of ketimine reductase/CRYM by thyroid hormones

Archives of Biochemistry and Biophysics 517 (2012) 20–29

Contents lists available at SciVerse ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

New insights into the metabolism of organomercury compounds: Mercury-containing cysteine *S*-conjugates are substrates of human glutamine transaminase K and potent inactivators of cystathionine γ-lyase

Christy C. Bridges [a,1], Boris F. Krasnikov [b,*,1], Lucy Joshee [a], John T. Pinto [b], André Hallen [c], Jianyong Li [d], Rudolfs K. Zalups [a], Arthur J.L. Cooper [b,*]

[a] *Division of Basic Medical Sciences, Mercer University School of Medicine, 1550 College Street, Macon, GA 31207, USA*
[b] *Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY 10595, USA*
[c] *Department of Chemistry and Biomolecular Sciences, Macquarie University, Balaclava Road, North Ryde, NSW 2109, Australia*
[d] *Department of Biochemistry, Virginia Tech, Blacksburg, VA 24061, USA*

ARTICLE INFO

Article history:
Received 22 August 2011
and in revised form 31 October 2011
Available online 10 November 2011

Keywords:
Cystathionine γ-lyase
Glutamine transaminase K
Kynurenine aminotransferase isozyme I
Mercury cysteine *S*-conjugate
Methylmercury cysteine *S*-conjugate
Sulfur-containing amino acids

ABSTRACT

Anthropogenic practices and recycling in the environment through natural processes result in release of potentially harmful levels of mercury into the biosphere. Mercury, especially organic forms, accumulates in the food chain. Mercury reacts readily with sulfur-containing compounds and often exists as a thiol *S*-conjugate, such as the L-cysteine (Cys)-*S*-conjugate of methylmercury (CH_3Hg-*S*-Cys) or inorganic mercury (Cys-*S*-Hg-*S*-Cys). These *S*-conjugates are structurally similar to L-methionine and L-cystine/L-cystathionine, respectively. Bovine and rat glutamine transaminase K (GTK) catalyze transamination of sulfur-containing amino acids. Recombinant human GTK (rhGTK) has a relatively open catalytic active site, and we report here that this enzyme, like the rat and bovine enzymes, can also utilize sulfur-containing L-amino acids, including L-methionine, L-cystine, and L-cystathionine as substrates. The current study extends this list to include mercuric *S*-conjugates, and shows that CH_3Hg-*S*-Cys and Cys-*S*-Hg-*S*-Cys are substrates and reversible inhibitors of rhGTK. The homocysteine *S*-conjugates, Hcy-*S*-Hg-*S*-Hcy and CH_3Hg-*S*-Hcy, are also inhibitors. Finally, we show that $HgCl_2$, CH_3Hg-*S*-Cys and Cys-*S*-Hg-*S*-Cys are potent irreversible inhibitors of rat cystathionine γ-lyase. The present study broadens our knowledge of the biochemistry of mercury compounds by showing that Cys *S*-conjugates of mercury interact with enzymes that catalyze transformations of biologically important sulfur-containing amino acids.

© 2011 Elsevier Inc. All rights reserved.

Introduction

Environmental mercury may exist in elemental (Hg^0), inorganic (Hg^{2+}) or organic (Hg(I), Hg(II)) forms [1]. When mercury vapor (Hg^0) and Hg(II) compounds in the atmosphere settle into bodies of water, organic mercury (primarily monomethylmercury) is formed by cobalamin-dependent methylation, which is mediated by a variety of microorganisms [1]. For simplicity, monomethylmercury will be referred to as methylmercury in the current manuscript; however, it is important to note that methylmercury, in aqueous solutions, often reacts with anions such as Cl^- to form covalent bonds [2].

Methylmercury accumulates readily in tissues of numerous aquatic organisms, especially large, predatory fish [1]. It is the predominant form of organic mercury in the environment and humans are exposed to this organometallic species primarily via consumption of contaminated fish and/or water [1–3]. Inasmuch as methylmercury exposure can be detrimental to multiple tissues and organs, including the brain and kidneys, it is recommended that certain species of fish, particularly those at the top of the food chain, be consumed sparingly, especially by pregnant women and young children [4–8]. Despite this recommendation, 6–8% of women of child-bearing age in the US may have unacceptably high levels of mercury [7,8]. Because of high lipid solubility, methylmercury can readily cross the placenta and accumulate in fetal tissues [1], thus raising the possibility that a sizable population of newborns may be exposed to mercury *in utero*.

Because of concerns about the toxicity of mercury, it is important to identify the forms of mercury capable of being transported by target organs and cells. Mercuric ions have a strong bonding affinity for reduced sulfur atoms [9]. Consequently, mercuric ions within biological systems are converted primarily to conjugates of one or more sulfur (thiol)-containing biomolecules, such as

---

* Corresponding authors. Fax: +1 914 594 4058.
E-mail address: Arthur_cooper@nymc.edu (A.J.L. Cooper).

[1] These authors contributed equally.

0003-9861/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2011.11.002

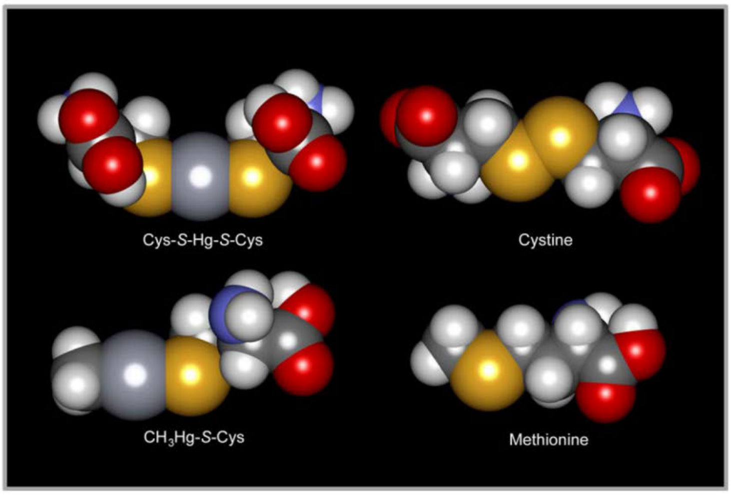


Fig. 1. Space-filled models showing the structural similarity of two mercuric *S*-conjugates with commonly occurring sulfur-containing amino acids. Structural similarity exists between methionine and the cysteine (Cys)-*S*-conjugate of methylmercury (CH_3Hg-*S*-Cys), and between cystine and the Cys-*S*-conjugate of Hg^{2+} (Cys-*S*-Hg-*S*-Cys). The models were generated with MolPOV 2.0 and POV Ray 3.5. Atoms: O, red; N, blue; C, dark gray (small); H, light gray; S, yellow; Hg, dark gray (large).

glutathione (GSH),[2] L-cysteine (Cys), and L-homocysteine (Hcy). Some of these mercury conjugates are similar structurally to certain endogenous amino acids [10–12]. For example, the structure of the Cys *S*-conjugate of methylmercury (i.e. CH_3Hg-*S*-Cys) is similar to that of methionine (Fig. 1). CH_3Hg-*S*-Cys may be formed by direct reaction of Cys with methylmercury. Alternatively, methylmercury may react with GSH to form a glutathione *S*-conjugate (i.e. CH_3Hg-*S*-G), which can then be catabolized to CH_3Hg-*S*-Cys by the sequential actions of γ-glutamyltransferase and cysteinylglycinase/aminopeptidase M located in the luminal membranes of intestinal and renal epithelial cell [11,13] (Fig. 2). Due to the presence of a large hydrophobic side group and structural similarity to methionine, CH_3Hg-*S*-Cys may be transported into cells via the amino acid transporter, system L [13]. This transporter has an affinity for large hydrophobic amino acids, including L-methionine [13].

The similarity between CH_3Hg-*S*-Cys and methionine has been suggested by a number of authors [14–16] to be an example of "molecular mimicry" whereby CH_3Hg-*S*-Cys is a mimic of methionine at the site of the system L transporter. Molecular mimicry has been defined as the phenomenon whereby one molecule/compound can act as a structural and/or functional molecule of another endogenous molecule/compound [11,15]. However, the concept that cysteine *S*-conjugates of mercury are molecular mimics of sulfur-containing amino acids has been criticized by Hoffmeyer and colleagues [17]. These authors' definition of molecular mimicry is restricted to isosteric compounds with similar electronic distributions [17]. Here, we simply point out the similarities between the cysteine *S*-conjugates of mercury and other amino acids, including sulfur-containing amino acids.

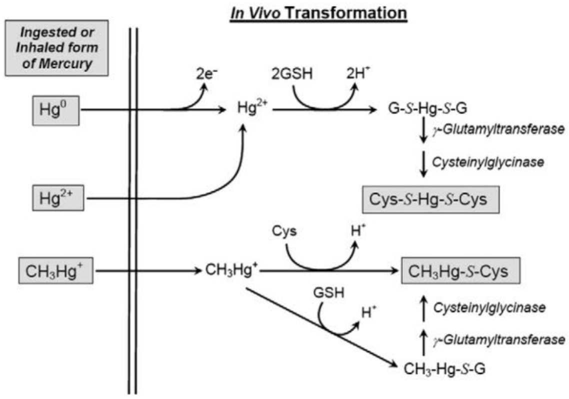


Fig. 2. Transformation of mercury species in mammalian tissues. Mercuric ions that are ingested, absorbed or inhaled may bond with glutathione (GSH) and/or cysteine (Cys) to form G-*S*- and Cys-*S*-conjugates of Hg^{2+} (G-*S*-Hg-*S*-G, Cys-*S*-Hg-*S*-Cys, respectively) and methylmercury (CH_3Hg-*S*-G, CH_3Hg-*S*-Cys, respectively). G-*S*-Hg-*S*-G and CH_3Hg-*S*-G are processed further by γ-glutamyltransferase and cysteinylglycinase to yield Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys, respectively. Based on endogenous concentrations of intracellular glutathione (1–10 mM), these cysteine *S*-conjugates of mercury are likely to be among the major species of Hg present in cellular systems. It should be noted that inhaled Hg^0 is oxidized rapidly in blood and tissues [18]. For convenience, methylmercuric species are depicted here as CH_3Hg^+ even though it is recognized that methylmercuric species are normally found bonded to an anion or sulfur-containing molecule.

Following ingestion of methylmercury, there is evidence indicating that some of this species undergoes biotransformation in intracellular or extracellular compartments to yield inorganic mercury [3]. Thus, thiol-*S*-conjugates of both Hg^{2+} and methylmercury may be present in target cells and organs of biological systems. Interestingly, the Cys-*S*-conjugate of Hg^{2+} (i.e. Cys-*S*-Hg-*S*-Cys), which is similar structurally to L-cystine (Fig. 1) (and L-cystathionine) and Hcy-*S*-Hg-*S*-Hcy (similar to L-homocystine) is taken up at the luminal membrane of renal proximal tubular cells by the amino acid transporter, system $b^{0,+}$ [18–20]. System $b^{0,+}$ is an important absorptive transporter of L-cystine. In addition, the glutathione *S*-conjugate of Hg^{2+} (G-*S*-Hg-*S*-G) is similar structurally to glutathione disulfide (GSSG) and may be a substrate/inhibitor of proteins that interact with GSSG. GSSG is critical for post-translational modification of proteins involved in redox signaling [21,22].

---

[2] *Abbreviations used*: CH_3Hg-*S*-Cys, L-cysteine *S*-conjugate of methylmercury; CH_3Hg-*S*-Hcy, L-homocysteine *S*-conjugate of methylmercury; Cys-*S*-Hg-*S*-Cys, L-cysteine *S*-conjugate of inorganic mercury; DTT, dithiothreitol; GTK, glutamine transaminase K; GSH, glutathione; G-*S*-Hg-*S*-G, glutathione *S*-conjugate of inorganic mercury; GSSG, glutathione disulfide; Hcy-*S*-Hg-*S*-Hcy, L-homocysteine *S*-conjugate of inorganic mercury; KAT I, kynurenine aminotransferase isozyme I; KMB, α-keto-γ-methiolbutyrate; MPA, metaphosphoric acid; PLP, pyridoxal 5′-phosphate; rhGTK, recombinant human GTK.

Given that select thiol-*S*-conjugates of Hg^{2+} and methylmercury are similar structurally to endogenous compounds, we hypothesized that these conjugates may also function as substrates of certain intracellular enzymes (in addition to the membrane-bound external enzymes γ-glutamyltransferase and cysteinylglycinase/aminopeptidase M). To investigate this possibility we chose to study the interaction of Cys- and Hcy-*S*-conjugates of Hg^{2+} and methylmercury with kidney glutamine transaminase K [GTK; also known as kynurenine aminotransferase isozyme I (KAT I)] and cystathionine γ-lyase (γ-cystathionase). The rationale for these investigations evolved from the observation that rat kidney GTK utilizes α-amino acid and α-keto acid substrates of the general structure $Y(CH_2)_nCH(NH_3^+)CO_2^-$ and $Z(CH_2)_nC(O)CO_2^-$, respectively, where $n = 1$ or 2 and Y (or Z) is generally a hydrophobic or uncharged moiety that can be relatively large [23,24] (Fig. 3, Eq. (1)). (An exception is cystine and some other sulfur-containing amino acids where Y possesses a terminal charged grouping.) Thus, for example, rat kidney GTK catalyzes transamination with L-glutamine (as the name implies), L-methionine and L-phenylalanine, and their corresponding α-keto acids [23,24]. Glutamine transaminases purified from bovine kidney [25] and brain [26] exhibit activity toward L-glutamine, L-methionine and L-phenylalanine, but also toward large sulfur-containing amino acids (e.g. L-cystine, L-homolanthionine and L-cystathionine). Highly purified rat kidney GTK exhibits activity toward L-cystine, L-homocystine, L-cystathionine and L-lanthionine when phenylpyruvate is used as the co-substrate (amine acceptor) [27].

X-ray crystallographic studies of recombinant human GTK/KAT I (rhGTK) have shown that this enzyme has a remarkably open configuration at the active site. The enzyme substrate binding site possesses a striking crown of aromatic residues that adorns the relatively large active site [28]. Although turnover is somewhat slow, rhGTK was shown to catalyze transamination of two positional isomers of β-naphthyl-L-alanine [29], consistent with a large, relatively open active site. Fig. 4 depicts a ribbon model of hGTK monomer (the active enzyme is a homodimer) showing the position of the pyridoxal 5′-phosphate (PLP) coenzyme bound in Schiff base linkage (internal aldimine) to a lysine residue in the active site. Surface representations of the monomer reveal that the active site is spacious (Fig. 4). Fig. 4 also illustrates the positions of all six cysteine moieties within the monomer. It is readily apparent that the active site does not contain a cysteine residue. One cysteine residue (C127) is 6.8 Å away from the PLP coenzyme; other cysteine residues are 12.5–20.4 Å away from the coenzyme epicenter.

Transamination of L-cystathionine, L-lanthionine, and L-cystine *in vitro* yields α-keto acids that can non-enzymatically cyclize to ketimines (reviewed in [24,30]). Some of these compounds and their reduced forms (cyclic amines) have been reported to be present in brain, plasma and/or urine and may be neuroactive (discussed in [24]). Furthermore, GTK activity is present in human brain [31]. Therefore, GTK may be responsible for generating sulfur-containing neuroactive cyclic ketimines in human brain. Because the active site of rhGTK is large [28], it is also possible that the enzyme will catalyze transamination in human tissues with thiol-*S*-conjugates of Hg (Fig. 3, Eq. (1) where $n = 1$ or 2, and $Y = CH_3HgS$- or Cys–*S*–HgS–). This hypothesis is supported by the finding that *Se*-methyl-L-selenocysteine is a substrate of rhGTK [29] and this conjugate and other selenocysteine *Se*-conjugates, many of which contain large aromatic moieties, are substrates of rat GTK [32]. On the other hand, whereas *Se*-methyl-L-selenocysteine and L-methionine are relatively good substrates of rhGTK, the closely related amino acid, L-selenomethionine, is an extremely poor substrate (<0.1% as affective as L-methionine) [29]. Thus, due to these subtleties in binding geometries it was not certain *a priori* that the enzyme would catalyze transamination of thiol-*S*-conjugates, despite the relatively open active site. The present work, however, does indeed show that rhGTK catalyzes transamination

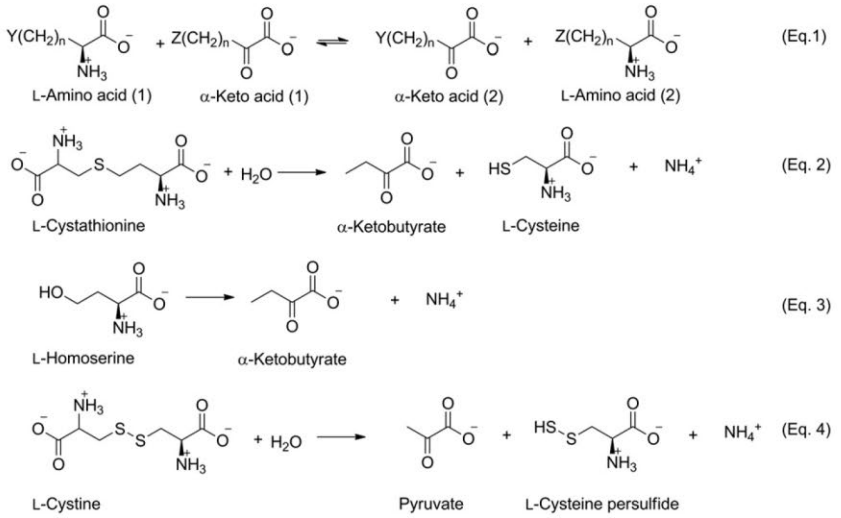


Fig. 3. Specificity of glutamine transaminase K (GTK) and cystathionine γ-lyase. Transamination reactions between amino acids and α-keto acids catalyzed by GTK are depicted in Eq. (1), where $n = 1$ or 2, and Y and Z can range in size from H- (e.g. α-ketobutyrate, $n = 2$) to relatively large hydrophobic moieties such as CH_3S- (KMB, $n = 2$; L-methionine, $n = 2$) or C_6H_5- (phenylpyruvate, $n = 1$; L-phenylalanine, $n = 1$). Cystine [Y or Z = $-SSCH_2CH(NH_3^+)CO_2^-$, $n = 1$] and some other sulfur-containing amino acids, in which Y or Z possesses a terminal charged grouping, are also substrates. Cystathionine γ-lyase catalyzes several γ-elimination, β-elimination and replacement reactions. Some of the more important reactions that are pertinent to the current study are shown here. The enzyme catalyzes a γ-elimination reaction with L-cystathionine (Eq. (2)) and with L-homoserine (Eq. (3)). In both cases, the α-keto acid product is α-ketobutyrate. Cystathionine γ-lyase also catalyzes a β-elimination reaction with L-cystine (Eq. (4)). In this case, the α-keto acid product is pyruvate.

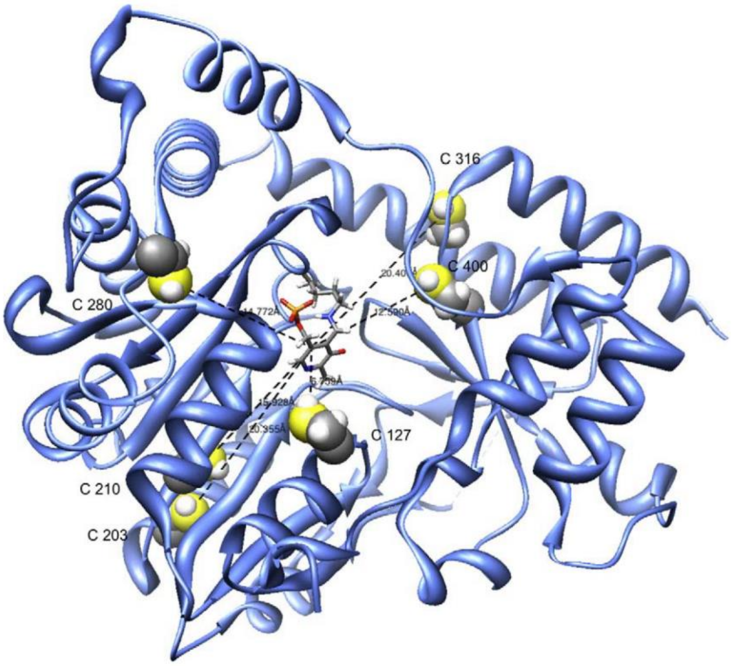


Fig. 4. Ribbon model of rhGTK monomer showing pyridoxal 5′-phosphate in the active site. The diagram shows a relatively large active site. Cysteine residues are emphasized. Atoms: S, yellow; H, light gray. The model was produced using the UCSF Chimera program (http://www.cgl.ucsf.edu/chimera/).

of large sulfur-containing amino acids as well as the Cys-*S*-conjugates of Hg^{2+} and methylmercury.

We next turned our attention to cystathionine γ-lyase. This enzyme catalyzes a γ-lyase reaction with L-cystathionine, generating α-ketobutyrate, cysteine (eliminated fragment) and ammonia (Fig. 3, Eq. (2)). L-Homoserine is also a γ-lyase substrate, generating α-ketobutyrate and ammonia (Fig. 3, Eq. (3)). In this case the hydrolysis reaction is balanced by elimination of H_2O. Thus, the net reaction does not include an H_2O term. The enzyme can also catalyze a β-lyase reaction with L-cystine (Fig. 3, Eq. (4)), generating pyruvate, the persulfide analogue of cysteine (thiocysteine) and ammonia [33,34].

Rat liver cystathionine γ-lyase has a cysteine residue within the active site [35] that is highly susceptible to inactivation by a number of sulfhydryl reagents including *p*-chloromercuribenzoate, iodosobenzoate and *N*-ethylmaleimide [36,37]. Therefore, we considered the possibility that Cys-*S*-conjugates of mercury, particularly Cys-*S*-Hg-*S*-Cys, would interact with this sulfhydryl in the active site of cystathionine γ-lyase. The present work shows that Cys-*S*-Hg-*S*-Cys is a strong irreversible inhibitor of cystathionine γ-lyase.

Materials and methods

Reagents

Methylmercuric chloride (CH_3HgCl) and $HgCl_2$ were obtained from Aldrich, Milwaukee, WI. Mercuric *S*-conjugates were generated by mixing $HgCl_2$ with Cys or Hcy in a 1:2.5 ratio. Methylmercury *S*-conjugates were generated by mixing CH_3HgCl with Cys or Hcy in a 1:1.25 ratio. The conjugates were incubated for five minutes at room temperature prior to use. Ammediol (2-amino-2-methyl-1,3-propanediol), dithiothreitol (DTT), GSH, sulfur-containing amino acids, L-glutamine, L-phenylalanine, L-homoserine, phenylpyruvate, sodium α-keto-γ-methiolbutyrate [sodium α-keto-(γ-methylthio)butyrate; KMB] were obtained from Sigma, St. Louis, MO. 2,4-Dinitrophenylhydrazine was purchased from MP Biochemicals, Irvine, CA.

Enzymes

Highly purified rhGTK [1.8 mg/ml in 20% glycerol, 10 mM potassium phosphate buffer (pH 7.4); specific activity 18 U/mg in the standard reaction assay mixture, see below] was obtained by the method of Han et al. [38]. Cystathionine γ-lyase (specific activity 24 U/mg, 20 mM L-homoserine as substrate) was purified from rat liver as described by Pinto et al. [39]. One unit of enzyme activity is the amount of enzyme that generates 1 μmol of product per min at 37 °C under standard reaction conditions.

Enzyme activity measurements

The standard GTK assay mixture (50 μl) contained 100 mM ammediol buffer (pH 9.0), 5 mM KMB and 20 mM L-phenylalanine [40]. After incubation at 37 °C (5–10 min), 150 μl of 1 M NaOH was added and the absorbance at 322 nm due to phenylpyruvate enol ($\varepsilon_{322nm} = 16{,}000\ M^{-1}\ cm^{-1}$) was determined within 5 min against a blank consisting of complete reaction mixture lacking enzyme. The activity of rhGTK in a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.4) in place of ammediol buffer in the standard reaction mixture is ~50% that exhibited at pH 9.0 [29]. The standard cystathionine γ-lyase assay mixture (50 μl) contained 100 mM potassium phosphate buffer (pH 7.4) and 20 mM L-homoserine [39]. After incubation at 37 °C (10 min), the reaction was stopped by addition of 20 μl of 5 mM 2,4-dinitrophenylhydrazine in 2 M HCl. After further incubation at 37 °C for 10 min, 130 μl of 1 M NaOH was added and the absorbance was read within 5 min at 430 nm against a blank consisting of reaction mixture lacking L-homoserine (or enzyme) (ε_{430nm} α-ketobutyrate 2,4-dinitrophenylhydrazone, 15,000 M^{-1} cm^{-1}). To measure aminotransferase activity of GTK toward various amino acids (other than the mercury *S*-conjugates) the reaction mixture (50 μl), except where noted, contained 100 mM potassium phosphate buffer (pH 7.4), 1.0 mM L-amino acid (50 nmol) and 0.4 mM phenylpyruvate (20 nmol) and GTK. In the case of L-cystine the concentration was 0.4 mM and the buffer was 50 mM sodium pyrophosphate, pH 9.2. The reaction mixture was incubated at 37 °C and the amount of phenylpyruvate remaining was determined (as described above) against a blank containing complete reaction mixture lacking enzyme. A similar procedure was used to measure the rhGTK-catalyzed transamination of mercury *S*-conjugates, except that the concentration of phenylpyruvate was 0.2 mM. Blanks contained the complete reaction mixture (including mercuric conjugates) without enzyme.

Spectrophotometric determinations were conducted with a Tecan Infinite M1000 96-well plate spectrophotometer (Tecan, Durham, NC) or with a SpectraMax 96-well plate spectrophotometer (Molecular Devices, Sunnyvale, CA).

HPLC measurement of L-methionine and KMB

The HPLC system consisted of a liquid chromatograph equipped with an 8-channel coulometric array (CoulArray) detector (ESA, Inc., Chelmsford, MA) [39,41]. The enzyme activity in the reaction mixtures (50 μl) was terminated by addition of 15 μl of 25% w/v metaphosphoric acid (MPA). The resulting 5% w/v MPA homogenates were injected directly onto a Bio-Sil ODS-5S, 5-μm particle size, 4.0 × 250 mm, C18 column (Bio-Rad, Life Science Research Group, Hercules, CA) and eluted with a mobile phase consisting of 50 mM NaH_2PO_4, 50 μM octane sulfonic acid, and 1% (v/v) acetonitrile (pH 2.52) at a flow rate of 1 ml/min. All buffers, following preparation, were routinely degassed, filtered through a 0.2-μm Millipore nylon filter, and the pH re-adjusted, if necessary. PEEK™ (polyetheretherketone) tubing was used throughout the HPLC system, and a 0.2-μm PEEK™ filter was placed pre- and post-column to protect both column and flow cells, respectively, from any particulate matter. A Rheodyne injection valve with a 5-μl sample loop was used to manually introduce samples. The 8-channels of the CoulArray detector were set at 100, 200, 300, 400, 500, 600, 700 and 800 mV, respectively. Elution times (min) and detection potential ranges (mV) are: MPA (1.6), L-methionine (9.6, 600–800 mV) and KMB (11.1, 600–800 mV). Other sulfur-containing compounds eluted at times that did not interfere with analysis of L-methionine or KMB.

Data analyses

For measurements of the effect of $HgCl_2$ and CH_3HgCl on the rhGTK-catalyzed transamination of L-phenylalanine with KMB, differences between two means were analyzed using the Mann–Whitney *U* test. For measurements of inactivation of cystathionine γ-lyase by various mercury-containing compounds, differences among means were analyzed using a two-way Analysis of Variance, followed by Tukey's post hoc testing. Each set of respective data was analyzed first with the Kolmogorov–Smirnov test for normality, followed by the Levene test for homogeneity of variances. A *p*-value of <0.05 was considered statistically significant. Data are presented as the mean ± SD. Except where noted, measurements were carried out at least in triplicate.

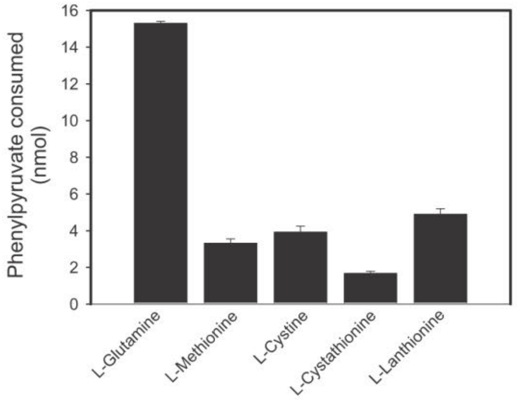


Fig. 5. rhGTK-catalyzed transamination of phenylpyruvate with glutamine and various sulfur-containing amino acids. Except where indicated, the reaction mixture (50 μl) contained 1 mM amino acid, 0.4 mM phenylpyruvate, 100 mM potassium phosphate buffer (pH 7.4) and enzyme (2.15 mU). In the case of L-cystine the concentration was 0.4 mM and the buffer was 50 mM sodium pyrophosphate (pH 9.2). After incubation for 1 h at 37 °C the amount of phenylpyruvate remaining in solution was determined relative to a blank reaction mixture lacking enzyme; *n* = 3.

Results

Sulfur-containing amino acids are substrates of rhGTK

Fig. 5 shows that the sulfur-containing amino acids, L-methionine, L-cystine, L-cystathionine, and L-lanthionine are substrates of rhGTK when 0.4 mM phenylpyruvate is used as an amine acceptor. Except as noted, the buffer was 100 mM potassium phosphate (pH 7.4). After incubation of the reaction mixture containing 2.15 mU of enzyme at 37 °C for 1 h the amount of phenylpyruvate remaining was determined. Under the conditions of the assay, L-methionine (1.0 mM), L-cystine (0.4 mM; 50 mM sodium pyrophosphate buffer, pH 9.2), L-cystathionine (1.0 mM) and L-lanthionine (1.0 mM) are about 12–30% as effective as L-glutamine (1.0 mM) as substrates. (Cystine has very limited solubility at pH 7.4, but is more soluble at higher pH values. Even at pH 9.2, however, the maximum concentration in the assay mixture is about 0.4 mM. The higher pH is not a major limitation for enzyme activity as GTK has a pH optimum of ~8.5–9.0) [23,38].

In order to provide additional evidence for the ability of rhGTK to catalyze transamination of sulfur-containing amino acids, 0.5 mM KMB was used as an α-keto acid substrate in place of phenylpyruvate, and the L-methionine generated by transamination was determined by HPLC with CoulArray detection. The advantage of this technique is that redox-active compounds can be characterized directly without the need to derivatize them and they can be identified by both their column retention times and the voltage required to produce a signal (i.e. the voltage required to oxidize (remove an electron from) the analyte). As a result of the presence of a sulfur ether moiety, which is readily oxidizable under the HPLC conditions, the substrate (KMB) and product (L-methionine) are both redox responsive and detectable by coulometry. Except in the case of L-cystine, the reaction mixture (50 μl) contained 100 mM potassium phosphate buffer (pH 7.4), 1.0 mM amino acid, 0.5 mM KMB and 2.15 mU rhGTK. After incubation for 60 min at 37 °C the reaction was terminated by the addition of 12.5 μl of 25% w/v MPA. The L-methionine content of the mixture was measured relative to mixtures that lacked enzyme (blanks). (The concentration of L-cystine was 0.4 mM and the buffer used was 50 mM sodium pyrophosphate, pH 9.2.) The amount of L-methionine generated (nmol; average of two determinations) with various amino substrates was as follows: L-glutamine (16.0), L-cystine (1.4), L-cystathionine (0.9), L-lanthionine (0.6). The formation of L-methionine in reaction mixtures containing L-glutamine was accompanied by an approximately equimolar loss of KMB.

The values for rhGTK-catalyzed transamination of L-cystine and L-cystathionine with KMB are somewhat lower than those obtained with phenylpyruvate as an α-keto acid substrate. As was previously demonstrated for bovine [26] and rat kidney [27] glutamine transaminases, the critical issue here is that rhGTK catalyzes transamination of L-methionine, L-cystine and L-cystathionine with suitable α-keto acid acceptors. Thus, because CH_3Hg-*S*-Cys and Cys-*S*-Hg-*S*-Cys are similar to L-methionine and L-cystine/L-cystathionine insofar as both conjugates contain sulfur and a relatively large side grouping, we considered the possibility that CH_3Hg-*S*-Cys and Cys-*S*-Hg-*S*-Cys would be substrates and/or inhibitors of rhGTK.

Inhibition of rhGTK by mM concentrations of $HgCl_2$ and CH_3HgCl

As discussed below, cystathionine γ-lyase is strongly inhibited by μM (or less) concentrations of mercury-containing compounds. By contrast our results show that rhGTK, despite the fact that both enzymes contain cysteine residues, is much more resistant to inhibition. Fig. 6 shows the time course for phenylpyruvate production at 37 °C in a reaction mixture (50 μl) containing 2.6 mU rhGTK, 5 mM KMB, 20 mM L-phenylalanine, and 100 mM potassium phosphate buffer (pH 7.4). The transamination reaction is freely reversible (Fig. 3, Eq. (1)). Thus, the slower reaction rate at 60 min relative to the initial rate is presumably a result of the back reaction competing with the forward reaction. When 10 μM $HgCl_2$ or 10 μM CH_3HgCl was included in the reaction mixture no difference in the rate of phenylpyruvate formation compared to the control was noted (data not shown). However, when 1 mM $HgCl_2$ or 1 mM CH_3HgCl was included in the reaction mixture, transamination of L-phenylalanine was inhibited at all time points relative to the control that lacked mercury compound (Fig. 6).

One possible explanation for the findings is that slow binding of Hg^{2+} and CH_3HgCl to the enzyme occurred over a 20 min period followed by establishment of a greatly slowed transamination rate between 30 and 60 min. However, due to the nature of the plot shown in Fig. 6 it was difficult to discern whether there was still some residual enzyme activity at 60 min or at later time points. Therefore, to investigate the possibility of residual activity in enzyme exposed to Hg^{2+} or CH_3HgCl, rhGTK (~2.6 mU) was incubated in complete reaction mixture (50 μl) lacking KMB in the presence or absence of 1 mM CH_3HgCl. After incubation for 1 h at 37 °C, transamination was initiated by addition of 2.5 μl of 100 mM KMB. After a further incubation for 30 min at 37 °C, the formation of phenylpyruvate was measured. The amount of phenylpyruvate formed by rhGTK that was exposed to 1 mM CH_3HgCl for 1 h was 11.7 ± 2.1% relative to the control (100 ± 13%) ($n = 6$). A similar experiment was carried out with $HgCl_2$. In this case, the amount of phenylpyruvate formed by rhGTK that was exposed to 1 mM $HgCl_2$ for 1 h was 6.9 ± 1.5% relative to the control (100 ± 10%) ($n = 6$).

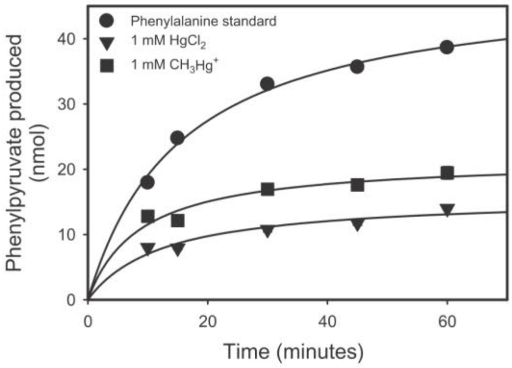


Fig. 6. Effect of $HgCl_2$ and CH_3HgCl on the rhGTK-catalyzed transamination of L-phenylalanine with KMB. The reaction mixture (50 μl) contained 20 mM L-phenylalanine, 5 mM KMB, 2.6 mU of enzyme, 100 mM potassium phosphate buffer (pH 7.4) in the presence or absence of 1 mM $HgCl_2$ or CH_3HgCl. Samples were incubated at 37 °C and phenylpyruvate formation was measured at the times shown ($n = 3$).

There are at least two possible explanations for the findings of residual activity in rhGTK exposed to CH_3HgCl or Hg^{2+}. First, binding of the mercury compound causes 100% irreversible inactivation, but 5–10% of the enzyme population is resistant to binding of the mercury compound and remains fully active. Secondly, 100% of the enzyme is strongly modified within about 20 min and all modified enzyme species are active, albeit at a reduced efficiency. To distinguish between the two possibilities, 0.6 mU of enzyme was incubated for 1 h at 37 °C in 40 μl of 100 mM potassium phosphate buffer (pH 7.4) containing 0, 0.5, 1 or 2 mM $HgCl_2$. Thereafter, L-phenylalanine and KMB were added to yield final concentrations of 20 mM and 5 mM, respectively (final volume 50 μl). After an additional 30-min incubation at 37 °C, the amounts of phenylpyruvate formed were 8.96 ± 0.66, 1.42 ± 0.42, 0.76 ± 0.26, and 0.52 ± 0.20 nmol, respectively ($n = 3$). In another series of experiments, 0.6 mU of enzyme was incubated for an hour at 37 °C in 40 μl of 100 mM potassium phosphate buffer (pH 7.4) containing 2 mM $HgCl_2$. Thereafter, 5 μl of 100 mM DTT was added and the mixture was incubated for 30 min at 37 °C. Then, 10 μl of 100 mM L-phenylalanine and 5 μl of 100 mM KMB were added (final volume 55 μl). After an additional 30-min incubation at 37 °C, phenylpyruvate was measured. The amount of phenylpyruvate formed was 7.12 ± 0.16 nmol ($n = 3$). Thus, after addition of a 6.25-fold molar excess of DTT over that of $HgCl_2$, the inhibition by Hg^{2+} was largely (~80%) reversed.

The data suggest that reaction with mM concentrations of Hg^{2+} ions or CH_3HgCl is a relatively slow process, but that the interaction eventually substantially lowers the activity of rhGTK. The activity, however, can be largely restored by treatment with DTT. Possibly, covalent binding of the mercury species to one or more cysteine sulfhydryls alters the conformation of the enzyme so that the enzyme cannot bind substrate or binds substrate much less effectively. The DTT restores the original sulfhydryl groups with recovery of activity. Alternatively, S-Hg exchange involving DTT results in an Hg conjugate that can no longer bind to the enzyme. As shown below, the results are in marked contrast to those obtained with cystathionine γ-lyase, where the enzyme is rapidly and irreversibly inactivated by μM (or lower) concentrations of $HgCl_2$, Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys. It should be noted that the use of mM concentrations of mercuric species is not biologically relevant. The experiments were designed to show the markedly different susceptibility of cystathionine γ-lyase to various forms of mercury compared to GTK despite the fact that both enzymes utilize sulfur-containing amino acids as substrates and possess cysteine residues.

rhGTK-catalyzed transamination of CH_3Hg-S-Cys and Cys-S-Hg-S-Cys

As noted above, CH_3Hg-*S*-Cys and Cys-*S*-Hg-*S*-Cys were hypothesized to be substrates of GTK. In order to provide evidence for this hypothesis, enzyme-catalyzed disappearance of phenylpyruvate was measured in a reaction mixture containing 2.6 mU rhGTK, 0.2 mM phenylpyruvate, 1 mM mercury *S*-conjugate and 100 mM potassium phosphate buffer (pH 7.4). The corresponding homocysteine *S*-conjugates were included for comparison. Lower concentrations of phenylpyruvate were included in this experiment compared to those used to determine the substrate specificity toward physiologically important sulfur-containing amino acids (Fig. 5). Significant disappearance of absorbance of phenylpyruvate (enol) relative to the blank should be more readily apparent at lower concentrations of phenylpyruvate. The low phenylpyruvate concentration in this experiment (0.2 mM) should not be limiting for enzyme activity because rat kidney GTK (and presumably rhGTK) has a high affinity for phenylpyruvate [23].

No significant rhGTK activity was detected with 1 mM Hcy-*S*-Hg-*S*-Hcy (Fig. 7). The enzyme appears to have some activity with the homocysteine derivative, CH_3Hg-*S*-Hcy, but the activity value did not quite reach significance. On the other hand, significant enzymatic activity was found with the cysteinyl derivatives, Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys (Fig. 7).

Inhibition of rhGTK activity by Cys-S-Hg-S-Cys, Hcy-S-Hg-S-Hcy, CH_3Hg-S-Cys and CH_3Hg-S-Hcy

Because CH_3Hg-*S*-Cys and Cys-*S*-Hg-*S*-Cys have similarities with methionine and cysteine/cystathionine, respectively, and are substrates of rhGTK, they were predicted to be inhibitors of the standard L-phenylalanine – KMB transaminase reaction catalyzed by rhGTK. We initially carried out experiments to determine the K_m for L-phenylalanine exhibited by rhGTK under conditions used for the inhibition studies. Fitting of data to the Michaelis–Menten equation showed that the K_m exhibited by rhGTK for KMB in the presence of 40 mM L-phenylalanine (in which the KMB concentrations were varied between 1 and 20 mM) and 100 mM potassium phosphate buffer (pH 7.4) at 37 °C is 1.8 ± 0.6 mM (Sigma plot; $n = 3$ for each value of v). A similar analysis showed that the K_m exhibited toward L-phenylalanine in the presence of 5 mM KMB (in which the L-phenylalanine concentration was varied between 5 and 20 mM) was 7.3 ± 1.3 mM (average of four separate experiments in which $n \geqslant 3$ for the determination of each value of v). The K_m for L-phenylalanine noted here is higher than that reported previously for rhGTK at pH 7.5 (1.7 mM) [38]. It should be noted that the previous study utilized 16 mM α-ketobutyrate as the α-keto acid substrate. Aminotransferases catalyze a ping-pong reaction. A feature of this type of reaction is that the apparent K_m value of one of a pair of substrates is strongly dependent on the nature and concentration of the other substrate.

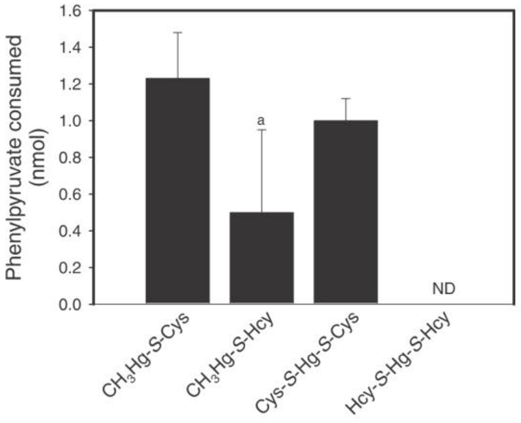


Fig. 7. Substrate specificity of rhGTK toward Cys- and Hcy-*S*-conjugates of Hg^{2+} and methylmercury. The reaction mixture (50 µl) contained 0.2 mM phenylpyruvate, 1.0 mM mercury *S*-conjugate, 100 mM potassium phosphate buffer (pH 7.4) and 2.6 mU of enzyme. After incubation for 2 h at 37 °C the disappearance of phenylpyruvate was measured in comparison to a blank lacking enzyme; ND not detectable. [a]Not significantly different from the blank values ($n = 5$).

Next, we determined the effect of various mercury *S*-conjugates on rhGTK-catalyzed transamination of L-phenylalanine with KMB. Accordingly, reaction mixtures (50 µl) containing enzyme (2 mU), various concentrations of L-phenylalanine, 5 mM KMB, and 100 mM potassium phosphate buffer (pH 7.4) were incubated for 30 min at 37 °C in the presence or absence of mercury *S*-conjugate (i.e. Cys-*S*-Hg-*S*-Cys, Hcy-*S*-Hg-*S*-Hcy, CH_3Hg-*S*-Cys or CH_3Hg-*S*-Hcy) and the amount of phenylpyruvate determined. The relative amounts of phenylpyruvate formed at three concentrations of L-phenylalanine, ranging from 10 mM (a concentration slightly above the K_m value) to 50 mM (a concentration considerably above the K_m value) in the presence and absence of various mercury *S*-conjugates are shown in Table 1. This table shows that, under the conditions of the assay, the mercury *S*-conjugates, at a concentration of 0.5–1 mM, are strongly inhibitory. Because Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys are substrates of rhGTK (Fig. 7) part of the inhibition noted with these two compounds must be competitive with respect to L-phenylalanine. If the inhibition, however, were strictly competitive then the relative inhibition would be less as the concentration of L-phenylalanine was increased relative to the concentration of conjugate. This was not the case for these two conjugates and also for Hcy-*S*-Hg-*S*-Hcy and CH_3Hg-*S*-Hcy. In fact, within experimental error there was little change in the degree of inhibition exerted by the mercury *S*-conjugates from 10 to 50 mM L-phenylalanine (Table 1). This finding suggests a strong noncompetitive component. However, it was not possible to carry out kinetic experiments with concentrations of mercury *S*-conjugates at concentrations >1 mM because this is the limit of the solubility of these conjugates. Moreover, the non-linearity of the reaction over the 30 min incubation (Fig. 6) complicates interpretation of data. The exact type of inhibition remains to be determined, but it is worth noting that the mercury conjugates appear to bind more strongly to rhGTK (strong inhibition at ⩽1 mM) than L-phenylalanine (K_m ~7.6 mM).

Inhibition of rat liver cystathionine γ-lyase by µM concentrations of $HgCl_2$, Cys-S-Hg-S-Cys and CH_3Hg-S-Cys

As noted above, cystathionine γ-lyase catalyzes a β-elimination reaction with L-cystine in a reaction that generates pyruvate ([33,34]; Fig. 3, Eq. (4)). Therefore, we considered the possibility that the enzyme might be able to catalyze pyruvate formation from Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys. However, we were unable to detect pyruvate formation (as the 2,4-dinitrophenylhydrazone derivative) within the sensitivity of the assay (~0.2 nmol), when 1 mU cystathionine γ-lyase was incubated for 1 h at 37 °C in a reaction

Table 1
Inhibition of rhGTK-catalyzed transamination of L-phenylalanine with KMB by Cys- and Hcy-*S*-conjugates of Hg^{2+} and methylmercury.

Inhibitor	L-Phenylalanine concentration		
	10 mM	20 mM	50 mM
	Relative phenylpyruvate formation (%)		
None	[100]	[100]	[100]
CH_3Hg-*S*-Cys (0.5 mM)	72 ± 26	55 ± 11	63 ± 25
CH_3Hg-*S*-Hcy (0.5 mM)	49 ± 11	40 ± 4	50 ± 3
Cys-*S*-Hg-*S*-Cys (1 mM)	31 ± 4	29 ± 8	33 ± 6
Hcy-*S*-Hg-*S*-Hcy (0.5 mM)	43 ± 5	35 ± 4	50 ± 7

The reaction mixture contained varying concentrations of L-phenylalanine, 5 mM KMB, 100 mM potassium phosphate buffer (pH 7.4) and enzyme (2 mU) in a final volume of 50 µl in the presence or absence of mercury *S*-conjugate. After incubation for 30 min at 37 °C, phenylpyruvate formation was determined. $N = 4$ or 5.

mixture (50 μl) containing 1 mM Cys-*S*-Hg-*S*-Cys (or CH_3Hg-*S*-Cys) and 100 mM potassium phosphate buffer, pH 7.4.

As also noted above, rat liver cystathionine γ-lyase was previously shown to be highly susceptible to inactivation by a number of sulfhydryl reagents including *p*-chloromercuribenzoate. This reaction and that with $HgCl_2$ are formally depicted below, where P = protein. Thus, it was predicted that $HgCl_2$ would also be a strong inactivator of cystathionine γ-lyase and this was found to be the case (see below).

$$\mathrm{PSH + ClHgAr \rightarrow PSHgAr + HCl}$$

$$\mathrm{PSH + ClHgCl \rightarrow PSHgCl + HCl}$$

The previous findings with *p*-chloromercuribenzoate [36] and the present findings with $HgCl_2$ indicate that the active site cysteine is reactive. It was then of interest to determine whether this reactive cysteine residue can interact with mercury cysteine conjugates that are substrate analogues. If the conjugates bind simply as substrate analogues then it is expected that they will be reversible inhibitors. However, if they can bind in such a manner as to come in close contact with this reactive cysteine residue then it was predicted that the mercury cysteine conjugates would be strong irreversible inhibitors of the enzyme. This was found to be the case.

When the effect of various mercury-containing compounds on the activity of purified rat liver cystathionine γ-lyase was determined, significant inhibition of cystathionine γ-lyase occurred when μM amounts of these compounds were added to the standard reaction mixture containing 20 mM L-homoserine (Fig. 8). (L-Homoserine (K_m = 20 mM) [42] is used in routine analytical assays for cystathionine γ-lyase (Fig. 3, Eq. (3).) Interestingly, the extent to which inactivation occurred with the various mercury-containing compounds was as follows: Cys-*S*-Hg-*S*-Cys > $HgCl_2$ > CH_3Hg-*S*-Cys. Possibly, the difference between Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys reflects the fact that Cys-*S*-Hg-*S*-Cys bears closer resemblance to the substrates L-cystathionine and L-cystine than does CH_3Hg-*S*-Cys. CH_3Hg-*S*-Cys resembles methionine, which is not a cystathionine γ-lyase substrate. It should be noted that the amount of mercury present in the 50-μl reaction mixture at the lowest concentration (0.2 μM) was 10 pmol. Based on the specific activity of cystathionine γ-lyase and the fact that the enzyme is a homotetramer (subunit M_r ~44,000), the amount of cystathionine γ-lyase monomers in the incubation mixture was 75 fmol. Thus, the mercury-containing compounds were in large excess over cystathionine γ-lyase in these experiments. Another point of concern is whether the mercury-containing compounds interfere with the 2,4-dinitrophenylhydrazone assay for the quantitation of pyruvate (β-lyase reaction) or α-ketobutyrate (standard cystathionine β-lyase reaction). In control experiments it was shown that the presence of 2 mM $HgCl_2$ or 2 mM Cys-*S*-Hg-*S*-Cys had no effect on the yield of α-keto acid 2,4-dinitrophenylhydrazone when standard cystathionine γ-lyase reaction mixtures (0.05 ml) were spiked with 10 nmol pyruvate and incubated at 37 °C for 1 h followed by a further 10 min incubation with the 2,4-dinitrophenylhydrazine reagent.

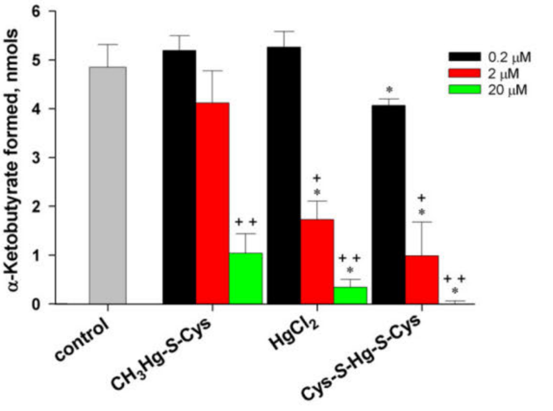


Fig. 8. Inactivation of cystathionine γ-lyase by various mercury-containing compounds. The reaction mixture (50 μl) contained 20 mM L-homoserine, 100 mM potassium phosphate (pH 7.4) and, where indicated, Hg-containing compound. Enzyme (0.08 mU; 75 fmol of enzyme subunit) was added last. After incubation for 1 h at 37 °C, α-ketobutyrate was measured by the 2,4-dinitrophenylhydrazine method. The control contained complete reaction mixture plus enzyme, but no added Hg-containing compound. *Significantly different from the same treatment group exposed to 0.2 μM Hg-containing compound (10 pmol/50 μl reaction mixture) ($P < 0.05$). **Significantly different from the same treatment group exposed to 0.2 or 2.0 μM Hg-containing compound ($P < 0.05$) ($n \geqslant 3$).

The present findings suggest that positioning a mercuric *S*-conjugate as a substrate analogue in close proximity to the active site of cystathionine γ-lyase facilitates modification of the cysteinyl moiety within the enzyme active site. Despite the fact that the S–Hg bond has a high stability constant (~0.22 kJ/mol) [9,43], the coordination bonds of mercury are kinetically quite labile [43–45]. Thus, the greater potency of Cys-*S*-Hg-*S*-Cys compared to $HgCl_2$ as an inactivator of cystathionine γ-lyase (Fig. 7) may be due to binding of Cys-*S*-Hg-*S*-Cys as an analogue of L-cystathionine/L-cystine followed by a rapid S-Hg exchange reaction with a protein-bound cysteine moiety within the active site of the enzyme.

In a separate set of experiments, 2.1 mU of cystathionine γ-lyase was incubated for 1 h at 37 °C in the presence of 100 mM potassium phosphate buffer (pH 7.4) and 20 μM of either $HgCl_2$ or Cys-*S*-Hg-*S*-Cys (final volume 20 μl). At the end of the incubation 1 M potassium phosphate (pH 7.4) and 100 mM L-homoserine were added, such that the final volume was 50 μl and the concentrations of buffer and L-homoserine were 100 mM and 20 mM, respectively. After an additional 30-min incubation at 37 °C, α-ketobutyrate formation was measured. Only 5% of the activity relative to a control (2.1 mU of enzyme incubated in phosphate buffer in the absence of mercury compound) was detected for enzyme that had been exposed to $HgCl_2$, whereas no activity could be detected with enzyme that had been exposed to Cys-*S*-Hg-*S*-Cys. In both cases, addition of DTT to a final concentration of 5 mM did not restore any enzyme activity, even after incubation for 5 h at 37 °C. However, the choice of DTT in this experiment may not have been optimal. For example, it is known that in the case of bacterial MerB (organomercurial lyase; catalyzes the reaction: RHg(I) → RH + Hg(II)), DTT binds to the Hg-S enzyme adduct to form a stable 3-coordinate Hg complex [46,47]. Therefore, the experiment was repeated to determine whether 20 mM cysteine or 20 mM GSH (final concentration) in place of DTT could reactivate enzyme that had been inactivated by $HgCl_2$ or Cys-*S*-Hg-*S*-Cys. No activity was restored after incubation of $HgCl_2$- or Cys-*S*-Hg-*S*-Cys-inactivated enzyme for two hours at 37 °C in the presence of 20 mM L-cysteine or 20 mM GSH.

Discussion

As mentioned in the Introduction, L-methionine, L-cystine and L-cystathionine are substrates of rat kidney GTK. Consistent with this finding, the present work shows that these sulfur-containing amino acids are also substrates of the human counterpart (rhGTK) (Fig. 5). Moreover, this work also shows that the sulfur-containing mercury conjugates Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys are transaminase substrates of this enzyme (Fig. 7). The ability to transaminate these conjugates presumably is a result in part of the relatively open active site and the ability of the enzyme to utilize large amino acid substrates. However, under the conditions of our assay these mercury conjugates are not as active as the "natural" *in vivo* substrates L-glutamine, L-cystine, L-methionine and L-phenylalanine.

The question arises as to whether these transamination reactions with Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys are biologically relevant. Transamination of Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys was demonstrated at 1 mM concentration, but it is doubtful that this concentration could be attained *in vivo* even after severe mercury poisoning. Moreover, there will be strong competition with endogenous amino acid substrates naturally present at much higher concentrations. However, aminotransferases generally exhibit K_m values in the mM or tens of mM range and this is true of GTK ([38]; present work). Thus, it is possible that GTK is not fully saturated with endogenous amino acid substrates and that some binding of Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys will occur even when these conjugates are present at low concentrations. There is precedent for transamination of a metabolite at very low levels *in vivo* despite the presence of much higher levels of other endogenous amino acid substrates. For example, neuroactive kynurenate is obtained by transamination of kynurenine. Two aminotransferases have been extensively studied as contributing to the formation of kynurenate from kynurenine *in vivo*, namely KAT I and KAT II. As noted above, KAT I is identical to GTK. KAT II is identical to glutamate – α-aminoadipate aminotransferase (for a review of the role of KATs in the brain see [48]). The concentration of kynurenine and its transamination product kynurenate in the brain are in the 400 nM range and 1 nM range, respectively [48,49]. Thus, kynurenate can be generated in brain through transamination of kynurenine despite the low levels of precursor kynurenine and high levels of endogenous alternative substrates – e.g. glutamine (KAT I) and glutamate (KAT II). Thus, we suggest that it is entirely possible that Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys even at nM concentrations could be transaminated *in vivo*.

Transamination of Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys are predicted to yield the corresponding mercury-containing α-keto acids. However, it is not clear to what extent these α-keto acids might accumulate *in vivo* given the large pool of GSH (mM) in most tissues and the potential for Hg–S exchange. If the exchange is relatively slow, then it is possible that the α-keto acids derived from Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys may contribute to the toxic effects *in vivo* in individuals exposed to organic mercury via food intake, especially since GTK is of relatively high specific activity in the kidney [23], and the kidney is a major site of accumulation of Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys [13]. It is possible that if the α-keto acid analogues of Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys are produced *in vivo* they may react with/inhibit α-keto acid-utilizing enzymes thereby contributing to the toxicity. However, the predicted mercury-containing α-keto acids have not yet been characterized and their biological and toxicological properties must await further studies. Finally, if there is some Hg–S exchange between GSH and the α-keto acid analogues of Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys, mercaptopyruvate may be one of the products formed. Mercaptopyruvate is a donor of sulfane sulfur to a suitable acceptor in a reaction catalyzed by mercaptopyruvate sulfurtransferase [50]. Addition of sulfane sulfur to cysteine residues has the potential to modify protein structure and function [50].

In addition, our data show that Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys (and the corresponding Hcy conjugates) are strongly inhibitory relative to the substrate phenylalanine. However, the exact kinetic mechanism remains to be elucidated. One possibility is that the mercury *S*-conjugates react covalently (participate in Hg–S exchange) with one or more cysteine residues in rhGTK. Reaction with the cysteinyl moiety (C127) near the active site (or possibly with more distant cysteines) may account for the apparent noncompetitive type of inhibition observed during our kinetic studies. Planned X-ray crystallographic studies of rhGTK crystallized in the presence of either $HgCl_2$ or Cys-*S*-Hg-*S*-Cys should provide additional information.

It is important to note that the activity of many enzymes, including, for example, certain aminotransferases [51,52] and dehydrogenases [53,54], contain cysteine residues that, when modified, can significantly alter enzyme activity. Thiol/disulfide bonds are critical for maintaining the structural, catalytic and allosteric integrity of a vast number of enzymes and signal proteins. In addition, they are key components involved in the maintenance of redox balance and redox-sensitive reaction pathways, and in mediating protein signaling events [21,22,55–57]. Binding of mercury compounds to sulfhydryl centers of exposed cysteine residues may lead to reversible and/or irreversible inhibition of the enzyme, enzyme denaturation and/or protein aggregation. For example, a recent study provides data suggesting that $HgCl_2$ and CH_3HgCl irreversibly inhibit the activity of arylamine *N*-acetyltransferase-1, an intracellular enzyme involved in the biotransformation of aromatic and heterocyclic amines [58]. Similarly, the current study indicates that $HgCl_2$ and CH_3HgCl are able to inhibit the intracellular enzymes, GTK and, much more potently and irreversibly, cystathionine γ-lyase. In previous work it was shown that after inactivation of crystalline rat liver cystathionine γ-lyase by *p*-chloromercuribenzoate, activity could only be partially restored by addition of 2,3-dimercaptopropionate [36].

Although more detailed studies are required to firmly establish the mechanism, the present findings of inhibition of rhGTK by $HgCl_2$ and CH_3HgCl have the characteristics of a slow, but reversible noncompetitive inhibition. There is a good precedent for such an occurrence. Frasco et al. [59] showed that human butyrylcholinesterase, which does not possess a cysteine residue sensitive to thiol reagents, is slowly, but reversibly, inhibited (minutes) by mM amounts of $HgCl_2$ in a noncompetitive manner. Human butyrylcholinesterase crystallized in the presence of $HgCl_2$ was found to contain two Hg binding sites with variable Hg occupancy. No Hg was found in the active site or was bound to cysteine sulfur [59]. As noted above, the crystal structure of rhGTK is known [28]. Subsequent studies from our group will focus on rhGTK crystallized in the presence of $HgCl_2$ and mercury *S*-conjugates. As also noted above, mM concentrations of CH_3HgCl and $HgCl_2$ are not biologically relevant. Nevertheless, the results are helpful because they indicate that the compounds will be useful in X-ray crystallographic studies of rhGTK. Moreover, they highlight the different susceptibilities of rhGTK and cystathionine γ-lyase to mercury-containing compounds as discussed below.

$HgCl_2$, Cys-*S*-Hg-*S*-Cys and CH_3HgCl strongly inhibit cystathionine γ-lyase at μM, or lower, concentrations (Fig. 8). This inhibition is irreversible for $HgCl_2$ and Cys-*S*-Hg-*S*-Cys (and probably also for CH_3Hg-*S*-Cys). These findings are markedly different from those noted for rhGTK, where strong inhibition by $HgCl_2$ and CH_3HgCl requires mM concentrations. Moreover, inhibition of rhGTK by $HgCl_2$ was shown to be reversible by addition of DTT. [The reversibility of the inhibition by CH_3HgCl was not investigated.] As noted above, the sensitivity of cystathionine γ-lyase to inhibition by $HgCl_2$ is probably due to a reactive cysteine in the vicinity of the active site. Again, there is a good precedent in the work of Frasco et al. [59]. These authors showed that, unlike human butyrylcholinesterase, *Torpedo californica* acetylcholinesterase possesses a cysteine residue sensitive to sulfhydryl reagents, and is irreversibly inhibited in a pseudo-first-order process by μM amounts of $HgCl_2$.

The earlier discovery that mercury species bond avidly with thiol-containing biomolecules to form *S*-conjugates such as Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys [9] was instrumental in expanding our understanding of the toxicity of mercury compounds within target organs and cells. When these *S*-conjugates of mercury act as molecular analogues of endogenous, sulfur-containing amino acids, they broaden the scope of the toxicological consequences of mercury exposure. Indeed, the present study shows that Cys-*S*-Hg-*S*-Cys is a potent irreversible inhibitor of cystathionine γ-lyase, while both Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys are aminotransferase substrates and reversible inhibitors of GTK. In rodents, the specific activity of cystathionine γ-lyase is highest in the liver followed by kidney, with much lower levels in other organs [60]. On the other hand, the specific activity of GTK is highest in kidney, but the enzyme is also notably active in liver and, to a lesser extent, in brain and other organs [23,24]. Thus, the differences in organ toxicity of Cys-*S*-Hg-*S*-Cys versus CH_3Hg-*S*-Cys may be related, in part, to differences in the way the compounds interact with enzymes/transporters. For example, CH_3Hg-*S*-Cys, but not Cys-*S*-Hg-*S*-Cys, has been shown to cross the blood–brain barrier via the amino acid transporter, system L [61,62]. Given that the human brain contains large amounts of cystathionine [63], the neurotoxicity of CH_3Hg-*S*-Cys may be related, in part, to its transport through the blood–brain barrier and its subsequent targeting of cystathionine metabolism/turnover/function in the human brain. These mercury-containing organosulfur conjugates are the forms to which humans would be most often likely exposed following consumption of contaminated fish that are high in the food chain. Thus, the metabolic burden of processing inorganic or organic forms of mercury, such as monomethyl *S*-conjugates, would be expected to occur within tissues of autotrophic species and/or animals lower in the food web that are initially exposed to mercury from the atmosphere or that settled in water.

Clearly human exposure to mercury-containing compounds has numerous consequences, not only at the organ level, but also at the cellular/molecular level. Our results emphasize the fact that mercury not only binds covalently and indiscriminately to thiol moieties in proteins, but once conjugated with sulfur-containing amino acids and peptides, particularly cysteine and GSH, forms compounds that can interact with enzymes, thereby potentially broadening the profile associated with overall mercury toxicity. These compounds may represent the major portion of organomercury that contributes to the metabolic burden following consumption of mercury-contaminated foods.

In summary, we have demonstrated that cysteine *S*-conjugates of mercury *in vitro* are a) substrates/inhibitors of GTK, and b) participate in enzyme inactivation (e.g. cystathionine γ-lyase). However, given the high concentration of GSH in most tissues and the propensity of mercury to undergo exchange reactions with sulfhydryl-containing compounds, the α-keto acid products of the GTK reaction on Cys-*S*-Hg-*S*-Cys and CH_3Hg-*S*-Cys may become diluted within the GSH pool. Nevertheless, we wish to emphasize that as a result of exposure to mercury-containing amino acids and subsequent *in vivo* transformation, mercury may be more mobile in its ability to distribute among various organic forms and to interact with more enzymes (as substrates/inhibitors) than previously appreciated. Future studies on the toxicity of mercury should take into account these biological transformations.

Acknowledgments

This work was supported by grants from the National Institutes of Health RO1 [ES8421] (to AJLC), RO1 [ES5980] (to RKZ), RO1 [ES11288] (to RKZ), NS062836 (to JL), RO3 [ES15511] (to CCB), R15 [ES19991] (to CCB) and CA111842 (to JTP).

References

[1] Agency for Toxic Substances and Disease Registry: Toxicological Profile for Mercury, TP-93/10 (United States Department of Health and Human Services, 2010).
[2] H.H. Harris, I.J. Pickering, G.N. George, Science 301 (2003) 1203.
[3] T.W. Clarkson, L. Magos, Crit. Rev. Toxicol. 36 (2006) 609–662.
[4] S.E. Schober, T.H. Sinks, R.L. Jones, P.M. Bolger, M. McDowell, J. Osterloh, E.S. Garrett, R.A. Canady, C.F. Dillon, Y. Sun, C.B. Joseph, K.R. Mahaffey, J. Am. Med. Assoc. 289 (2003) 1667–1674.
[5] M.A. McDowell, C.F. Dillon, J. Osterloh, P.M. Bolger, E. Pellizzari, R. Fernando, R. Montes de Oca, S.E. Schober, T. Sinks, R.L. Jones, K.R. Mahaffey, Environ. Health Perspect. 112 (2004) 1165–1171.
[6] K.R. Mahaffey, Trans. Am. Climatol. Assoc. 116 (2005) 127–153.
[7] A.F. Castoldi, T. Coccini, L. Manzo, Rev. Environ. Health 18 (2003) 19–31.
[8] L. Trasande, P.J. Landrigan, C. Schecter, Environ. Health Perspect. 113 (2005) 590–596.
[9] B. Fuhr, D.L. Rabenstein, J. Am. Chem. Soc. 95 (1973) 6944–6950.
[10] T.W. Clarkson, Ann. Rev. Pharmacol. Toxicol. 32 (1993) 545–571.
[11] C.C. Bridges, R.K. Zalups, Toxicol. Appl. Pharmacol. 204 (2005) 274–308.
[12] T.A. Simmons-Willis, A.S. Koh, T.W. Clarkson, N. Ballatori, Biochem. J. 367 (2002) 239–246.
[13] R.K. Zalups, Pharmacol. Rev. 52 (2000) 113–143.
[14] D.H. Roos, R.O. Puntel, M. Farina, M. Aschner, R.K. Bohrer, J.B.T. Rocha, N.B.V. Barbosa, Toxicol. Appl. Pharmacol. 252 (2011) 28–35.
[15] T.W. Clarkson, Annu. Rev. Pharmacol. Toxicol. 33 (1993) 545–571.
[16] N. Ballatori, Environ. Health Perspect. 110 (Suppl. 5) (2002) 689–694.
[17] R.E. Hoffmeyer, S.P. Singh, C.J. Doonan, A.R. Ross, R.J. Hughes, I.J. Pickering, G.N. George, Chem. Res. Toxicol. 19 (2006) 753–759.
[18] T.W. Clarkson, J.B. Vyas, N. Ballatori, Am. J. Ind. Med. 50 (2007) 757–764.
[19] C.C. Bridges, C. Bauch, F. Verrey, R.K. Zalups, J. Am. Soc. Nephrol. 15 (2004) 663–673.
[20] C.C. Bridges, R.K. Zalups, Am. J. Pathol. 165 (2004) 1385–1394.
[21] I. Dalle-Donne, R. Rossi, D. Giustarini, R. Colombo, A. Milzani, Free Radic. Biol. Med. 43 (2007) 883–898.
[22] A.J.L. Cooper, J.T. Pinto, P.S. Callery, Expert Opin. Drug Metab. Toxicol. 7 (2011) 891–910.
[23] A.J.L. Cooper, A. Meister, Comp. Biochem. Physiol. 69B (1981) 137–145.
[24] A.J.L. Cooper, Neurochem. Int. 44 (2004) 557–577.
[25] G. Ricci, M. Nardini, G. Federici, D. Cavallini, Eur. J. Biochem. 157 (1986) 57–63.
[26] M. Costa, B. Pensa, B. Di Costanzo, R. Coccia, D. Cavallini, Neurochem. Int. 10 (1987) 377–382.
[27] A.J.L. Cooper, M.W. Anders, Ann. N. Y. Acad. Sci. 585 (1990) 118–127.
[28] F. Rossi, Q. Han, R. Li, R. Li, M. Rizzi, J. Biol. Chem. 279 (2004) 50214–50220.
[29] A.J.L. Cooper, J.T. Pinto, B.F. Krasnikov, Z.V. Niatsetskaya, Q. Han, J. Li, D. Vauzour, J.P.E. Spencer, Arch. Biochem. Biophys. 474 (2008) 72–81.
[30] D. Cavallini, G. Ricci, S. Duprè, L. Pecci, M. Costa, R.M. Matarese, B. Pensa, A. Antonucci, S.P. Solinas, M. Fontana, Eur. J. Biochem. 202 (1991) 217–223.
[31] A.J.L. Cooper, M.J. Gross, J. Neurochem. 28 (1977) 771–778.
[32] J.N.M. Commandeur, I. Andreadou, M. Rooseboom, M. Out, L.J. de Leur, E. Groot, N.P.E. Vermeulen, J. Pharmacol. Exp. Ther. 294 (2000) 753–761.
[33] D. Cavallini, C. De Marco, B. Mondovi, B.G. Mori, Enzymologia 22 (1960) 161–173.
[34] A.E. Braunstein, E.V. Goryachenkova, Adv. Enzymol. Relat. Areas Mol. Biol. 56 (1984) 1–89.
[35] C.W. Fearon, J.A. Rodkey, R.H. Abeles, Biochemistry 21 (1982) 3790–3794.
[36] Y. Matsuo, D.M. Greenberg, J. Biol. Chem. 234 (1959) 507–515.
[37] F.C. Brown, M.C. DeFoor, Eur. J. Biochem. 46 (1974) 317–322.
[38] Q. Han, J. Li, J. Li, Eur. J. Biochem. 271 (2004) 4804–4814.
[39] J.T. Pinto, B.F. Krasnikov, A.J.L. Cooper, J. Nutr. 136 (2005) S835–S841.
[40] A.J.L. Cooper, Anal. Biochem. 89 (1978) 451–460.
[41] J.T. Pinto, T. Khomenko, S. Szabo, G.D. McLaren, T.T. Denton, B.F. Krasnikov, T.M. Jeitner, A.J.L. Cooper, J. Chromatogr, B. Analyt, Technol. Biomed. Life Sci. 877 (2009) 3434–3441.
[42] W. Washtien, A.J.L. Cooper, R.H. Abeles, Biochemistry 16 (1977) 460–463.
[43] D.L. Rabenstein, Acc. Chem. Res. 11 (1978) 100–107.
[44] I. Erni, G. Geier, Helv. Chim. Acta 62 (1979) 1007–1015.
[45] D.L. Rabenstein, M.T. Fairhurst, J. Am. Chem. Soc. 97 (1975) 2086–2092.
[46] K.E. Pitts, A.O. Summers, Biochemistry 41 (2002) 10287–10296.
[47] P. Di Lello, G.C. Benison, H. Valafar, K.E. Pitts, A.O. Summers, P. Legault, J.G. Omichinski, Biochemistry 43 (2004) 8322–8332.
[48] M.C. Potter, G.I. Elmer, R. Bergeron, E.X. Albuquerque, P. Guidetti, H.Q. Wu, R. Schwarcz, Neuropsychopharmacology 35 (2010) 1734–1742.
[49] K. Saito, S. Fujigaki, M.P. Heyes, K. Shibata, M. Takemura, H. Fujii, H. Wada, A. Noma, M. Seishima, Am. J. Physiol. Renal Physiol. 279 (2000) F565–F572.
[50] J.I. Toohey, Anal. Biochem. 413 (2011) 1–7.
[51] W. Birchmeier, K.J. Wilson, P. Christen, J. Biol. Chem. 248 (1973) 1751–1759.
[52] T.G. Kalogerakos, N.G. Oikonomakos, C.G. Dimitropoulos, I.A. Karni-Katsadima, A.E. Evangelopoulos, Biochem. J. 167 (1977) 53–63.
[53] K. Pamp, T. Bramey, M. Kirsch, H. De Groot, F. Petrat, Free Radic. Res. 39 (2005) 31–40.
[54] G.G. Chang, R.Y. Hsu, Biochemistry 16 (1977) 311–320.
[55] L.K. Moran, J.M. Gutteridge, G.J. Quinlan, Curr. Med. Chem. 8 (2001) 763–772.
[56] B. Schmidt, L. Ho, P.J. Hogg, Biochemistry 45 (2006) 7429–7433.
[57] D. Summa, O. Spiga, A. Bernini, V. Venditti, R. Priora, S. Frosali, A. Margaritis, D. Di Giuseppe, N. Niccolai, P. Di Simplicio, Proteins 69 (2007) 369–378.
[58] N. Ragunathan, F. Busi, B. Pluvinage, E. Sanfins, J.M. Dupret, F. Rodrigues-Lima, J. Dairou, FEBS Lett. 584 (2010) 3366–3369.
[59] M.F. Frasco, J.P. Colletier, M. Weik, F. Carvalho, L. Guilhermino, J. Stojan, D. Fournier, FEBS J. 274 (2007) 1849–1861.
[60] I. Ishii, N. Akahoshi, X.-N. Yu, Y. Kobayashi, K. Namekata, G. Komaki, H. Kimura, Biochem. J. 381 (2004) 113–123.
[61] Z. Yin, H. Jiang, T. Syversen, J.B. Rocha, M. Farina, M. Aschner, J. Neurochem. 107 (2008) 1083–1090.
[62] M. Aschner, N.B. Eberle, S. Goderie, H.K. Kimelberg, Brain Res. 521 (1990) 221–228.
[63] H.H. Tallan, S. Moore, W.H. Stein, J. Biol. Chem. 230 (1958) 707–716.

Pages 138-155 (Appendices A2) of this thesis have been removed as they contain published material under copyright. Removed contents published as:

Cooper A. J. L. et al. (2015) Role of Glutamine Transaminases in Nitrogen, Sulfur, Selenium, and 1-Carbon Metabolism. In: Rajendram R., Preedy V., Patel V. (eds) *Glutamine in Clinical Nutrition. Nutrition and Health*. Humana Press, New York, NY.
https://doi.org/10.1007/978-1-4939-1932-1_3

A3

Letter to the Editor: μ-Crystallin/CRYM functions as a ketimine-reducing enzyme and plays a role in thyroid hormone bioavailability due to strong inhibition/regulation by thyroid hormones

André Hallen[1] and Arthur J. L. Cooper[2]

DOI: http://dx.doi.org/10.1210/jc.2014-3963
Received: October 30, 2014
Accepted: November 22, 2014
Published Online: January 05, 2015

We have read with interest the recent article by Serrano et al (1) regarding the thyroid hormone-binding protein μ-crystallin (CRYM). Serrano et al state, "Recently, the spectrum of functions of this hormone-binding protein has been broadened since a recent report considers μ-crystallin as a versatile enzyme with a reductase activity able to accommodate various substrates such as ketimine, thyroid hormones or alanine dehydrogenase ligands pyruvate and alanine." We wish to comment on this statement.

1. The report that the authors cite describes only the structure of CRYM (μ-crystallin) with T_3 bound in the presence of NADPH (2).
2. The word "ketimine" refers to compounds containing the functional grouping [RC(=NH)R′] and doesn't refer to any compound in particular. CRYM is an enzyme that reduces cyclic ketimines. This enzymatic property of CRYM was discovered by us (3), but our work is not mentioned by Serrano et al (1). CRYM reduces cyclic ketimines such as Δ^1-piperideine-2-carboxylate originating from lysine metabolism as well as sulfur-containing analogs such as aminoethylcysteine ketimine derived from the metabolism of certain sulfur-containing amino acids. The products of these reductions are L-pipecolate and thiomorpholine-3-carboxylate, respectively (3).
3. The thyroid hormone T_3 is an enzyme inhibitor, not an enzyme substrate. We discovered that T_3 acts as an exceptionally strong inhibitor of CRYM-catalyzed cyclic ketimine reductase activity and suggested that the intracellular bioavailability of T_3 is related to enzyme catalysis (2). Again, this is not mentioned by Serrano et al (1).
4. CRYM is phylogenetically related to alanine dehydrogenase, but CRYM does not possess alanine dehydrogenase activity.

Serrano et al (1) state, "As the main function of μ-crystallin is proposed to be transport of intracellular thyroid hormone…. " We also wish to comment on this statement. We would like to clarify that CRYM has numerous functions; however, it is primarily an enzyme as noted above. Its function in intracellular thyroid hormone transport is related to its role as a ketimine reductase. The level of *free* thyroid hormone in the cytosol will depend in part on the strength of its binding to CRYM. As noted above, T_3 acts as a strong inhibitor/regulator of CRYM enzyme activity. Conversely, binding of cyclic ketimine substrate will compete with binding of T_3.

We appreciate the interesting statement of Serrano et al (1) that CRYM “might mediate the interaction of thyroid function and sensitivity.” However, the authors fail to recognize the true enzyme function of CRYM and the significance of enzyme catalysis on the bioavailability of T_3/T_4. The bioavailability of T_3/T_4 will depend not only on the cytosolic levels of NADPH, but also on the cytosolic concentration of cyclic ketimines. Cyclic ketimines, including several sulfur-containing cyclic ketimines, have long been known to occur in the brain, but their biological importance was initially somewhat of a mystery (4). However, it is now becoming apparent that some cyclic ketimines may be neuroactive (5). Thus, it is becoming apparent that cyclic ketimines are not just incidental waste products of amino acid metabolism but have hitherto unsuspected biological roles, not only as neuroactive agents, but also through binding to CRYM, in the regulation of free cytosolic thyroid hormone levels.

Disclosure Summary: The authors have nothing to disclose.

André Hallen[1] and Arthur J. L. Cooper[2]
[1] Department of Chemistry and Biomolecular Sciences
Macquarie University
N Ryde
NSW 2109
Australia
[2] Department of Biochemistry and Molecular Biology
New York Medical College
Valhalla
New York 10595

Abbreviations:

CRYM	μ-crystallin.

References

1. Serrano M, Moreno M, Ortega FJ, et al. Adipose tissue μ-crystallin is a thyroid hormone-binding protein associated with systemic insulin sensitivity. J Clin Endocrinol Metab. 2014;99:E2259–E2268. [Abstract] [Medline]
2. Borel F, Hachi I, Palencia A, Gaillard MC, Ferrer JL. Crystal structure of mouse mu-crystallin complexed with NADPH and the T3 thyroid hormone. FEBS J. 2014;281:1598–1612. [CrossRef] [Medline]
3. Hallen A, Cooper AJ, Jamie JF, Haynes PA, Willows RD. Mammalian forebrain ketimine reductase identified as μ-crystallin; potential regulation by thyroid hormones. J Neurochem. 2011;118:379–387. [CrossRef] [Medline]
4. Cavallini D, Ricci G, Duprè S, et al. Sulfur-containing cyclic ketimines and imino acids. A novel family of endogenous products in the search for a role. Eur J Biochem. 1991;202:217–223. [CrossRef] [Medline]
5. Hensley K, Venkova K, Christov A. Emerging biological importance of central nervous system lanthionines. Molecules 2010;15:5581–5594. [CrossRef] [Medline]