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Stereochemical Evidence for the Evolution of Pyridoxal-phosphate Enzymes of Various Function from a Common Ancestor

(tryptophan synthase B protein/transamination/stereochemistry)

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ABSTRACT **Several pyridoxal-phosphate-dependent enzymes can convert the bound cofactor to pyridoxamine phosphate. This conversion may be an obligatory part of the normal catalytic sequence, as with transaminases, or may be an abnormal path, inactivating the enzyme. This conversion requires protonation of the C_4' carbon of the cofactor, which has now been shown to proceed stereospecifically and *with the same absolute stereochemistry* in seven quite different pyridoxal-phosphate enzymes. We report on one of these, tryptophan synthase B protein. This regularity in protonation stereochemistry suggests a remarkable regularity in the geometry of cofactor binding to the apoenzyme. This regularity is interpreted as evidence for the evolution of this entire family of enzymes from a common progenitor which, through the course of evolution, could not invert its original, arbitrary binding stereochemistry without passing through catalytically inactive conformations.**

Pyridoxal-phosphate-dependent enzymes catalyze a very broad range of reactions of amino acids. It is believed that all of these reactions proceed by formation of a Schiff base between the cofactor and the amino group of the substrate, followed by cleavage of one of three bonds to the substrate alpha carbon. The nature of the substrate and the influence of the apoenzyme dictate which bond is broken and control the nature of the final product.

In many pyridoxal-phosphate-dependent reactions, the bound cofactor is converted to pyridoxamine phosphate. This conversion may be an obligatory part of the normal catalytic sequence, as with transaminases, or may be an abnormal path, inactivating the enzyme. The key step in this reaction is the protonation of the bound substrate–pyridoxal-phosphate Schiff base at the cofactor C_4' carbon after loss of one of the groups at the substrate C_α.* The absolute stereochemistry of this protonation has been determined for six different pyridoxal-phosphate-dependent enzymes. They are the four transaminases: glutamate aspartate transaminase (1, 2), pyridoxamine pyruvate transaminase (3), dialkyl amino-acid transaminase (4), and alanine transaminase (D. Arigoni, personal communication); the pyridoxal-phosphate-dependent L-glutamate decarboxylase, which undergoes transamination and inactivation under certain conditions (5); and serine hydroxymethylase, which is inactivated by transamination in the presence of D-alanine (6). In all cases, the cofactor C_4' carbon is protonated from the *si* face of the planar intermediate, yielding pyridoxamine or pyridoxamine phosphate with the enzyme labile proton in the *pro*-S configuration (7).

We now report the stereochemistry of this protonation in a seventh system, the transamination of the bound pyridoxal-phosphate of tryptophan synthase B protein by L-serine in the presence of 2-mercaptoethanol. This abnormal transamination was discovered by E. W. Miles *et al.* (8) and is believed to proceed as shown in Fig. 1. The normal α-β elimination–readdition path (path I) leading to tryptophan is catalyzed by the complete tryptophan synthase oligomer. The B-protein component of that oligomer binds pyridoxal-phosphate and will catalyze the dehydration of serine (path II). In the presence of mercaptoethanol, the intermediate (*A*) is formed, which can follow the abnormal path III, protonating at C_4' and hydrolyzing to pyridoxamine phosphate.

Analysis of the product of this transamination (Fig. 2) showed that the C_4' protonation in this system also proceeds from the *si* face of the C_4' imine carbon, yielding pyridoxamine phosphate with the C_4' *pro*-S proton in the enzyme-labile position (Fig. 3).

The invariant stereochemistry of protonation shown by these seven pyridoxal-phosphate enzymes was unexpected, since the seven include enzymes that labilize each of the bonds to C_α of the amino-acid substrate. We believe that this regularity has important implications for the early evolutionary history of this family of enzymes.

EXPERIMENTAL

Tryptophan synthase B protein was a gift of E. W. Miles.

All radioactivity counting was done on a Packard Tri-Carb Scintillation Spectrometer model 3320 with a scintillation

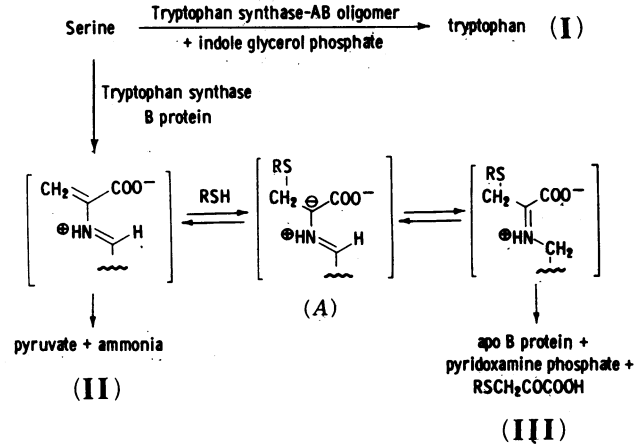

FIG. 1. Reactions of tryptophan synthase (from ref. 8).

* For numbering system, see Fig. 3.

$$\text{RSH} + \text{L-serine} + [C_4'\text{-}{}^3\text{H}]\text{Pyridoxal-phosphate} \xrightarrow[\text{B protein}]{\text{tryptophan synthase}} \text{RSCH}_2\text{COCOOH} + [C_4'\text{-}{}^1\text{H}{}^3\text{H}]\text{Pyridoxamine phosphate}$$

$$[C_4'\text{-}{}^1\text{H}{}^3\text{H}]\text{Pyridoxamine phosphate} \xrightarrow[\text{phosphate}]{\text{alkaline}} [C_4'\text{-}{}^1\text{H}{}^3\text{H}]\text{Pyridoxamine}$$

$$[C_4'\text{-}{}^1\text{H}{}^3\text{H}]\text{Pyridoxamine} + \text{pyruvate} \xrightarrow[\text{transaminase}]{\text{pyridoxamine-pyruvate}} [C_4'\text{-}{}^3\text{H}]\text{Pyridoxal} + \text{L-alanine}$$

FIG. 2. Stereochemistry of cofactor protonation during thiol-dependent transamination catalyzed by tryptophan synthase B protein

fluid mixture containing 330 ml of Triton X-100 (Packard), 28 ml of Liquiflor (New England Nuclear Corp.), and 642 ml of toluene (9). The counting efficiency was 26%.

Pyridoxal-phosphate specifically labeled with ^{3}H at C_4' was prepared by reduction of pyridoxal-phosphate imine with NaB^3H_4 followed by reoxidation of the resulting [^{3}H]pyridoxamine phosphate with MnO_2, as described by Voet *et al.* (6). The specific activity of the [^{3}H]pyridoxal phosphate was 83 μCi/μmole.

RESULTS

The apoenzyme of tryptophan synthase B protein was prepared by dialyzing the enzyme for 24 hr against 50 mM L-serine, 50 mM 2-mercaptoethanol in 0.1 M phosphate (pH 7.8). One milligram (0.01 μmole) of dialyzed enzyme was brought to 1.0 ml with the same buffer. About 0.01 μmole of [^{3}H]pyridoxal phosphate (specific activity 83 μCi/μmole) was added, and the mixture was incubated 25 min at 25°.

Transamination was observed spectrally by the decrease in absorption at 418 nm and the increase at 322 nm. After transamination was complete, carrier pyridoxamine phosphate was added and the reaction mixture was diluted to 3.0 ml. Protein was removed by ultrafiltration, and the pyridoxamine phosphate was isolated on a Dowex-1-acetate column, as described by Voet *et al.* (6). The configuration of the tritium at C_4' was determined as in ref. 6 by dephosphorylation followed by reverse transamination with pyruvate and pyridoxamine-pyruvate transaminase. The path of the tritium during these transformations is shown in Fig. 2.

Under the conditions of the enzymatic transamination, no measurable nonenzymatic transamination was observed. However, if 0.1 M DL-alanine and 10 μM [^{3}H]pyridoxal phosphate are incubated for 80 min at 25° in 1.0 M acetate buffer at pH 5.0, almost complete transamination is observed. The stereochemistry of this nonenzymatic transamination was determined just as with the enzymatic reaction. As shown in Table 1, the racemic [^{3}H]pyridoxamine formed in the nonenzymatic transamination retains only half of its radioactivity on enzymatic reverse transamination to pyridoxal, while the tritium label of the tryptophan synthase-derived pyridoxamine is almost completely retained in the pyridoxal product of that reverse transamination.

Since pyridoxamine-pyruvate transaminase is known to labilize the *pro* S proton of pyridoxamine (3), it follows that a solvent ^{1}H occupied this position in the [^{3}H]pyridoxamine from tryptophan synthase B protein transamination. Thus, solvent ^{1}H was added to the *si* face of the C_4' carbon during that transamination.

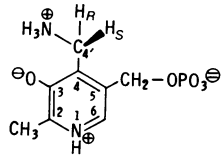

FIG. 3. Pyridoxamine phosphate.

DISCUSSION

In the seven pyridoxal-phosphate enzymes that have been examined, transamination of the bound cofactor proceeds in all cases with protonation of the *si* face of the C_4' carbon in the substrate–cofactor Schiff base. Assuming that the C_4–C_4' carbon–carbon bond exists in that conformation which allows the phenolic hydroxyl group to hydrogen bond to the imine nitrogen, we can draw Fig. 4 *A* to represent the face of the bound substrate–cofactor Schiff base that is protonated in each of the seven enzymes. It seems reasonable to assume that this is in fact the outward, more aqueous face of this bound Schiff base and that the invariant protonation stereochemistry is the result of invariance in the arrangement of binding sites on the enzyme surface for the substrate–cofactor Schiff base. We will refer to this outward face of the cofactor complex as the *si* face, and the opposite, bound face as the *re* face†. Although the cofactor is itself achiral, it has enantiotopic faces between which any chiral substance can distinguish. The question is, why do seven different pyridoxal-phosphate enzymes, which catalyze quite different reactions, all have an active site geometry that binds the *re* face of the cofactor–substrate Schiff base?

The answer must lie either in a selective advantage attached to the binding of one face, or in the evolution of all pyridoxal-phosphate enzymes from a common ancestral protein.

In the former case, convergent evolution of pyridoxal-phosphate enzymes of various origin would have eliminated those binding the *si* face. This is similar to the subtilisin–chymotrypsin case, where nearly identical arrangements of catalytic groups in the active site are found in enzymes of markedly different tertiary structure (10).

The second possibility, evolution from a common ancestor, views the regularity as a result of the binding of the *re* face by the first functional pyridoxal-phosphate enzyme and the preservation of that original choice throughout evolution.

† In the absence of substrate, most pyridoxal-phosphate-dependent enzymes bind the cofactor in Schiff base linkage to an active site lysine residue. In one case, reduction of the pyridoxal-phosphate–lysine Schiff base with borohydride shows either a lack of stereospecificity (in the absence of substrate) or reduction of the *re* face of C_4' (in the presence of a substrate analog) (D. Arigoni, personal communication). This suggests that the pyridoxal-phosphate–lysine Schiff base is not held in a rigid geometry and that formation of the pyridoxal-phosphate–substrate Schiff base can involve a change in the exposed face of C_4'. However, this discussion concerns only the cofactor–substrate Schiff base that is directly involved in the catalytic act and that appears to be bound with an invariant stereochemistry.

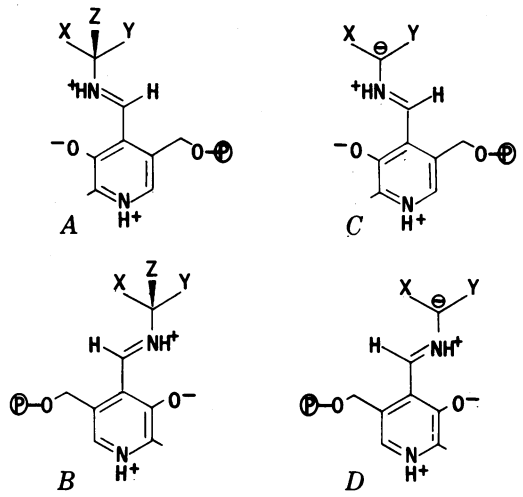

FIG. 4. Alternate binding stereochemistry for cofactor–substrate Schiff bases.

This differs from the usual pattern of divergent evolution found in many protein families in that the structural feature preserved (ginding symmetry) may have no relationship to efficiency. While divergent evolution is normally seen as the preservation, for reasons of efficiency, of a structure that evolved very early under the pressure of efficiency, the pyridoxal-phosphate enzyme regularity may reflect an early, completely arbitrary choice between *re* and *si* that was preserved, not because the other choice is less efficient, but because the first choice, once made, is immutable.

A choice between these two alternatives depends on a chemical judgement as to the possible selective advantage of one binding symmetry over the other. In Fig. 4, structures *A–D* show the possible paths for a pyridoxal-phosphate enzyme-catalyzed loss of a group "Z" from an amino acid alpha carbon. The C_α-Z bond is presumed to lie in a plane perpendicular to the cofactor pi system (11). Thus, the face from which Z leaves must resemble structure *A* or *B* and, after loss of Z, the anions *C* or *D* will result. Neither *A* and *B* nor *C* and *D* have the same energy because of different relationships of the groups X and Y to the —CH=N-bond. For a specific reaction, where X, Y, and Z are defined by the reaction catalyzed and by the structure of the substrate, the paths A → C → products and B → D → products *must* differ in efficiency, which would allow evolution to eliminate the enzyme using the less efficient path.

However, if this analysis is to explain the existence of the A → C path in seven cases, which include examples of each of the reaction types of Fig. 3, it would have to be argued that A → C → products happens to be the most efficient path independent of the nature of Z (and of X and Y). Furthermore, within each reaction type the A → C path would have to be most efficient independent of the exact nature of the amino acid R group.

We find it very unlikely that the path, A → C → products, should be the most efficient in all these cases and interpret the regularity in protonation symmetry as very good evidence for the evolution of all pyridoxal-phosphate enzymes from a common ancestor. It is possible that the ancestral protein's choice of *re* face binding was itself the product of evolutionary selection during a period when only one of the reactions of Fig. 4 was important. However, we find it more likely that the original binding was a chance and unique event and that the ancestral protein had fixed cofactor binding but low reaction and substrate specificity. It is easy to imagine this relatively nonspecific enzyme giving rise to the whole family of present pyridoxal-phosphate enzymes by a series of small changes in the amino acid binding geometry. It is much more difficult to imagine a series of mutations that would lead to the binding of the other face of the Schiff base without including intermediate structures of low catalytic efficiency. The groups of the cofactor–substrate Schiff base that interact most strongly with the enzyme are the C_5' phosphate, the amino acid residue attached at C_4', and the pyridine ring nitrogen. These groups form a rough triangle, which must correspond to a triangular arrangement of binding sites on the enzyme. Once established, the inversion of this triangle of binding sites seems improbable. Thus the original, randomly chosen binding stereochemistry of the substrate–pyridoxal-phosphate Schiff base could well have been frozen throughout biochemical evolution by the lack of viable paths leading to enzymes of inverted binding symmetry.

There are very few phenomena in biology that represent the preservation of an early choice between equally probable alternatives. These few cases are very good evidence for the unique event formulation of the origin of life. Rose has discussed one such case, the uniformity of protonation stereochemistry in the enzyme-catalyzed reactions of phospho*enol*-pyruvate (12). We believe that pyridoxal-phosphate binding symmetry represents a second example.

To date, the sequence of only one pyridoxal-phosphate-dependent enzyme has been completely determined (13), and no pyridoxal-phosphate enzyme structure has been determined by x-ray analysis. Thus, comparisons within this group are limited to the studies reported here, optical rotatory dispersion measurements (14), general spectral properties, and limited sequences of amino acids surrounding the active-site lysine residue (15, 16). As other sequences are determined, it may be possible to detect evidence of a common origin by sequence comparison. It is also possible that the common ancestor is so

TABLE 1. *Radioactivity released from [C_4'-$^1H^3H$]pyridoxamine during transamination with pyruvate by pyridoxamine-pyruvate transaminase*

	Source of [C_4'-$^1H^3H$]pyridoxamine	
	Enzymatic[a]	Nonenzymatic[b]
cpm in pyridoxamine	2.80×10^5	3.66×10^4
cpm recovered in pyridoxal	2.82×10^5	1.87×10^4
cpm present in water	0.09×10^5	1.76×10^4

[a] Tryptophan synthase B protein catalyzed transamination of [C_4'-^{3}H]pyridoxal phosphate by L-serine in the presence of 2-mercaptoethanol followed by enzymatic dephosphorylation of the [^{3}H]pyridoxamine phosphate.

[b] Nonenzymatic transamination of 0.1 M DL-alanine and 10 μM [C_4'-^{3}H]pyridoxal phosphate in 1.0 M acetate buffer (pH 5.0) followed by enzymatic dephosphorylation.

remote that certain common features of tertiary structure and the common stereochemistry of substrate–cofactor Schiff base binding will be the best clues to a common origin.

The regularity of protonation stereochemistry in this group of pyridoxal-phosphate enzymes is in marked contrast to the NAD-NADH oxidoreductases, where about equal numbers of enzymes are specific for alpha as for beta hydrogen transfer from NADH (17). The nicotinamide ring's 2-fold axis is destroyed only by the 3-carboxyamide group. In contrast to the pyridoxal-phosphate case, one can easily imagine a series of minor changes in active site geometry that would allow 180° rotation about the N_1-C_4 axis of the nicotinamide ring without serious loss of enzymatic efficiency.

No other cofactor molecules undergo enzymatic transformations that produce enantiotopic groups. However, thiamine pyrophosphate is achiral and lacks anything approaching a 2-fold axis. If a technique were available to determine which face of thiamine pyrophosphate is exposed in various thiamine pyrophosphate-dependent enzymes, that cofactor might also show evidence of an early, arbitrary choice of binding symmetry preserved in all present day thiamine pyrophosphate-dependent enzymes.

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1. Dunathan, H. C., Davis, L., Kury, P. G. & Kaplan, M. (1968) *Biochemistry* **7**, 4532–4537.
2. Besmer, P. & Arigoni, D. (1969) *Chimia (Switz.)* **23**, 190.
3. Ayling, J. E., Dunathan, H. C. & Snell, E. E. (1968) *Biochemistry* **7**, 4537–4542.
4. Bailey, G. B., Kusamrarn, T. & Vuttivej, K. (1970) *Fed. Proc.* **29**, 857.
5. Sukhareva, B. S., Dunathan, H. C. & Braunstein, A. E. (1971) *FEBS Lett.* **15**, 241–244.
6. Voet, J. G., Hindenlang, D. M., Blanck, T. J. J., Ulevitch, R. J., Kallen, R. G. & Dunathan, H. C. (1973) *J. Biol. Chem.* **248**, 841–842.
7. Hanson, K. R. (1966) *J. Amer. Chem. Soc.* **88**, 2731–2742.
8. Miles, E. W., Hatanaka, M. & Crawford, I. P. (1968) *Biochemistry* **7**, 2742–2753.
9. Patterson, M. S. & Greene, R. C. (1965) *Anal. Chem.* **37**, 854–857.
10. Robertus, J. D., Alden, R. A., Birktoft, J. J., Kraut, J., Powers, J. C. & Wilcox, P. E. (1972) *Biochemistry* **11**, 2439–2449.
11. Dunathan, H. C. (1966) *Proc. Nat. Acad. Sci. USA* **55**, 712–716.
12. Rose, I. A. (1970) *J. Biol. Chem.* **245**, 6052–6059.
13. Ovchinnikov, Yu. A., Egorov, C. A., Aldanova, N. A., Feigina, M. Y., Lipken, V. M., Abdulaev, N. G., Grishin, E. V., Kiselev, A. P., Modyanov, N. N. & Braunstein, A. E. (1973) *FEBS Lett.* **29**, 31–35.
14. Dunathan, H. C. (1971) *Advan. Enzymol.* **35**, 79–134.
15. Snell, E. E. & DiMari, S. J. (1970) in *The Enzymes*, ed. Boyer, P. D. (Academic Press, New York), 3rd ed., Vol. 11, p. 366.
16. Boeker, E. A. & Snell, E. E. (1972) in *The Enzymes*, ed. Boyer, P. D. (Academic Press, New York), 3rd. ed., Vol. VI, p. 247.
17. Bentley, R. (1970) in *Molecular Asymmetry in Biology* (Academic Press, New York), Vol. II, pp. 6–9.