Studies on Tyrosine Phenol Lyase

MODIFICATION OF ESSENTIAL HISTIDYL RESIDUES BY DIETHYLPYROCARBONATE

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SUMMARY

Tyrosine phenol-lyase of Escherichia intermedia is inactivated by treatment with diethylpyrocarbonate at pH 6.0 and 4°. Spectrophotometric studies show that the inactivation is stoichiometric, with a modification of 2 histidyl residues per molecule of the enzyme. Finding that this inactivation is largely reversed by treatment with hydroxylamine indicates that the inactivation is mainly due to modification of the histidyl residues. No changes in the sulphydryl content or in the aromatic amino acids are observed as a result of this modification. The modified tyrosine phenol-lyase retains most of its ability to form a nearly normal complex with its coenzyme, pyridoxal phosphate. This has been shown by studies of its absorption, by the determination of pyridoxal phosphate, and by reduction of the holoenzyme with tritiated sodium borohydride. The modified enzyme also appears to form a Schiff base intermediate with L-alanine.

The modified enzyme fails to catalyze the exchange of the α-hydrogen of L-alanine with tritium from tritiated water. This is consistent with a catalytic role for modified histidyl residues at the active site of the enzyme; this role is the removal of the α-hydrogen of substrates.

Tyrosine phenol-lyase is an enzyme which catalyzes the stoichiometric conversion of L-tyrosine to pyruvate, ammonia, and phenol, and requires pyridoxal phosphate as a cofactor (1-3). A crystalline preparation of the enzyme was prepared in our laboratory from cells of Escherichia intermedia grown in a bouillon medium supplemented with L-tyrosine (4, 5). The enzyme has a molecular weight of 170,000 and binds with 2 mol of pyridoxal phosphate per mol of enzyme. The crystalline preparation of the enzyme catalyzes a series of α,β elimination (Equations 1 to 3) (4, 5), β replacement (Equations 4 to 6) (6-8), and racemization reactions (Equation 7) (9).

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\begin{align*}
L-\text{Tyrosine} + H_2O &\rightleftharpoons \text{pyruvate} + NH_3 + \text{phenol} \quad (1) \\
S-\text{Methyl-L-cysteine} + H_2O &\rightleftharpoons \text{pyruvate} + NH_3 + CH_2SH \quad (2) \\
L-\text{Serine} &\rightleftharpoons \text{pyruvate} + NH_3 \quad (3) \\
L-\text{Tyrosine} + \text{pyrocatechol} &\rightleftharpoons 3,4-\text{dihydroxyphenyl-L-alanine} + \text{phenol} \quad (4) \\
L-\text{Serine} + \text{phenol} &\rightleftharpoons L-\text{tyrosine} + H_2O \quad (5) \\
L-\text{Serine} + \text{pyrocatechol} &\rightleftharpoons 3,4-\text{dihydroxyphenyl-L-alanine} + H_2O \quad (6) \\
L-\text{or D-Alanine} &\rightleftharpoons \text{DL-alanine} \quad (7)
\end{align*}
\]

Recently, we proved that this enzyme catalyzes the synthesis of L-tyrosine from pyruvate, ammonia, and phenol by the reversal of the α,β elimination reaction (Equation 1) (10). These reactions are explainable by adopting the general mechanism for pyridoxal-dependent reactions proposed by Braunitz and Shemyakin (11) and by Metzler et al. (12) (Scheme I).

Although L-alanine is not a substrate of tyrosine phenol-lyase, it does act as a competitive inhibitor of tyrosine degradation by the enzyme (4). L-Alanine cannot undergo β elimination to form an EA species (Scheme I), but it can proceed through ES to an EX species (13). This quasi-substrate was used in this study to investigate the effect of modification on the enzyme.

REFERENCES

1. Melchior, H., and Fahrney, C. (19) found that the reagent can also react irreversibly with amino groups in ribonuclease and with the active site serine in chymotrypsin.

EXPERIMENTAL PROCEDURE

Materials Crystalline tyrosine phenol-lyase was prepared from cells of Escherichia intermedia A-21 grown on a tyrosine-supplemented medium, as previously described (4). Diethylpyrocarbonate was purchased from Tokyo Kasel Kogyo. Pyridoxal 5'-phosphate was kindly provided by Dainippon Pharmaceutical Co. Other chemicals used in this work were commercial products.

Enzyme Assay—α,β Elimination reaction was assayed by meas-
using the amount of pyruvate liberated from L-tyrosine under conditions described in a previous paper (4). One unit of activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of pyruvate per min under standard assay conditions.

L-Tyrosine formation was measured by determining the incorporation of [14C]phenol into L-tyrosine. The reaction mixture contained 75 μmol of pyruvate; 37.5 μmol of ammonium sulfate; pH 8.0; 0.1 μmol of pyridoxal phosphate; 50 μmol of NH$_2$-NH$_2$Cl buffer, pH 9.25; 12.5 μmol of [14C]phenol (185,000 cpm); and 0.06 unit of crystalline enzyme in a volume of 1.0 ml. The reaction was carried out for 30 min at 30°C and was terminated by the addition of 0.2 ml of 30% trichloroacetic acid solution. The denatured enzyme was removed by brief centrifugation, and 4.0 ml of toluene was added to 1.0 ml of the supernatant solution. This extraction was repeated five times, then 0.5 ml of water layer was added with 10 ml of Bray's scintillation solution, and the whole was counted in a model 102 liquid scintillation spectrometer (Packard).

The assay of the β replacement reaction was carried out by determining the incorporation of [14C]phenol into L-tyrosine. The reaction mixture of 1 ml contained 200 μmol of 1-tyrosine; 0.1 μmol of pyridoxal phosphate; 12.5 μmol of [14C]phenol; 50 μmol of potassium phosphate buffer, pH 7.8; and the crystalline enzyme. The reaction was carried out at 30°C for 30 min and was terminated by the addition of 0.2 ml of 30% trichloroacetic acid solution. The remaining phenol was extracted with toluene, as above, then the radioactivity in 0.5 ml of the aqueous layer was counted as described above.

**Protein Determination**—The protein determination of tyrosine phenol-lyase was performed spectrophotometrically by measuring the absorbance at 280 nm. An E of 8.57 for 10 mg per ml and for a 1-cm light path was used throughout (4).

**Spectrophotometric Determination**—Spectrophotometric determinations and the recording of absorption spectra were carried out with a Beckman model DB-G recording spectrophotometer and a Hitachi model 124 spectrophotometer.

**Treatments with Diethylpyrocarbonate and with Hydroxylamine**—Diethylpyrocarbonate was freshly diluted to 0.06 M with cold ethanol for each experiment. Protein samples were prepared as a solution of about 1 mg per ml in 0.1 M potassium phosphate buffer, pH 7.0; they were then dialyzed against the same buffer for 16 hours at 4°C.

**Phenol-lyase**—The reaction mixture of 1 ml contained 75 μmol of pyruvate; 10 mM p-mercaptoethanol, and 1 mM EDTA for 15 hours at 4°C. The rate of formation of carbethoxyhistidine was followed by measuring the absorbance at 412 nm of each solution and of a reagent blank by brief centrifugation, and 4.0 ml of toluene was added to 1.0 ml of the supernatant solution. This extraction was repeated five times, then 0.5 ml of water layer was added with 10 ml of Bray's scintillator and counted in a liquid scintillation spectrometer. Another 50-μl portion was diluted with 4.85 ml of water; then 100 μl of the solution was subjected to the L-tyrosine assay using the ninhydrin method (24).

**RESULTS**

**Inactivation by Diethylpyrocarbonate**—Fig. 1 shows that tyrosine phenol-lyase is inactivated by rather low concentrations of diethylpyrocarbonate at pH 6.0 and that pyridoxal phosphate has no effect on the extent of its inactivation. L-Alanine has shown a protective effect at a relatively high concentration.

Studies of the effects of diethylpyrocarbonate on the spectrum of the enzyme were carried out on the apoenzyme to avoid any changes which might occur in the spectrum of the bound pyridoxal phosphate on modification of the enzyme.

Fig. 2A shows a spectrum of the apoenzyme before and after treatment with 1.0 mM diethylpyrocarbonate for 60 min, and a difference spectrum during the reaction of enzyme solutions. No changes in the spectra were detected above 270 nm. This indicates that no modification of the tyrosyl residue had occurred since O-carbethoxytyrosine absorbs between 270 and 280 nm (17). Large changes in absorbance at 242 nm were observed as shown in Fig. 2A and B. This absorbance is characteristic of the N-carbethoxyhistidyl residues in proteins (18). The number of histidyl residues modified is shown in Fig. 2C as a

**Measurement of Tritium Incorporation into L-Alanine**—The incorporation of tritium into L-alanine from tritiated water was measured according to the method of Morino and Snell (25). A reaction mixture containing 50 μmol of L-alanine; 0.1 ml of H$_2$O (2 × 10$^4$ cpm); 2 mg (3.8 units) of crystalline apoenzyme; 10 μmol of potassium phosphate, pH 8.0; 0.25 μmol of β-mercaptoethanol, and 0.6 μmol of pyridoxal phosphate was in a total volume of 0.5 ml was incubated at 30°C for various periods. At appropriate time intervals, 0.1 ml of 30% trichloroacetic acid was added to the reaction mixture. The denatured enzyme was removed by brief centrifugation, and 0.5 ml of the supernatant solution was then added to 5.0 ml of acetone at 0°C. After standing for 10 min at 0°C, the precipitated l-alanine was collected by centrifugation. This procedure was repeated five times and the precipitated L-alanine was dissolved in 0.6 ml of water, and a 0.5-ml portion was then mixed with 10 ml of Bray's scintillator and counted in a liquid scintillation spectrometer. Another 50-μl portion was diluted with 4.85 ml of water; then 100 μl of the solution was subjected to the L-alanine assay using the ninhydrin method (24).

**FIG. 1. Inhibition of tyrosine phenol-lyase by treatment with diethylpyrocarbonate. Reaction mixture contained in a final volume of 1.0 ml, 1.1 mg of apoenzyme, potassium phosphate buffer, pH 6.0 (30 μmol) and diethylpyrocarbonate (0 to 1 μmol) at the indicated concentrations. After incubation for 30 min at 0°C, aliquots (0.01 ml) of the reaction mixtures were diluted with 3.0 ml of the standard tyrosine phenol-lyase assay mixture (see "Experimental Procedure") to which 1 mM β-mercaptoethanol had been added and were subsequently assayed after addition of 1 ml of 2.5 mM L-tyrosine.**
Fig. 2. Effects of diethylpyrocarbonate on the spectrum, the enzymatic activities, and the histidine content of tyrosine phenol-lyase. Apoenzyme, 3.0 mg in 2.5 ml of 0.1 M potassium phosphate buffer, pH 6.0, was placed in each of two cuvettes in the reference and sample compartments of a Hitachi 124 Spectrophotometer which was cooled with circulating water at 4°. A solution of diethylpyrocarbonate in ethanol (0.050 ml) was added to the sample cell to give a final concentration of 1.0 mM and ethanol (0.050 ml) was added to the reference cell. Difference spectra were recorded at intervals between 280 nm and 240 nm and an aliquot (0.01 ml) at the treated enzyme was removed immediately after the absorption at 240 nm was recorded, and added to the reaction mixture for assays of α,β elimination activity. A, difference spectra with 1 mM diethylpyrocarbonate recorded at 1, 2, 5, 10, 15, and 30 min (a). The times refer to the time when the spectrum was completed and an aliquot was removed for assay. Spectra were also recorded of the enzyme solutions before and after treatment for 30 min with 1 mM diethylpyrocarbonate using a buffer blank (b). B, effect of incubation time with 1 mM diethylpyrocarbonate on the α,β elimination activity (●) or difference absorbance at 240 nm (○) under the conditions given above. C, relationship between the number of histidyl residues modified and α,β elimination activity after treatment with 1 mM diethylpyrocarbonate for various times.

Function of Activity. Extrapolation of L-tyrosine phenol-lyase activity to zero corresponds to the carbethoxylation of 2 histidyl residues per mol of apoenzyme. The reasonably good stoichiometry between the loss of activity and the modification of 2 histidyl residues no longer holds when less than 20% of the initial activity remains; complete loss of activity was associated with the modification of 8 or more histidyl residues. Tyrosine phenol-lyase modified to the extent of 20% of the residual activity (3 histidyl residues modified per mol of enzyme) was used as a reasonable sample of modified enzyme in this work.

Various activities of the enzyme, modified to the extent of about 3.0 histidyl residues per mol of enzyme, are compared. Both α,β elimination and its reverse reaction are reduced to 15 to 20% of the control, whereas activity in the β replacement reaction is 6% of the control.

Reversal of Inactivation by Hydroxylamine.—Melchior and Fahrney (19) found that 0.5 M hydroxylamine, pH 7.0, removes the N-carbethoxy group from imidazole in several minutes. Several diethylpyrocarbonate-inhibited enzymes have also been found to be reactivated by hydroxylamine (16, 19, 21-23).

Fig. 3 shows that the enzyme, in which 3 or fewer histidyl residues have been modified, can be largely reactivated by treatment with hydroxylamine.

Stability of Modified Tyrosine Phenol-lyase.—Melchior and Fahrney studied the pH dependence of hydrolysis for the model compound N-carbethoxyimidazole (N-ethoxyformylimidazole) and found it to have a half-life of about 60 hours at pH 7.8 or 23 hours at pH 8.3 (19). We have dialyzed the enzyme modified to various degrees and the control enzyme against 0.1 M phosphate buffer, pH 8.0, containing 0.1 mM pyridoxal...
reactivated to 88% activity by treatment with hydroxylamine activity was found (Fig. 3). The modified enzyme could still be solutions was assayed after 1 and 7 days, and a slight restoration of ROSINE PHENOL-LYASE ACTIVITY OF MODIFIED AND CONTROL ENZYME so-phosphate, 0.1 mM EDTA, and 0.5 mM β-mercaptoethanol. Ty-

Figure 4: Absorption spectra of untreated and diethylpyrocarbonate-treated tyrosine phenol-lyase in the presence and absence of L-alanine. Spectra of the enzyme solutions (1.5 mg per ml in 0.1 M potassium phosphate buffer, pH 8.0, containing 0.1 mM pyridoxal phosphate, 0.5 mM β-mercaptoethanol, and 0.1 mM EDTA) were recorded at 25°C against a buffer blank before and immediately after the addition of L-alanine to a final concentration of 0.1 mM to both the enzyme and blank solutions. The modified enzyme had been previously treated with 0.8 mM diethylpyrocarbonate until 70% inhibited and then dialyzed against the above buffer.

phosphate, 0.1 mM EDTA, and 0.5 mM β-mercaptoethanol. Ty-
roside phenol-lyase activity of modified and control enzyme sol-
itions was assayed after 1 and 7 days, and a slight restoration of
activity was found (Fig. 3). The modified enzyme could still be reactivated to 88% activity by treatment with hydroxylamine after 7 days of dialysis. We concluded that the modified enzyme is stable, for at least a few days, under these conditions and so we used modified enzymes after a dialysis of 12 hours in this study.

Binding of Pyridoxal Phosphate to Modified Tyrosine Phenol-
yase—The amount of pyridoxal phosphate bound by modified apoenzyme was determined after dialysis of the enzyme, 1.91 mg of protein, against 0.1 M potassium phosphate buffer, pH 8.0, containing 5 mM pyridoxal phosphate. After 16 hours, the concentration of pyridoxal phosphate inside and outside the dialysis bag was determined. An excess concentration of pyridoxal phosphate was found within the dialysis bag, which corre-
sponded to the binding of 1.8 mol of pyridoxal phosphate by 170,000 g of the modified apoenzyme. The untreated enzyme bound 2.2 mol of coenzyme under the same conditions.

Effect of Modification by Diethylpyrocarbonate on Sulhydryl Content—The total sulhydryl content of the enzyme solutions was determined as described under “Experimental Procedure.” Untreated tyrosine phenol-lyase and tyrosine phenol-lyase treated with diethylpyrocarbonate, and having 22% residual activity, each contained 9.8 sulhydryl residues per molecule of

Table I

| Enzyme                        | Activity | Incorporation |
|-------------------------------|----------|---------------|
| Unmodified + PLP              | 43.3     | 30,100        |
| Unmodified + PLP + L-alanine  | 65.0     | 13,700        |
| Unmodified                    | 94.0     | 2,940         |
| Modified + PLP                | 12.1     | 19,200        |
| Modified + PLP + L-alanine    | 15.7     | 7,110         |
| Modified                      | 16.7     | 3,060         |

The addition of L-alanine caused increased absorbance at 500 nm in the control enzyme, while in modified enzyme it gave proportional height to the residual activity. The increase in absorbance at 430 nm on the addition of pyridoxal phosphate to the enzyme has been reported and attributed to the formation of an enzyme-bound pyridoxal phosphate Schiff base. The species absorbing at 500 nm represents a deprotonated enzyme-L-alanine complex at the α-carbon of L-alanine (ES → EX⁺ in Scheme I). Modified enzyme, though it can bind with pyridoxal phosphate nearly normally, fails to produce the intermediate which has an absorption maximum at 500 nm. Most of the absorbance at 500 nm in modified enzyme is due to unmodified enzyme present.

Sodium Borohydride Reduction of Native Enzyme and Modified Enzyme—Sodium borohydride reduces the Schiff base linkage between pyridoxal phosphate and the ε-amino group of a lysyl residue in the enzyme (5). When the holoenzyme is treated with small amounts of sodium borohydride, the absorbance at 430 nm decreases to about 90% of the initial value, then decreases no further unless a large excess of sodium borohydride is used. We found that the minimal amount of sodium borohydride required to reduce the absorbance of the enzyme by 80%, has very little effect on the spectrum of the enzyme in the presence of L-alanine. L-Alanine significantly protects the activity of the enzyme and decreases the incorporation of tritium into the enzyme (Table I). Thus, the enzyme-bound pyridoxal phosphate-alanine Schiff base linkages seems to be more resistant to sodium borohydride reduction than is the enzyme-pyridoxal phosphate Schiff base linkage. Table I shows that sodium borohydride also reduced the pyridoxal phosphate bound to the diethylpyrocarbonate-modified enzyme which resulted in the incorporation of the tritium label. L-Alanine decreased the incorporation of tritium into the protein by 60%. These data provide evidence for the formation of a pyridoxal phosphate-alanine Schiff base by the modified enzyme (ES in Scheme I).

Effect of Modification by Diethylpyrocarbonate on Proton Exchange Activity of Tyrosine Phenol-Lyase—The first step in the tyrosine phenol-lyase catalyzing α,β elimination reaction is labilization of the α-hydrogen atom of an amino acid (ES → EX⁺, in Scheme I). Since studies on the modified enzyme based on absorption spectra and reduction with sodium borohydride support
**Scheme I.** Possible mechanism for the reactions catalyzed by tyrosine phenol-lyase. Enzyme protein is omitted in the intermediates, EX and EA. \( R, -\text{OH}, -\text{SCH}_2, \) and phenolyl.

Studies on Absorption Spectra of Diethylpyrocarbonate-treated Enzyme in Reversal Reaction—Degradation of L-tyrosine to phenol, pyruvate, and ammonia by tyrosine phenol-lyase is readily reversible at high concentrations of pyruvate and ammonia (10). The addition of phenol, pyruvate, and ammonia to the holotyrosine phenol-lyase resulted in the appearance of a spectral band near 500 nm (Fig. 6) (10) similar to that observed when \( L \)-alanine was added to the enzyme. This absorption peak can be attributed to the deprotonated substrate enzyme intermediate, \( EX \) or \( EX' \) in Scheme I. The absorption exhibited in the reversal reaction at 500 nm by the native enzyme is changed with time, increasing at first then decreasing slowly as shown in Fig. 6. The same absorption band was shown by diethylpyrocarbonate-treated tyrosine phenol-lyase. In contrast to a similar peak formed by the native enzyme, this band was high and showed slight change in absorbance after long standing (Fig. 6).

**DISCUSSION**

Tyrosine phenol-lyase contains pyridoxal phosphate bound in a Schiff base linkage to an amino group of the lysyl residue (5) (Scheme I, \( E \)). This amino group is displaced by the substrate to form a second Schiff base (Scheme I, \( ES \)). The first catalytic step in all parts of the \( \alpha, \beta \) elimination reaction is the removal of the \( \alpha \)-hydrogen from the amino acid to give an intermediate represented as \( EX \) in Scheme I. Although this step is greatly facilitated by electron withdrawal through the conjugated system of pyridoxal, it probably requires the presence of a base group on the protein to accept the hydrogen. Just such a role for a hist...
tions on the basis of model (14) and enzymatic (26) studies.

The reaction conditions are described in the text.

Recently, Miles and Kumagai (16) have investigated the essential histidyl residues in the β₂ subunit of tryptophan synthetase which catalyzes the α,β elimination and β replacement reactions of amino acids (28). It is difficult to check the exchange of the α-hydrogen of substrates by the β₂ subunit of tryptophan synthetase to determine the role of modified histidine, since extraction of the hydrogen from the substrate is the rate-determining step in the α,β elimination reaction by tryptophan synthetase (29). Thus, substrates which exchanged an α-hydrogen with labeled H₂O could not be found in the reaction mixture. With tyrosine phenol-lyase, the proton exchange reaction is readily measured using L-alanine in tritium labeled water. Incorporation of the proton into the α position of L-alanine takes place during the reaction $EX \rightarrow ES \rightarrow E + S \text{ in Scheme I.}$

Tyrosine phenol-lyase exhibits an intense absorption band at 500 nm in the presence of L-alanine. Similar peaks near 500 nm have been observed in many pyridoxal phosphate-dependent enzymes (25, 30) and have been ascribed to the deprotonated intermediate, $EX$, in Scheme I, or to a species in equilibrium with this intermediate, $EX'$ (25). L-Alanine was used throughout this study to investigate the role of modified histidine residues because of its convenient properties described above.

Current studies show that inactivation of tyrosine phenol-lyase by diethylpyrocarbonate is stoichiometric with modification of 2 histidyl residues per mol of the enzyme, and inactivation is largely reversed by hydroxylysinamide. Reduction of the rate of inactivation by L-alanine and pyridoxal phosphate indicates that the histidine residue is located in the active site region. Although these data support the specific modification of the essential histidyl residue at the active site, they do not eliminate the possibility of carboxylation of active amino groups which might exist in the enzyme. The ε-amino group of lysine at the binding site of pyridoxal phosphate does not seem to be damaged to an extent that would explain the loss of enzyme activity because pyridoxal phosphate did not protect the modification and the modification did not greatly affect the absorption at 430 nm.

The modified tyrosine phenol-lyase binds pyridoxal phosphate nearly stoichiometrically and in an environment almost similar to that of the untreated enzyme. This has been shown by a determination of the pyridoxal phosphate bound to the enzyme using the absorption spectrum, and by reduction with tritium-labeled sodium borohydride.

Interaction of the untreated and modified enzyme with L-alanine has been investigated using absorption spectra and by treatment with sodium borohydride, in the presence and absence of L-alanine. The modified enzyme exhibits about 85% of the absorbance shown by the native enzyme at 430 nm. The ability of the modified enzyme to bind with pyridoxal phosphate and to make a complex with L-alanine is less than that of unmodified enzyme, though loss of this ability is not enough to explain the loss of enzymatic activity. These differences between the modified and unmodified enzyme may be caused by a slight change in the conformation of the modified protein at the active site by excess modification of the histidyl residues, or by partial modification of amino groups at the coenzyme binding site.

The modified enzyme has shown only 6% of the tritium exchange activity of the control enzyme. Then, the histidyl residue which has been modified may have a catalytic role in the exchange of the α-proton of L-alanine. The absorption spectrum of the modified enzyme in the presence of pyruvate, ammonia, and phenol shows a remarkable accumulation of the $ES \rightarrow EX'$ species, i.e. the blockage in the addition step of the α-proton. These results obtained from the studies on absorption spectra and on the tritium exchange reaction suggest that modification blocks the conversion of $ES$ to $EX$ in tyrosine degradation reaction and the conversion of $EX$ to $ES$ in the reversal reaction.

These studies show that histidyl modification by diethylpyrocarbonate results in a modified enzyme which can form nearly normal complexes with pyridoxal phosphate and L-alanine. A
role for this modified histidyl residue, in the abstraction of the α-hydrogen of the substrate has been proposed.

Recently, it was reported by Miles that an essential, photosensitive histidyl residue is present in the pyridoxal phosphate-bound peptide in the β₁ subunit of tryptophan synthetase (31).

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