Stereochemistry of Pyridoxal Phosphate Catalyzed Enzyme Reactions

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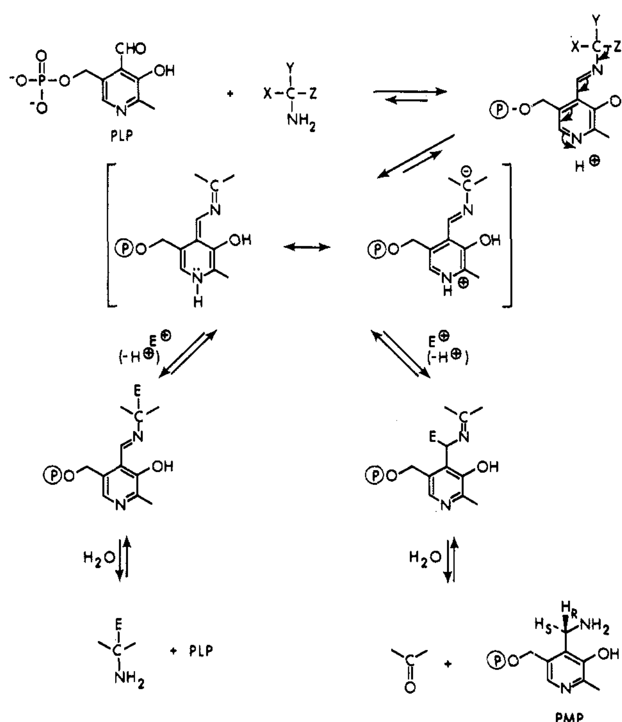
All living organisms use pyridoxal phosphate (PLP), the biochemically functional derivative of vitamin B_6, to synthesize, degrade, and interconvert amino acids. In the presence of the appropriate enzymes this cofactor plays a pivotal role in connecting carbon and nitrogen metabolism as well as in providing an entry into the "one-carbon pool" and catalyzing formation of biogenic amines.[1,2] Although this versatile coenzyme enables enzymes to perform a host of different reactions, most of these processes depend on a common structural and mechanistic principle. Basically PLP behaves like a chemical pawnbroker; it stores electrons in cleaving substrate bonds until new molecules arrive to claim them.

Pyridoxal phosphate readily condenses in a reversible fashion with the primary amino groups of proteins or substrates (e.g., amino acids) to form conjugated imines. The imines derived from α-amino acids are prone to cleavage of one of three bonds to the α carbon since the conjugated pyridine system acts as an electron sink, especially if the ring nitrogen is protonated. Attack by electrophiles on the resulting stabilized "carbanionic" intermediate leads to a new imine which upon hydrolysis can release the transformed amino acid derivative (Scheme I). If the electrophile is a proton, it can also add to C-4′ of the cofactor to generate an isomeric imine which will be hydrolyzed to pyridoxamine phosphate (PMP) and a carbonyl compound. Since the steps are reversible and certain enzymes will accept a limited variety of substrates, this system permits nitrogen transfer (transamination) from one carbon chain to another, with PMP temporarily storing the nitrogen.

An interesting feature of PLP catalysis is the stabilization of either cationic or anionic character at substrate carbons β to nitrogen in the carbanionic intermediate. As shown in Scheme II, this facilitates α,β-elimination, β-replacement, and β,γ-elimination reactions. Condensation with this remarkable cofactor can activate the enzyme substrate at least to the carbon γ to nitrogen.

Scheme I



What then is the function of the protein? Although model systems utilizing metal ions and the coenzyme can mimic many of the biological reaction types,[3] enzymes provide a rate enhancement and specificity which is currently unattainable in their absence. Certainly their major functions must include increased acid–base catalysis in a push–pull system, anchoring of the phosphate group, and recognition of the correct reaction partners.

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(1) For general reviews of various aspects of pyridoxal phosphate catalysis, see: (a) W. P. Jencks, "Catalysis in Chemistry and Enzymology", McGraw-Hill, New York, 1969, pp 133–146; (b) H. C. Dunathan, *Adv. Enzymol.*, **35**, 79–134 (1971); (c) A. E. Braunstein, *Enzymes*, **9**, 379–481 (1973); (d) C. Walsh, "Enzymatic Reaction Mechanisms", W. H. Freeman, San Francisco, 1979, pp 777–833; (e) D. E. Metzler, *Adv. Enzymol.*, **50**, 1–40 (1979).

(2) Certain specialized aspects of pyridoxal phosphate enzymes are discussed in *Methods Enzymol.*, **62**, Part D, Section VI (1979).

(3) For recent examples see: (a) Y. Karube and Y. Matsushima, *J. Am. Chem. Soc.*, **99**, 7356–7358 (1977); (b) K. Tatsumoto and A. E. Martell, *ibid.*, **100**, 5549–5553 (1978); (c) J. W. Ledbetter, H. W. Askins, and R. S. Hartman, *ibid.*, **101**, 4284–4289 (1979); (d) H. Kuzuhara, M. Iwata, and S. Emoto, *ibid.*, **99**, 4173–4175 (1977); (e) J. R. Fischer and E. H. Abbott, *ibid.*, **101** 2781–2782 (1979); (f) H. U. Meisch, H. Hoffmann, and W. Reinle, *Z. Naturforsch. C*, **33C**, 623–628 (1978).

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Scheme II

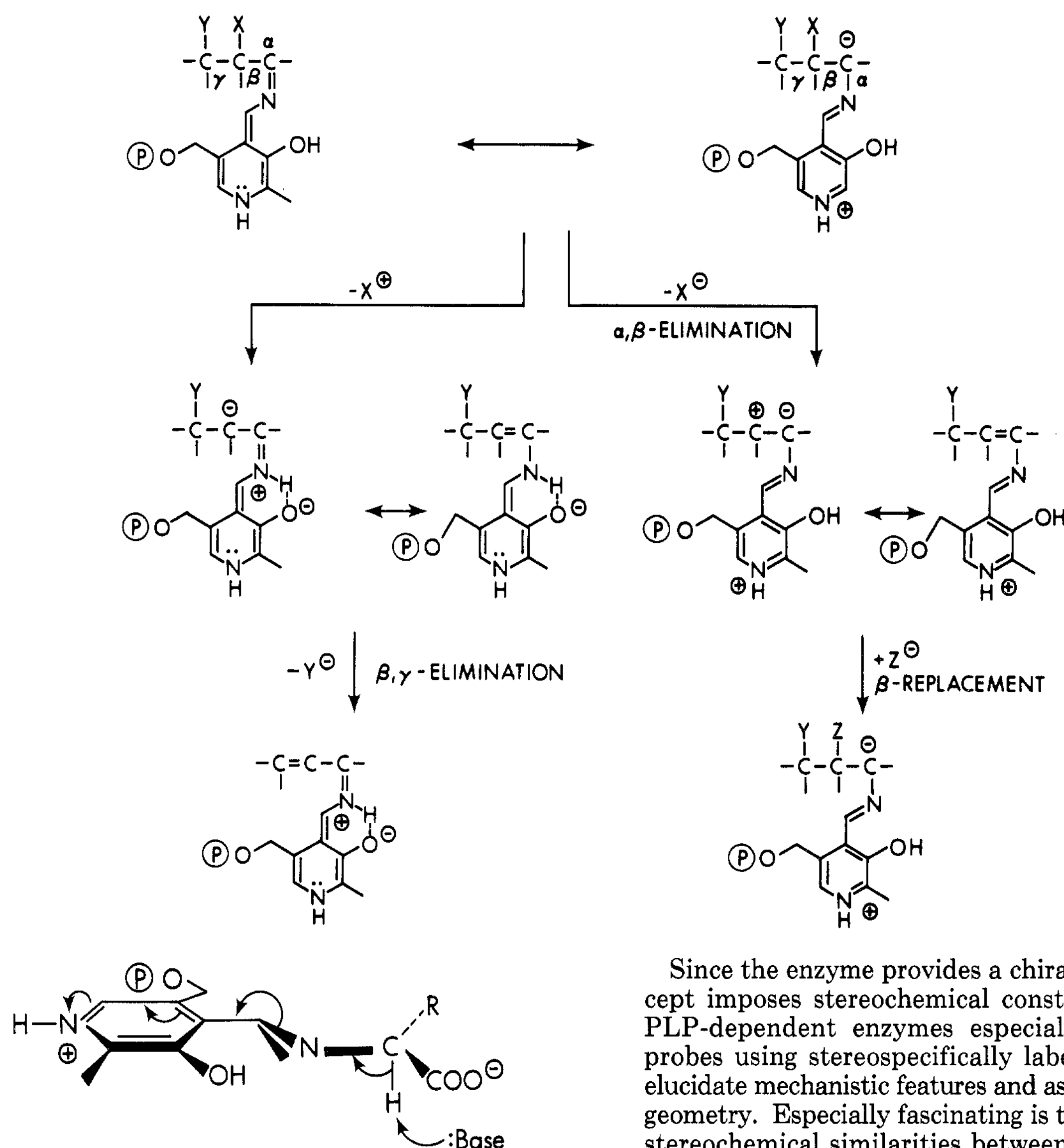


Figure 1.

In 1966 Dunathan proposed that a key enzyme function was orientation of the bond to be broken in the substrate–cofactor complex perpendicular to the plane of the extended conjugated system[4] (Figure 1). It is in this conformation that the breaking σ bond achieves maximal orbital overlap with the π system.[5] Our recent model studies show that in the absence of enzyme the rate of racemization and hydrogen exchange at the α carbon of amino acid–pyridoxal Schiff bases is determined by the proportion of conformer having the C_α–H_α bond orthogonal to the π system.[6] Such stereoelectronic requirements enable PLP-dependent enzymes to enhance reaction rates and control specificity of bond cleavage (and formation) by proper conformational orientation. If an enzyme binds the relatively rigid PLP cofactor at the pyridine nitrogen and at the phosphate, attachment of a single distal group on the substrate results in three-point binding of the substrate–cofactor imine.

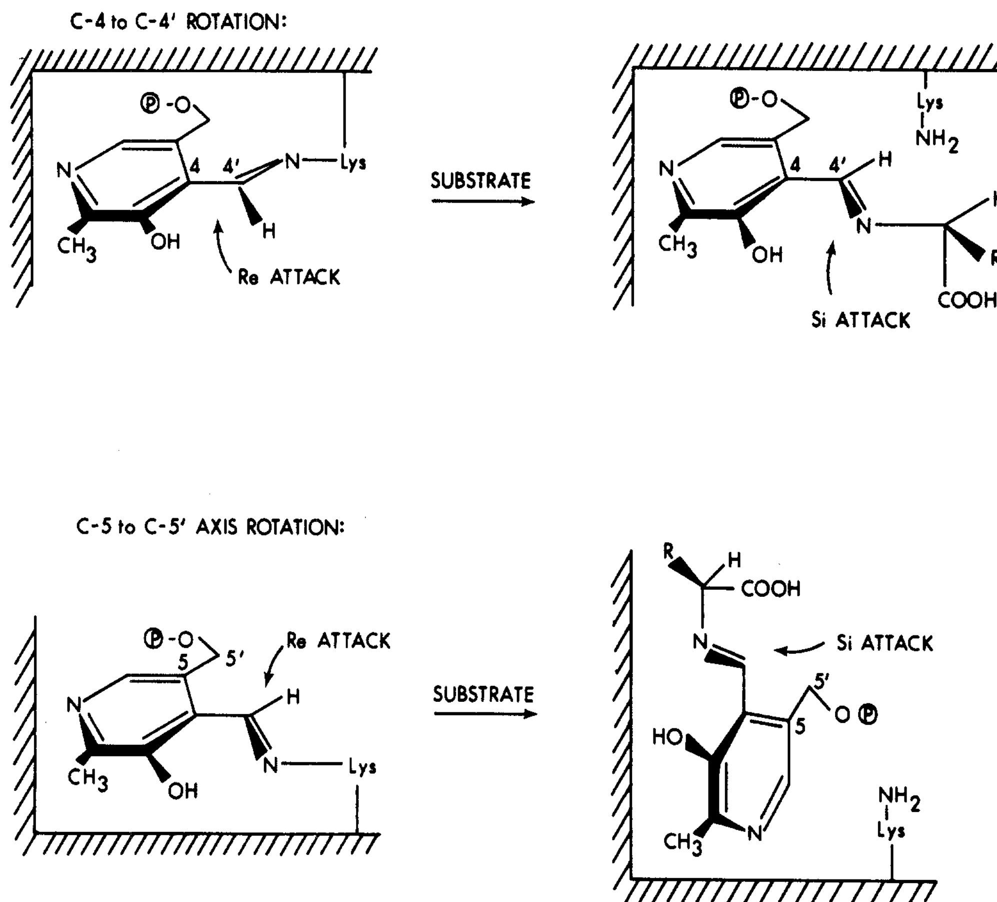
Since the enzyme provides a chiral matrix, this concept imposes stereochemical constraints and makes PLP-dependent enzymes especially susceptible to probes using stereospecifically labeled substrates to elucidate mechanistic features and aspects of active-site geometry. Especially fascinating is the suggestion that stereochemical similarities between PLP enzymes of widely varying function result from chemical evolution from a common progenitor, a "grandfather enzyme".[1b,7] Homology of amino acid residues at the active sites of these proteins strongly supports this concept.[8] In every PLP-dependent enzyme examined so far, the ϵ-amino group of a lysine residue binds the cofactor as a Schiff base. In combination with protein structural studies, detailed stereochemical examination of PLP-dependent enzymes can test the validity of the single progenitor hypothesis.

Reactions at C_α of Amino Acids

According to Dunathan's hypothesis,[1b] aminotransferases (transaminases) orient the amino acid–PLP complex so that the α hydrogen is nearly orthogonal to the plane of the conjugated pyridine ring (Figure 1).[1c,9] The enzymes accept only a single enantiomer of an amino acid to form an α-keto acid (Scheme I). They then generate the analogous enantiomer of a new amino acid from a different α-keto acid.[1c,9] In the enzymes

(4) H. C. Dunathan, *Proc. Natl. Acad. Sci. U.S.A.*, **55**, 713–716 (1966).
(5) (a) E. J. Corey and R. A. Sneen, *J. Am. Chem. Soc.*, **78**, 6269–6278 (1956); (b) R. R. Fraser and P. J. Champagne, *ibid.*, **100**, 657–658 (1978).
(6) M. D. Tsai, H. J. R. Weintraub, S. R. Byrn, C. Chang, and H. G. Floss, *Biochemistry*, **17**, 3183–3188 (1978).
(7) H. C. Dunathan and J. G. Voet, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 3888–3891 (1974).
(8) (a) S. Tanase, H. Kojima, and Y. Morino, *Biochemistry*, **18**, 3002–3007 (1979). (b) H. Kagamiyama, R. Sakakibara, S. Tanase, Y. Morino, and H. Wada, *J. Biol. Chem.*, **255**, 6153–6159 (1980).
(9) A. E. Braunstein, *Mol. Biol.*, **11**, 947–963 (1977).

Scheme III



examined so far, the intermediate imine is always protonated from the *si* face[10] at C-4' of the cofactor so that the *pro-S* hydrogen (H_s)[10] of PMP is added or removed.[7,11] A significant amount of the α hydrogen of L-alanine is incorporated at C-4' by alanine aminotransferase, thereby indicating that a single basic group on the enzyme accomplishes an intramolecular suprafacial 1,3-prototropic shift.[12] Transaldimination[13] of the cofactor–enzyme complex by L-alanine frees an ϵ-amino group of lysine near the active site, but apparently this group is not essential to retain the stereochemistry of protonation in closely related aspartate aminotransferase for which the results suggest involvement of an active-site histidine.[14]

If an enzyme orients the amino acid–PLP complex such that the carboxyl group is perpendicular to the conjugated system, stereoelectronic effects favor decarboxylation. Considerable evidence has accumulated that α-decarboxylases control the conformation about the α-carbon–nitrogen bond by binding a distal group in the fully extended side chain of the amino acid moiety.[15] The intermediate imine normally protonates at the α carbon to ultimately release an amine.[16] Although the protonation could, in theory, óccur from either side of the planar imine, the decarboxylations of L-tyrosine,[17] L-lysine,[18] L-glutamate,[19] and L-histidine[20] by their respective decarboxylases have been shown to proceed in a retention mode. Sometimes a small amount of protonation occurs at C-4' of the cofactor to ultimately produce an inactive pyridoxamine phosphate–enzyme complex. In the case of glutamate decarboxylase this protonation at C-4' occurs from the *si* face just as in the transaminases.[21] It thus appears that all reactions occur on a single side of the cofactor–substrate complex, the other being inaccessible.

To test the implication that one face of the complex is covered and the other, reactive face is exposed, we recently reduced several Schiff base complexes of tyrosine decarboxylase from *Streptococcus faecalis* with

(10) Stereochemical symbols outlined by K. R. Hanson, *J. Am. Chem. Soc.*, 88, 2731 (1966), are employed, with meanings briefly (and somewhat approximately) as follows: *pro-R*: the one of paired ligands at a prochiral center which, if elevated in priority over the other, would result in a center with *R* chirality; *pro-S*: the other of the paired ligands at a prochiral center; *re* face: the face of a trigonal atom from which the three ligands attached to it appear to be arranged in clockwise order of decreasing priority; *si* face: the face of a trigonal atom opposite to the *re* face.

(11) J. G. Voet, D. M. Hindenlang, T. J. J. Blanck, R. J. Ulevitch, R. G. Kallen, and H. C. Dunathan, *J. Biol. Chem.*, **248**, 841–842 (1973).

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(13) P. S. Tobias and R. G. Kallen, *J. Am. Chem. Soc.*, **97**, 6530–6539 (1975).

(14) M. Martinez-Carrion, J. C. Slebe, and M. Gonzalez, *J. Biol. Chem.*, **254**, 3160–3062 (1979).

(15) (a) G. B. Bailey, D. Chotamangsa, and K. Vuttivej, *Biochemistry*, 9, 3243–3248 (1970); (b) M. L. Fonda, *ibid.*, **11**, 1304–1309 (1972); (c) M. H. O'Leary and G. J. Piazza, *J. Am. Chem. Soc.*, **100**, 632–633 (1978).

(16) For a review of decarboxylases, see: E. A. Boeker and E. E. Snell, *Enzymes, 3rd Ed.*, 6, 217–253 (1972).

(17) (a) B. Belleau and J. Burba, *J. Am. Chem. Soc.*, **82**, 5751–5752 (1960); (b) A. R. Battersby, E. J. T. Chrystal, and J. Staunton, *J. Chem. Soc., Perkin Trans. 1*, 31–42 (1980).

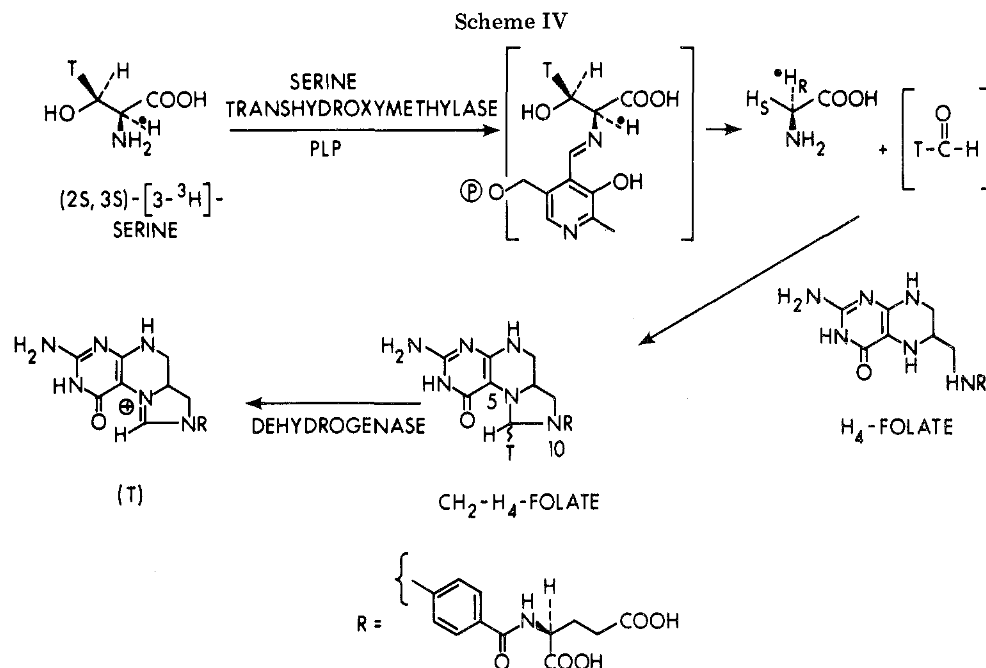
(18) (a) E. Leistner and I. D. Spenser, *J. Chem. Soc., Chem. Commun.*, 378–379 (1975); (b) H. J. Gerdes and E. Leistner, *Phytochemistry*, **18**, 771–775 (1979).

(19) (a) H. Yamada and M. H. O'Leary, *Biochemistry*, **17**, 669–672 (1978); (b) E. Santaniello, M. G. Kienle, A. Manzocchi, and E. Bosisio, *J. Chem. Soc., Perkin Trans. 1*, 1677–1679 (1979).

(20) A. R. Battersby, R. Joyeau, and J. Staunton, *FEBS Lett.*, **107**, 231–232 (1979).

(21) B. S. Sukhareva, H. C. Dunathan, and A. E. Braunstein, *FEBS Lett.*, **15**, 241–248 (1971).

Scheme IV



sodium boro[^{3}H]hydride.[22] Attack on the imine bond should occur from the exposed side of C-4′ of the cofactor to generate a new chiral center bearing tritium. Surprisingly, reduction of the enzyme–PLP complex in the absence of substrates occurred primarily from the *re* face. However, after addition of either L-tyrosine or the product, tyramine, to form enzyme-bound substrate–PLP complexes, reduction proceeded mostly from the opposite side (*si* face) at C-4′.

This unusual result could be explained by either of two conformational changes occurring upon substrate binding (Scheme III). In the first model,[1c,23] the imine nitrogen is always on the same side of the C-4 to C-4′ bond as the phenolic OH (cisoid conformation), and rotation about an axis through the C-5 to C-5′ bond during substrate binding exposes the other face of both the imine bond and the pyridine ring. Model studies and calculations show that in the absence of enzyme, pyridoxal Schiff bases prefer a cisoid conformation.[23a,24] Another possibility is that the pyridine ring keeps the same side exposed while rotation about the C-4 to C-4′ bond during substrate binding exposes a new face of the imine bond; this is a transoid to cisoid reorientation. Such a conformational change has been suggested for aspartate aminotransferase on the basis of absorption spectra and X-ray diffraction studies.[25] Modifications of aspartate aminotransferases have also indicated conformational changes during substrate binding,[26] and Austermühle-Bertola has shown by analogous sodium boro[^{3}H]hydride reductions that these changes are similar to those in tyrosine decarboxylase.[27] However, Zito and Martinez-Carrion recently observed that reduction of the complex between L-aspartate and carbamoylated aspartate aminotransferase occurs from the *re* rather than the *si* face.[28] This modified form of the enzyme still catalyzes the transamination of L-aspartate to PMP and in the process protonates the cofactor from the *si* face.[14] At present it is not possible to distinguish between the two possible conformational reorientations or to define unequivocally their relationship to the catalytic process. Obviously the occurrence of such phenomena in PLP-dependent enzymes of widely varying function would support the hypothesis of evolution from a single progenitor. We have detected similar conformational changes in tryptophanase,[29] an enzyme which will be discussed later.

Serine transhydroxymethylase catalyzes the reversible reaction of L-serine with tetrahydrofolate (H_4folate) to form 5,10-methylenetetrahydrofolate (CH_2-H_4folate) and glycine (Scheme IV), thereby providing an important physiological entry into the C_1 pool. This PLP-dependent enzyme also cleaves several other β-hydroxy amino acids to glycine and the respective carbonyl compounds.[30] During the cleavage of L-threonine to acetaldehyde the oxygen is conserved.[31] A large amount of additional evidence supports a retro-aldol mechanism to form a PLP–glycine anion with the rate being increased by the presence of H_4folate.[32] Model systems using metal ions and pyridoxal phosphate also

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(23) (a) V. G. Tumanyan, D. K. Mamaeva, A. C. Bocharov, V. I. Ivanov, M. Karpeisky, and G. I. Yakovlev, *Eur. J. Biochem.*, **50**, 119–127 (1974); (b) V. I. Ivanov and M. Y. Karpeisky, *Adv. Enzymol.*, **32**, 21–53 (1969).
(24) M. D. Tsai, S. R. Byrn, C. Chang, H. G. Floss, and J. R. Weintraub, *Biochemistry*, **17**, 3177–3182 (1978).
(25) (a) T. L. Fisher and D. E. Metzler, *J. Am. Chem. Soc.*, **91**, 5323–5328 (1969); (b) C. M. Metzler, D. E. Metzler, D. S. Martin, R. Newman, A. Arnone, and P. Rogers, *J. Biol. Chem.*, **253**, 5251–5254 (1978); (c) G. Eichele, G. C. Ford, M. Glor, J. N. Jansonius, C. Mavrides, and P. Christen, *J. Mol. Biol.*, **133**, 161–180 (1979); (d) G. C. Ford, G. Eichele, and J. N. Jansonius, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 2559–2563 (1980).
(26) (a) H. Gehring and P. Christen, *J. Biol. Chem.*, **253**, 3158–3163 (1978); (b) K. Pfister, J. H. R. Kägi, and P. Christen, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 145–148 (1978).
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(28) S. W. Zito and M. Martinez-Carrion, *J. Biol. Chem.*, **255**, 8645–8649 (1980).
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(30) (a) M. S. Chen and L. Schirch, *J. Biol. Chem.*, **248**, 3631–3635 (1973); (b) R. J. Ulevitch and R. G. Kallen, *Biochemistry*, **16**, 5355–5363 (1977).
(31) P. M. Jordan, H. A. El-Obeid, D. L. Corina, and M. Akhtar, *J. Chem. Soc., Chem. Commun.*, 73–74 (1976).
(32) (a) L. Schirch, C. M. Tatum, and S. J. Benkovic, *Biochemistry*, **16**, 410–419 (1977); (b) L. Schirch, S. Slagel, D. Barra, F. Martini, and F. Bossa, *J. Biol. Chem.*, **255**, 2986–2989 (1980), and references therein.

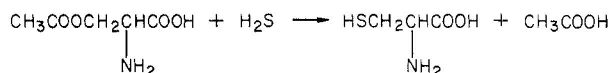
catalyze such conversions.[33] Reprotonation of the intermediate glycine–PLP anion complex again occurs in a retention mode; serine transhydroxymethylase removes exclusively the *pro*-2*S* hydrogen of glycine and leaves the *pro*-2*R* hydrogen in the product L-serine.[34] In accord with the configurational position occupied by the hydroxymethylene group of L-serine, the enzyme catalyzes α-hydrogen exchange and transamination of D-alanine,[35] with reprotonation at C-4′ of the intermediate again coming from the *si* face.[11] Surprisingly, some (but not all) L-amino acids not only exchange their α hydrogen but also react with formaldehyde to form new amino acids (presumably β-hydroxymethyl derivatives).[36] Although L-alanine reacts in this way and D-alanine undergoes transamination, the D antipodes of all other amino acids tested exhibit no reaction or hydrogen exchange of any sort.[36,37] In contrast, the enzyme shows no specificity for the heterotopic carboxyl groups in the decarboxylation of aminomalonate.[38] If we assume that stereoelectronic control requires orientation of the cleaving bond perpendicular to the cofactor plane, these unusual results suggest that the substrate–PLP complex may be bound to the enzyme in two different C_α–N conformations, possibly due to the presence of two binding sites for the distal group. If this idea is correct, each antipode of racemic alanine or stereospecifically carboxyl-labeled aminomalonate should react with retention of configuration at the α carbon.

Although the nature of the bound glycine species has been extensively studied, the nature of the aldehyde species involved in the enzymatic reaction and the role of H_4folate are still unclear. Initial studies by Biellmann and Schuber on the serine generated in tissue slices from [^{3}H]formate (presumably via methenyl-H_4folate and CH_2-H_4folate) showed that 72% of the tritium at C-3 of serine was in the *pro-S* position.[39] To examine the reasons for partial stereospecificity we investigated[40] this problem using purified enzymes and L-serine samples which were stereospecifically tritiated at C-3 (>98% isomeric purity) prepared from [1-^{3}H]-glucose and [1-^{3}H]mannose.[41,42] Serine transhydroxymethylase converted these substrates under single turnover conditions into CH_2-H_4folate, which in a coupled reaction with yeast CH_2-H_4folate dehydrogenase was oxidized to methenyl-H_4folate to stereospecifically remove one of the hydrogens from the 5,10-methylene bridge (Scheme IV). Surprisingly, the results using pure serine transhydroxymethylase are in excellent agreement with those obtained with rat liver tissue. Hence, there is a 24% crossover of label from each serine isomer into the CH_2-H_4folate isomer with the methylene unit of opposite configuration which is a function of the reaction. Apparently the enzyme catalyzes the cleavage of serine by two parallel pathways which are undetectable unless the methylene group is stereospecifically labeled. Other studies show that serine transhydroxymethylase cleaves both threonine and allothreonine as well as the L isomers of *erythro*- and *threo*-β-phenylserine.[36,43] This indicates considerable latitude in the steric requirements at the β carbon and suggests that the reaction may proceed from two different conformations. An alternative possibility is that crossover occurs through the formaldehyde intermediate, which is bound on a single face of the enzyme immediately after its formation, but may be occasionally released and rebound on the opposite face.

Reactions at C_β of Amino Acids

As outlined in Scheme II, condensation with pyridoxal phosphate in certain cases permits replacement of a substituent at the β position of amino acids by a nucleophile. At least three possible mechanisms immediately spring to mind: direct S_N2 displacement, displacement by enzyme followed by displacement by the nucleophile (double displacement), and α,β elimination followed by Michael addition of the nucleophile to the conjugated system. The first mechanism implies inversion of configuration at the β carbon, the second would result in retention, and the third could give either retention or inversion. We examined the stereochemistry of a series of such enzyme reactions to help elucidate the mechanism and to discover possible additional common features of PLP enzymes (Scheme V).

O-Acetylserine sulfhydrase (cysteine synthase) catalyzes the last step in cysteine biosynthesis in many microorganisms and plants by conversion of *O*-acetylserine and hydrogen sulfide to cysteine and acetate:



In our study[44] the (2*S*,3*S*)- and (2*S*,3*R*)-[3-^{3}H]serine samples used earlier were acetylated and transformed by *O*-acetylserine sulfhydrase from *E. coli* into cysteine. Chemical degradations and stereochemical analysis of the products demonstrated that the *O*-acetylserine sulfhydrase reaction proceeds with retention of configuration at the β carbon (C-3), thereby eliminating the direct S_N2 process as a possible mechanism. A kinetic study by Cook and Wedding[45] on *O*-acetylserine sulfhydrase from *Salmonella typhimurium* shows that the enzyme catalyzes a Bi-Bi Ping-Pong reaction[46] between *O*-acetylserine and sulfide; i.e., after binding of the first substrate the first product must leave the enzyme before the second substrate can bind. Taken together, these results strongly support an elimination–addition mechanism in which the leaving group exits from the

(33) (a) J. A. Marcello, A. E. Martell, and E. H. Abbott, *J. Chem. Soc., Chem. Commun.*, 16–17 (1975); (b) Y. N. Belokon, V. M. Belikov, S. V. Vitt, T. F. Savel'eva, V. M. Burbelo, V. I. Bakhmutov, G. G. Aleksandrov, and Y. T. Struchkov, *Tetrahedron*, 2551–2564 (1977).
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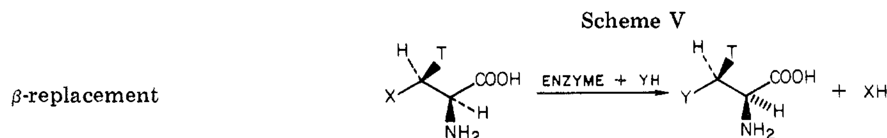
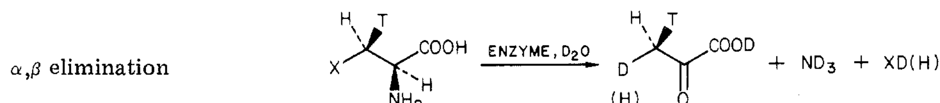
Scheme V

β-replacement



enzyme	substrate	product	ref
O-acetylserine sulfhydrase	(2*S*,3*S*)-*O*-acetylserine X = OAc	(2*R*,3*S*)-cysteine Y = SH	44
β-cyanoalanine synthetase	(2*R*,3*S*)-cysteine X = SH	(2*S*,3*S*)-cyanoalanine Y = CN	47
tyrosine phenol-lyase	(2*S*,3*S*)-serine X = OH	(2*S*,3*R*)-tyrosine Y = 4′-hydroxyphenyl	51a
		(2*S*,3*R*)-dihydroxyphenylalanine Y = 2′,4′-dihydroxyphenyl	51b
tryptophanase	(2*S*,3*S*)-serine X = OH	(2*S*,3*R*)-tryptophan Y = 3′-indolyl	52
tryptophan synthetase, or β_2 subunit	(2*S*,3*S*)-serine X = OH	(2*S*,3*R*)-tryptophan Y = 3′-indolyl	42, 53

α,β elimination

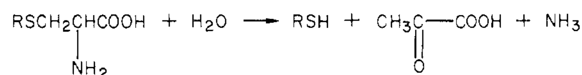


enzyme	substrate	ref
S-alkylcysteine lyase	(2*R*,3*S*)-cystine X = $HO_2CCH(NH_2)CH_2SS$	47
tyrosine phenol-lyase	(2*S*,3*S*)-serine X = OH	51c
	(2*S*,3*R*)-tyrosine X = 4′-hydroxyphenyl	
tryptophanase	(2*S*,3*S*)-serine X = OH	52
	(2*S*,3*R*)-tryptophan X = 3′-indolyl	
tryptophan synthetase	(2*S*,3*S*)-serine X = OH [pyruvate contains 100% (H)]	53b

same side of the planar aminoacrylate–PLP imine intermediate as the nucleophile approaches.

Having obtained [3-^{3}H]cysteine of known absolute configurtion, we embarked[47] on a study of a PLP-dependent enzyme from higher plants, β-cyanoalanine synthetase, which catalyzes the transformation of cysteine and hydrogen cyanide to β-cyanoalanine and hydrogen sulfide. Degradation of samples of β-cyanoalanine produced from the stereospecifically labeled [3-^{3}H]cysteine isomers defined the configuration at C-3 and showed that the β-cyanoalanine synthetase reaction occurs with retention of configuration at the amino acid β carbon. On the basis of their observation that α-hydrogen exchange of substrate in the absence of cyanide is slower than the normal overall reaction, Braunstein and co-workers have suggested a direct displacement of the β substituent by the incoming nucleophile.[48] To be compatible with our stereochemical results, this displacement would have to be a double displacement involving an anionic group of the enzyme on the "protein side" of the coenzyme–substrate complex. Although both double displacement and elimination–addition mechanisms are in agreement with experimental results, there is no evidence for the former process in any PLP-catalyzed β-replacement reaction, whereas kinetic evidence supports the latter mechanism for the *O*-acetylserine sulfhydrase reaction.[45] It is likely that efficient labilization of α hydrogen and overall reaction require a ternary complex and that cyanide binding results in an enzyme conformational change which favors cleavage of the α-hydrogen bond.

The stereospecifically tritiated cysteine samples could also be used to study *S*-alkylcysteine lyase,[47] a PLP enzyme from higher plants which catalyzes an α,β elimination of *S*-alkylcysteines, including cystine, to form a thiol and an aminoacrylate–PLP imine (see Scheme II). This intermediate then hydrolyzes to pyruvate and ammonia:



The cysteine samples were oxidized to cystines which were incubated with the enzyme in D_2O to determine the steric course of protonation at C-3. Coupled reduction of the pyruvate with lactate dehydrogenase gave lactate samples having chiral methyl groups. Oxidation of the lactates to chiral acetates and stereochemical analysis of the methyl groups by established procedures[49] showed that solvent protonation of the planar aminoacrylate intermediate occurs from the same side from which the β substituent leaves. Such a retention mode of pyruvate formation is also observed with D-serine dehydratase[50] as well as with the enzymes discussed below (Scheme V).

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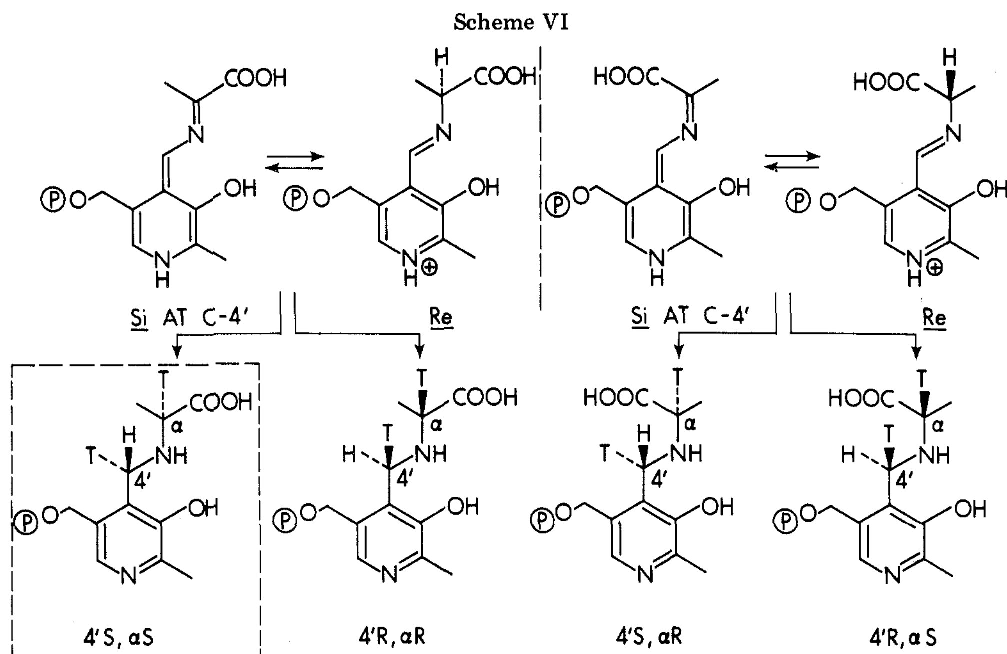
(48) (a) T. N. Akopyan, A. E. Braunstein, and E. V. Gorayachenkova, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 1617–1621 (1975); (b) E. A. Tolosa, R. N. Maslova, E. V. Gorayachenkova, I. H. Willhardt, and A. E. Braunstein, *Eur. J. Biochem.*, **53**, 429–436 (1975).

(49) (a) J. W. Cornforth, J. W. Redmond, H. Eggerer, W. Buckel, and C. Gutschow, *Eur. J. Biochem.*, **14**, 1–13 (1970); (b) J. Lüthy, J. Retey, and D. Arigoni, *Nature (London)*, **221**, 1213–1215 (1969); (c) M. D. Tsai and H. G. Floss, *Adv. Enzymol.*, **50**, 243–302 (1979).

(50) (a) J. Y. Yang, Y. Z. Huang, and E. E. Snell, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **34**, 496 (1975); (b) Y. F. Cheung and C. Walsh, *J. Am. Chem. Soc.*, **98**, 3397–3398 (1976).

Scheme VI



A number of PLP-dependent enzymes perform both α,β elimination (pyruvate formation) as well as β replacements on suitable substrates. Of these, tyrosine phenol-lyase (β-tyrosinase),[51] tryptophanase,[52] and tryptophan synthetase[53] have been examined to determine the stereochemical outcome of both processes. Reaction of serine isomers which are stereospecifically labeled at C-3 with tyrosine phenol-lyase and phenol[51a] or resorcinol[51b] in the presence of a large excess of ammonium ions produces L-tyrosine or 2,4-dihydroxyphenyl-L-alanine, respectively, in a retention mode at C-3. In the absence of excess ammonium ions, this enzyme degrades both serine and tyrosine to pyruvate, ammonia, and water or phenol. Treatment of (2*S*,3*R*)- and (2*S*,3*S*)-[3-^{3}H]serine with tyrosine phenol-lyase in coupled reaction with lactate dehydrogenase in D_2O forms lactates with chiral methyl groups.[51c] Stereochemical analysis showed that the initial replacement of the β substituent by a proton again occurs in a retention mode. Retention was also observed during conversion of (2*S*,3*R*)-[3-^{2}H]-tyrosine, prepared according to Kirby and Michael,[54] into lactate and phenol in tritiated water. Interestingly, prolonged incubation of the serines eventually leads to racemization at the β carbon. This may relate to the ability of tyrosine phenol-lyase to deaminate both D and L enantiomers of tyrosine and serine[55] or to racemize alanine.[56]

(51) (a) C. Fuganti, D. Ghiringhelli, D. Giangrasso, and P. Grasselli, *J. Chem. Soc., Chem. Commun.*, 726–727 (1974); (b) S. Sawada, H. Kumagai, H. Yamada, and R. K. Hill, *J. Am. Chem. Soc.*, **97**, 4334–4337 (1975); (c) H. Kumagai, H. Yamada, S. Sawada, E. Schleicher, K. Mascaro, and H. G. Floss, *J. Chem. Soc., Chem. Commun.*, 85–86 (1977).
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(53) (a) C. Fuganti, D. Ghiringhelli, D. Giangrasso, P. Grasselli, and A. S. Amisano, *Chim. Ind.*, **56**, 424 (1974); (b) M. D. Tsai, E. Schleicher, R. Potts, G. E. Skye, and H. G. Floss, *J. Biol. Chem.*, **253**, 5344–5349 (1978).
(54) G. W. Kirby and J. Michael, *J. Chem. Soc., Perkin Trans. 1*, 115–120 (1973).
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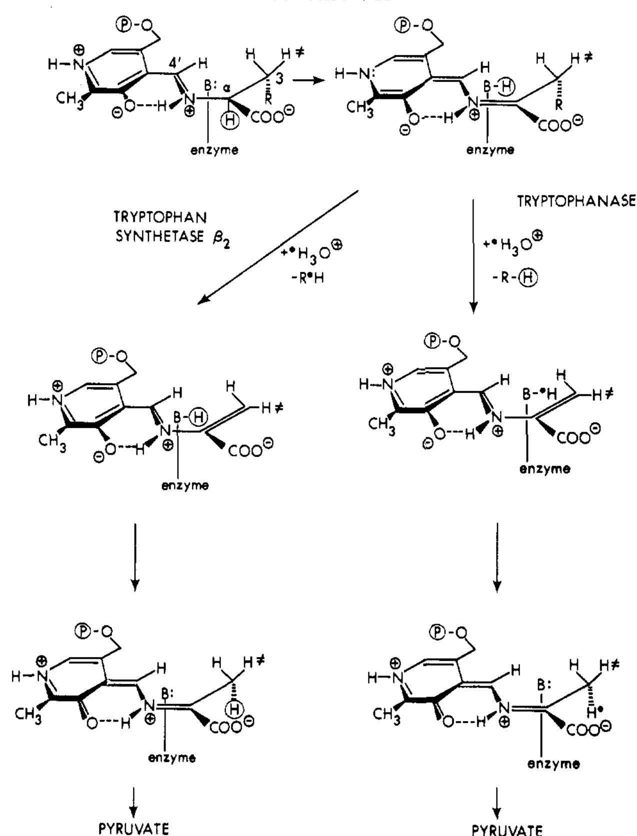
Tryptophanase is the protein primarily responsible for bacterial catabolism of L-tryptophan to indole, pyruvate, and ammonia.[57] We investigated this reaction as well as the analogous deamination of L-serine and the β-replacement synthesis of L-tryptophan from L-serine and indole.[52] Conversion of stereospecifically C-3 tritiated serine and tryptophan[42,44] by trytophanase to pyruvate in D_2O again proceeds in a retention mode. To test the possibility that the α proton of tryptophan which is removed in the initial step is involved in protonation of the aminoacrylate intermediate or the indolyl group, L-[2-^{3}H]tryptophan was incubated with tryptophanase and lactate dehydrogenase. Although less than 0.05% of the tritium could be detected in the methyl group of lactate, there was significant transfer of tritium from C-2 of tryptophan to C-3 of indole. Further experiments demonstrated that the hydrogen migration is at least predominantly intramolecular. These results suggest that a single base accomplishes a suprafacial 1,3 shift of the α hydrogen and that the elimination is a syn process. Since the resulting aminoacrylate intermediate is protonated from the same side as indole leaves, the same base may also be involved in this proton transfer. If this is true, hydrogen exchange of the base with solvent must then be more rapid than decomposition of the intermediate to pyruvate. Since the β-replacement reaction (formation of tryptophan from serine and indole) also proceeds in a retention mode, all reactions appear to occur on a single side of the substrate–cofactor complex.

To further define the geometry of this intermediate, we reduced the tryptophanase–PLP–L-alanine complex with sodium boro[^{3}H]hydride. The L-alanine is a competitive inhibitor which exchanges its α hydrogen and generates an aldimine–ketimine equilibrium on the enzyme surface (Scheme VI).[58] Degradation of the resulting pyridoxylalanine to alanine and pyridoxamine and stereochemical analysis showed that reduction proceeds predominantly from the *re* face at C_α and the *si* face at C-4′. These results define the ketimine bond

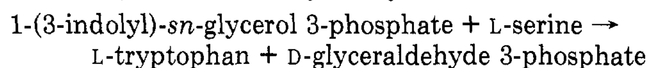
(57) E. E. Snell, *Adv. Enzymol.*, **42**, 287–333 (1975).
(58) Y. Morino and E. E. Snell, *J. Biol. Chem.*, **242**, 2800–2809 (1967).

Scheme VII



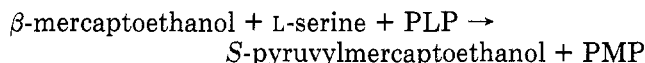
as E and identify the exposed "solvent side" at C-4′ as *si*. The overall mechanistic and geometrical picture is shown in Scheme VII. Reduction of the tryptophanase–PLP complex in the absence of substrate shows a greater exposure of the *re* face at C-4′,[29] indicating conformational changes upon binding of substrate similar to those described previously for tyrosine decarboxylase.

A catalytically related enzyme, tryptophan synthetase,[59,60] is a tetrameric protein having an $\alpha_2\beta_2$ subunit structure, which normally catalyzes the reaction


1-(3-indolyl)-*sn*-glycerol 3-phosphate + L-serine → L-tryptophan + D-glyceraldehyde 3-phosphate

The enzyme can also synthesize tryptophan from L-serine and indole. Although its β_2 subunit is unable to catalyze the first reaction, it does carry out the latter process in addition to a number of other reactions:

L-serine → pyruvate + ammonia


β-mercaptoethanol + L-serine + PLP → *S*-pyruvylmercaptoethanol + PMP

Our group and Fuganti and co-workers independently confirmed that the β-replacement syntheses of tryptophan by the native enzyme or its β_2 subunit proceed in a retention mode at C-3 of the amino acid.[42,53] Surprisingly, the α,β elimination of (2*S*,3*S*)- and (2*S*,3*R*)-[3-^{3}H]serine isomers by tryptophan synthetase β_2 protein in D_2O gave pyruvate (isolated as lactate) in which the methyl groups were achiral or racemic. Closer inspection indicated that there was no deuterium incorporation into the methyl group and that the third hydrogen must originate from within the enzyme–substrate complex. Further experiments with labeled serine isomers showed the process to be a quantitative intramolecular 1,2 shift of the α hydrogen to the β carbon in a retention mode at the β position. During the abortive transamination of unlabeled serine and mercaptoethanol in D_2O by the β_2 protein, only about 0.25 atom of deuterium per mole appears at C-4′ of the product pyridoxamine phosphate (PMP). Since the rest of the newly added hydrogen must originate from within the enzyme–substrate complex and be added from the *si* face at C-4′,[7] the reaction is probably a suprafacial 1,3-hydrogen shift from the α carbon of serine to C-4′ of the cofactor. Such a shift has been observed in pyridoxamine–pyruvate transaminase.[1b,12] Some internal proton transfer may also take place in the β-replacement reaction. Incubation of native tryptophan synthetase with unlabeled indole and L-serine in D_2O produces L-tryptophan with about 10% ^{1}H at the α carbon. The absence of an isotope effect for this reaction in a medium of H_2O and D_2O (1:1) suggests[61] that a monoprotic base, probably histidine, accomplishes these proton transfers.[60,62]

If we accept the reasonable assumption[63] that the conformation about the C-4 to C-4′ bond is syn to allow hydrogen bonding between nitrogen and the phenolic hydroxyl, the mechanistic-stereochemical picture in Scheme VII emerges. The enzyme controls the C_α–N conformation to place the C_α-hydrogen bond perpendicular to the plane of the conjugated system. A monoprotic base (e.g., histidine) removes this hydrogen to initiate a syn elimination of the β substituent and generate a PLP–aminoacrylate intermediate. The now protonated base can add hydrogen from a single face to one of three sites: C-3 to form pyruvate (α,β elimination), C-4′ to produce pyridoxamine (abortive transamination), or C_α (following nucleophilic addition from the same side at C-3) to synthesize a new amino acid (β replacement). Since tryptophan synthetase β_2 and tryptophanase share many catalytic and stereochemical properties, the geometries of their coenzyme–substrate complexes (and of related enzymes) are very similar. However, tryptophanase uses the α hydrogen to protonate the leaving group, whereas β_2 protein can quantitatively transfer it to the complex at C-3. The probable difference lies in the nature of the base groups and their precise location relative to the PLP-substrate complex.

General Aspects and Conclusions

One may wonder whether during evolution many diverse enzymes perfected their use of pyridoxal phosphate to achieve optimal catalysis (convergent evolution) or whether current functional differences are results of mutation of a single "grandfather enzyme" (divergent evolution). Although a final answer will not be possible until a number of detailed three-dimensional protein structures become available, recent stereochemical studies support the concept of pyridoxal phosphate enzyme evolution from a single ancestral protein. The generality of protonation from the expo-

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sed *si* face at C-4′ of PLP–substrate imines and the similarity of conformational changes upon substrate binding document the relatedness of these proteins but offer no obvious catalytic advantage. Examination of the cryptic stereochemistry of their reactions has given a conformational picture of the PLP–substrate complexes in the active sites and provided insight into mechanistic aspects, especially intramolecular proton transfers. With the exception of racemases,[64] PLP enzymes generally force retention modes of operation at the α and β carbons of substrates for processes as diverse as transamination, decarboxylation, retroaldolization, β replacement, α,β elimination, and β,γ elimination.[65] This allows in many cases the mediation of multiple proton transfers in a catalytic cycle by a single base; internal proton recycling can then reduce the number of diffusion-controlled steps. This bias favors similar conformations for the bound coenzyme–substrate complexes in the active sites of many of these enzymes. Predominantly, reactions seem to occur on only one side of the enzyme-bound substrate–cofactor complex. Work is already in progress in several laboratories to determine how α-racemases, ω-aminotransferases,[66] enzymes operating on D-amino acids, and the "anomalous" reactions of serine transhydroxymethylase fit into this concept and the single progenitor hypothesis.

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Tricarbonyl(diene)iron Complexes: Synthetically Useful Properties

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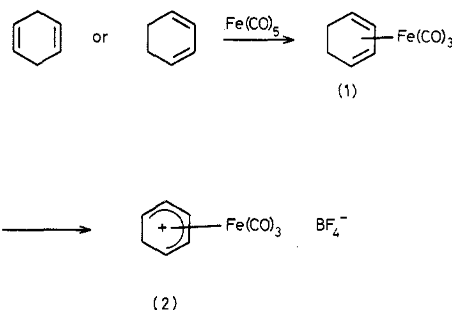
Synthetic application of organometallic complexes is both an exciting and a challenging area of research: exciting because of the enormous areas of chemistry still awaiting exploration, and challenging because, to a large extent, the behavior of such complexes has still to be put on a firm mechanistic basis. While not forgetting some of the earlier pioneering work of Pettit, Lillya, Whitesides, Lewis,[1] and Birch (references) this account mainly concentrates on our own efforts to develop reactions of tricarbonyl(diene)iron complexes which may be applied to the synthesis of fairly complex natural products. Although most of our targets are still far from being realized, we hope to convey the considerable potential of these compounds.

Synthesis of Tricarbonylcyclohexadienyliron Salts

Reaction of either cyclohexa-1,4-diene or cyclohexa-1,3-diene with pentacarbonyliron gives tricarbonyl(cyclohexa-1,3-diene)iron (1) in moderate yield.[2] This complex undergoes hydride abstraction by treatment with triphenylmethyl (trityl) tetrafluoroborate in dichloromethane to give tricarbonylcyclohexadienyliron tetrafluoroborate (2), precipitated quantitatively by addition of diethyl ether.[3] Birch et al. have studied complex formation between pentacarbonyliron and substituted cyclohexa-1,4-dienes, readily available by metal–ammonia reduction of aromatic compounds.



Predictably, this reaction results in the formation of mixtures, since the 1,4-diene can be conjugated in two or more ways. Examples which concern us are the complexes (3 and 4) derived from 2,5-dihydroanisole, and (5 and 6) from 4-methyl-2,5-dihydroanisole.[4]

Anthony J. Pearson is currently recipient of a Science Research Council Advanced Fellowship at the University of Cambridge. After undergraduate studies at Leeds University and postgraduate research at Aston University, he spent 3 postdoctoral years with A. J. Birch in Canberra.

(1) See, for example: J. E. Mahler and R. Pettit, *J. Am. Chem. Soc.*, **85**, 3955 (1963); G. F. Emerson, L. Watts and R. Pettit, *ibid.*, **87**, 131 (1965); G. F. Emerson and R. Pettit, *ibid.*, **84**, 4591 (1962); G. F. Emerson, J. E. Mahler, and R. Pettit, *Chem. Ind. (London)*, 836 (1964); R. Pettit and J. Henery, *Org. Synth.*, **50**, 21 (1970); R. Pettit, J. C. Barborak, and L. Watts, *J. Am. Chem. Soc.*, **88**, 1328 (1966); C. P. Lillya and R. A. Sahatjian, *J. Organometal. Chem.*, **32**, 371 (1971); R. E. Graf and C. P. Lillya, *J. Am. Chem. Soc.*, **94**, 8282 (1972); *J. Organometal. Chem.*, **47**, 413 (1973); *J. Chem. Soc., Chem. Commun.*, 271 (1973); *J. Organometal. Chem.*, **122**, 377 (1976); T. H. Whitesides and R. W. Arhart, *J. Am. Chem. Soc.*, **93**, 5296 (1971); T. H. Whitesides, R. W. Arhart, and R. W. Slaven, *ibid.*, **95**, 5792 (1973); T. H. Whitesides and J. P. Neilan, *ibid.*, **95**, 5811 (1973); **98**, 63 (1976); T. H. Whitesides and R. W. Slaven, *J. Organometal. Chem.*, **67**, 99 (1974); B. F. G. Johnson, K. D. Karlin, and J. Lewis, *ibid.*, **145**, C23 (1978); and references cited therein.
(2) B. F. Hallam and P. L. Pauson, *J. Chem. Soc.*, 642 (1958).
(3) E. O. Fischer and R. D. Fischer, *Angew. Chem.*, **72**, 919 (1960).
(4) (a) A. J. Birch, P. E. Cross, J. Lewis, and D. A. White, *Chem. Ind. (London)*, 838 (1964); (b) A. J. Birch, P. E. Cross, J. Lewis, D. A. White, and S. B. Wild, *J. Chem. Soc. A*, 332 (1968); (c) A. J. Birch, K. B. Chamberlain, M. A. Haas, and D. J. Thompson, *J. Chem. Soc., Perkin Trans. 1*, 1882 (1973).

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