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Imine Reductases: A Comparison of Glutamate Dehydrogenase to Ketimine Reductases in the Brain

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Abstract

A key intermediate in the glutamate dehydrogenase (GDH)-catalyzed reaction is an imine. Mechanistically, therefore, GDH exhibits similarities to the ketimine reductases. In the current review, we briefly discuss (a) the metabolic importance of the GDH reaction in liver and brain, (b) the mechanistic similarities between GDH and the ketimine reductases, (c) the metabolic importance of the brain ketimine reductases, and (d) the neurochemical consequences of defective ketimine reductases. Our review contains many historical references to the early work on amino acid metabolism. This work tends to be overlooked nowadays, but is crucial for a contemporary understanding of the central importance of ketimines in nitrogen and intermediary metabolism. The ketimine reductases are important enzymes linking nitrogen flow among several key amino acids, yet have been little studied. The cerebral importance of the ketimine reductases is an area of biomedical research that deserves far more attention.

Keywords

Glutamate dehydrogenase; Ketimine reductase; Imine

Introduction

We are honored to provide a contribution to this Special Issue of Neurochemical Research in honor of Professor Andreas Plaitakis. Much of Dr. Plaitakis's early scientific career began in the mid 1970s in the Department of Neurology at the Mount Sinai School of Medicine where he published extensively with two distinguished neurochemists— William Niklas and Sol Berl. Sol Berl and his colleagues had earlier carried out a series of novel tracer experiments [1, 2] that laid the foundation for the later establishment of the cerebral glutamine cycle in brain [glutamate (neurons; neurotransmission) → glutamate (astrocytes) → glutamine (astrocytes) → glutamine (neurons) → glutamate (neurons)]. This cycle is

maintained in part as a result of the unique astrocytic localization of glutamine synthetase (Eq. 1) in the brain [3, 4] and strong (but not unique) glutaminase activity in neurons. (Recent studies suggest complex expression of glutaminase forms in the brain [5].) Although not stoichiometric (glutamate has many biological roles in addition to its function as a neurotransmitter in the brain [6]) flux through the cerebral glutamine cycle in the brain is impressive—perhaps 20 % that of the flux through the tricarboxylic acid (TCA) cycle. For a recent review on the brain glutamine cycle and its relationship to the TCA cycle see Rothman et al. [7].

An important source of ammonia¹ for the glutamine synthetase reaction is the glutamate dehydrogenase (GDH) reaction (Eq. 2; back reaction). Dr. Plaitakis and colleagues have extensively studied the GDH reaction in brain. These highly innovative studies have emphasized the importance of the GDH reaction as contributing to cerebral nitrogen homeostasis and the possible derangement of neurotransmitter glutamate metabolism/GDH activity in various neurodegenerative diseases. Dr. Plaitakis and colleagues have also published extensively on a second active form of GDH (GLUD2) that has arisen via gene duplication in higher primates and is particularly strongly expressed in the brain. For some recent reviews on the metabolic role of GDH (GLUD1) and GLUD2 from the Plaitakis group see refs [8, 9].

In this review we briefly summarize the metabolic importance of the GDH reaction in liver and brain, and its catalytic mechanism. We then turn our attention to ketimine reductases that catalyze an almost identical type of reaction to that catalyzed by GDH – a topic that is in keeping with the theme of the current Special Issue. We then discuss metabolic pathways involving lysine, glutamate, proline and ornithine. Outlines of these interrelated pathways may be found in many biochemical textbooks. Thus, these pathways will not be covered in depth. Rather we concentrate on the central importance of cyclic ketimines in these pathways. Finally, we finish with a review of the enzymes involved in the reduction of these cyclic ketimines and the neurochemical consequences of disruption of these reductive processes.

Metabolic Importance of the GDH Reaction— Transreamination and Transdeamination

The terms transreamination and transdeamination were introduced by Alexander Braunstein [10], who discovered transamination in the 1930s [11]. For discussions on this topic see references [12–14]. Braunstein [10] pointed out that coupling of the GDH reaction (Eq. 2) to an α -ketoglutarate-linked transaminase (aminotransferase) (Eq. 3) can be used to incorporate ammonia nitrogen into an amino acid (transreamination) or to remove it (transdeamination). The net reaction is shown in Eq. 4.

¹Ammonia free base (NH₃) has a pK_a of ~9.2. Thus, under normal intracellular physiological conditions (pH 7.2–7.4) ammonia exists predominantly (~99%) as the conjugate acid, ammonium (NH₄⁺). For convenience, unless otherwise stated, the term ammonia is used throughout the text to indicate the sum of NH₃ plus NH₄⁺.

The forward direction of Eq. 4 is transamination. The back reaction is transdeamination. In the liver these reactions are especially important for the catabolism of excess branched-chain amino acids and tyrosine. The reactions are also important in regulating levels of the non-essential amino acids alanine, glutamate and aspartate.

Urea synthesis in the liver requires incorporation of one equivalent of N as ammonia and one equivalent of N as aspartate. This can be accomplished by coupling the aspartate aminotransferase reaction (Eq. 5) with the GDH reaction (Eq. 2). The back reaction of Eq. 2 provides ammonia for urea synthesis, whereas the forward reaction coupled to the aspartate aminotransferase reaction provides aspartate for urea synthesis (Eq. 6).

Coupling of an α -ketoglutarate/amino acid-linked aminotransferase (back reaction of Eq. 3) to the aspartate aminotransferase reaction (Eq. 5) permits transfer of an amino acid nitrogen to aspartate (Eq. 7).

Thus, GDH, α -ketoglutarate/glutamate-linked aminotransferases and aspartate aminotransferase may be linked in the liver to rapidly shuttle nitrogen [15, 16] according to the following scheme (Scheme 1).

The urea cycle is present in periportal hepatocytes, whereas glutamine synthetase is located exclusively in a thin rim of perivenous hepatocytes. As previously demonstrated by studies with ^{13}N -labeled ammonia and amino acids [12, 13] (^{13}N , positron-emitter $t_{1/2}$ 9.96 min), this arrangement allows for efficient removal of ammonia arriving in the liver from the portal vein first by incorporation into urea in the periportal cells and then into glutamine via a back-up system in the perivenous cells [8, 14]. In the liver, GDH is present in both periportal and perivenous hepatocytes, but is especially enriched in the perivenous cells, whereas glutaminase (Eq. 5) is enriched in the periportal cells [15]. The perivenous cells release glutamine to the circulation whereas the periportal cells take up glutamine. The liver

thus acts as a glutamine rheostat controlling the levels of circulating glutamine and at the same time providing a source of ammonia for urea synthesis [16].

In summary, in the liver α -ketoglutarate-linked aminotransferases coupled to the aspartate aminotransferase reaction and to the GDH reaction can provide ammonia and aspartate for urea synthesis (Scheme 1). The GDH reaction can also provide ammonia for the glutamine synthetase reaction. These processes are aided by the remarkable compartmentation of enzymes involved in nitrogen homeostasis in the liver sinusoid. An important point to note is that the mitochondrial GDH reaction is very active in the liver [17] so that in this organ, the GDH reaction can *rapidly* serve as both a net source of ammonia for urea synthesis (and glutamine synthesis) and as a net source of ammonia for the synthesis of certain non-essential amino acids as dictated by the ever-changing needs of the liver and the body. But what is the role of GDH in the brain?

The GDH reaction is also very active in the brain [18] and therefore can theoretically be used as both a source of ammonia or in the removal of ammonia. At physiological pH values (7.2–7.4) the equilibrium greatly favors reductive amination in vitro (forward direction of Eq. 2). For the reaction to favor glutamate oxidation in vitro, very high levels of glutamate and/or high pH values (>10.0) are typically required. Nevertheless, the reaction can proceed in the direction of glutamate oxidation in vivo if ammonia is rapidly removed, as can occur in the liver when ammonia is removed by incorporation into urea or glutamine. Studies with [^{13}N]ammonia showed that the overwhelming metabolic fate (>95 %) of ammonia entering the rat brain from the blood or cerebrospinal fluid is incorporation into the amide position of glutamine [16]. The major fate of blood-derived [^{13}N]ammonia in the brains of acutely hyperammonemic (urease-treated) rats and chronically hyperammonemic (portacaval-shunted) rats was also found to be incorporation into the amide position of glutamine [19]. Only relatively small quantities of label were incorporated into brain glutamate in the normal and hyperammonemic rats [16, 19]. When rats were pre-treated with the glutamine synthetase inhibitor L-methionine-*S,R*-sulfoximine (MSO), such that the brain enzyme was inhibited by 85 %, a dramatic decrease in the relative amount of label in glutamine was noted, but only a modest increase in label in glutamate [16]. Most of the [^{13}N]ammonia entering the brain from the circulation is metabolized to glutamine in the astrocytes; very little label enters the neurons. However, in the MSO-treated animals the metabolic compartmentation was no longer evident and the label was incorporated into glutamate via the GDH reaction in the neuronal compartment. But even under this situation, label incorporation into glutamate was modest. In conclusion, the ^{13}N studies showed that (a) the major metabolic routes for ammonia metabolism in the rat brain are much simpler than those in the rat liver, and (b) unlike the situation in the rat liver where the GDH reaction can be used for either ammonia assimilation or dissimilation, the GDH reaction in the rat brain is normally a major means for the net dissimilation of ammonia (mainly for glutamine synthesis), but not for the assimilation of ammonia. Assimilation of ammonia is almost entirely via the glutamine synthetase reaction under normal conditions. Even under hyperammonemic conditions the glutamine synthetase reaction predominates. However, the GDH reaction under hyperammonemic conditions may also contribute to a smaller extent (see ref [20]). Some of the studies utilizing [^{13}N]ammonia as a tracer and the metabolic importance of GDH in cerebral nitrogen homeostasis have recently been reviewed [21, 22].

Glutamate Dehydrogenase Catalysis Proceeds via an Imine Intermediate

von Euler et al. in the 1930s were the first to suggest that the GDH enzyme mechanism proceeds via an imine intermediate [23]. Strong but indirect evidence for an imine intermediate was later obtained by Hochreiter et al. [24, 25]. These authors showed that highly purified bovine liver GDH, in the absence of NAD(P)H, but in the presence of α -

ketoglutarate, ammonia and the reducing agent sodium borohydride, catalyzes the formation of more glutamate than is formed in a control lacking enzyme. Moreover, most of the generated glutamate was found to be in the L configuration, showing that the enzyme intermediate formed under these conditions is reduced stereospecifically at the active site [24]. More direct evidence for an imine intermediate in the GDH-catalyzed reaction was obtained from the findings that GDH catalyzes the reversible reduction of the imine bond of the cyclic ketimine Δ^1 -pyrroline-2-carboxylate (Pyr2C), resulting in the formation of L-proline [26] (Fig. 1). Pyr2C is formed by spontaneous cyclization of α -keto- δ -aminovaleic acid (KAV). The equilibrium position strongly favors the cyclic ketimine at neutral pH [27].

As was noted for Pyr2C, Δ^1 -piperidine-2-carboxylate (P2C) also exists almost exclusively in its cyclic ketimine form at neutral pH and is formed from the spontaneous cyclization of α -keto- δ -aminocaproate (KAC). Meister showed that the cyclic ketimine P2C can be non-enzymatically reduced by pyridine nucleotides to pipecolate [28]. Later it was shown that Pyr2C could also be non-enzymatically reduced by dihydropyridines to proline [29]. Interestingly, the mechanism by which Pyr2C is reduced was shown to involve the direct transfer of a hydride ion from the C-4 position of the dihydropyridine to the C-2 position of Pyr2C. Additional strong evidence for the hypothesis that an imine is an intermediate in the GDH-catalyzed-reaction was the finding that GDH catalyzes the reduction of Pyr2C, and to a lesser extent P2C, at the same catalytic site as that used for the reductive amination of α -ketoglutarate [26, 30]. It was also demonstrated that the protonated iminium ion is the favored state for reduction and unprotonated L-proline is the favored state for oxidation [26].

Pyr2C is, however, poorly bound to bovine liver GDH. For example, the rate of GDH-catalyzed reduction of Pyr2C to L-proline was found to be linearly proportional to Pyr2C concentration from 0.01 M to 1.0 M, indicating a K_m value >100 mM [30]. Finally, both α -ketoglutarate and L-glutamate were found to be strong inhibitors of the GDH-catalyzed reduction of Pyr2C [30]. Thus, although GDH can catalyze the reduction of cyclic ketimines at a site that normally binds α -iminoglutarate, cyclic ketimines such as Pyr2C and P2C, are unlikely to be physiologically relevant substrates of mammalian GDH. This conclusion is also true for the bacterium *Escherichia coli*. Thus, Lewis et al. showed that Pyr2C is a very poor substrate of overexpressed GDH in this bacterium and not a discernable substrate of overexpressed pyrroline-5-carboxylate (Pyr5C) reductase [31]. In conclusion, it is likely that in vivo catalytic reductions of cyclic imines are likely to be carried out by ketimine reductases distinct from GDH. Moreover, it is also likely that specific reductases can discriminate between the double bond positional isomers Pyr5C and Pyr2C [31, 32] (see below).

The Central Importance of Cyclic Imines in the Metabolism of L-Lysine

The metabolism of L-lysine in mammalian systems has been investigated over many years. Historically, Weissman and Schoenheimer in 1941 demonstrated that L-lysine, unlike many other amino acids, does not undergo reversible deamination, and suggested that a unique pathway exists for its catabolism [33]. In hindsight, we can appreciate the fact that transamination (or amine oxidation) of L-lysine at either the α - or ϵ -position results in oxidation products (KAC and α -aminoadipate δ -semialdehyde, respectively) that are almost entirely in the form of cyclic imines at neutral pH. Thus, transdeamination and transamination pathways involving lysine are unfavorable. Rothstein and Miller further showed, using ^{15}N as a tracer, that L-lysine is converted in the rat to L-pipecolate, and this process involves the loss of the α -amino group [34].

The metabolic breakdown of lysine is complex, but two pathways are thought to predominate, namely the L-pipecolate and L-saccharopine pathways (Fig. 2). P2C is an intermediate in the breakdown of L-lysine via the pipecolate pathway. However, the formation of L-pipecolate from L-lysine in mammalian tissues is not well understood. There are at least three possible pathways: (1) a transamination reaction involving the α -amino group, yielding KAC which in turn cyclizes to P2C; (2) a transamination involving the ϵ -amino group, yielding L-aminoadipate δ -semialdehyde, which in turn cyclizes to Δ^1 -piperidine-6-carboxylate (P6C); and (3) an L-amino acid oxidase (LAAO) reaction at the α -position, yielding P2C. L-Pipecolate can then be produced by enzymatic reduction of either P2C or P6C. A discussion of each possibility follows.

Pathway 1: We are aware of only a limited number of reports demonstrating enzyme-catalyzed transamination of the α -amino group of lysine. For example, Coccia et al. [35] showed that lysine is a substrate of bovine liver glutamine transaminase. However, in our hands lysine is a poor substrate of rat kidney glutamine transaminase (unpublished data). Ogawa et al. [36] showed that alanine: glyoxylate aminotransferase II can catalyze transamination of L-ornithine and L-lysine. In the case of ornithine, transamination was shown to involve the α -amino group, suggesting that transamination of L-lysine also involves the α -amino group. However, L-lysine is a poor substrate compared to L-alanine [36]. Thus, the contribution of pathway 1 to pipecolate is still uncertain. *Pathway 2:* Transamination of the terminal (i.e. δ)-amino group of L-ornithine, resulting in formation of L-glutamate γ -semialdehyde, was discovered by Meister in 1954 [37]. Transamination of L-ornithine at the δ -amino group is now regarded as part of the major route for the metabolism of ornithine, and the term ornithine aminotransferase now generally applies to an enzyme that catalyzes transamination of the δ -amino group of ornithine. The rat liver enzyme has a narrow amino acid substrate specificity and L-lysine is not a substrate [38]. The fact that L-lysine is not a substrate of L-ornithine δ -aminotransferase is consistent with the previous findings mentioned above that L-lysine is converted in the rat to L-pipecolate with loss of the α -amino group (but not the ϵ -amino group). Thus, pathway 2 to pipecolate is excluded. *Pathway 3:* LAAO of rat kidney and liver has a very high pH optimum. The activity at physiological pH values (pH $\sim$ 7.2) is very low and L-lysine is not a substrate [39]. Thus, these findings rule out kidney and liver LAAO as a source of KAC. However, an L-lysine oxidase is present in mouse brain, but the enzyme responsible has not been purified or fully characterized [40]. Thus, the exact mechanism by which L-lysine is converted to KAC still remains to be established although it seems probable that an LAAO is involved. Nevertheless, it appears that the KAC pathway of lysine catabolism predominates in the brain, whereas the saccharopine pathway predominates in extracerebral tissues [41]. The saccharopine pathway is, however, active in the early stages of brain development [42]. This pronounced difference in L-lysine metabolism between cerebral and extracerebral tissues suggests novel neurochemical roles for the enzymes and metabolites involved in the pipecolate pathway—we return to this topic later in the review.

The pipecolate and saccharopine pathways converge at L- α -aminoadipate- δ -semialdehyde, and thereafter follow the same degradation pathway, ultimately forming acetoacetate (Fig. 2) [41]. As noted above, P2C generated from L-lysine is enzymatically reduced to form L-pipecolate. Alternatively P6C can be reduced L-pipecolate by Δ^1 -pyrroline-5-carboxylate reductase [43]. It is interesting to note, however, that P2C can also arise by the action of D-amino acid oxidase (DAAO) in peroxisomes on D-lysine, and in rat experiments it was shown that both L- and D-lysine are metabolized to L-pipecolate [44].

The Central Importance of Cyclic Ketimines in the Interrelated Metabolism of 5-Carbon Amino Acids

The interconversion of C and N among the 5-C amino acids L-ornithine, L-glutamate and L-proline in mammalian tissues is shown in Fig. 3 (enzyme reactions 1–4). These reactions have been known for many years [e.g., 31, 34, 39 and references cited therein] and are shown in most biochemistry textbooks. Thus, only a few key points regarding this section of Fig. 3 will be discussed here. However, we do add to this diagram a DAAO reaction and a more recently discovered pathway that are not covered in biochemistry texts, and these will be discussed here. As mentioned in the previous section, a key enzyme linking L-ornithine metabolism to L-glutamate and L-proline metabolism is L-ornithine δ -aminotransferase (or more simply ornithine aminotransferase). The product L-glutamate γ -semialdehyde spontaneously cyclizes to the cyclic ketimine Pyr5C, which is well known to be a precursor of L-proline [via reaction 3 (Fig. 3); catalyzed by Pyr5C reductase] and a product of L-proline catabolism [via reaction 4 (Fig. 3), catalyzed by L-proline oxidase]. As also mentioned in the previous section, there is some evidence that Pyr2C (the double bond positional isomer of Pyr5C) may be generated by mammalian aminotransferases (Fig. 3, reaction 5). Alanine-glyoxylate aminotransferase isozyme II has been shown to transaminate L-ornithine with pyruvate, or glyoxylate, to form Pyr2C, but, the high K_m (70 mM) suggests that this is not a quantitatively important reaction under physiological conditions [36]. However, we cannot rule out the possibility that other aminotransferases responsible for this catalysis may exist. Thus, the quantitative importance of the ornithine α -aminotransferase reaction versus ornithine δ -aminotransferase reaction in mammalian tissues is not clear at the present time. There is, however, strong evidence that the conversion of L-ornithine to L-proline favors the ornithine α -aminotransferase pathway in several plant species (Fig. 3, enzyme reactions 5 followed by enzyme reaction 3) [45, 46]. Another possible route for the formation of KAV/Pyr2C is via oxidation of L-ornithine at the α -carbon by an LAAO reaction or by a dehydrogenase reaction. The latter process has apparently been observed to occur in homogenates of turkey liver [47], but to our knowledge has not been demonstrated in mammalian tissues. Interestingly, Costilow and colleagues ([48], and references cited therein) have isolated an enzyme from *Clostridium sticklandii* which they named ornithine cyclase (deaminating) [more recent terminology, L-ornithine cyclodeaminase (OCD)] that converts L-ornithine to L-proline and ammonia. The enzyme catalyzes an oxidation–reduction cycle at the active site in which L-ornithine is oxidized to Pyr2C followed by a reduction in which Pyr2C is converted to L-proline. The prosthetic group promoting this redox cycle is tightly bound NAD^+ . Subsequently, this enzymatic activity has been found to be present in a number of prokaryotes and plants [49–51].

The findings of an enzyme in rat liver that reduces Pyr2C to L-proline that is distinct from a Pyr5C reductase [32, 52] could be interpreted as conclusive evidence for a biologically important pathway to L-proline via Pyr2C arising from enzymes (aminotransferase, LAAO or dehydrogenase) that convert the α -amine functional group of L-ornithine to an α -keto functional group. However, additional routes from ornithine to Pyr2C are now known that do not involve conversion of an L- α -amino function to an α -keto acid function, including a DAAO reaction on D-proline. To appreciate the role of DAAO a discussion of its specificity is germane. It is interesting to note that, unlike LAAO, which cannot oxidize secondary amino acids such as L-proline and L-pipecolate, these secondary amino acids in the form of their D-enantiomers are excellent substrates of DAAO [53–55]. The cyclic ketimine Pyr2C is the product of the oxidation of D-proline by DAAO [54, 55]. Thus, the enzymatic reduction of Pyr2C to L-proline (Fig. 3, reaction 8) together with DAAO may be regarded as a salvage mechanism for converting D-proline, arising from the diet or intestinal bacteria, to L-proline—the more biological useful enantiomer in mammals. Pyr2C has also recently

been shown to be formed from collagen-derived *trans*-3-hydroxy-L-proline by a specific *trans*-3-hydroxy-L-proline dehydratase [56]. It is often stated that about 30 % of the protein in the body is represented by collagen [57]. Thus, it makes considerable biological sense for nature to “devise” a salvage system to convert *trans*-3-hydroxy-L-proline resulting from the turnover of collagen to biochemically useful L-proline.

In conclusion, Pyr2C can now take “center stage” together with Pyr5C as crucial intermediates in the anabolic formation of L-proline. This concept is important in our discussion below of the ketimine reductases.

The Discovery of Specific Imine Reductases

Meister et al. were the first to investigate specific ketimine reductases catalyzing the reduction of the imine double bond of Pyr2C and P2C, resulting in the formation of L-proline and L-pipecolate, respectively [28, 32, 52]. These authors provided evidence that both Pyr2C and P2C are reduced by the same enzyme [28]. P2C/Pyr2C reductase was partially purified from rat tissues and was found to have a pH optimum of approximately 6 and the products were shown to be the L-enantiomers [28]. Pyr5C and P6C were determined not to be enzyme substrates. Thus, the enzyme is specific for the positional isomers in which the carboxylate of Δ^1 -pyrroline carboxylate and Δ^1 -piperidine carboxylate is in the 2 position. The P2C/Pyr2C reductase was found to be present in rat kidney, liver, brain, testis, cardiac muscle, and spleen. Rat kidney was found to have the highest specific activity followed by brain. Enzyme activity was also noted to be present in extracts derived from plants (*Pisum sativum* and *Phaseolus radiatus*), yeast (*Neurospora crassa*) and bacteria (*E. coli* and *Aerobacter aerogenes*). Mutant strains of *N. crassa*, in which the reduction of Pyr5C to L-proline had been genetically blocked, were used to show that the enzyme that catalyzes the reduction of P2C/Pyr2C is different from the enzyme that catalyzes the reduction of P6C/Pyr5C. In later work, Petrakis and Greenberg [58] obtained a partially purified preparation of P2C/Pyr2C reductase from porcine kidney of higher specific activity than that obtained previously by Meister et al. As noted by Meister et al. [52], Petrakis and Greenberg found that both NADH and NADPH are equally effective as cofactors. A pH optimum of 5 was demonstrated and Pyr2C was found to be a better enzyme substrate than P2C (~3 times more effective). Cu^{2+} was found to significantly stimulate enzyme activity, whereas EDTA inhibited the reaction by 50 %, suggesting a metal cofactor requirement.

P2C/Pyr2C reductase from *Pseudomonas putida* has been purified to apparent homogeneity using multiple chromatographic steps [59]. This enzyme exhibits markedly different properties to those of the mammalian enzyme. For example, a pH optimum of 8 (P2C as substrate) was found, which is different from that exhibited by mammalian P2C/Pyr2C reductase (pH 5–6). Also, in contrast to mammalian P2C/Pyr2C reductases, NADPH was found to be a much better reductant than NADH. Finally, metal cations were shown to be inhibitory in contrast to the stimulatory effect noted with the mammalian enzyme. Further research identified the gene and it was recombinantly expressed in *E. coli* [60]. Sequence analysis suggested that the enzyme belongs to a novel NADP-dependent oxidoreductase superfamily, and this was confirmed by X-ray crystallography [61].

Garweg et al. demonstrated the presence of P2C/Pyr2C reductase in brain [60]. The optimal pH was found to be 5.4 with P2C and 5.2 with Pyr2C as substrates. Considerable differences in regional distribution of enzyme activity were noted, with minimal activity in the cerebellum [62]. Interestingly, these findings do not correlate with an earlier report that, in the brain, L-pipecolate concentration is highest in the cerebellum [63]. However, Garweg et al. employed NADH (and not NADPH) as a cofactor and this may have contributed to the apparent discrepancy. This anomaly was solved when a specific cytosolic ketimine

reductase was partially purified from bovine cerebellum by Nardini et al. [64] and characterized. The enzyme was found to have maximal activity with NADPH as a cofactor when P2C was used as the ketimine substrate. Maximal activity was found in the cerebellum and cerebral cortex, and, as with purified or partially purified ketimine reductases studied to date, it was found to be highly unstable. A different cytosolic ketimine reductase was later purified from porcine kidney [65] and found to have a M_r of ~70,000 (no data were presented on subunit composition), which is distinct from the cerebellum ketimine reductase, which was determined to be a homodimer (monomer M_r ~ 50,000) [64]. Whereas the cerebellum enzyme “prefers” NADPH as cofactor, NADH is the “preferred” cofactor for the kidney enzyme. In addition to P2C, both ketimine reductases were shown to accept additional substrates, namely sulfur-containing cyclic ketimines (Pyr2C was not tested as a substrate for either enzyme). Of the cyclic ketimines investigated, P2C was found to be a better substrate for cerebellum ketimine reductase than any of the sulfur-containing cyclic ketimines tested [64]. However, of the sulfur-containing cyclic ketimines investigated, aminoethylcysteine ketimine (AECK, an analogue of P2C in which a ring $-CH_2-$ is replaced with S; see below) was found to be a better substrate than P2C for kidney ketimine reductase [65]. Both ketimine reductases were found to exhibit a pH optimum of 5–6, which is consistent with the findings reported by previous researchers for P2C/Pyr2C reductases [28, 58, 64, 65].

Sulfur-Containing Cyclic Ketimines: Novel Metabolic Intermediates with Potentially Important Neurochemical Properties

Sulfur-containing cyclic ketimines are metabolites derived from sulfur-containing amino acids, such as cystine, lanthionine, thialysine (*S*-2-aminoethyl-L-cysteine), and cystathionine (Fig. 4) [66]. These amino acids are transaminated by glutamine transaminases, generating α -keto acids that cyclize to sulfur-containing cyclic ketimines [65–67]. [Naming of these cyclic ketimines is complex. Consequently, the authors who first described these reactions devised simple trivial names based on the amino acid from which the ketimine is derived, a practice we continue here.] Thus, transamination of cystathionine, cystine, thialysine and lanthionine results in the formation of the corresponding α -keto acids that cyclize to cyclic ketimines given the trivial names cystathionine ketimine (CysK), cystine ketimine (CK), aminoethylcysteine ketimine (AECK), and lanthionine ketimine (LK), respectively (Fig. 5) [67–69]. Thialysine is generated in mammalian tissues by enzymatic decarboxylation of lanthionine [70] or from the condensation of cysteamine (a product of pantetheine metabolism) with serine (or cysteine) in a reaction catalyzed by cystathionine β -synthase (CbS) [71]. In a similar manner, lanthionine is formed by CbS using either a) cysteine and serine, or b) two equivalents of cysteine as substrates [72]. Another route that may be important for the formation of lanthionine, particularly in older individuals, is the Michael addition of free cysteine, the cysteine moiety of glutathione or cysteine residues in proteins to non-enzymatically generated dehydroalanine residues in proteins, followed by proteolysis of the adducted protein [73].

The sulfur-containing ketimines (AECK, LK, CysK, and CK) exist in three different forms that are in equilibrium with each other; namely an open-chain form, a cyclic (closed) enamine form, and a cyclic (closed) ketimine form [74]. Under strongly acidic conditions the open-chain form predominates, under moderately acidic conditions the cyclic ketimine form is predominant, and under neutral or alkaline conditions the cyclic enamine form is the favored species [66]. Ketimine reductases are believed to act only on the cyclic ketimine form [64, 65], which, as noted above, is not the dominant form of the sulfur-containing cyclic ketimines at neutral pH. P2C and PyrC only exist in ketiminic forms at neutral pH [27] and this may explain why P2C is a better substrate under physiological (neutral pH)

conditions for cerebellum ketimine reductase than the sulfur-containing cyclic ketimines [64].

Several sulfur-containing ketimines, and/or their reduced products, have been reported to be present in mammalian brain; namely, (a) CysK and LK in human brain [75], (b) AECK, LK and CysK in bovine brain [76–78], (c) cyclothionine (the reduced form of CysK) in bovine brain [79], and (d) 1,4-thiomorpholine-3,5-dicarboxylate (the reduced form of LK) in bovine brain [80]. Of some note, is the report that CysK occurs in human brain [75] as cystathionine concentrations are known to be relatively high in autopsied human brain (1–2.5 $\mu\text{mol/g}$ wet weight), with concentrations 10–50 times greater than in other tissues [81]. A neurological role for these sulfur-containing cyclic ketimine substrates is implied by the demonstration of binding of LK to isolated bovine brain membranes, resulting in a marked elevation in adenylate cyclase activity and a subsequent marked increase in basal intracellular cAMP levels [82, 83]. This binding is displaced by catecholamines, such as dopamine, adrenaline, noradrenaline, and the sulfur-containing ketimines AECK and CysK. To this date, however, no specific receptors have been identified. AECK and LK have been tested for *N*-methyl-D-aspartate (NMDA) receptor-interaction and no excitatory or inhibitory activity was determined (unpublished results—Dr. Steven Traynelis, Emory University, USA). The imidazoline receptors apparently have little or no affinity for AECK and LK (unpublished results—Dr. Alan Hudson, University of Alberta, Canada). CysK, AECK, and LK have been shown to significantly enhance reactive oxygen species (ROS) production in neutrophils, via NADPH oxidase (NOX) [84–86]. This activation of NOX is inhibited by the tyrosine kinase inhibitor genistein. NOX is also present in neurons [87], and the potential production of free radicals implies a role in immune responses and also a possible role in neurodegeneration. AECK is a powerful inhibitor of DAAO [88]. D-Serine is a modulator of the NMDA receptor [84]. Thus, AECK has the potential to inhibit DAAO activity, which could lead to increased D-serine levels and a subsequent increased glutamate NMDA receptor activity. Hensley et al. [89] have shown that LK has notable neurochemical properties. LK and its ethyl ester were found to promote neurite extensions in duck dorsal root ganglia and increase the length and number of neurites in NSC-34 motor neuron-like cells [89, 90].

In conclusion, although several interesting cyclic sulfur-containing compounds have been reported to be present in the mammalian brain, the findings, except in a few instances, have been largely overlooked by neurochemists. More work is needed to independently verify the occurrence of these interesting compounds in the brain (and other tissues) and to establish the biological and neurological roles of these compounds. We believe that this is an exciting area for future research.

μ -Crystallin: The First Definitively Identified Mammalian Ketimine Reductase

Hallen et al. recently purified a cytosolic lamb forebrain ketimine reductase to apparent homogeneity [91]. The authors also demonstrated similar enzymatic activity with the recombinantly expressed human orthologue. The purified lamb brain ketimine reductase was found to be a homodimer (M_r monomer, ~36,000).

Similar assays to those employed by Hallen et al. were used by Cavallini and colleagues previously to purify porcine kidney [65] and bovine cerebellum [64] ketimine reductases. Notably, the pH of the assay system was maintained at 5 and NADH was used as reductant. The enzyme purified by Hallen et al. displayed similar enzyme substrate specificity to that reported earlier for kidney ketimine reductase [65], with relative activity toward various substrates in the order: AECK>P2C>CysK with NADH as cofactor. In the study of the lamb forebrain ketimine reductase, the likely enzyme substrates, LK and Pyr2C, were not

evaluated as substrates. Nevertheless, comparative analysis of the reported findings suggested that there are notable differences between the ketimine reductases purified from lamb brain [91] and porcine kidney [65], on the one hand, and bovine cerebellum ketimine reductase [64], on the other hand. For example, bovine cerebellum ketimine reductase demonstrated maximal activity with P2C and NADPH as cofactor [64] in contrast to lamb brain/bovine kidney ketimine reductase, which, as noted above, is more active with AECK and NADH. Thus, there exists the strong possibility that mammalian brain contains at least two ketimine reductases.

At this point it is worth commenting on the fact that sequence analysis of the ketimine reductase isolated from lamb forebrain revealed an unexpected multiplicity of functions. Thus, among diurnal marsupials, the ketimine reductase orthologue is a structural protein in the lens (previously assigned the name μ -crystallin; CRYM) [92]. Perhaps even more interesting (and unexpected!) was the finding that the sequence of the purified ketimine reductase is identical to that of the main cytosolic thyroid hormone binding protein [93]. Thus, forebrain ketimine reductase/CRYM as well as possessing important enzymatic properties (ketimine reductase) and structural properties (in diurnal marsupial lens) is involved in the redox-dependent intracellular binding and transport of 3,5,3'-triiodothyronine (T3) [94].

The T3-binding protein was originally purified on the basis of its strong T3 binding in the presence of NADPH [95]. When the T3-binding protein was first discovered the mechanism by which NADPH elicits strong binding of T3 was not apparent. However, our studies showing that the T3-binding protein is identical to NAD(P)H-dependent ketimine reductase provides a rational explanation [91]. Evolution has apparently co-opted the NADPH-binding site not only to bind cofactor for the enzymatic reduction of cyclic ketimines, but also to assist in the correct geometry for the binding of T3. In this regard, we found that T3 is a very strong competitive inhibitor (with respect to AECK as substrate) of enzymatic activity at pH 5 (K_i , 278 nM) [91]. However, a similar kinetic analysis at physiological neutral pH was not possible. At this pH, the reported K_d for binding of T3 to T3-binding protein is 0.3 nM [96]. Since the concentration of enzyme in the assay mixture is much greater than this (~44 nM), competitive inhibition studies were not possible at pH 7.0. At this pH, T3 has the hall-mark of a tight-binding inhibitor, such that the enzyme activity of ketimine reductase/CRYM/T3-binding protein would be tightly regulated by sub nM concentrations of T3. Conversely, the concentration of cyclic ketimine substrates will influence the ability of the protein to bind T3. T3-binding protein has previously been implicated in regulating the bioavailability of T3 and redox-dependent translocation of T3 to the nucleus [94]. These processes must now be evaluated in the context of ketimine reductase activity, where increased availability of cyclic ketimine substrate has the potential to decrease the binding of T3 and subsequent translocation to the nucleus. This is a novel concept where the bioavailability of T3 as a transcription factor is regulated by the concentration of enzyme substrates derived from amino acid metabolism. Under conditions of protein deprivation, and subsequent decreased amino acid and ketimine substrate bioavailability, T3 would presumably remain bound to ketimine reductase and not be bioavailable in the cell.

Cytosolic thyroid binding proteins have been intensely researched and, in addition to ketimine reductase/T3-binding protein isolated from lamb brain, a T3-binding protein has been isolated from *Xenopus laevis* [97] liver and identified as aldehyde dehydrogenase [98]. T3 is an inhibitor of aldehyde dehydrogenase activity. A number of other NAD⁺-dependent dehydrogenases are inhibited by thyroxine hormones or their analogues; notably alcohol dehydrogenase [99], glycerol-3-phosphate dehydrogenase [100], malate dehydrogenase [101], and GDH [102].

Forebrain ketimine reductase/CRYM/T3-binding protein is a homologue of a number of prokaryote enzymes, notably lysine cyclodeaminase (LCD) [103], OCD [104], and alanine dehydrogenase (AlaDH [105]. The reactions catalyzed by LCD, OCD, and AlaDH have all been postulated to proceed via imine intermediates [103, 106]. Interestingly, however, the *Putida syringae* P2C/Pyr2C reductase, whose X-ray structure has been solved [61], is not a mammalian ketimine reductase/CRYM/T3-binding protein homologue in spite of the fact that it catalyzes the same imine reductions. The X-ray crystal structure of human CRYM has also been solved with no bound ligand (apart from NADPH) [104]. At the time of this study, the enzyme binding function of CRYM was unknown. The studies need to be repeated with crystals of ketimine reductase/CRYM/T3-binding protein in the presence of a bound inhibitor. Nevertheless, the studies reveal no similarity in structure between human and bacterial ketimine reductases. Thus, bacterial P2C/Pyr2C reductase and ketimine reductase/CRYM/T3-binding protein can be regarded as examples of convergent evolution with no sequence or protein fold similarities.

Neurochemical Importance of Ketimine Reductase/CRYM/T3-Binding Protein

Prior to our work identifying CRM as a ketimine reductase/T3 binding hormone, CRYM had been implicated in a number of neurological and psychiatric disorders [[107–110] and references cited therein]. These findings must now be evaluated in the context of the possible neuroactivity of the enzyme substrates. The CRYM gene is upregulated in a murine model of familial amyotrophic lateral sclerosis (ALS), and it is suspected that this up-regulation is associated with the pathology of ALS [111]. Hensley et al. found beneficial effects of the administered cyclic ketimine LK (and its ethyl ester) in a murine model of ALS [89]. This finding further strengthens the possibility of ketimine reductase involvement in ALS. Up-regulation of the CRYM gene has also been reported in multiple sclerosis [108], and increased protein expression has been noted in facioscapulohumeral muscular dystrophy in muscle biopsies from a cohort of affected patients [109]. Altered CRYM gene expression may represent a common pathological mechanism in a number of neurodegenerative and neuromuscular diseases. The CRYM gene is substantially under-expressed in a mouse model of Alzheimer disease associated with an increase in amyloid- β [112]. Hyperglycemia has been shown to elevate CRYM expression in vivo and in vitro [113], and this would result in decreased ketimine substrate levels. Although it is highly speculative given the severe neuropathological phenotype associated with mutations of CRYM, it is possible that decreased levels of ketimines contribute to diabetic neuropathy. Gene expression profiling of schizophrenia prefrontal cortex has revealed CRYM to be consistently decreased compared to that in controls [114]. Moreover, low levels of the CRYM protein have been found in medication-naïve schizophrenia prefrontal cortex [107]. These changes may not be causal but instead may represent a secondary affect—for example, decreased transcriptional/translational events associated with lower concentrations of ketimine reductase substrates. Inasmuch as mutations in CRYM have been associated with deafness the intriguing possibility arises that auditory hallucinations, so common in paranoid schizophrenia, may also be related to concentrations of ketimine substrates.

CRYM mRNA is highly expressed in human inner ear [115]. Two point mutations of CRYM are known, namely X315Y and K314T, both of which are associated with nonsyndromal deafness, [116]. Both mutations are remote from the active site of the enzyme and at the C-terminus. The K314T mutation is reported to abolish NADPH-dependent T3 binding to CRYM and is associated with severe deafness. On the other hand, the X315Y mutation has no effect on T3 binding and the disease phenotype is not as severe [110]. The

effects of these mutations on enzyme activity must now be evaluated. The inference is that levels of CRYM substrates are important determinants in hearing.

ROS derived from the enhancement of NOX activity by sulfur-containing ketimines may have implications in neurological pathologies. NOX has been associated with neurodegeneration in temporal lobe epilepsy [117], and NOX-derived ROS have also been found to cause dopaminergic neuronal death in Parkinson disease [118]. NOX inhibitors are of current therapeutic interest in treating stroke as reviewed by Cairns et al. [119].

Summary

Considerable evidence indicates that the GDH-catalyzed reaction proceeds via an imine intermediate. Consistent with this mechanism is the fact that GDH catalyzes the reduction of cyclic ketimines, such as P2C and Pyr2C. These reactions are, however, unlikely to be of physiological significance. Instead, specific reductases catalyze the NAD(P)H-dependent reduction of P2C/Pyr2C (and other) cyclic ketimines. Cyclic ketimines are at the center of interconnecting metabolic pathways involving lysine, ornithine, proline and glutamate. Thus, enzymes involved in the metabolism of cyclic ketimines are of crucial importance for the maintenance of nitrogen and carbon homeostasis. Metabolically important cyclic ketimines possess either a double bond between the ring N and C2 [$-\text{CH}_2\text{N} = \text{C}(\text{CO}_2^-)-$] or between the ring N and C5 or C6 [$-\text{CH} = \text{NC}(\text{CO}_2^-)\text{H}-$]. In the current review, we mention both types of enzymes, but have concentrated our discussion on the enzyme-catalyzed reduction of the double bond of cyclic ketimines that possess the $-\text{CH}_2\text{N} = \text{C}(\text{CO}_2^-)-$ configuration. We present evidence that there are at least two enzymes in mammals that catalyze the reduction of these ketimines and emphasize the biological importance of these enzymes. Of interest is the finding that sulfur-containing ketimines that possess the $-\text{CH}_2\text{N} = \text{C}(\text{CO}_2^-)-$ configuration are substrates. We have emphasized the neurochemical importance of the cyclic ketimines, including the sulfur-containing ketimines. For example, some ketimine reductase substrates are potential neurotransmitters/neuromodulators and bind to brain membranes with subsequent enhancement of cAMP levels. Moreover, some ketimine reductase substrates have been shown to enhance ROS formation through activation of NOX and therefore may play a role in neurological pathologies in which ROS is implicated. Of considerable importance is the identification of the ketimine reductase purified from lamb forebrain as μ -crystallin (CRYM) and its strong regulation by T3. Thus, the forebrain ketimine reductase now joins the ranks of multifunctional ("moonlighting") enzymes, and suggests a previously unsuspected link between nitrogen metabolism and regulation of hormone levels. Finally, CRYM (i.e. ketimine reductase) has been implicated in various neurological and psychiatric disorders, suggesting that the ketimine substrates play a role in these pathologies.

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Abbreviations

AECK	Aminoethylcysteine ketimine
AlaDH	Alanine dehydrogenase
ALS	Amyotrophic lateral sclerosis
CbS	Cystathionine β -synthase

CRYM	μ -Crystallin
CK	Cystathionine ketimine
CysK	Cystathionine ketimine
DAAO	D-Amino acid oxidase
GDH	Glutamate dehydrogenase
KAC	α -Keto- δ -aminocaproate
KAV	α -Keto- δ -aminovalerate
LAO	L-Amino acid oxidase
LCD	Lysine cyclodeaminase
LK	Lanthionine ketimine
NOX	NADPH oxidase complex
NMDA	N-Methyl-D-aspartate
OCD	Ornithine cyclodeaminase
P2C	Δ^1 -Piperidine-2-carboxylate
P6C	Δ^1 -Piperidine-6-carboxylate
Pyr2C	Δ^1 -Pyrroline-2-carboxylate
Pyr5C	Δ^1 -Pyrroline-5-carboxylate
ROS	Reactive oxygen species
T3	353'-Triiodothyronine
TCA	Tricarboxylic acid

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Fig. 1.

Enzymatic pathways for the formation of cyclic ketimines and their reduction by GDH. The first step in the metabolism of L-ornithine is a transamination reaction involving either the α -amino group [product, α -keto- δ -aminovalerate (KAV)] or the δ -amino group [product, {L-glutamate- γ -semialdehyde (not shown)}]. However, it is thought that transamination of the δ -amino group is the preferred route in mammalian tissues (see the text). The enzymes involved in conversion of L-lysine to α -keto- ϵ -aminocaproate (KAC) have not been fully characterized. Note also that the reduction of Δ^1 -pyrroline-2-carboxylate (Pyr2C) and Δ^1 -piperidine-2-carboxylate (P2C) to L-proline and L-pipecolate, respectively, catalyzed by GDH, is probably not physiologically important, but is shown here to make the point that the physiological GDH reaction (i.e. Eq. 2) is consistent with an imine as an intermediate. For original reference see the text

Fig. 2.

Lysine catabolism in mammalian tissues. The saccharopine pathway (indicated by →A) predominates in extracerebral tissues, whereas the L-pipecolate pathway (indicated by →B) predominates in the brain. Enzymes/enzyme pathways: 1. Oxidation of L-lysine at the α-amino group, most probably by an L-amino acid/L-lysine oxidase, but some contribution from transamination reactions cannot be ruled out; 2. P2C/Pyr2C ketimine reductase(s); 3. L-pipecolate oxidase; 4. saccharopine dehydrogenase; 5. α-amino adipate δ-semialdehyde dehydrogenase (antiquitin); 6. D-amino acid oxidase; 7. L-lysine-α-ketoglutarate reductase; 8. enzyme pathway leading to acetoacetate; 9. Δ¹-pyrroline-5-carboxylate reductase/Δ¹-piperidine-5-carboxylate reductase. Note the central importance of the cyclic ketimines, Δ¹-piperidine-2-carboxylate and Δ¹-piperidine-6-carboxylate, in lysine metabolism. In addition, several bacteria and plant species have been shown to possess an enzyme (L-lysine cyclodeaminase, LCD) that catalyzes the conversion of L-lysine to L-pipecolate and ammonia [103]. For simplicity not all cofactors or reactants are shown. The diagram is meant to show the main metabolic fates of the lysine backbone. α-KG α-ketoglutarate, L-Glu L-glutamate

Fig. 3.

Metabolic interconversions of 5-C amino acids in mammalian tissues showing the central importance of the cyclic ketimines, Δ^1 -pyrroline-2-carboxylate (Pyr2C) and Δ^1 -pyrroline-5-carboxylate (Pyr5C). Enzymes/enzyme pathways: 1. L-Ornithine- δ -aminotransferase; 2. Δ^1 -pyrroline-5-carboxylate dehydrogenase; 3. Δ^1 -pyrroline-5-carboxylate reductase; 4. L-proline oxidase; 5. possible conversion of the α -amino function of L-ornithine to an α -keto function by transamination, an L-amino acid oxidase reaction or a dehydrogenase reaction (see the text); 6. *trans*-3-hydroxy-L-proline dehydratase; 7. D-amino acid oxidase; 8. P2C/Pyr2C/ketimine reductase. Note that certain bacteria and plants possess an enzyme that can convert L-ornithine directly to L-proline (L-ornithine cyclodeaminase, OCD). For original references see the text. For simplicity not all cofactors or reactants are shown

Fig. 4.

Metabolic pathways leading to the formation of sulfur-containing cyclic ketimines: *CysK* cystathionine ketimine, *LK* lanthionine ketimine, *CK* cystine ketimine, *AECK* aminoethylcysteine ketimine. Enzymes: 1. glutamine transaminases; 2. cystathionine- β -synthase (CbS); 3. pantetheinase; 4. Michael addition of cysteine/cysteine residues to dehydroalanine residues followed by proteolytic digestion; 5. uncharacterized decarboxylase; 6. cystathionine- γ -lyase. For simplicity not all cofactors or reactants are shown. For a discussion of some of these pathways see [67]

Fig. 5.

Reduction of sulfur-containing cyclic ketimines by ketimine reductases: *AECK* aminoethylcysteine ketimine, *CysK* cystathionine ketimine, *LK* lanthionine ketimine, *CK* cystine ketimine, *TMA* 1,4-L-thiomorpholine-3-carboxylate, *TMDA* 1,4-L-thiomorpholine-3,5-dicarboxylate. NADPH is the preferred cofactor for cerebellum ketimine reductase, whereas NADH is the preferred cofactor for both kidney ketimine reductase and cerebral μ -crystallin/ketimine reductase. In addition, among the sulfur-containing ketimines investigated, AECK (a monocarboxylate) is a better substrate than are the dicarboxylate ketimines LK, CysK, and CK [64, 65, 91]

Scheme 1.

Shuttling of amino acid nitrogen to urea via linked aminotransferases and the GDH reaction