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A Structural and Mechanistic Comparison of Pyridoxal 5<latex>$^{\prime}$</latex>-phosphate Dependent Decarboxylase and Transaminase Enzymes

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Phil. Trans. R. Soc. Lond. B 1991 **332**, 131-139
doi: 10.1098/rstb.1991.0041

A structural and mechanistic comparison of pyridoxal 5′-phosphate dependent decarboxylase and transaminase enzymes†

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SUMMARY

Stereochemical studies of three pyridoxal phosphate dependent decarboxylases and serine hydroxymethyltransferase have allowed the dispositions of conjugate acids that operate at the C^{α} and C-4′ positions of intermediate quinoids to be determined. Kinetic work with the decarboxylase group has determined that two different acids are involved, a monoprotic acid and a polyprotic acid. The use of solvent kinetic isotope effects allowed the resolution of chemical steps in the reaction coordinate profile for decarboxylation and abortive transamination and pH-sensitivities gave the molecular pK_a of the monoprotic base. Thus the ε-ammonium group of the internal aldimine-forming lysine residue operates at C-4′-*si*-face of the coenzyme and the imidazolium side chain of an active site histidine residue protonates at C^{α} from the 4′-*si*-face. Histidine serves two other functions, as a base in generating nitrogen nucleophiles during both transaldimination processes and as a binding group for the α-carboxyl group of substrates. The latter role for histidine was determined by comparison of the sequences for decarboxylase active site tetrapeptides (e.g. —S—X—H—K—) with that for aspartate aminotransferase (e.g. —S—X—A—K—) where it was known, from X-ray studies, that the serine and lysine residues interact with the coenzyme. By using the Dunathan Postulate, the conformation of the external aldimine was modified, and without changing the tetrapeptide conformation, the alanine residue was altered to a histidine. This model for the active site of a pyridoxal dependent decarboxylase was consistent with all available stereochemical and mechanistic data. A similar model for serine hydroxymethyltransferase suggested that previous reports of stereochemical infidelity with decarboxylation substrates were incorrect. A series of careful experiments confirmed this. Hence, no actual examples of non-stereospecific α-amino acid decarboxylation by pyridoxal enzymes exist.

1. INTRODUCTION

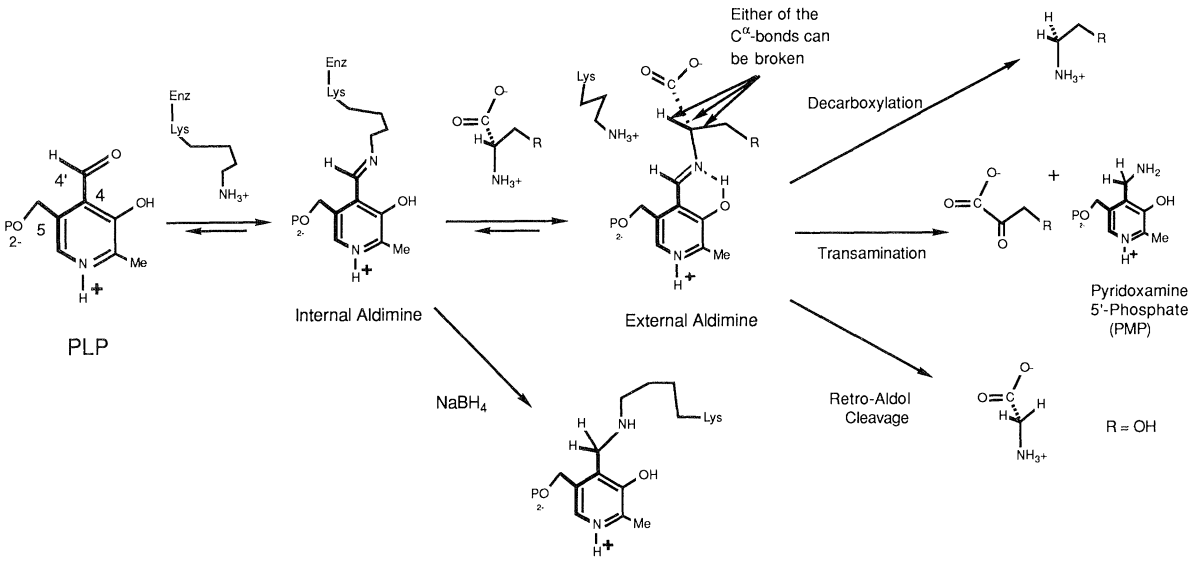
Pyridoxal 5′-phosphate is a coenzyme for a vast number of important enzyme-catalysed transformations in amino acid metabolism. Although many PLP-dependent enzymes are known which catalyse chemistry at the β- and γ-carbon atoms of amino acid substrates, our discussion here concentrates on reactions at C^{α}. The four most common of these are transamination, racemization, decarboxylation and retroaldol cleavage. Because racemization is a rather special reaction, in which active site bases and conjugate acids must act upon both faces of the coenzyme, these reactions will not be considered here. The remaining three types of reaction; transamination, decarboxylation and retroaldol cleavage (scheme 1) will now be considered in detail. Our aims are to learn how the apoenzyme controls the chemistry of the coenzyme such that specific bonds connected to C^{α} are broken.

In 1966 Dunathan proposed, on the basis of stereoelectronic arguments, that the bonds to be broken at C^{α} of PLP-amino acid Schiff's bases should be held at 90° to the plane of the extended conjugated system (Dunathan 1966), see scheme 1. In the early 1980s when the first X-ray crystal structure for a PLP-dependent enzyme, aspartate aminotransferase (AAT), was published it was evident that Dunathan's ideas were correct, at least for AAT. However, no X-ray crystal structures had been reported for a decarboxylase or for serine hydroxymethyltransferase (SHMT). Because the prospects for obtaining structures seemed poor, we decided to use stereochemical and kinetic techniques to discover how these other enzymes might control the chemistry of the cofactor. Before embarking on a detailed analysis of the chemistry catalysed by decarboxylases and SHMT, it is useful to summarize the important features of AAT catalysis.

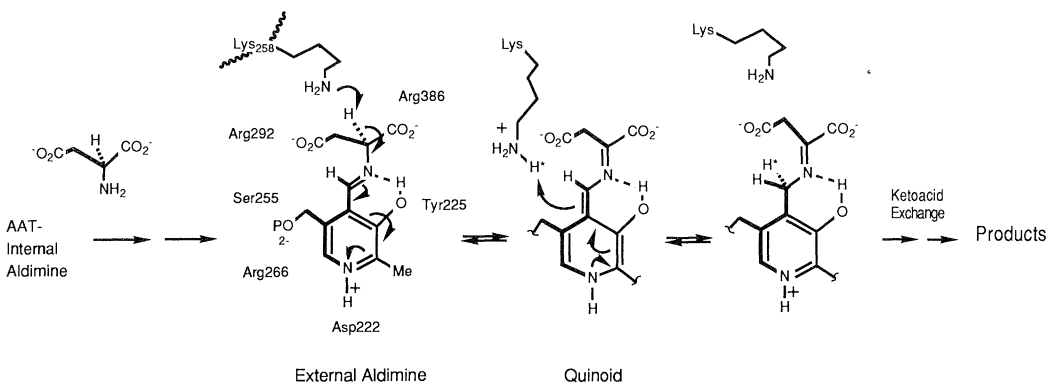
2. ASPARTATE AMINOTRANSFERASE

Transaminases are the best understood PLP-dependent enzymes. Much of the early work in the area was concerned with assessing the stereochemical course of the reaction with respect to C^{α} of the substrate and C-4′ of the coenzyme. However, the most significant contributions came from more recent X-ray crystal

† The earlier parts of this work were done in the Chemistry Department at Southampton University.



Scheme 1.



Scheme 2.

work on chicken heart mitochondrial (Kirsch *et al.* 1984) and *Escherichia coli* aspartate aminotransferase and now it is possible to envisage the three-dimensional catalytic function of the enzyme for the entire transamination process, see scheme 2.

The protein is composed of two identical subunits (relative molecular mass, $M_r \approx 45\,000$) 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 and products (namely, aspartate, glutamate, oxaloacetate and α-keto-glutarate) are bound by two arginine residues (386 and 292 from adjacent subunits). Substrate specificity is determined mainly 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, 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 that 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 residues. The protonated pyridinium ring is hydrogen bonded to Asp 222. 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 show 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 borohydride reducing agents (see scheme 1). The internal and external aldimines are reduced at the C-4′-*re*- and C-4′-*si*-faces, respectively (see Gani 1990).

The 5′-phosphate ester group of the coenzyme 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 offsets the double negative charge (scheme 2). Significantly, the side-chain of Ser 255 (only four residues removed from Lys 258) forms another H-bond to the phosphate ester. This serine residue is conserved in all reported

† 1 Å = 10^{-10} m = 10^{-1} nm.

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

E. coli	I	V	A	S	S	Y	S	**K**	N	F	G	L	Y
chicken, mitochondrial	V	L	S	Q	S	Y	A	**K**	N	M	G	L	Y
turkey, mitochondrial	V	L	S	Q	S	Y	A	**K**	N	M	G	L	Y
pig, mitochondrial	C	L	C	Q	S	Y	A	**K**	N	M	G	L	Y
rat, mitochondrial	C	L	C	Q	S	Y	A	**K**	N	M	G	L	Y
human, mitochondrial	C	L	C	Q	S	Y	A	**K**	N	M	G	L	Y
chicken, cytosolic	F	C	A	Q	S	F	S	**K**	N	F	G	L	Y
pig, cytosolic	F	C	A	Q	S	F	S	**K**	N	F	G	L	Y

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

arginine (*E. coli*)		A	T	H	S	T	H	**K**	L	L	N	A	L
glutamate (*E. coli*)	S	I	S	A	S	G	H	**K**	F				
histidine (*Morganella morganii*)	S	I	G	V	S	G	H	**K**	M	I	G	S	P
lysine (*E. coli*)	Y	E	T	E	S	T	H	**K**	L	L	A	A	F
lysine (*Hafnia alvei*)	Y	E	T	Q	S	T	H	**K**	L	L	A	A	F
ornithine (*E. coli*)						V	H	**K**	Q	Q	A	G	Q
dopa (*Drosophila*)	S	F	N	F	N	P	H	**K**	W	M	L	V	N
dopa (pig)			N	F	N	P	H	**K**	W				
glutamate (feline)	S	V	T	W	N	P	H	**K**	M	M	G	V	L
glycine (chicken)	V	S	H	L	N	L	H	**K**	T	F	C	I	P
SHMT (*E. coli*)	V	V	T	T	T	T	H	**K**	T	L	A	G	P
SHMT (rabbit, cytosolic)	V	V	T	T	T	T	H	**K**	T	L	R	G	C
SHMT (rabbit, mitochondrial)	V	V	T	T	T	T	H	**K**	T	L	R		

sequences for aspartate aminotransferase enzymes (table 1; Smith *et al.* 1991) and, indeed, there is almost 100% homology for all regions of the protein which are involved in binding to the substrate or the coenzyme for each of the sequenced proteins.

3. α-AMINO ACID DECARBOXYLASES

(*a*) *Stereochemical course at* C^{α}

By comparison to AAT, 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 (Smith *et al.* 1991) for some decarboxylases were known (table 2) and the stereochemical courses of decarboxylation had been reported for some systems (Gani 1985). From these studies it was evident that an active site lysine residue was involved in the formation of a coenzyme internal aldimine, as for transaminases and that, in general, PLP-dependent decarboxylases catalysed the decarboxylation of L-amino acid substrates with retention of configuration at C^{α}. Nevertheless, α,ω-*meso*-diaminopimelate decarboxylase from two different species catalysed the decarboxylation of the D-amino acid centre of α,ω-*meso*-diaminopimelic acid with inversion of configuration at C^{α} to give L-lysine (Kelland *et al.* 1985) and the decarboxylase activity of SHMT catalysed the decarboxylation of stereospecifically labelled 2-aminomalonic acid non-stereospecifically to give racemic glycine (Palekar *et al.* 1973). Although it was not clear that only one binding mode was available to the substrate in its interaction with SHMT, it was quite clear that the stereochemical course of PLP-dependent amino acid α-decarboxylations with respect to C^{α} could not be assumed.

(*b*) *Coenzyme face selectivity*

To compare the active site of decarboxylases and SHMT with AAT, it would, eventually, be necessary to determine upon which face of the coenzyme decarboxylations occurred. This objective poses a difficult problem because decarboxylases and SHMT, unlike transaminases, do not catalyse chemistry at the C-4′ positions of the quinoid intermediates derived from their physiological substrates, which might allow the reaction at C^{α} to be assigned to a particular face of the coenzyme (see scheme 2). Fortunately, in the presence of substrate analogues both decarboxylases and SHMT can be forced to catalyse abortive transamination reactions. For example, in the presence of D-alanine, SHMT protonates the coenzyme on the C-4′-*si*-face, the same result as for bona fide transaminases (Voet *et al.* 1973). While the observation shows that the enzyme can catalyse at 1,3-proton transfer, it cannot be assumed that the same enzyme-bound base or conjugate acid interacts at both sites. The ambiguity arises because of the uncertainty in knowing how the enzyme binds to D-alanine in the light of Palekar's work (1973) given also that the enzyme can bind to L-alanine (Shostack & Schirch 1988). However, it was evident that formation of PMP occurred stereospecifically and from the same face of the coenzyme as for transaminases.

The situation was even more ambiguous for the decarboxylases where 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 (Sukhareva & Braunstein 1971). The results showed that a proton was transferred to the C-4′-*si*-face of the coenzyme. Again, it could not be assumed that the same stereochemical course would have been followed in the much less frequent transamination reaction which occurs with the physiological substrate, or that the apparent 1,5- or 1,3-proton shift occurred suprafacially (Yamada & O'Leary 1977).

(*c*) L-*methionine decarboxylase*

In view of the confusion and uncertainty regarding the stereochemical courses of events which occur at C^{α} and C-4′ in decarboxylases and SHMT, a system eminently suited for stereochemical and mechanistic study was sought. L-Methionine decarboxylase (MD)

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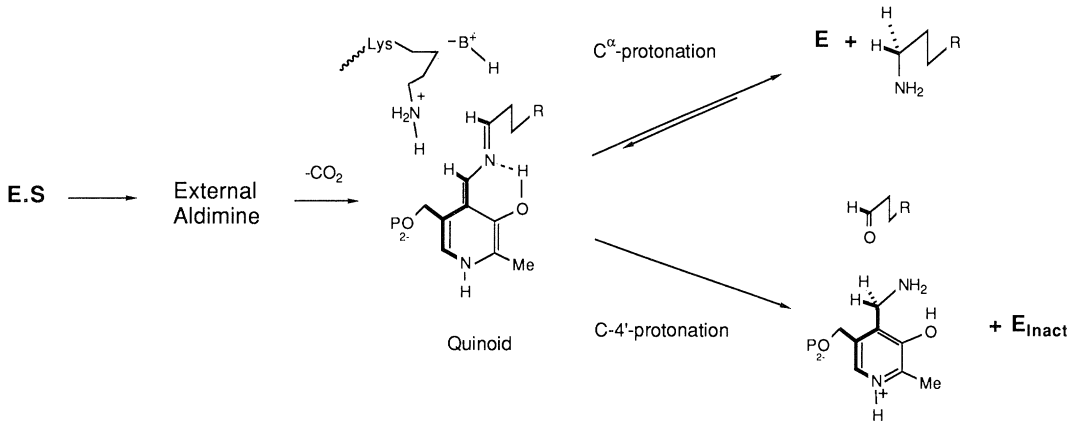

Scheme 3.

from the fern *Dryopteris filix-mas* and Streptomyces were identified as possessing suitable properties. Each of the proteins was purified to near homogeneity and was shown to be a homodimer ($M_r \approx 110\,000$). The two proteins showed similar activities at the optimum pH for V_{max} with methionine as substrate and were both able to catalyse the decarboxylation of a range of straight and branched chain hydrophobic amino acids (Stevenson *et al.* 1990*a*, *b*).

To determine the stereochemical course of the decarboxylation reaction with respect to C^{α}, incubations of the enzyme with unlabelled and labelled substrate were conducted in deuterium oxide and protium oxide respectively, and the products were isolated and derivatized as their camphanamide derivatives. Comparison of the 360 MHz ^{1}H-NMR spectra of these samples with synthetic samples showed that each enzyme catalysed the decarboxylation of methionine with retention of configuration at C^{α}. Thus the enzymes displayed the more usual stereochemical course for α-amino acid decarboxylation (Stevenson *et al.* 1990*a*, *b*).

When the stereochemical courses of the decarboxylation of several other substrates was determined for each methionine decarboxylase, retention of configuration was observed. Furthermore, each of the products showed a high chiral integrity. An interesting finding considering the diverse structures of the substrates.

Upon incubation of the enzymes with L-methionine in the absence of excess coenzyme, fern methionine decarboxylase, but not the *Streptomyces* enzyme, displayed activity loss. The addition of coenzyme to these incubations restored full activity and it appeared that enzyme was catalysing occasional abortive transamination events (see scheme 3). This explanation was verified in two different ways. First, [^{35}S]methionine was incubated with the enzyme and the expected transamination product, labelled 3-methylthiopropionaldehyde was detected and isolated. Second, PMP was isolated and characterized from similar incubations.

To determine the stereochemical course of the abortive transamination with the physiological substrate L-methionine, large-scale incubations were set up with the fern enzyme in which excess C-4′ tritiated coenzyme was added. The tritiated PMP generated during these incubations was isolated and purified and was treated with alkaline phosphatase to remove the phosphate ester group. The resulting tritiated pyridoxamine was incubated with freshly prepared apoaspartate aminotransferase (which is known to exchange the 4′-*pro*-S hydrogen of pyridoxamine with the solvent, (Voet *et al.* 1973) and the tritium content of the water and the solid residue was determined at time intervals against controls containing racemic tritiated pyridoxamine. From the results it was evident that the tritium occupied the 4′-*pro*-R position of the pyridoxamine derived from the abortive transamination reaction and, therefore, that proton transfer to the C-4′ position occurred from the *si*-face. Thus we had established, without ambiguity, that methionine decarboxylase showed the same stereochemical course for transamination of the coenzyme as AAT (Stevenson *et al.* 1990*a*).

To check the generality of the stereospecificity for C-4′ protonations in decarboxylases, the stereochemical course of the transamination reaction catalysed by *E. coli* glutamate decarboxylase (GAD) was determined with L-glutamic acid, the physiological substrate. The result was consistent with the emerging trend, that protonation occurred from the C-4′ *si*-face of the coenzyme (Smith *et al.* 1991).

Given that a lysine residue in all PLP-dependent enzymes forms an aldimine linkage with the C-4′ carbonyl group of the coenzyme, it seemed likely that this lysine residue was responsible for the protonation at C-4′ and for the stereospecificity of the protonation. Thus one common feature for these different enzymes appeared to be emerging, that the ε-ammonium group of the active site was disposed on the 4′-*si*-face of the coenzyme. However, verification was needed.

(*d*) *Does lysine protonate at* C^{α}*?*

To determine whether the same or different conjugate acids protonate the quinoid intermediate at C^{α} and C-4′ (see scheme 3), the enzyme was incubated with methionine and excess coenzyme in tritium oxide of high specific radioactivity. The tritium content of the 3-methylthio-1-aminopropane decarboxylation product, after crystallization to constant specific

activity, was found to match that of the original incubation medium. The specific activity of the isolated PMP was, however, ≈ 40–50 times lower than that of the solvent. Taken at face value, the results showed that a monoprotic acid operates at C^{α}, while a polyprotic acid operates at C-4′ (Akhtar *et al.* 1990). The latter result was rather pleasing, since it was consistent with the proposed role of the lysine ε-ammonium group. Furthermore, there could be no ambiguity in interpretation because the tritium content was so different to that of the solvent. The very slow rate of the abortive reaction and its predicted kinetic dominance by a chemical step, proton transfer, requires the observation of the intrinsic tritium isotope effect, statistically adjusted for the number of equivalent protons on the conjugate acid.

(*e*) *Conjugate acid for* C^{α}

The interpretation that a monoprotic acid operates at C^{α}, however, was not so clearcut since if the protonation step was followed by a very slow step, the proton transfer step would be brought into equilibrium, a situation in which the isolated product would display the specific activity of the solvent, regardless of the number of equivalent protons.

To determine whether the same or different bases or conjugate acids operated at C^{α} and C-4′, a series of kinetic and kinetic isotope effect experiments were performed by using the normal decarboxylation, and the abortive transamination reactions, as probes (Akhtar *et al.* 1990). It was reasoned that if the partition ratio between decarboxylation and transamination events varied with pH, the result would be consistent with the operation of two bases whereas, a pH-insensitive partition ratio would be consistent with the operation of a single base or conjugate acid.

The experiments allowed a detailed analysis of the kinetic properties of the fern MD system and in particular, the kinetic resolution of pre-decarboxylation events (those leading-up to the first irreversible step, carbon dioxide desorption) and post-decarboxylation steps. First, the partition ratio (v_{am}/v_{ab}, where v_{am} and v_{ab} are the rates of amine and PMP formation, respectively) and V/K were pH-sensitive and showed sharp increases at pH 6.25. The profiles, therefore, showed that a group on the enzyme, in its unprotonated state, simultaneously suppresses the abortive reaction and enhances the physiological reaction. The results strongly suggested that the deprotonations of the ammonium groups of the lysine residue and the substrate (by the conjugate base of an acid possessing a molecular pK_a of ≈ 6.25) to generate the nucleophiles for each of the two transaldimination processes were responsible for the observed profiles (Akhtar *et al.* 1990).

Experiments conducted in deuterium oxide allowed the solvent isotope effect (SIE) for the partition ratio and for the abortive reaction to be determined. The SIE for the partition ratio increased sharply from 0.6 to 1.7 at pL 6.25 while that for the abortive reaction decreased from 1.7 to 0.7 indicating that a post-decarboxylation step on the normal reaction pathway requires a catalytic group on the enzyme to be protonated. This step was probably quinoid protonation at C^{α} (Akhtar *et al.* 1990).

^{1}H-NMR (nuclear magnetic resonance) spectroscopic analysis of 3-methylthio-1-aminopropane isolated from incubations conducted in 50 (molar) % deuterium oxide at pL 4.8 and at pL 6.5, where the step for protonation of the quinoid at C^{α} was expected to be kinetically significant in the post-decarboxylation part of the reaction, showed that 50 % of deuterium was incorporated into the 1-*pro*-R position. The result shows that the proton donor was monoprotic and, in consideration of its kinetic titration, is the imidazolium sidechain of a histidine residue (Akhtar *et al.* 1990).

To determine if the two conjugate acids were disposed on the same faces of the coenzyme, or on different faces, the stereochemical courses for the decarboxylation were re-examined. The high chiral integrity of the monodeuteriated amine products for the diverse structural range of tested substrates suggested that the acids were disposed on the same face. If they were not, and the histidine was disposed on the C-4′-*re*-face, the bulkier substrates might have been expected to allow the lysine ε-ammonium group to occasionally protonate at C^{α} from the C-4′-*si*-face. Such events would give the deuteriated amine of the opposite configuration and would reduce the overall enantiomeric excess of the product. Given that the ability of the histidine to act as an acid would be diminished above pH 6.25 but, that of the ε-ammonium group would not, any protonation due to the action of lysine at C^{α} of the quinoid intermediate should be enhanced at pH 6.25 or higher. When the stereochemical courses for the decarboxylation of methionine and isoleucine were assessed at pL 7.0 in deuterium oxide the isolated (R)-monodeuteriated amines were completely pure. Thus as the lysine residue resides on the 4′-*si*-face of the coenzyme, the histidine must also.

4. COMPARISON OF ACTIVE SITE PEPTIDES

To compare the α-decarboxylases and SHMT to AAT, the X-ray crystal structures of the active-site of AAT were re-examined and the residues involved in binding to the coenzyme or to the substrate, or in catalysing specific chemical steps were identified (see table 2). Analysis of the regions of AAT which interact with the coenzyme showed that a tetrapeptide unit —S—X—X—K— contained two completely conserved residues, a serine (Ser 255) which forms a sidechain H-bond with the 5′-phosphate ester group of PLP, and the active site lysine (Lys 258), 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; see scheme 2).

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 serine, asparagine, or a threonine residue, all H-bond donors. In particular, the active site tetrapeptides for decarboxylases and SHMT were

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

well conserved (table 2) and contained the sequence —S(N,T)—X—H—K—, unless there were rationalizable mechanistic reasons for why they should not be conserved. For example, all decarboxylases known to catalyse the decarboxylation of (2S)-amino acids with retention of stereochemistry showed the conserved sequence but, *meso*-α,ω-diaminopimelate decarboxylase (which catalyses the decarboxylation at the (2R)-centre of its substrate with inversion of configuration) and mouse, rat, trypsanoma and yeast ornithine decarboxylases, some of which are the most unstable enzymes known, did not (Smith *et al.* 1991). Thus it appeared that for the decarboxylases which operate in a retentive mode, the serine, asparagine, or threonine residue might hydrogen bond to the 5′-phosphate ester group of the coenzyme, as in AAT, and that the histidine residue might be the catalytically important residue involved in quinoid protonation at C^{α}.

Given that the conformation of the homologous tetrapeptide section of each enzyme might be similar, it was instructive to note that histidine modifying reagents react with decarboxylases in the absence of substrate to give inactive proteins that possess unaltered binding affinities for their substrates. The result shows that the modified histidine residue is catalytically essential but, not important in the formation of the Michaelis complex (Dominici *et al.* 1985; Mishin & Sukhareva 1986). Gratefully, these interpretations are completely consistent with the role for histidine which we had proposed (Akhtar *et al.* 1990).

5. LOCATION OF DISTAL BINDING GROUPS

The final part of the comparison concerned the position of the distal substrate binding groups for decarboxylases and SHMT relative to those for the transaminases.

In decarboxylases the side chain could bind to a similarly positioned distal site as for transaminases (e.g. Arg 292, which is on the 5′ phosphate ester side on the coenzyme and 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. Since typical decarboxylases catalyse the decarboxylation of L-amino acids with retention of configuration at C^{α} and as the histidine residue which protonates the quinoid intermediate at C^{α} appears to be disposed on the 4′-*si*-face (Akhtar *et al.* 1990), decarboxylases should possess a distal binding group that is located on the 3′-phenol side of the coenzyme ring. This is opposite to the situation for transaminases.

To check the validity of the analysis, some simple modelling experiments were performed. It was expected that minor changes to the distal binding groups in the external aldimine of AAT would give the correct disposition of catalytically functional groups and the correct external aldimine conformation for a decarboxylase.

Accordingly, the coordinates for the tetrapeptide —S—Y—A—K— and the substrate–coenzyme aldimine were excised from the X-ray crystal structure of

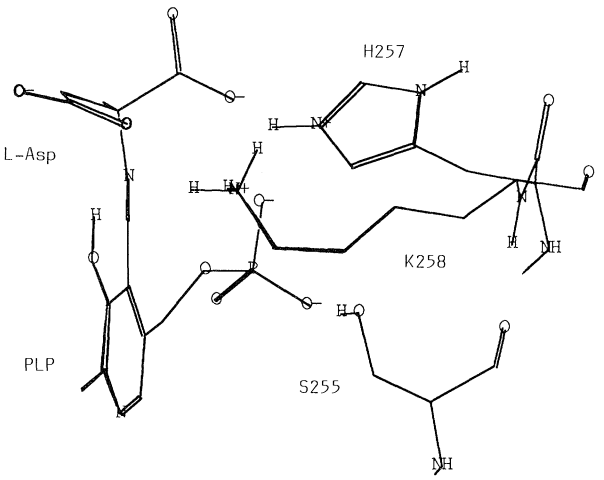

Figure 1. Result of the replacement of the methyl group of Ala 257 in the external aldimine form in chicken mitochondrial AAT by an imidazolium group, and rotation about the C^{α}-bond by 120°, and removal of the arginine residues. This conformation which was minimized, as described by Smith (1990), allows the formation of a hydrogen-bond between with the α-carboxylate group of the substrate and the N^{δ}-proton of the nascent His 257 residue.

chicken mitochondrial AAT. Without changing any distances or torsional angles in the tetrapeptide, the C^{α}-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 (Dunathan 1966). Without any further change, the position of Ala 257 was examined. The sidechain pointed directly towards the substrate. The alanine residue, in keeping with the decarboxylase motif, was then altered by modelling to histidine and the interactions were energy minimized by using Macromodel (see figure 1). The α-carboxylate group formed an H-bond with the protonated histidine ε-N-atom and the entire system appeared to be optimally set up to catalyse decarboxylation. (These interactions were also modelled with all of the amino acid residues which interact with the external aldimine of AAT in place except Arg 386. Here the configuration of the substrate was inverted to the D-antipode so that its α-carboxylate group was disposed on the 4′-*si*-face of the coenzyme, see Smith (1990)).

From this analysis it is expected that the histidine 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. Following decarboxylation, and generation of the quinoid intermediate, the imidazolium sidechain then protonates the quinoid 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 rationalization 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

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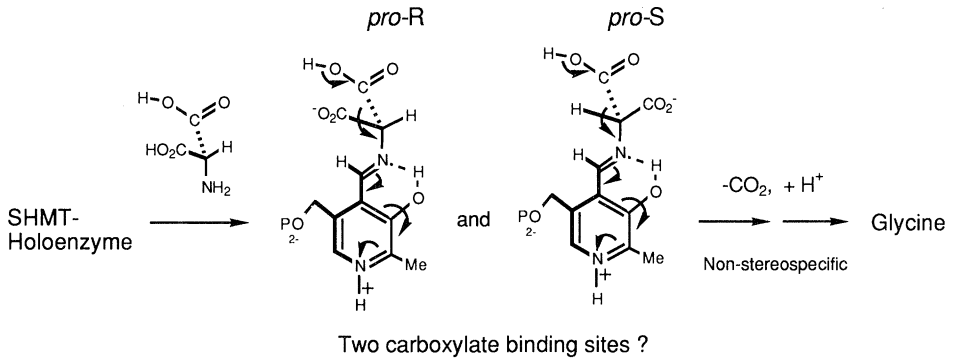

Scheme 4.

by sidechain binding and, in decarboxylases, by the presence of the positively charged imidazolium sidechain of a histidine residue which interacts electrostatistically with the α-carboxylate group. Our cumulative results show that histidine serves two other functions; as a base in the deprotonation of the ammonium group of the substrate and lysine residue immediately before transaldimination reactions, and; as a proton donor for the quinoid intermediate at C^{α}. These findings are completely in accord with the Dunathan postulate and show that it should be possible to design new decarboxylases starting from AAT (Smith 1990). Researches directed towards these goals are underway.

6. SERINE HYDROXYMETHYLTRANSFERASE

SHMT shows many similarities to α-amino acid decarboxylases, including the ability to catalyse decarboxylation and, the S(T,N)—X—H—K— tetrapeptide decarboxylase motif. However, the enzyme is unusual in that it shows a low regard for reaction-type specificity with α-amino acid substrates and catalyses many of these reactions non-stereospecifically (Thomas *et al.* 1990).

As noted earlier, Palekar *et al.* (1973) showed that in tritiated water the decarboxylation of aminomalonic acid by SHMT gave both (2R)- and (2S)-tritiated glycine and that incubation with specific carboxyl-labelled [^{14}C]aminomalonate confirmed that the reaction was non-stereospecific. To explain the apparent lack of stereospecificity, Palekar *et al.* (1973) proposed that the substrate might bind in two conformations at the active site of the enzyme, such that each of the two carboxyl groups was positioned correctly for decarboxylation (scheme 4). If each conformer was equally populated and, the decarboxylation and subsequent protonation steps occurred stereospecifically for each form, then apparent non-stereospecific decarboxylation would be observed. However, if the enzyme was able to catalyse the racemization of the substrate before decarboxylation, the same observations might have been expected. The latter scenario, before racemization, may have seemed unlikely to Palekar *et al.* in view of their earlier finding that PLP-dependent aspartate β-decarboxylase catalysed the stereospecific decarboxylation of aminomalonate (Palekar *et al.* 1971). To unravel the mechanistic and stereochemical ambiguities, a new substrate was sought, preferably one that could not racemize (Thomas *et al.* 1990).

2-Amino-2-methylmalonic acid was prepared and was tested as a substrate for cytosolic rabbit liver 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 characterized by H-NMR spectroscopy (Thomas *et al.* 1990).

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. Analysis revealed that only (2R)-alanine was formed initially (note: SHMT is able to catalyse the racemization of (2R)-alanine upon prolonged incubation (Shostack & Schirch 1988)). Hence, one stereochemical aspect of the decarboxylation had been solved, and whichever carboxyl group was lost, the resulting quinoid 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 quinoid intermediate a synthesis of the enantiomers of [1-^{13}C]2-amino-2-methylmalonic acid was devised by using Schollkopf *bis*-lactim ether methodology (Thomas *et al.* 1990; Thomas & Gani 1991).

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 showed that the (2R)-2-amino-2-methylmalonate gave unlabelled alanine (δ_H 1.21 p.p.m.; d, $J_{H\text{-}2,3} = 6.8$ Hz, in D_2O at pH 10) whereas the (2S)-enantiomer gave [1-^{13}C]alanine (δ_H 1.21 p.p.m.; dd, $J_{H\text{-}2,3} = 6.8$ Hz, $J_{H\text{-}2,C\text{-}1} = 4$ Hz). 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 occurred with retention of configuration (Thomas *et al.* 1990). The same stereochemical course was observed for the *E. coli* enzyme (Thomas 1990).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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 and our model for the active-site of PLP-dependent α-decarboxylase enzymes.

In conclusion, our work with SHMT showed that the findings of Palekar *et al.* (1973) are best rationalized in terms of the prior enzymic or non-enzymic racemization 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 more than 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 almost evenly distributed between the 2-*pro*-R and 2-*pro*-S positions of the glycine, implying that racemization occurs before decarboxylation. In accord with this conclusion, control experiments, which were done under identical conditions, but which contained no enzyme showed that the exchange of protium from the substrate was very rapid with half lives for exchange of ≈ 10–20 min over the pH range of interest (Thomas 1990).

When incubations containing high concentrations of SHMT and low concentrations of 2-aminomalonic acid were performed in deuterium oxide, a highly stereoselective decarboxylation reaction was observed (Thomas 1990). The predominant product was (2S)[2-^{2}H]glycine and, again, the major side product was the dideuterio isotopomer. Thus there is no evidence to suggest that SHMT catalyses non-stereospecific decarboxylation for any substrate (see scheme 4). The implication of these findings in the light of the model for the α-decarboxylases is that the conserved histidine residue in SHMT (see table 2) 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 SHMT (Shostack & Schirch 1988) is in accord with the notion that the side chain of the histidine should be protonated and should not serve as a base. Furthermore, it appears that the binding site for the α-carboxyl group of L-serine, and D-alanine (an abortive transamination substrate) and for the *pro*-S carboxyl groups of 2-aminomalonic acid decarboxylation substrates should be disposed on the 5′-phosphate ester side of the coenzyme, in a similar but slightly nearer position to that of Arg 292 in AAT. Reactions of L-alanine with SHMT cannot be rationalized at the present time and extensive further studies of SHMT will be required to elucidate the kinetic influence and mechanistic role of the formaldehyde acceptor, tetrahydrofolic acid, in the physiological reaction.

I thank my co-workers, the researchers who actually did the work, Dr M. Akhtar, Dr D. E. Stevenson, Dr D. M. Smith and Dr N. R. Thomas, and for their constant enthusiasm and dedication. We are all indebted to Professor J. Jansonius for providing the X-ray coordinates for chicken mitochondrial AAT, to Professor V. Schirch for providing SHMT enzymes and to S. Chamberlin for help with running Macromodel. We thank the SERC for financial support and for studentships to D. M. S. and N. R. T. and the Royal Society for a Royal Society University Fellowship to D. G. for 1983–88.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES