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The mechanism of action of phenylalanine ammonia-lyase: The role of prosthetic dehydroalanine

(transient Friedel–Crafts acylation/β-proton activation/4-nitrophenylalanine as substrate)

BIRGID SCHUSTER AND JANOS RÉTEY*

Lehrstuhl Biochemie im Institut für Organische Chemie der Universität Karlsruhe, Kaiserstrasse 12, D-76128 Karlsruhe, Germany

Communicated by Klaus Hahlbrock, Max-Planck-Institut für Züchtungsforschung, Cologne, Germany, June 5, 1995 (received for review April 7, 1995)

ABSTRACT **Phenylalanine ammonia-lyase (EC 4.3.1.5) from parsley is posttranslationally modified by dehydrating its Ser-202 to the catalytically essential dehydroalanine prosthetic group. The codon of Ser-202 was changed to those of alanine and threonine by site-directed mutagenesis. These mutants and the recombinant wild-type enzyme, after treatment with sodium borohydride, were virtually inactive with L-phenylalanine as substrate but catalyzed the deamination of L-4-nitrophenylalanine, which is also a substrate for the wild-type enzyme. Although the mutants reacted about 20 times slower with L-4-nitrophenylalanine than the wild-type enzyme, their V_{max} for L-4-nitrophenylalanine was two orders of magnitude higher than for L-phenylalanine. In contrast to L-tyrosine, which was a poor substrate, DL-3-hydroxyphenylalanine (DL-*m*-tyrosine) was converted by phenylalanine ammonia-lyase at a rate comparable to that of L-phenylalanine. These results suggest a mechanism in which the crucial step is an electrophilic attack of the prosthetic group at position 2 or 6 of the phenyl group. In the resulting carbenium ion, the β-H_{Si} atom is activated in a similar way as it is in the nitro analogue. Subsequent elimination of ammonia, concomitant with restoration of both the aromatic ring and the prosthetic group, completes the catalytic cycle.**

Phenylalanine ammonia-lyase (PAL) is an important plant enzyme that converts L-phenylalanine into *trans*-cinnamic acid, which in turn is the precursor of various phenylpropanoids, such as lignins, flavonoids, and coumarins (1, 2). It has been known for a long time that PAL contains a catalytically essential dehydroalanine residue (3). Although the latter occurs in a number of natural peptides (4, 5) and has been postulated as an intermediate in the conversion of L-serine residues into D-alanine (6), it is shared only by one related enzyme, histidine ammonia-lyase (HAL) (7–10).

The electrophilic property of the prosthetic dehydroalanine has been demonstrated by addition of various nucleophiles, such as nitromethane (7–11), cyanide (12), and sodium borohydride (3, 8). All these reagents caused inactivation and, using radiolabel, incorporation of the radioisotope into the expected product. Twenty-five years ago a mechanism was proposed for the PAL and HAL reactions involving as an initial step the nucleophilic attack at the dehydroalanine residue by the amino group of the substrate (3, 7). Such a reaction should enhance the leaving ability of the amino group. Recently, this laboratory has identified the precursor of the dehydroalanine in HAL from *Pseudomonas putida* and PAL from parsley to be Ser-143 and Ser-202, respectively (9, 10, 13). Heterologous expression allowed the production of mutants by site-directed mutagenesis. The PAL mutant S202A and the HAL mutants S143A and S143T were virtually inactive with L-phenylalanine and L-histidine as substrates, respectively (9, 10, 13, 14).

Surprisingly, L-5-nitrohistidine, known to be a moderately good substrate of HAL, reacted with the dehydroalanine-less mutants S143A and S143T and with wild-type HAL to the same extent. This rules out the mechanism that all investigations have relied upon for the last 25 years and suggests a chemically more plausible mechanism (15).

These results prompted us to reinvestigate the PAL reaction with alternative substrates and dehydroalanine-less mutant enzymes. Here we report the results of our experiments that led us to postulate a completely different mechanism of the PAL reaction involving a transient Friedel–Crafts-like acylation of the phenyl ring.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions. *Escherichia coli* TG1 cells were used for the isolation of single-stranded DNA from M13 phages to carry out site-directed mutagenesis. Cells were grown and infected as described (16). *E. coli* BL21(DE3) served for the expression of either wild-type or mutant PALs. For overexpression, cells were grown in 1 liter of Luria–Bertani (LB) medium supplemented with ampicillin (85 μg/ml) at 37°C. At an OD_{600} of 1.0, 400 mM isopropyl β-D-thiogalactoside was added (17) and cells were harvested 4 h after induction.

The phage M13BM21 was purchased from Boehringer Mannheim.

The expression vector pT7.7 was generously provided by Stanley Tabor (18).

The *PAL1* gene from a cDNA library from elicitor-treated parsley (*Petroselinum crispum* L.) cells and antibodies against PAL were generous gifts of K. Hahlbrock (Max-Planck-Institut, Cologne, Germany) (19) and N. Amrhein (Eidgenössiche Technische Hochschule, Zurich), respectively. pT7.7PAL was constructed as described by Schuster and Rétey (13).

DL-*m*-Tyrosine and L-4-nitrophenylalanine were purchased from Fluka and their quality was checked by ^{1}H NMR spectroscopy.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed by following the protocol of the Amersham mutagenesis kit (Sculptor). PALMutS202A and PALMutS202T were constructed as described by Schuster and Rétey (13). The oligonucleotide sequence used in this mutagenesis reaction was 5′-CACTGCTACTGGTGATC-3′.

Transformation. Either *E. coli* TG1 or *E. coli* BL21(DE3) cells were grown in 0.5 liter of LB medium to an OD_{600} of 0.90–0.95 (20). The cells were collected by centrifugation at 4500 × *g*, and the cell pellet was resuspended in 0.5 liter of ice-cold 10% (vol/vol) glycerol solution. The cells were made competent in five steps by reduction of the suspension volume of 10% glycerol to a final volume of 1 ml. All steps were

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Abbreviations: PAL, phenylalanine ammonia-lyase; HAL, histidine ammonia-lyase.

*To whom reprint requests should be addressed.

performed at 4°C. Aliquots (140 μl) of the concentrated competent cells were stored at −70°C for 2 months without loss of competence. Transformation was performed by electroporation by using a Gene Pulser from Bio-Rad. The competent cell suspension (70 μl) was transformed with 20 ng of vector DNA in a 0.4-mm cuvette at 2.5 kV and 0.2 kΩ (6.25 kV/cm) for 4.4 ms. Recombinant phages were selected by blue/white screening (16). Recombinant bacteria were detected either by blue/white screening or by restriction analysis.

Purification. Transformed *E. coli* BL21 cells were grown in 1 liter of LB medium containing ampicillin (85 μg/ml) to an OD_{600} of 1.0. Then 0.4 mM isopropyl β-D-thiogalactoside was added. Cells were harvested 4 h after induction, by centrifugation at 4500 × *g*. The cell pellet was resuspended in 10 ml of 50 mM potassium phosphate (pH 7.0) containing 5 mM phenylalanine, 40 units of Benzonase (Merck), 5 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride. Sonication (Branson model 450, 70% power setting, 10 min in ice bath) was followed by centrifugation at 30,000 × *g* for 30 min. The clear supernatant was heat-treated in a 50°C bath for 10 min to precipitate soluble proteins (21). The suspension was again centrifuged at 30,000 × *g* for 30 min. The filtered supernatant (0.45-μm pore-size filter) was then applied to a HILoad 16/10 Q-Sepharose high-performance column at a flow rate of 3 ml/min, and 50 mM potassium phosphate (pH 7.0) supplemented with 5 mM phenylalanine as start buffer and 400 mM potassium phosphate (pH 7.2) as eluent was used. PAL was eluted at ≈120 mM potassium phosphate. Those fractions containing activity were pooled and concentrated in a semipermeable stirred cell, with a molecular mass limit of 50 kDa, to a final volume of 50 ml. PAL was further purified by anion-exchange chromatography with a TSK (North Bend, WA) DEAE-3SW Ultrapac column at a flow rate of 3 ml/min by using 10 mM potassium phosphate (pH 6.6) as buffer A and 400 mM potassium phosphate (pH 7.2) as buffer B. PAL was eluted at a concentration of 130 mM potassium phosphate. The active fractions were pooled, concentrated to 0.5 ml (Centricon-30, Amicon), and mixed with 0.5 ml of 100% glycerol. The enzyme was stored at −20°C.

SDS/PAGE, Western Blot Analysis, Protein Determination, and Enzyme Assay. SDS/PAGE using a 10% polyacrylamide gel was performed according to Laemmli (22) with Coomassie brilliant blue R250 staining. Western blot analysis was performed by using the protocol of Symington *et al.* (23). Protein determination was carried out according to Warburg and Christian as reviewed by Lane (24). PAL activity was determined spectrophotometrically as described by Zimmermann and Hahlbrock (25); the conversion of L-phenylalanine, L-tyrosine, and DL-*m*-tyrosine was monitored at 290 nm and that of L-4-nitrophenylalanine was monitored at 340 nm.

Preparation of Sodium Borohydride-Treated Wild-Type PAL. Reduction of the active site dehydroalanine in PAL was achieved by treating the enzyme with 20 mg of $NaBH_4$ in 0.1 M Tris (pH 8.8) at 4°C for ≈15 h. The excess $NaBH_4$ was then removed by dialysis against 0.1 M Tris (pH 8.8). Finally, the volume of the enzyme solution was reduced to obtain a final concentration of 7 mg/ml with Centricon-30 (Amicon).

RESULTS

Expression and Purification of Recombinant Wild-Type PAL and the Mutants S202A and S202T. These procedures for recombinant wild-type PAL and the mutant S202A have been described in detail (13). The PAL mutant S202T was constructed and overexpressed by an analogous method using the pT7.7 expression system. In all cases, the yield of recombinant protein was approximately the same, i.e., 4–8 mg per liter of *E. coli* culture. Western blot analysis of the SDS/PAGE gels of the recombinant wild-type PAL and the mutants S202A and S202T (Fig. 1) shows that all three proteins are indistinguishable by their molecular masses.

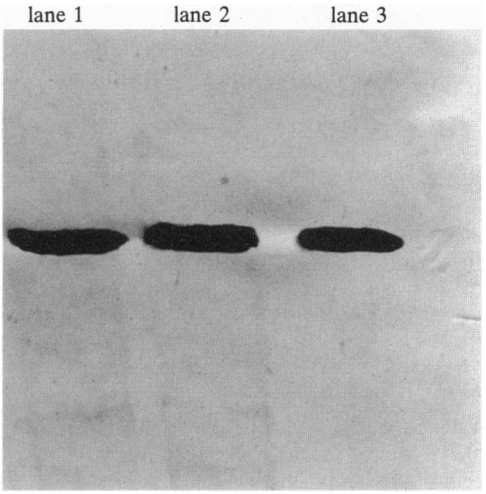

FIG. 1. Western blot of a SDS/PAGE gel showing overexpressed wild-type PAL (lane 1), mutant S202A (lane 2), and mutant S202T (lane 3), demonstrating that all three proteins are indistinguishable by their molecular masses.

Purification of the recombinant PAL proteins was carried out by a slight modification of the procedure as described (21, 25). This included sonification of the cell suspension and heat treatment of the cell extract followed by HPLC on a HILoad 16/10 Q-Sepharose and a TSK DEAE-3SW Ultrapac column. The purified recombinant wild-type enzyme had a specific activity of 0.56 unit/mg, comparable to PAL from soybean (26) or *Rhizoctonia solani* (27). Although the resulting preparations of wild-type PAL, $NaBH_4$-treated wild-type PAL, and the mutants S202A and S202T were not completely homogeneous, they were, however, of the same level of purity and adequate for comparison of our kinetic results.

Comparison of Kinetic Behavior of Wild-Type PAL, the Mutants S202A and S202T, and the Sodium Borohydride-Treated PAL with L-Phenylalanine as Substrate. The kinetic measurements were carried out using the spectrophotometric enzyme assay. The substrate concentrations were varied between 0.05 mM and 5 mM and the K_m and V_{max} values were determined by using the double reciprocal plot. As reported (13), with L-phenylalanine, mutant S202A showed a 5000 times lower V_{max} value, while its K_m value was of the same magnitude as that of the wild-type enzyme. Table 1 also shows that the V_{max} values determined for the mutant S202T and the $NaBH_4$-treated wild-type PAL were 21,000 and 1200 times lower, respectively, than that of fully active PAL.

Kinetics of the Reaction of L-Tyrosine and DL-*m*-Tyrosine Catalyzed by Wild-Type PAL. These measurements were carried out as described for L-phenylalanine as substrate. The substrate concentrations varied between 0.05 mM and 7 mM. A much higher enzyme concentration was required for L-tyrosine than for the other two substrates. The results are listed in Table 2. Whereas the kinetic constants for DL-*m*-tyrosine were very similar to those for the natural substrate L-phenylalanine, L-tyrosine showed a 4 times higher K_m and a

Table 1. Comparison of kinetic parameters from wild-type (wt) PAL, the mutants S202A and S202T, and the $NaBH_4$-treated wild-type PAL (wt PAL red.) with L-phenylalanine as a substrate

Enzyme	K_m, mM	V_{max}, units/mg	$V_{maxwtPAL}/V_{maxMutPAL}$
wt PAL	0.17	0.56	
S202A	0.20	1.1×10^{-4}	5,000
S202T		2.7×10^{-5}	21,000
wt PAL red.		4.5×10^{-4}	1,200

Table 2. Kinetic parameters from wild-type PAL to the substrates L-phenylalanine, L-tyrosine, and DL-*m*-tyrosine

Substrate	V_{max}, units/mg	K_m, mM
Phenylalanine	0.42	—
DL-*m*-Tyrosine	0.50	0.20
L-Tyrosine	0.02	0.8

20–25 times lower V_{max}. Its V_{max}/K_m ratio is thus 100 times lower than that for the two other substrates.

Kinetic Behavior of Wild-Type PAL, the Mutants S202A and S202T, and the Sodium Borohydride-Treated PAL with L-4-Nitrophenylalanine as Substrate. Table 3 shows the K_m and V_{max} values of the various forms of PAL with L-4-nitrophenylalanine as substrate. The kinetic constants were determined spectrophotometrically at 340 nm by taking advantage of the difference in the absorption of the substrate and the product 4-nitrocinnamic acid. The double reciprocal plots are depicted in Fig. 2 and the calculated kinetic constants are listed in Table 3. The K_m value (0.32 mM) of wild-type PAL is comparable with those of PAL from maize and potato (29), whereas the K_m value of the modified enzymes are ≈3 times higher. More dramatic are the differences in the V_{max} values. Although the modified enzymes react ≈20 times slower with L-4-nitrophenylalanine than does wild-type PAL, their V_{max} values are one to two orders of magnitude higher for L-4-nitrophenylalanine than for L-phenylalanine.

To confirm the reactivity of our modified enzymes with L-4-nitrophenylalanine, we carried out experiments on preparative scale. The crystalline products were examined by ^{1}H NMR spectroscopy, which identified them as 4-nitrocinnamic acid.

DISCUSSION

The widely accepted mechanism for the ammonia lyase reaction catalyzed by PAL and HAL is depicted in Fig. 3. Although this mechanism includes chemically implausible steps, it has provided the basis for numerous mechanistic investigations for 25 years (1, 3, 12, 30–32). While a carbanionic intermediate was postulated, it was not explained how this could be formed. In a thorough investigation on isotope effects in the PAL reaction (32), it was noted that "for now the facility of enzymes in forming carbanions remains a challenging puzzle." Indeed, a base able to abstract a benzylic proton should be so strong that its existence in a protein environment appears to be doubtful.

Prompted by our discovery that in the HAL reaction the dehydroalanine prosthetic group functions as an electrophilic catalyst in the activation of the β-H atom (15), we examined the PAL reaction by using dehydroalanine-less mutants and alternative substrates.

Recently, we reported (13) the construction of the PAL mutant S202A that was virtually inactive with L-phenylalanine as substrate. In addition we have now prepared the S202T mutant. Examination of Table 1 reveals that both of these mutants as well as the $NaBH_4$-treated wild-type PAL exhibit

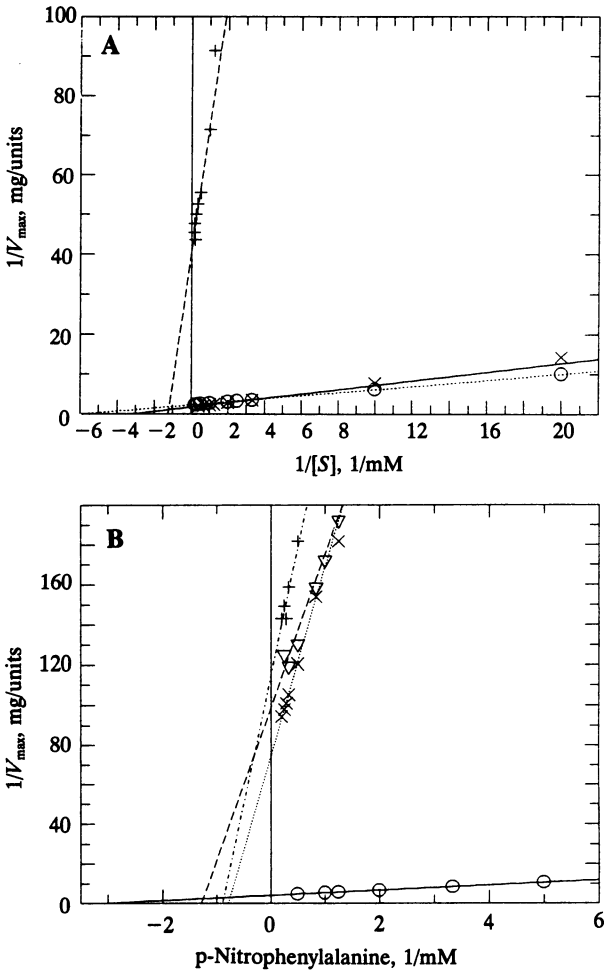

FIG. 2. (*A*) Lineweaver–Burk plot of wild-type PAL with DL-*m*-tyrosine (solid line), L-phenylalanine (dotted line), and L-tyrosine (dashed line) as substrates. The rate of reaction was determined spectrophotometrically at λ = 290 nm (2, 28). (*B*) Lineweaver–Burk plot of wild-type PAL (solid line), the mutants S202A (dashed line) and S202T (dotted line), and the $NaBH_4$-treated wild-type PAL (dotted/dashed line) with 4-nitrophenylalanine as substrate. The rate of reaction was determined spectrophotometrically at λ = 340 nm (29) based on ε = 9800 liter·cm^{-1}·mol^{-1} for 4-nitrocinnamate at pH 8.8.

V_{max} values that are 10^3–10^4 times reduced with L-phenylalanine as substrate.

The results of the kinetic experiments are summarized in Table 3. The fact that the dehydroalanine-less mutants react much faster with L-4-nitrophenylalanine (V_{max} is two orders of magnitudes higher than with the natural substrate) suggests that the 4-nitro group can partially substitute for the prosthetic dehydroalanine.

Since the 4-nitro group stabilizes the carbanion resulting from abstraction of the β-H atom of the amino acid (Fig. 4), we surmised an analogous role of the prosthetic dehydroala-

Table 3. Comparison of kinetic parameters from wild-type PAL, the mutants S202A and S202T, and the $NaBH_4$-treated wild-type PAL (wt PAL red.) with 4-nitrophenylalanine (4-NO_2-Phe) as substrate

Enzyme	K_m for 4-NO_2-Phe, mM	V_{max} for Phe, unit/mg	V_{max} for 4-NO_2-Phe, unit/mg	$V_{max4\text{-}NO_2\text{-}Phe}/V_{maxPhe}$	$V_{maxwtPAL}/V_{maxmod.PAL}$
wt PAL	0.32	0.56	0.17	0.3	
S202A	0.84	1.1×10^{-4}	0.008	70	21
S202T	1.14	2.7×10^{-5}	0.01	370	17
wt PAL red.	1.08	4.5×10^{-4}	0.007	16	25

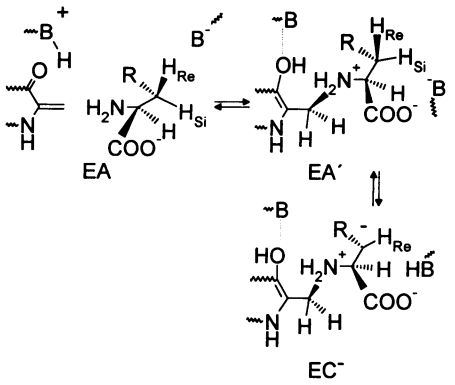

FIG. 3. Hypothetical mechanism of the PAL reaction proposed by Hanson and Havir (3) involving the carbanionic intermediate EC. E, enzyme; A, adduct; C, carbanion.

nine. Furthermore, the relative ease with which the PAL species lacking dehydroalanine react with L-4-nitrophenylalanine contradicts the mechanism in which nucleophilic attack of the α-amino group at the dehydroalanine is an essential step (Fig. 3). These considerations, combined with earlier results enlightening the mechanism of action of HAL and urocanase (33), where a transient electrophilic attack at the C-5 atom of imidazole modifies its reactivity, lead us to suggest an analogous role of the prosthetic dehydroalanine of PAL. This means an electrophilic attack at the phenyl group for which there is little if any precedence in enzymology. However, if the amide carbonyl group of the prosthetic dehydroalanine is protonated, this function becomes equivalent with a vinylogous acyl cation, which can attack the aromatic nucleus. In Fig. 5, we present a mechanism that begins with such a Friedel–Crafts type electrophilic acylation. As a result the positive charge in the aromatic ring facilitates abstraction of the β-proton by an enzymatic base. In intermediate ES′ (enzyme-substrate), ammonia elimination can occur with simultaneous rearomatization and regeneration of the prosthetic dehydroalanine.

Because of the unprecedented nature of such a mechanism further tests appeared necessary. It is known that L-tyrosine is an alternative, though reluctant, substrate of PAL (29). We confirmed this and showed that both K_m and V_{max} are impaired in comparison to L-phenylalanine (Table 2). If this mechanism is valid, then 3-hydroxyphenylalanine (*m*-tyrosine) should be a better substrate of PAL than L-tyrosine. This is indeed the case (Table 2). DL-*m*-Tyrosine turned out to be a slightly better substrate than L-phenylalanine in spite of the fact that its D-enantiomer content might impair the V_{max} values (29). As shown in Fig. 6, the phenolic OH group in position 3 should facilitate the electrophilic attack at the *para* or *ortho* (data not shown) position.

In the before-mentioned study by Hermes *et al.* (32), a very small kinetic deuterium isotope effect (V_{max}/K_m = 1.15) was found with L-(β-$^2H_2$)phenylalanine as substrate. This indicates that rather the electrophilic attack is the rate determining step than the subsequent β-proton abstraction by the enzymatic base. In contrast, in the HAL reaction, the proton abstraction is at least partially rate limiting, as revealed by a kinetic deuterium isotope effect of k_H/k_D = 1.5–2 (34, 35). Since the two enzymes exhibit considerable homology (12, 19) and are

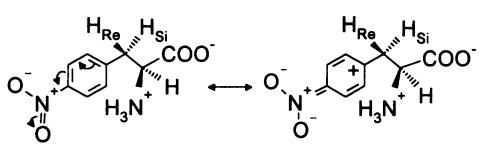

FIG. 4. Resonance structures of 4-nitrophenylalanine showing activation of the β-protons.

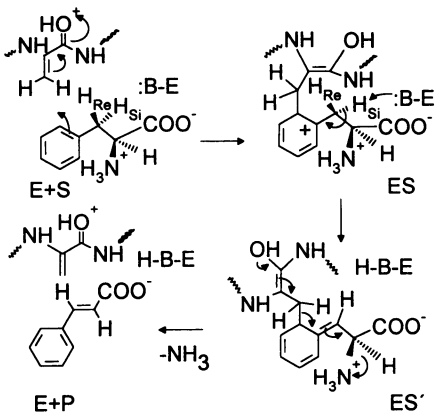

FIG. 5. Mechanism of the PAL reaction proposed in this work. Electrophilic attack at the phenyl nucleus by the dehydroalanine prosthetic group facilitates abstraction of the β-H_{Si} proton by an enzymic base. E, enzyme; S, substrate; P, product.

related in many respects, the lower specific activity of PAL may be due to the lower nucleophilicity of the phenyl compared to the imidazole ring. This would explain the difference in the rate-limiting step.

The question now arises, whether the electrophilic attack occurs at the *ortho* or *para* position. Although our results do not give a definitive answer, the findings of Hanson *et al.* (31) and Hermes *et al.* (32) that dihydrophenylalanine [3-(1,4-cyclohexadienyl)-DL-alanine] is a moderately good substrate of PAL supports an *ortho* attack (Fig. 7). The lower V_{max} value of this substrate may be due to the less-stabilized cationic intermediate, i.e., tertiary versus allylic tertiary carbenium ion (Fig. 5). An attack at the *para* position would lead to an even less stable secondary carbenium ion that would be isolated from the β-side chain. These results support an attack at the *ortho* position.

The none-substrate but weak inhibitory nature of cyclohexylalanine (32) is in agreement with our mechanism. A survey of the literature relevant to the mechanism of the PAL reaction did not bring to light any results that did not conform to the present proposal. Although Hermes *et al.* (32) interpreted their results on isotope effects in terms of the Hanson mechanism, these are not in conflict with the mechanism proposed in this paper. In particular the ^{15}N kinetic and thermodynamic isotope effects are not compelling for the old mechanism. The kinetic mechanism of the PAL reaction has been found to be "ordered Uni-Bi" (11), with release of ammonia occurring after the release of cinnamate. This was interpreted in terms

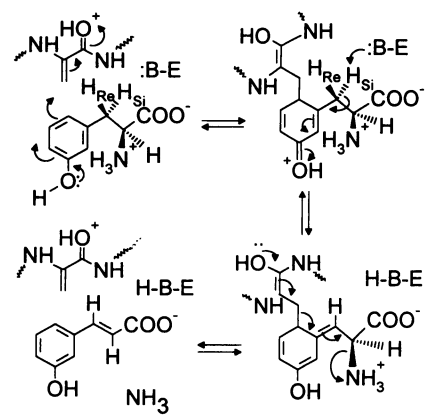

FIG. 6. Mechanism of the PAL reaction with *m*-tyrosine as substrate. In contrast to the *p*-OH group (tyrosine) the *m*-OH group facilitates electrophilic attack in the position *ortho* to the side chain.

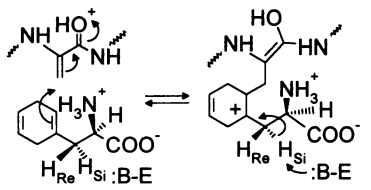

FIG. 7. Electrophilic attack at 3,6-dihydrophenylalanine leads to a tertiary carbenium ion in which abstraction of the β-H_{Si} proton is facilitated.

of binding ammonia to the prosthetic dehydroalanine. However, ionic binding of NH_4^+ to a carboxylate group of the protein could also be stable before cinnamate is released.

In conclusion, we have presented experimental data suggesting an alternative role for the prosthetic dehydroalanine residue. This mechanism is supported also by earlier results and by analogy to the HAL reaction. To our knowledge, there are no experimental results to contradict our present proposal.

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