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A comparison of pyridoxal 5′-phosphate dependent decarboxylase and transaminase enzymes at a molecular level

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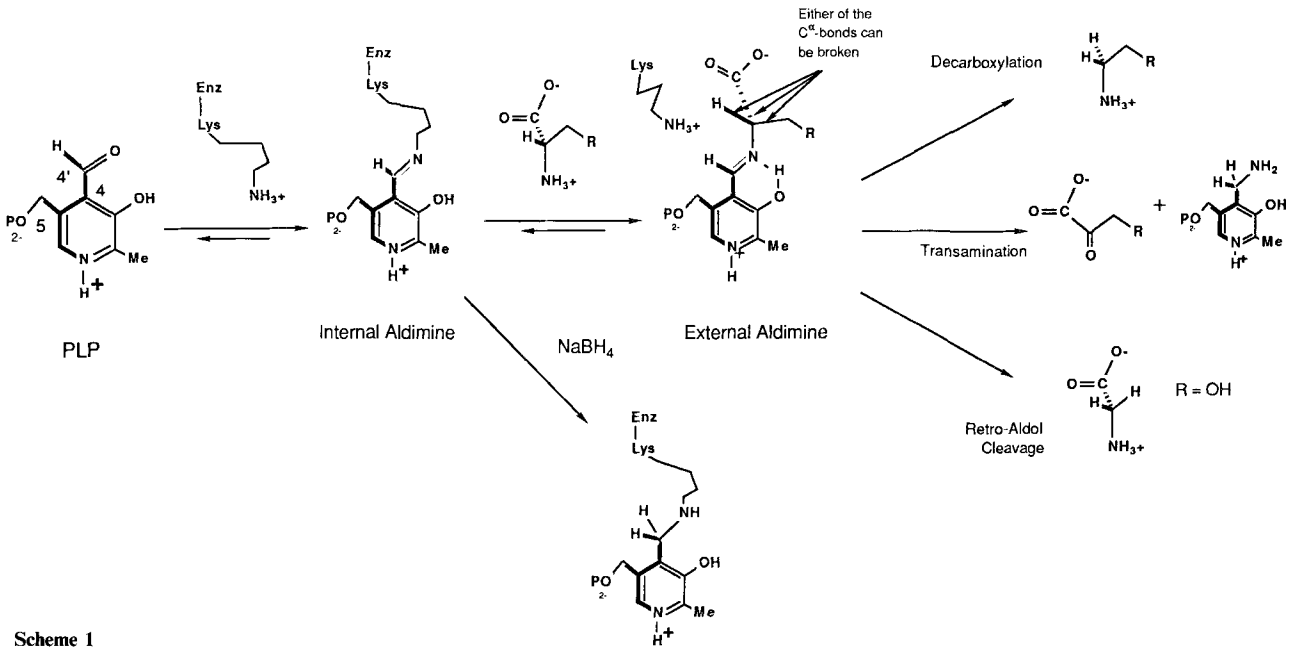
Abstract. Pyridoxal 5′-phosphate is a coenzyme for a number of enzymes which catalyse reactions at C^{α} of amino acid substrates including transaminases, decarboxylases and serine hydroxymethyltransferase. Using the X-ray coordinates for a transaminase, aspartate aminotransferase, and the results of stereochemical and mechanistic studies for decarboxylases and serine hydroxymethyltransferase, an active-site structure for the decarboxylase group is constructed. The structure of the active-site is further refined through active-site pyridoxyllysine peptide sequence comparison and a 3-D catalytic mechanism for the L-α-amino acid decarboxylases is proposed. The chemistry of serine hydroxymethyltransferase is re-examined in the light of the proposed decarboxylase mechanism.
Key words. Transaminase; decarboxylase; serine hydroxymethyltransferase; pyridoxal 5′-phosphate; enzyme mechanism; stereochemistry; kinetics.

Introduction

Pyridoxal 5′-phosphate (PLP) dependent enzymes catalyse a myriad of different types of chemical reaction in amino acid metabolism. Examples include α-amino acid-keto acid and ω-amine-aldehyde transamination; α-, and β-amino acid decarboxylation; α-amino acid racemisation; β-hydroxy amino acid retro-aldol cleavage; α-amino acid deamination; and elimination and replacement reactions at C^{β} and C^{γ}. Some examples are shown in scheme 1.

Of the PLP-enzyme catalyzed reactions, the α-amino acid transaminase and α-amino acid decarboxylase groups are the largest. In each group there are dozens of different enzymes which catalyse the conversion of specific substrates to specific products. These have different names and Enzyme Catalogue[24] (EC) numbers, for example, glutamate and histidine decarboxylase. There are also several quite distinct characterised enzymes, from different species, which catalyse the same specific reaction.



Scheme 1

These have the same name and EC numbers, although often, the enzyme molecules themselves are very different, for example, *E. coli* and mammalian brain glutamate decarboxylase. The transaminase and decarboxylase enzyme groups are ubiquitous in nature and often representatives are closely associated in metabolic pathways.

The PLP-dependent enzymes have diverse quaternary structures. Some are active as monomers[9] others as dimers[4, 35], and some as tetramers or hexamers (see Gani for a recent review[32]). The majority are homopolymeric and contain one mole of coenzyme per subunit but, others appear to have more complex structures.

While PLP serves as a coenzyme in each of the reactions, all of which involve the initial formation of an external aldimine via transaldimination of an internal lysine aldimine, scheme 1, the role of the protein in subsequent steps is poorly understood for all systems other than aspartate aminotransferase (AAT). For AAT, stereochemical, X-ray structural and specific-site alteration studies over the last fifteen years or so have greatly enhanced our perception of the mode of action of the transaminase group[20, 38, 39, 43]. However, the results of these studies have had little or no impact on our understanding of the other enzyme groups.

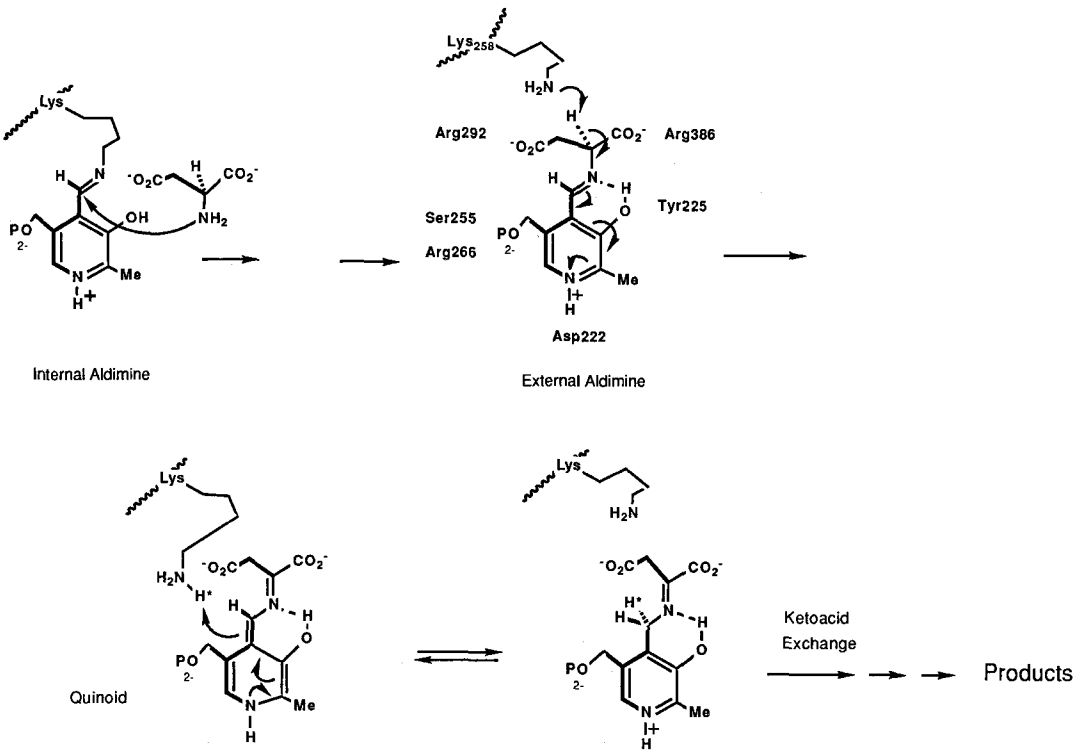
In particular, studies with AAT have provided no information on the structure or mechanism of the other large PLP-dependent enzyme group, the decarboxylases for which no X-ray structures have yet been published.

We had been particularly interested in the mechanism of the decarboxylase group and wished to undertake a detailed comparison of the transaminase and decarboxylase enzymes with the objective of uncovering shared features in addition to the utilisation of a common cofactor. The detection of such similarities might then allow the wealth of data available for AAT to be used in the construction of a model for the active-site of the less well studied decarboxylase enzymes and hence a rationalisation for the roles of the protein components in controlling the chemistry of the two large enzymes groups.

Aspartate aminotransferase

Transaminases are the best understood PLP-dependent enzymes. Much of the early work in the area was concerned with assessing the stereochemical and mechanistic course of the reaction with respect to C^{α} of the substrate and C-4′ of the coenzyme. This work has been reviewed[27], and Christen and Metzler have edited a book entitled "Transaminases"[72]. One of the most significant contributions came from an X-ray study of chicken heart mitochondrial aspartate aminotransferase and is now possible to envisage the three-dimensional catalytic function of the enzyme for the entire transamination process[43].

The protein is composed of two identical subunits (M_r ~ 45000) which consist of two domains. The coenzyme is bound to the larger domain in a pocket near the subunit interface. The proximal and distal carboxylate groups of the dicarboxylic acid substrates/products (viz., aspartate, glutamate, oxaloacetate and α-ketoglutarate) are bound by two arginine residues (386 and 292 from adjacent subunits). Substrate specificity is determined largely by these binding interactions. The mode of substrate binding not only ensures efficient catalysis but, also causes a bulk movement in the smaller domain which closes the active-site pocket and moves Arg-386 3 Å closer to the coenzyme. The transaldimination of the ε-amino group of Lys-258 by the substrate, aspartic acid,



Scheme 2

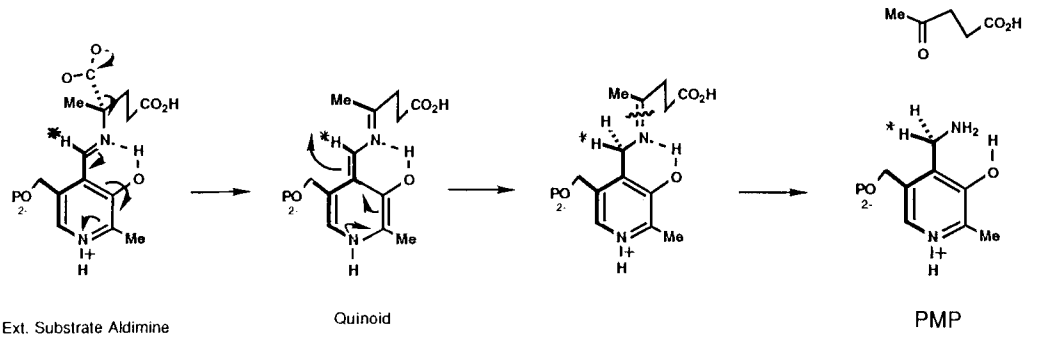
to form the substrate aldimine, occurs from the *re*-face at C-4′ and causes the coenzyme to tilt by ~30°. The released ε-amino group then serves as the enzyme-bound proton carrier for suprafacial 1,3-prototropic shifts which occur on the *si*-face of C-4′. After the formation and hydrolysis of the initial ketimine, the coenzyme tilt relaxes back slightly.

The coenzyme is held in place by several amino acid residues. The protonated pyridinium ring is hydrogen bonded to Asp-222 which forms a second hydrogen bond to His-143. The 2-methyl group is located in a pocket formed by eight amino acid residues and the 3′-oxygen atom is hydrogen bonded to the phenolic OH of Tyr-225. X-Ray data also indicate that a *cisoid* ε-lysine aldimine conformer exists in the absence of substrate (i.e. the aldimine N is on the 3′-OH side). As the substrate aldimine also exists in a *cisoid* conformation it seems probable that the conformational changes in the tilt angle of the coenzyme accounts for the differential C-4′-face selectivity of reducing agents. The internal and external aldimines are reduced at the C-4′-*re*- and C-4′-*si*-faces, respectively[8,27]. The 5′-phosphate ester group of the co-enzyme appears to be bound by seven or eight hydrogen bonds as the dianion. The guanidinium group of Arg-266 forms two of these H-bonds and largely offsets the double negative charge. The side-chain of Ser-255 forms another H-bond to the phosphate ester. It is also evident that the covalent bonds between the phosphate group and pyridine ring are strained. All of these results are in accord with stereochemical observations and it is now apparent that the dynamic model of transaminase catalysis based on solution properties complements the X-ray diffraction model (scheme 2).

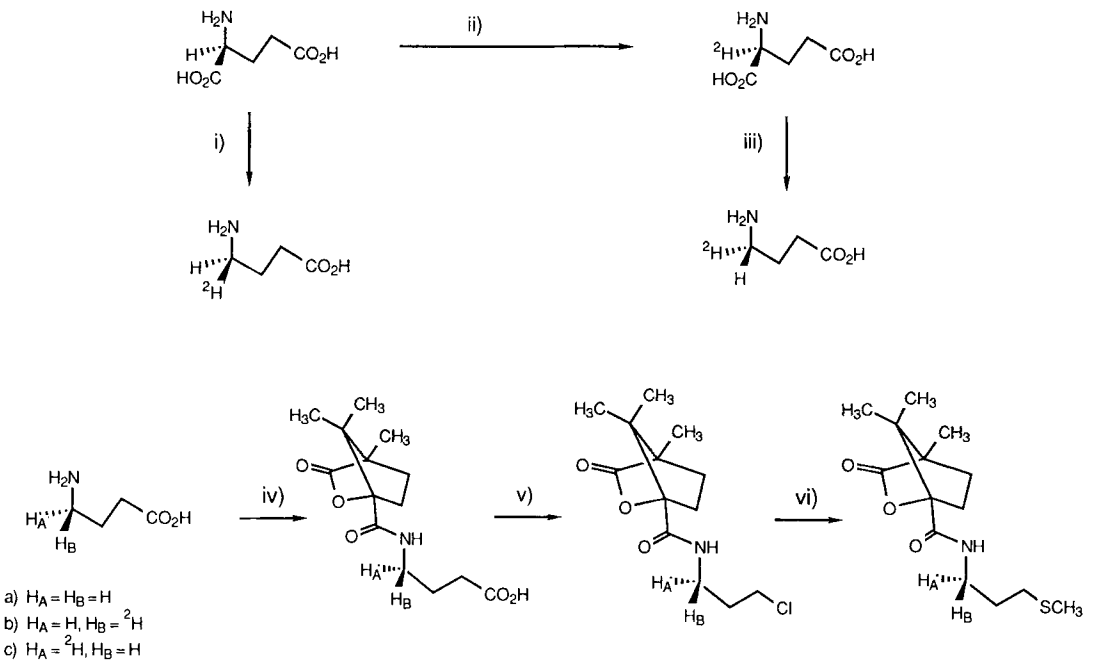
The primary structure of chicken heart mitochondrial enzyme was determined by Graf-Hausner et al.[33]. The sequences of several other aspartate aminotransferase[11,12,22,37,45,47,59] enzymes have been determined (see table 1 for residues adjacent to the internal aldimine, Lys-258) and these show almost 100% homology for the regions of the protein which correspond to substrate or coenzyme binding residues of the chicken mitochondrial enzyme, for which X-ray data are available[43]. Note: X-ray data is now also available for the other AAT enzymes, the derived structures are very similar[6,61].

α-Amino acid decarboxylases

The amount of stereochemical, mechanistic and structural information available for the decarboxylase group was sparse at the start of our studies. However, the active-site peptide sequences for some decarboxylases were known, table 2, and the stereochemical courses of decarboxylation had been reported for some systems[27,31]. From these studies it was evident that, in general, PLP-dependent carboxylases catalyse the decarboxylation of L-amino acid substrates with retention of configuration at C^{α}. However, α,ω-*meso*-diaminopimelate decarboxylase from two different species was shown to catalyse decarboxylation of the D-amino acid centre of the *meso*-substrate with inversion of configuration at C^{α} to give



Scheme 3



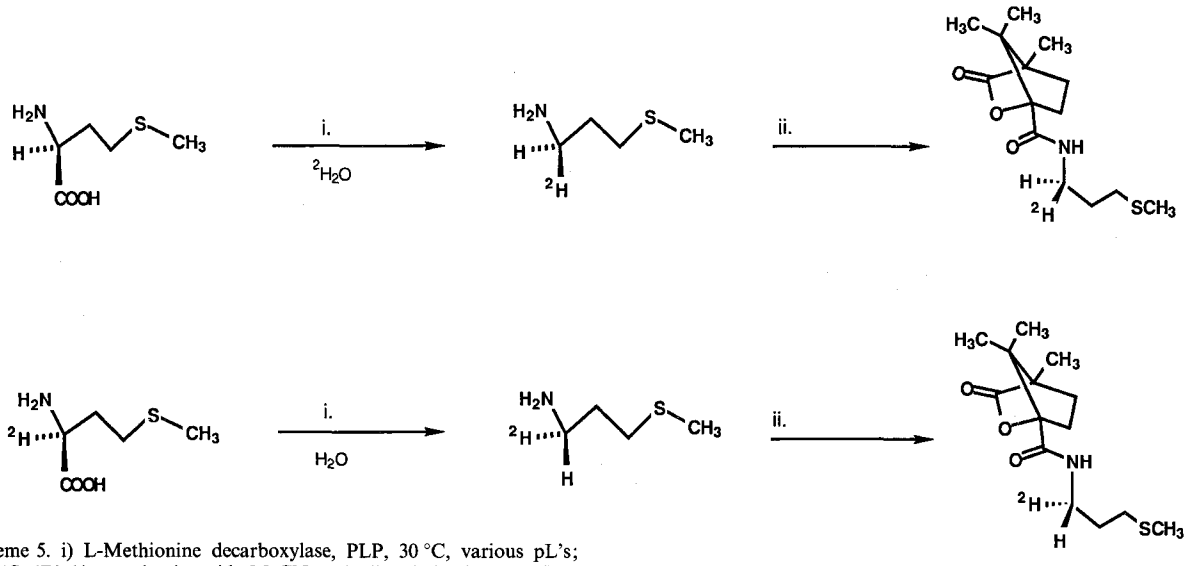
Scheme 4. Reagents: i) Glutamic acid decarboxylase, ^2H_2O; ii) Aspartate aminotransferase, ^2H_2O; iii) Glutamic acid decarboxylase, H_2O; iv) Camphanoyl chloride, $PhCH_3$, NaOH(aq); v) $Pb(OAc)_4$, LiCl, C_6H_6, reflux; vi) $NaSCH_3$, CH_3OH, reflux.

L-lysine[7,42]. Conversely, the aminomalonate decarboxylase activity of serine hydroxymethyltransferase was shown to catalyse apparently, non-stereospecific decarboxylation of stereospecifically labelled 2-aminomalonic acid to give racemic glycine[53]. Although it was not clear that only one binding mode was available to the substrate in its interaction with serine hydroxymethyltransferase, it was quite clear that the stereochemical course of PLP-dependent amino acid α-decarboxylations could not be assumed.

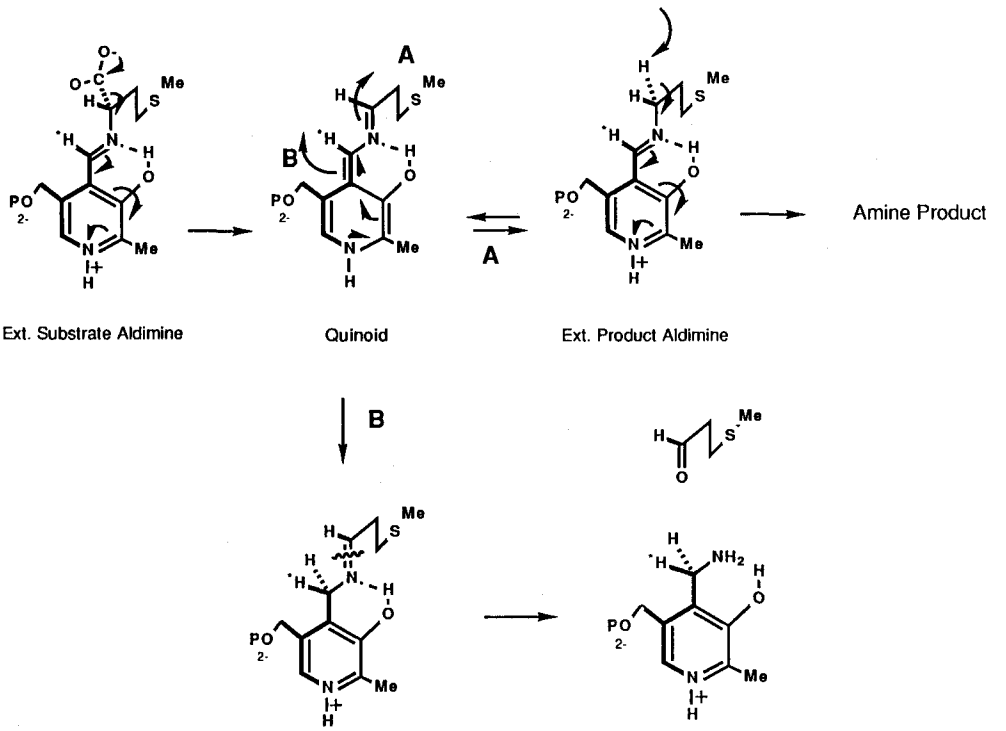
Transaminases appear to catalyse proton transfers on the C-4′-*si*-face of the coenzyme only, vide supra. Other PLP-dependent enzymes also utilise the C-4′-*si*-face of the coenzyme, including serine hydroxymethyltransferase[75]. However, the only reported study of the facial selectivity for a decarboxylase, *E. coli* glutamate decarboxylase, used a racemic non-physiological substrate, (2RS)-2-methylglutamic acid, to enhance the frequency of events leading to the formation of PMP via abortive decarboxylation-transamination[68]. The results of the study indicated that a proton was transferred to the C-4′-*si*-face of the coenzyme (scheme 3).

Unfortunately, it cannot be assumed that the same stereochemical course would have been followed in the much less frequent transamination reaction which occurs with the physiological substrate. Indeed, for *E. coli* glutamate decarboxylase there was some question as to whether the same or different proton-donating groups act upon the quinonoid intermediates derived from (2S)-glutamic acid and (2RS)-2-methylglutamic acid, during the formation of the respective amine products[76].

L-methionine decarboxylase from *Dryopteris filix-mas*[63] and *Streptomyces* sp.[64] were identified as excellent enzymes for chemical studies in view of their wide substrate structure tolerance and stabilities. Through the preparation of the (1S,4R)-camphanoyl derivatives of synthetic chirally deuteriated 3-methylthio-1-aminopropanes (scheme 4) it was possible to demonstrate that both en-



Scheme 5. i) L-Methionine decarboxylase, PLP, 30 °C, various pL's; ii) (1S, 4R)-(-)-camphanic acid, MeCN, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide, Et_3N.



Scheme 6

zymes catalyse the decarboxylation of methionine with retention of configuration at C^{α} (scheme 5)[63-65].

A number of straight chain and branched chain hydrophobic amino acids (six for the fern enzyme and three for the *Streptomyces* enzyme) were also decarboxylated with retention of configuration at C^{α} and it was noted that the reactions were completely stereospecific, even at extreme pH. For the fern enzyme it was further demonstrated, using [4'-^{3}H]-PLP, that the coenzyme was protonated from the C-4'-*si*-face of the coenzyme during an infrequent abortive decarboxylation transamination reaction promoted by the physiological substrate (scheme 6)[63]. Interestingly, PMP isolated from incubations performed in tritiated water containing the enzyme and unlabelled PLP contained very low levels of tritium.

The fact that the abortive reaction was essentially irreversible was established by showing that the inclusion of large excesses of each of the transamination products

(PMP and 3-methylthiopropionaldehyde) in incubations, separately and together, did not suppress the rate of the abortive reaction. It was also noted that the abortive reaction was promoted by the product amine, 3-methylthiopropylamine but, only at very high concentrations, indicating that the protonation at C^{α} was slightly reversible. Thus, the transamination reaction itself was irreversible, and stereochemically, the reaction was completely analogous to that catalysed by the bona fide transaminase enzymes. Indeed, by analogy with the bona fide transaminase enzymes, it seemed reasonable that the ε-ammonium side-chain of the active-site Lys residue, the residue involved in internal aldimine formation, should serve as the proton donor at the C-4′ position of the quinonoid intermediate. Nevertheless, this idea needed to be substantiated and the identity and position of the proton donor for C^{α} of the quinonoid intermediate needed to be established.

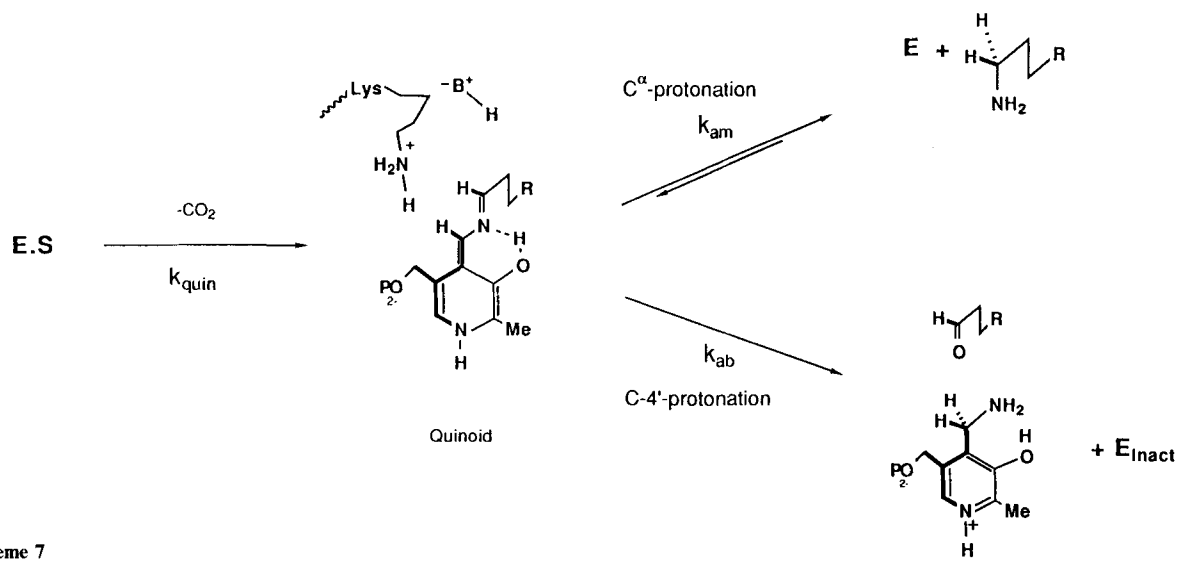
Identity and position of key functional groups

The abortive reaction occurs very infrequently compared to normal decarboxylation, approximately 10^4–10^5 times less often, and involves protonation at the C-4′ position of an intermediate, the quinonoid intermediate, which lies on the reaction coordinate profile for the normal reaction. Thus, the quinonoid partitions between protonation at C^{α} and C-4′, scheme 7.

From the known value of k_{cat} ($\sim 50\ s^{-1}$) for decarboxylation, and estimated k_{cat} for transamination ($\sim 0.001\ s^{-1}$, i.e. $3.6\ h^{-1}$) it was reasonable to propose that the rate limiting step in the transamination part of the reaction coordinate profile was the actual, essentially irreversible, transfer of a proton to C-4′, rather than extremely slow debinding of the transamination products[3]. Hence, it was reasonable to expect that k_{ab}, the first order rate constant for the proton transfer step, would determine k_{cat} for transamination and would possess low reaction commitments. Therefore, the transamination process should be expected to display large detectable isotope effects.

Very low levels of tritium were transferred from the solvent into PMP during the abortive transamination reaction catalysed by fern L-methionine decarboxylase, vide supra, ~2% of the tritium content of the buffer medium[63]. The result is consistent with the operation of a polyprotic proton donor at C-4′ of the quinonoid intermediate, possibly the ε-ammonium group of the lysine residue, since the transfer of tritium mediated by a monoprotic proton donor should reflect only the intrinsic tritium isotope effect and give ~10% incorporation, assuming $^1k/^3k = 10$.

The extent of tritium incorporation into the amine decarboxylation product, however, approximately matched the tritium content of the solvent. This result indicates that the transfer of a proton to the quinonoid intermediate at C^{α} is not rate-limiting for the overall decarboxylation reaction but, in isolation, does not reveal the nature of the proton donor. If the proton transfer step were followed by a slow step in the reaction coordinate profile, imposing a large reverse reaction commitment, the extent of tritium incorporation in the isolated product amine would reflect the tritium equilibrium isotope effect ($^3K_{eq}$) for the proton transfer. The value of $^3K_{eq}$ would be close to unity for any proton donor other than a cysteinyl thiol group[19]. If the proton transfer step were preceded by an irreversible step in the reaction coordinate profile, imposing a large forward reaction commitment, which is the case for decarboxylases[36], then the extent of tritium incorporation into the amine would also match the tritium content of the solvent; but, only for a monoprotic conjugate acid mechanism. Thus, the tritium incorporation result does not distinguish between the possibilities of mono- and polyprotic conjugate acids acting at C^{α} of the



Scheme 7

quinonoid intermediate in the absence of a knowledge of the magnitude of the reaction commitments for protonation.

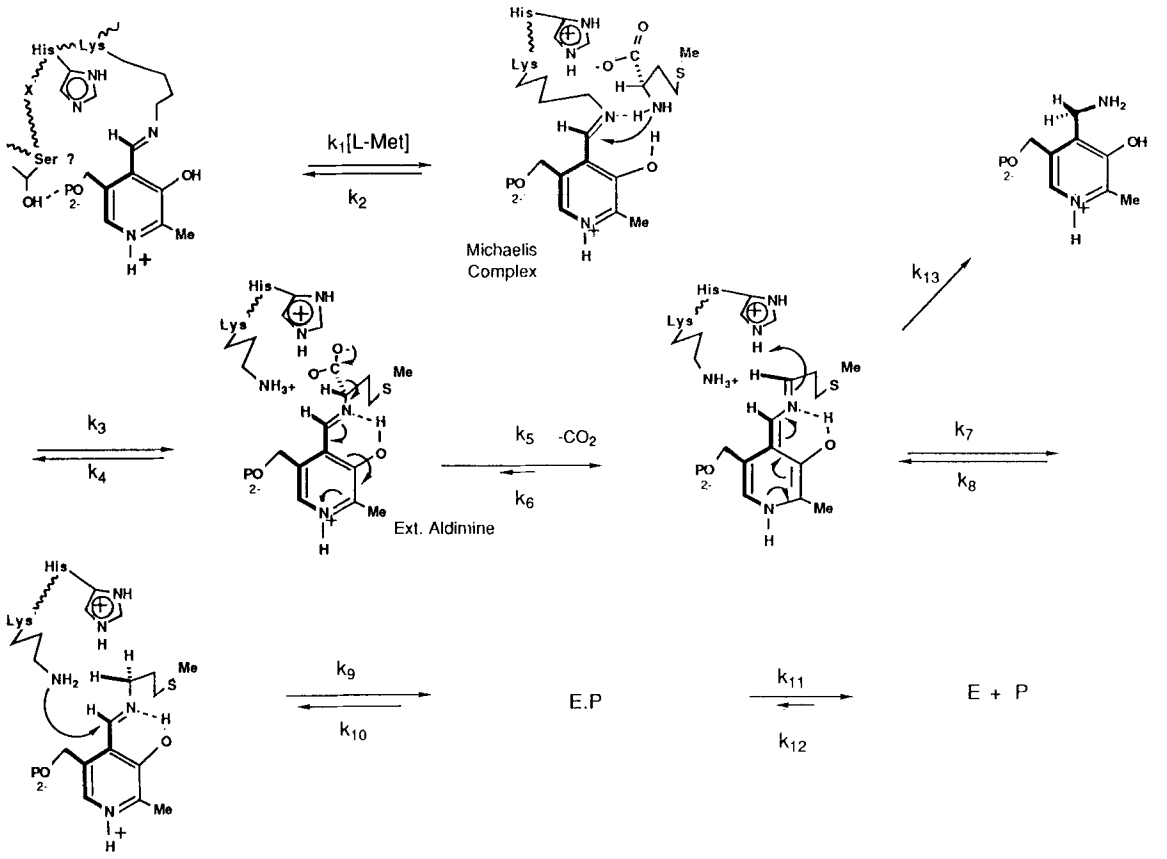
In order to determine the identity of the proton donor, the deuterium solvent isotope effects on V_{max} and V/K were determined for a range of substrates over the pH range 4.0–7.5. For L-methionine, ^{D}V varied from 2.3 at pL 5.5 to 1.1 at pL 6.0 and above while $^{D}(V/K)$ remained large and significant (~ 6) across the pL range of the study[3]. The actual value of V_{max} decreased by a factor of ~ 25 between pH 5.0 and 6.5 and titrated at $\sim$pH 5.1. Since V/K isotope effects are not influenced by events which occur after the first irreversible step, carbon dioxide desorption in this case, it appears that kinetically important steps possessing deuterium-sensitive transition states occur prior to the formation of the quinonoid intermediate. These steps must represent the substrate transaldimination process. The titration of V_{max} at $\sim$pH 5.1 together with the behaviour of ^{D}V is most consistent with a situation in which substrate transamination and $C-CO_2^-$ bond cleavage are both rate limiting and both require a protonated form of the coenzyme. If the pyridinium heteroatom is not associated with an ω-carboxyl group of an aspartate or glutamate residue, as it is in AAT[43], the titration would be expected to occur at $\sim$pL 5.1. Above pL 5.1, V_{max} would be governed by events which occur before quinonoid formation and thus, the value of ^{D}V would provide no information on the relative importance of transition states in the post-decarboxylation part of the reaction co-ordinate profile.

An interesting finding, that the pH profile of the partition ratio (decarboxylation events/transamination events) matched that for V/K, prompted us to examine the solvent isotope effects for the protonations at C^{α} and C-4′. The result was particularly interesting because the similar pH dependencies cannot arise from the same chemical event. The pH dependency of V/K can only report on pre-decarboxylation events, vide supra, while the partition ratio for an intermediate which has a low steady state concentration (this is implicit from the pH-dependent behaviour of V_{max}) can only report upon events which occur after the formation of the intermediate (i.e. on the rates of the protonations at C-4′ and C^{α} and the subsequent reactions leading to the regeneration of free enzyme). The most striking feature in each profile occurred at pH 6.2, corresponding to an increase in V/K and a decrease in the frequency of abortive events with increasing pH. Given there are good reasons to believe that only the proton transfer step itself is important in the transamination reaction, vide supra, the step should show a large solvent isotope effect. Also, since the value of ^{D}V is small across the pL range of the study, it is evident that there are no proton transfer steps in the post-decarboxylation part of the reaction which limit the overall rate of the decarboxylation. Thus, the pL dependencies of the solvent isotope effects on the two protonations should directly provide information on the post-decarboxylation part of the reaction coordinate profile for the decarboxylation reaction. Furthermore, the profiles should allow the molecular acid pK_a values of the proton donors to be estimated, if the proton donors for C^{α} and C-4′ of the quinonoid intermediate are, indeed, different species and titrations are observable.

The solvent deuterium isotope effects for the transamination reaction and the partition ratio were determined over the pL range 5.0 to 7.0[3]. The observed values for the transamination reaction were smaller than predicted on the basis of the tritium isotope effect alone, vide supra. This is the expected result for a system which contains deuterium-sensitive transition states in the post-decarboxylation part of the reaction coordinate profile which retard the formation of free enzyme and normal decarboxylation products in favour of the abortive reaction and thus suppress the real value of the isotope effect for protonation at C-4′ of the quinonoid intermediate. Note, these deuterium-sensitive steps do not need to be slow enough to affect the overall decarboxylation rate. The isotope effect for transamination titrated at pL 6.2 changing from 1.7 to 0.7, corresponding to an increase in the frequency of the abortive reaction in heavy compared to light water with increasing pL. The isotope effect for the partition ratio increased sharply from 0.6 to 1.7 at pL 6.2 corresponding to a decrease in the partition ratio in heavy water with increasing pL. These results indicate that the proton donors for C^{α} and C-4′ of the quinonoid intermediate are distinct, that protonation at C^{α} is kinetically significant in the post-decarboxylation part of the reaction coordinate profile, that the acid donor for C^{α} possesses a molecular pK_a of 6.2, and that in protium oxide, a base possessing a pK_a of 6.2 is required to facilitate the conversion of the quinonoid to decarboxylation products. In the light of the analysis of the similar profile for the pH dependence of V/K, it seems likely that the base is also involved in base catalysis during product aldimine transaldimination by the lysine residue. Clearly the imidazole side-chain of a single histidine residue, disposed on the 4′-*si*-face of the coenzyme, could fulfil all of these roles.

Incubations using methionine conducted in 50 atom % deuterium oxide solution at pL 4.5, 6.0 and 7.0 gave partially deuteriated 3-methylthiopropylamine products. In each case the deuterium content at the 1-*pro*-R position was $50 \pm 5\%$. Since the reverse reaction commitment to protonation at C^{α} of the quinonoid intermediate was now known to be small (see discussion above on the possibility of observing an equilibrium isotope effect), the result indicates that a monoprotic conjugate acid operates at C^{α}. Together with the observed pK_a value of 6.2 for the conjugate acid, the evidence for the operation of a histidine residue at C^{α} seemed quite compelling (scheme 8)[3].

In order to test the generality of the observation, a similar stereochemical and kinetic analysis was performed with *E. coli* glutamate decarboxylase using L-glutamic



Scheme 8

acid as the substrate. Again, as for the fern enzyme, it was observed that a proton was transferred to the C-4′ *si*-face of the coenzyme during the abortive transamination reaction [63], in accord with the result for bona fide transaminase enzymes. The finding is analogous to that obtained by Sukhareva [68] with the racemic substrate analogue 2-methylglutamic acid and suggests that the same proton donor operates at C^{α} of the quinonoid intermediate derived from both substrates vide supra. Since it was already established that borohydride reduction of the holoenzyme gave a pyridoxyllysine peptide [67], it appeared that the proton donor was the ε-ammonium group of the lysine residue.

For the *E. coli* enzyme, the kinetic analysis was hampered because subunit dissociation occurs above pH 6.0 and the kinetic parameters do not show sharp titrations below this pH [28, 50]. Nevertheless, it was possible to demonstrate that the large isotope effect of 5.0 observed for ^{D}V was due, in part, to partially rate-limiting protonation of the quinonoid intermediate at C^{α}. Note: $^{D}(V/K)$ is 2.6 and that our (Tilley and Gani, in preparation) analysis of the origins of the solvent isotope effects (based upon theory developed in ref. 3) is different to that reported by O'Leary [1, 50]. In O'Leary's treatment it is assumed that steps subsequent to decarboxylation are not kinetically important.

The finding that C^{α} protonation is partially rate limiting, together with O'Leary's reported observation, which showed that the incorporation of deuterium into the decarboxylation product, γ-aminobutyric acid, matched that available in partially deuteriated solvents [51], is in accord with the operation of a monoprotic conjugate acid. Thus, for fern methionine decarboxylase and *E. coli* glutamate decarboxylase it appears that lysyl ε-ammonium and histidyl imidazolium groups are disposed on the 4′-*si*-face of the coenzyme. Furthermore, it appears that these conjugate acids are responsible for the specific protonation of the quinonoid intermediate at C-4′ and C^{α}, respectively.

In view of the similarities, the reported active-site peptide sequences for PLP-dependent decarboxylases were examined.

Comparison of the active-site peptides of PLP-enzymes

In order to attempt to relate other groups of PLP-dependent enzymes, notably the α-decarboxylases, including SHMT, to the transaminases, and specifically to AAT, the X-ray crystal structures of the active-site of chicken mitochondrial AAT were scrutinised [43]. The active-site sequences of seven other AAT enzymes from a diverse range of species were then aligned with the regions of

Table 1. Schiff's base forming region of aspartate aminotransferase enzymes

E. coli[45]	I	V	A	S	S	Y	S	**K**	N	F	G	L	Y
Chicken, Mitochondrial[33]	V	L	S	Q	S	Y	A	**K**	N	M	G	L	Y
Turkey, Mitochondrial[11]	V	L	S	Q	S	Y	A	**K**	N	M	G	L	Y
Pig, Mitochondrial[12]	C	L	C	Q	S	Y	A	**K**	N	M	G	L	Y
Rat, Mitochondrial[37]	C	L	C	Q	S	Y	A	**K**	N	M	G	L	Y
Human, Mitochondrial[47]	C	L	C	Q	S	Y	A	**K**	N	M	G	L	Y
Chicken, Cytosolic[59]	F	C	A	Q	S	F	S	**K**	N	F	G	L	Y
Pig, Cytosolic[22]	F	C	A	Q	S	F	S	**K**	N	F	G	L	Y

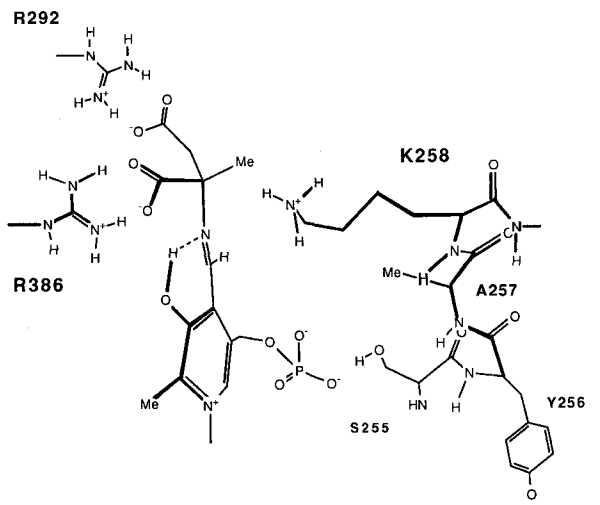


Figure 1. The active-site structure of the external aldimine form of chicken mitochondrial AAT with the inhibitor (2S)-2-methylaspartic acid showing the positions of the S-Y-A-K tetrapeptide side chains and the arginine residues which bind to the carboxylate groups of the substrate[43]. Note, in particular, the position of the side chain of Ala-257. This structure was used as the base set for further modelling.

chicken mitochondrial AAT which were either involved in binding to the substrate or to the coenzyme, or in catalysing specific chemical steps. Each residue deemed to be functionally important was completely conserved, vide supra.

In order to make a comparison with other enzyme types, the only known common property, the ability of the enzymes to bind to PLP was used as a starting point. Analysis of the regions of AAT which interact with the coenzyme indicated that a tetrapeptide unit -S-X-X-K- contained two completely conserved residues, a serine (S255) which forms a side-chain H-bond with the 5′-phosphate ester group of PLP, and the active-site lysine (K258) which forms the C-4′ internal aldimine with the coenzyme and which also serves to shuttle protons between C^{α} and C-4′ (table 1, fig. 1).

An examination of all of the available active-site sequences for PLP-dependent enzymes was then undertaken and, in many, the residue equivalent to Ser-255 in AAT was a Ser, Asn or a Thr residue, all H-bond donors. In particular the active-site tetrapeptides for decarboxylases were well conserved (table 2), and contained the sequence -S(N,T)-X-H-K-, unless there were rationalisable mechanistic reasons for why they should not be conserved. For example, all decarboxylases which are known to catalyse the decarboxylation of α-amino acids of the (2S)-absolute configuration are known to do so with retention of stereochemistry. These include glutamate, lysine, methionine, ornithine, arginine, histidine and dopa decarboxylase, some from more than one source. Moreover, for each of these enzymes for which the active-site peptide sequence had been determined, the His and Lys residues were completely conserved (table 2). For the others L-methionine decarboxylase from fern[63] and from *Streptomyces*[64] and also for some where the peptide sequence was known, for example, *E. coli* glutamate decarboxylase (see discussion above) and DOPA decarboxylase[21], there was chemical evidence to indicate a catalytic role for a His residue, vide infra. Indeed, within this group there were no reported examples of any exceptions to the consensus sequence. On the other hand *meso*-α,ω-diaminopimelate decarboxylase[66] (which catalyses the decarboxylation at the (2R)-centre of its substrate with inversion of configuration[7,42]) and mouse[34,40], rat[74], trypsanoma[54] and yeast[29] ornithine decarboxylases, some of which are the most unstable enzymes known, did not contain a histidine residue adjacent to a lysine residue. Thus, it appeared that for the L-α-amino acid decarboxylases which operate in a retentive mode, the Ser, Asn or Thr residue might hydrogen bond to the 5′-phosphate ester group of the coenzyme, as in AAT, and that the His residue might be the catalytically important residue which is involved in C^{α} quinonoid protonation. Note that the adjacent conserved Lys forms an aldimine with the coenzyme in each case and that, in general, sequence information was obtained on proteolytic pyridoxyllysine peptides after reduction with cyanoborotritide (see Morino and Nagashima[49] for a review of the earlier work).

Table 2. Schiff's base forming region of PLP-dependent decarboxylase enzymes

Arginine (*E. coli*)[15]		A	T	H	S	T	H	**K**	L	L	N	A	L
Glutamate (*E. coli*)[67]	S	I	S	A	S	G	H	**K**	F				
Histidine (*Morganella morganii*)[73]	S	I	G	V	S	G	H	**K**	M	I	G	S	P
Lysine (*E. coli*)[56]	Y	E	T	E	S	T	H	**K**	L	L	A	A	F
Lysine (*Hafnia alvei*)[26]	Y	E	T	Q	S	T	H	**K**	L	L	A	A	F
Ornithine (*E. coli*)[5]						V	H	**K**	Q	Q	A	G	Q
Dopa (*Drosophila*)[25]	S	F	N	F	N	P	H	**K**	W	M	L	V	N
Dopa (Pig)[16]			N	F	N	P	H	**K**	W				
Glutamate (Feline)[44]	S	V	T	W	N	P	H	**K**	M	M	G	V	L
Glycine (Chicken)[30]	V	S	H	L	N	L	H	**K**	T	F	C	I	P
SHMT (*E. coli*)[55]	V	V	T	T	T	T	H	**K**	T	L	A	G	P
SHMT (Rabbit, Cytosolic)[13]	V	V	T	T	T	T	H	**K**	T	L	R	G	C
SHMT (Rabbit, Mitochondrial)[13]	V	V	T	T	T	T	H	**K**	T	L	R		

Given that the conformation of the homologous tetrapeptide section of each enzyme might be similar, it was instructive to examine independent data regarding the location, nature and identity of catalytically important

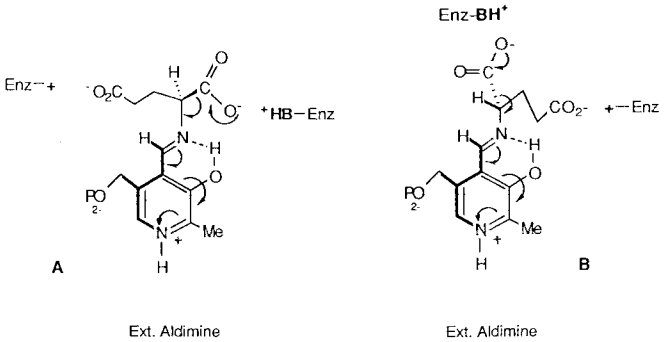
residues at the active site of decarboxylases. Studies of the extent of solvent deuterium incorporation into the product amine in incubations conducted in partially deuteriated water have indicated that a monoprotic acid serves to protonate the quinonoid intermediate at C^{α} in a number of decarboxylase systems including *E. coli* glutamate decarboxylase [51] (also see discussion above), and fern [63] and *Streptomyces* [64] methionine decarboxylase. Histidine modifying reagents react with PLP-dependent decarboxylases in the absence of substrate to give inactive proteins. For example, diethyl pyrocarbonate inactivated DOPA decarboxylase possesses an unaltered binding affinity for its substrates suggesting that the modified His residue is catalytically essential but, not important in the formation of the Michaelis complex [21]. Recent studies with *E. coli* L-glutamic acid decarboxylase have led to the same conclusion [48].

An alternative approach, using the pH-dependence of the partition ratio between normal decarboxylation and abortive transamination events has revealed that for fern L-methionine decarboxylase [3] and for *E. coli* glutamate decarboxylase, acids of different pK_a serve to protonate the quinonoid at C^{α} and C-4′, vide supra. For the fern enzyme the molecular acid dissociation constant for the most acidic acid, which operates at C^{α}, is within the expected range ($pK_a \sim 6.2$) for the protonated imidazolium side chain of a His residue. The pK_a of the other acid is greater than 7.5 (the high pH limit of the study) which is consistent with the expected value for the ε-ammonium group of Lys. Indeed, the protonation at C-4′ of the quinonoid by the Lys ε-ammonium group during abortive transamination is completely analogous to the normal course of events for bona fide transaminases and the stereochemical courses of the two processes are identical and involve protonation from the C-4′-*si*-face of the quinonoid intermediate, see discussion above. Thus, there are excellent reasons to believe that the tetrapeptide fragment -S(N,T)-X-H-K- of the decarboxylases contains the two catalytically important residues, from independent experimental data.

Location of distal substrate binding groups

The final part of the analysis concerns the position of the distal substrate binding groups for decarboxylases relative to those for the transaminases.

Typical PLP-dependent decarboxylases catalyse the decarboxylation of L-amino acids with retention of configuration at C^{α}. In deuterium oxide the products are (1R)[$^2H_1$]-deuteriated amines. The side chain of the substrate in decarboxylases could bind to a similarly positioned distal site as for transaminases, e.g. Arg-292, which binds the β- and γ-carboxylate groups of aspartate and glutamate in AAT, which would place the α-carboxyl group of the L-antipode of a substrate on the 4′-*re*-face of the coenzyme, or in an alternative mode which would place the carboxyl group of the 4′-*si*-face. The possible



Scheme 9. Possible binding modes for (2S)-glutamic acid at the active-site of glutamate decarboxylase. *A* Similar to the binding mode of AAT with the α-carboxylate group on the 4′-*re*-face of the coenzyme. *B* Alternative binding mode, opposite to that for AAT, with the α-carboxylate group positioned on the 4′-*si*-face and the γ-carboxylate group positioned on the 3′-phenol side of the coenzyme.

situations are depicted for L-glutamate decarboxylase which binds to the same substrate as AAT, L-glutamic acid (Scheme 9).

Given that abortive transamination occurs via proton transfer to the C-4′-*si*-face of the quinonoid and that the proton donor is the lysine which forms an internal aldimine at C-4′ of the coenzyme, it should be able to reach the C^{α} position of the quinonoid to provide a proton quite easily. If the other residue, histidine, were positioned on the 4′-*re*-face, it might be expected that at high pH, when the imidazole side chain is neutral, the stereospecificity of the protonation would be reduced through protonation by the Lys residue, especially when the quinonoid is composed of loose-fit or strained substrates. L-Methionine decarboxylase from two different sources shows complete stereospecificity in the protonation at C^{α} of the quinonoid intermediate with a range of substrates even at pH 7.0 [63]. There is also evidence for fern methionine decarboxylase to indicate that the His residue functions (in addition) as a base catalyst in the deprotonation of ammonium groups immediately prior to transaldimination reactions [3]. Both of these results indicate that the His residue is located on the 4′-*si*-face of the coenzyme and, furthermore, that decarboxylases possess a distal binding group for the side chain of their substrates which is located on the 3′-phenol side of the coenzyme ring. This is opposite to the situation for transaminases, Arg-292 is on the 5′-phosphohydroxymethyl side of the coenzyme [43]. Also note that for normal ω-transaminases (those which also act upon L-glutamic acid), the enzymes catalyse the exchange of the 1-*pro*-S hydrogen of amine substrates with the solvent to give (1S)[$^2H_1$]-deuteriated amines [2, 17, 18, 69].

In order to check the validity of the arguments presented above, some simple modelling experiments were performed. The theory was that if our reasoning was correct, minor changes to the active-site structure for the external aldimine of AAT would give the correct disposition of catalytically functional groups and the correct external

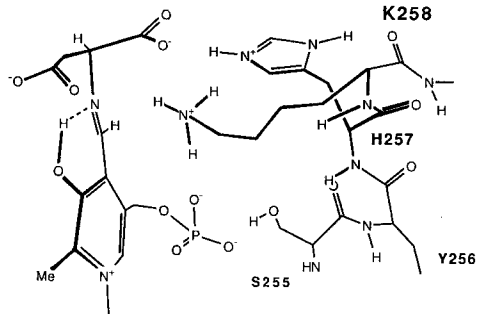


Figure 2. Result of the replacement of the methyl group of Ala-257 by an imidazolium group, and rotation about the C^{α}-N bond by 120°, and removal of the arginine residues starting with the structure in fig. 1. This conformation allows the formation of a hydrogen bond between with the α-carboxylate group of the substrate and the N^3-proton of the nascent His-257 residue.

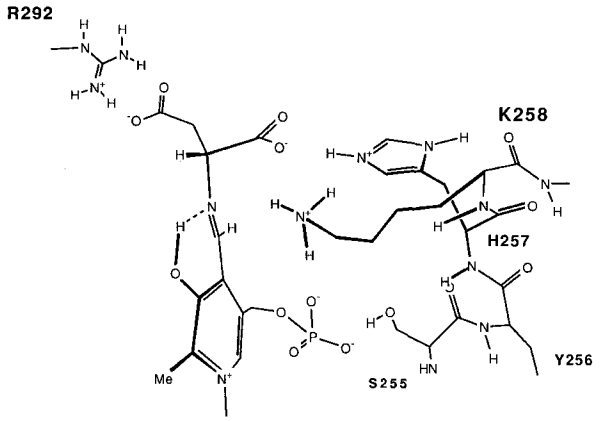


Figure 3. Result of inverting the chiral centre at C-2 of the substrate [so that it is (2R)-] and replacing the distal carboxylate binding group, Arg-292. This structure is essentially that expected for a D-amino acid decarboxylase.

aldimine conformation for a decarboxylase capable of satisfying all of the known catalytic, stereochemical and mechanistic requirements.

Accordingly, the coordinates for the tetrapeptide -S-Y-A-K- and the substrate-coenzyme aldimine were excised from the X-ray crystal structure of chicken mitochondrial AAT (fig. 1)[43]. Without changing any distances or torsional angles in the tetrapeptide, the C^{α}-N bond of the substrate-aldimine was rotated at 120° so that the α-carboxyl group of the substrate was on the 4′-*si*-face of the coenzyme at the angle required for maximum stereoelectronic assistance[23]. Without any further change, the position of Ala257 was examined. The side chain pointed directly towards the α-carboxylate group of the substrate. The Ala residue, in keeping with the known sequences for many decarboxylases, vida supra, was then altered by modelling to a His and the interactions were energy minimised[62] and then examined (fig. 2). The α-carboxylate group formed an H-bond with the ε-N-atom of the His residue and the entire system appeared to be optimally set up to catalyse decarboxylation.

From this analysis it is expected that the His residue does not serve as a catalytic base in the decarboxylation step but, in its protonated form, ensures that the conformation of the C^{α}-N bond is close to 90° to the plane of the conjugated π-electron system[23]. Following decarboxylation and generation of the quinonoid intermediate the imidazolium side chain then protonates the quinonoid from C-4′-*si*-face at C^{α} to give the product aldimine.

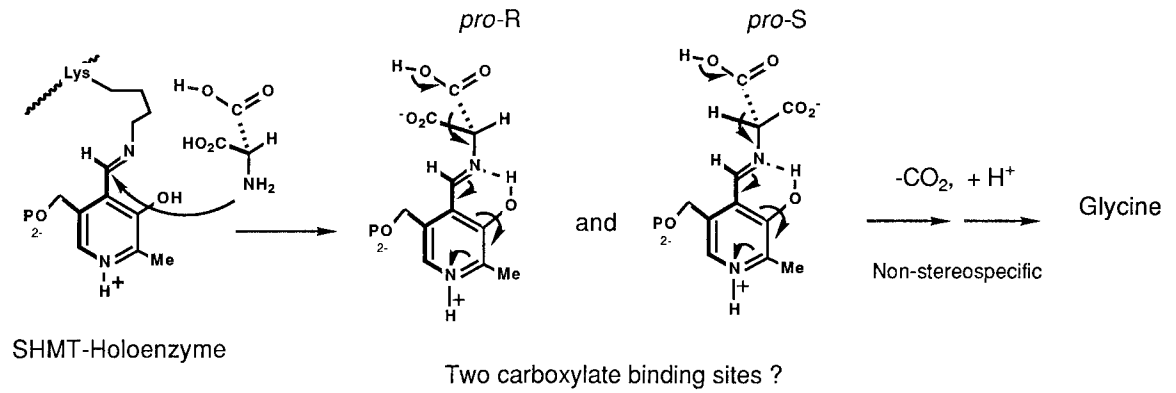
The findings reviewed and presented here allow a very detailed comparison of PLP-dependent enzymes and the rationalisation of the stereochemical courses of most of the reactions which have been studied to date but, not those for SHMT, vide infra. The major difference between transaminases and decarboxylases appears to be the conformation of the substrate aldimine C^{α}-N bond which is controlled by side chain binding and, in decarboxylases, by the presence of the positively charged imidazolium side chain of a His residue which interacts electrostatically with the α-carboxylate group. Our cumulative results indicate that His serves two other functions; as a base in the deprotonation of the ε-ammonium group of the Lys residue immediately prior to transaldimination reactions, and; as a proton donor for the quinonoid intermediate at C^{α}. These findings are completely in accord with the Dunathan postulate[23] and indicate that it should be possible to design new decarboxylases starting from AAT (fig. 3). Researches directed towards these goals are presently underway.

Serine hydroxymethyltransferase

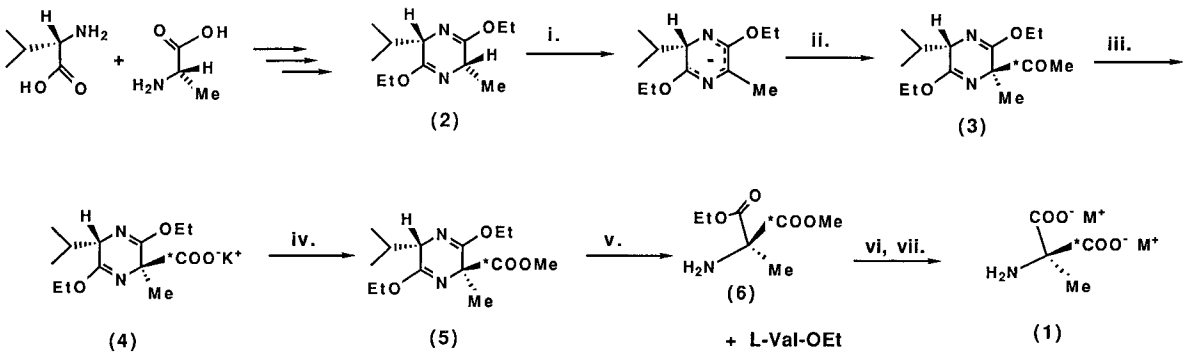
Serine hydroxymethyltransferase (SHMT) is a ubiquitous pyridoxal 5′-phosphate dependent enzyme which catalyzes the retro-aldol cleavage of L-serine to give glycine and formaldehyde[14]. The enzyme is unusual in that it shows a low regard for reactiontype specificity with α-amino acid substrates and is able to catalyse aldol/retro-aldol, transamination and decarboxylation reactions with the appropriate substrates. In addition, the enzyme catalyses many of these reactions non-stereospecifically[32, 58].

In 1973 Palekar, Tate and Meister showed that the decarboxylation of aminomalonic acid by SHMT in incubations conducted in tritiated water gave both (2R)- and (2S)- tritiated glycine[53]. Furthermore, studies with specific carboxyl-labelled [^{14}C]-aminomalonate confirmed that the enzyme decarboxylated the substrate in a non-stereospecific manner. The result was particularly interesting because it was the only reported example of a non-stereospecific decarboxylation catalysed by a PLP-dependent enzyme[32].

In order to explain the apparent lack of stereospecificity, two possible mechanistic courses can be invoked. In the first, the scheme originally proposed by Palekar et al.[53], it was envisaged that the aminomalonate substrate was able to bind in two conformations at the active-site of the enzyme, such that each of the two carboxyl groups could



Scheme 10



Scheme 11. i) n-BuLi,-80 °C, THF; ii) Me*COCl, – 80 °C, 2 h; iii) KOCl/NaOH, 4 h, Aqueous dioxan; iv) MeI, THF, 12 h; v) 0.01 M HCl, 6 h; vi) 2 M NaOH, 60 °C, 10 min; vii) Amberlite IR-120(H^+).

be positioned correctly for decarboxylation. If each conformer was equally populated and, the decarboxylation and subsequent protonation steps occurred stereospecifically for each form, then apparent non-stereospecific decarboxylation would have been observed (scheme 10).

On the other hand, if the enzyme was able to catalyse the racemisation of the substrate prior to decarboxylation, the same observations might have been expected. However, the latter scenario, prior racemisation, may have seemed unlikely to Palekar et al.[53] in view of their earlier finding that another PLP-dependent enzyme, aspartate β-decarboxylase, catalysed the stereospecific decarboxylation of aminomalonate, albeit at a much lower pH[52].

In order to unravel the mechanistic and stereochemical ambiguities, a new substrate was sought, preferably one which could not racemise.

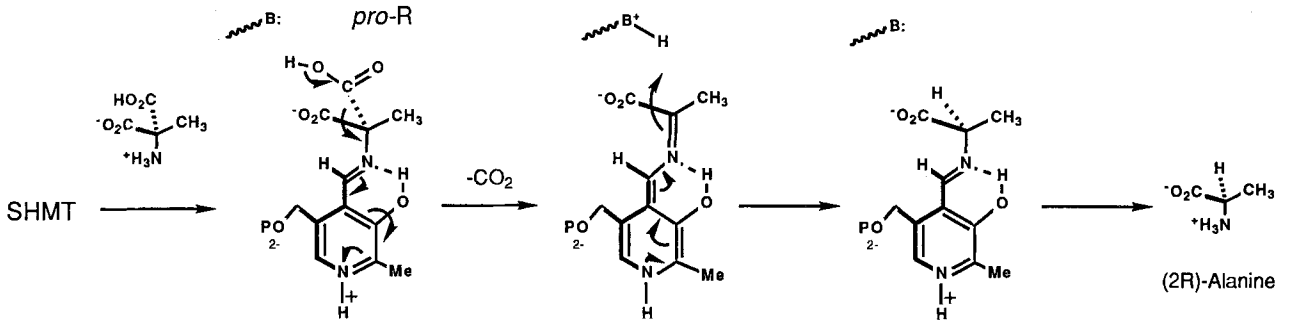
2-Amino-2-methylmalonic acid was prepared by the method of Bailey et al.[10] and was tested as a substrate for cytosolic rabbit liver[57] SHMT. At pH 7.5 significant enzymic decarboxylation occurred relative to control incubations containing PLP but, no enzyme. The formation of alanine was detected by thin layer chromatography, and the product was isolated, and characterised by ^{1}H-nmr spectroscopy[70].

The absolute stereochemistry of the alanine was determined by incubating aliquots of the reaction solution at various time intervals with enzyme cocktails containing either D-amino acid oxidase and lactate dehydrogenase or L-alanine dehydrogenase[13]. Analysis revealed that only (2R)-alanine was formed initially (note: SHMT is able to catalyze the racemisation of (2R)-alanine upon prolonged incubation[60]). Hence, one stereochemical aspect of the decarboxylation had been solved, and whichever carboxyl group was lost, the resulting quinonoid intermediate was protonated from the *si*-face at C^α to give (2R)-alanine.

To facilitate experiments to determine whether the cleavage of a unique carboxyl group or the cleavage of both carboxyl groups could give rise to a quinonoid intermediate a synthesis of the enantiomers of [1-^{13}C]-2-amino-2-methylmalonic acid (1) was devised[70,71].

To synthesise the (S)-enantiomer, the Schollkopf *bis*-lactim ether (2) was prepared from L-valine and L-alanine and then further reacted as outlined in scheme 11. The (R)-enantiomer was prepared in a similar manner, starting from the *bis*-lactim ether derived from D-valine.

The labelled chiral aminomalonates were each incubated with SHMT and PLP at pH 7.5 and the resulting alanines were isolated. Examination of the products by ^{1}H- and ^{13}C-nmr spectroscopy indicated that the (2R)-2-amino-2-methylmalonate gave unlabelled alanine (δ_H 1.21 ppm, d, $J_{H\text{-}2,3}$ = 6.8 Hz, in D_2O at pH 10) while the



Scheme 12

(2S)-enantiomer gave [1-^{13}C]-alanine (δ_H 1.21 ppm, dd, $J_{H\text{-}2,3}$ = 6.8 Hz, $J_{H\text{-}3,C\text{-}1}$ = 4 Hz, in D_2O at pH 10). Thus, the *pro*-R carboxyl group of the substrate was lost during the decarboxylation. Together with the finding that D-alanine was the decarboxylation product, it was evident that replacement of the *pro*-R carboxyl group by a proton occurs with retention of configuration [70]. The same stereochemical course was observed for the *E. coli* enzyme (scheme 12).

Interestingly, the *pro*-R carboxyl group of 2-amino-2-methylmalonic acid is expected to occupy the same position at the active-site of the enzyme as the hydroxymethyl group of the physiological substrate, L-serine, in complete accord with the Dunathan postulate [23] and our model for the active-site of PLP-dependent α-decarboxylase enzymes.

In conclusion, our work with SHMT indicated that Palekar, Tate and Meister's findings [53] are best rationalised in terms of the prior enzymic or non-enzymic racemisation of the 2-aminomalonic acid. Indeed, in our hands, glycine isolated from the incubation of cytosolic SHMT with 2-aminomalonic acid at pL 6.0 in deuterium oxide contained > 1.8 equivalents of deuterium at the C-2 position. Examination of the ^{1}H- and ^{2}H-nmr spectra of the camphanamide derivatives revealed that the deuterium was evenly distributed between the 2-*pro*-R and 2-*pro*-S positions of the glycine, implying that racemisation occurs before decarboxylation. In accord with this conclusion, control experiments which were performed under identical conditions but, which contained no enzyme, showed that the exchange of protium from the substrate was very rapid. At pL 6.0, the half life for exchange was ~10 minutes. Thus, there is no evidence to suggest that SHMT catalyses non-stereospecific decarboxylation for any substrate***. The implication of these findings in the light of the model for the active-site of α-decarboxylases is that the conserved His residue in the active-site peptide (see table 2) of SHMT should serve as an H-bond donor for the β-hydroxyl group in the retro-aldol cleavage of L-serine. The high level of activity observed for the cleavage of serine catalysed by an *E. coli* His-Asn mutant form of SHMT [60] is in accord with the notion that the side chain of the histidine should be protonated and should not serve as a base. Extensive further study of SHMT will be required to rationalise the kinetic influence and mechanistic role of the formaldehyde acceptor, tetrahydrofolic acid, in the physiological reaction.

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* Author for correspondence

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*** Since this review was written, the stereochemical course of the SHMT-mediated decarboxylation of 2-aminomalonic acid was determined to proceed with retention of configuration; see Thomas, N. R., Rose, J. E., and Gani, D., J. chem. Soc. chem. Commun. (1991) 908–909.

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Stereochemistry of enzymic processes in the biosynthesis of pyrrolizidine alkaloids

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Abstract. The harmonization of biosynthetic pathways with organic reaction mechanisms has relied heavily on stereochemical studies. The field of biosynthesis of pyrrolizidine alkaloids exemplifies these connections through a wide range of common organic reactions including oxidation, condensation, and decarboxylation. Further, the applications of biogenetic concepts and enzyme-catalysed reactions to synthesis are illustrated. The results are exciting, not only for their intrinsic scientific interest, but because they point the way to using plant enzymes to recognise structurally modified biosynthetic intermediates and hence open routes to the synthesis of new compounds that would otherwise be difficult to obtain.
Key words. Pyrrolizidine alkaloid; biosynthesis; stereochemistry; retronecine; rosmarinecine; diamine oxidase.

Introduction

Pyrrolizidine alkaloids are important natural products because of their widespread occurrence (e.g. ragworts are *Senecio* spp., family Asteraceae) and because many are hepatotoxic[26, 30]. Many deaths to grazing animals have resulted from ingestion of pyrrolizidine alkaloids. The most toxic pyrrolizidine alkaloids are those that contain a 1,2-unsaturated pyrrolizidine diol (necine) such as retronecine (**1**). This is usually found joined to a dicarboxylic acid (necic acid) to form a macrocyclic dilactone as in retrorsine (**2**), present in *S. isatideus*. The alkaloids exhibit their major toxic effect in the liver, where they are oxidised to the corresponding pyrroles, which are bifunctional alkylating agents[18]. Alkaloids such as rosmarinine (**3**) from *S. pleistocephalus*, which contains the base portion rosmarinecine (**4**), are not hepatotoxic. Pyrrolizidine alkaloids are also implicated in human liver disease when they are eaten by accident when foodstuffs are contaminated, or by design when used as herbal remedies. A