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ENZYME CATALYSED NON-OXIDATIVE DECARBOXYLATION OF AROMATIC ACIDS II. IDENTIFICATION OF ACTIVE SITE RESIDUES OF 2,3-DIHYDROXYBENZOIC ACID DECARBOXYLASE FROM *ASPERGILLUS NIGER*

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SUMMARY: In order to understand the mechanism of decarboxylation by 2,3-dihydroxybenzoic acid decarboxylase, chemical modification studies were carried out. Specific modification of the amino acid residues with diethylpyrocarbonate, N-bromosuccinimide and N-ethylmaleiimide revealed that at least one residue each of histidine, tryptophan and cysteine were essential for the activity. Various substrate analogs which were potential inhibitors significantly protected the enzyme against inactivation. The modification of residues at low concentration of the reagents and the protection experiments suggested that these amino acid residues might be present at the active site. Studies also suggested that the carboxyl and *ortho*-hydroxyl groups of the substrate are essential for interaction with the enzyme.

Many micro organisms carry out an unusual non-oxidative decarboxylation of aromatic acids and convert them to respective phenols (1). In bacteria, this reaction has been reported for 4-hydroxybenzoic acid (2), 4,5-dihydroxyphthalic acid (3,4), protocatechuic acid (2, 5), gallic acid (6), vanillic acid (7) and in fungi, 2,3-dihydroxybenzoic acid (DHBA) (8-10), 2,4-dihydroxybenzoic acid (11), salicylic acid (12) and 6-methylsalicylic acid (13) undergo similar decarboxylation. So far, the enzymes involved in such reactions have not been purified except 4,5-dihydroxyphthalate decarboxylase from pseudomonads(3,4)and DHBA decarboxylase from *Aspergillus niger* (8,9) and yeast(10). These enzymes neither require a cofactor for catalysis nor contain a bound carbonyl group and hence the mechanism of decarboxylation is different from other decarboxylases.

In our earlier communication (9) we reported the purification and spectroscopic properties of DHBA decarboxylase from *A.niger*. This enzyme catalyses the formation of catechol from DHBA without requiring a cofactor. Though it was suggested that basic amino acid residues (3, 10) might be involved in such an unusual decarboxylation, to date, there are no reports on the identification of the amino acids involved in these reactions. In this paper we report the presence of histidine, tryptophan and cysteine residues at the active site of DHBA decarboxylase.

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MATERIALS AND METHODS

All biochemicals were purchased from Sigma Chemical Co. St. Louis, MO.
Enzyme: Homogeneous DHBA decarboxylase was prepared from *A.niger* as described and assayed spectrophotometrically following the disappearence of DHBA (8, 9). One unit of enzyme is defined as the amount that catalysed the disappearance of one μmole of DHBA per min. Protein was estimated by the method of Lowry *et al* (14) using bovine serum albumin as the standard.

Modification of the enzyme by diethylpyrocarbonate (DEPC), N-bromosuccinimide (NBS) and N-ethylmaleiimide(NEM):-DHBA decarboxylase (2.25 μM) in 50 mM sodium phosphate buffer, pH 7.0, was mixed with different concentrations (1,2,3 and 4 mM) of DEPC (freshly prepared in ethanol) and incubated at room temperature (28 ^{o}C). Aliquotes (5 μl) were withdrawn at 2,4,6,8 and 10 min., diluted into the assay mixture (1 ml) and assayed for the residual activity (8, 9) which was expressed as percentage of the activity in the absence of the reagent.

Inactivation of the enzyme (1.5 μM) by NBS (0-25 μM) was carried out in 50 mM sodium acetate buffer pH 5.2. Aliquotes (5 μl) were taken out at 5,10,15,20,25 and 30 min. and assayed for residual activity.

Inactivation by NEM (0.5,1.0,2.5,4.0 and 5.0 mM) was done under the conditions described for DEPC and the aliquotes (5 μl) were taken out at various time points(5-30 min.) to assay for residual enzyme activity.

In all the reactions mentioned above, the amount of the unreacted reagents carried into the assay mixture were very low and at that concentration they had no effect on the activity.

Protection experiments using substrate analogs and product:- The protection of the enzyme activity by various aromatic compounds (2 mM) against inactivation by the reagents, were carried out under same conditions described above with a minor change. The enzyme was preincubated with the analogs for 5 min. before initiating the inactivation reaction.

Reversal of DEPC modification by hydroxylamine:- DHBA decarboxylase (5 μM)was inactivated with 4 mM DEPC in 50 mM sodium phophate buffer at pH 7.0 for 10 min. This resulted in the loss of more than 80% of the enzyme activity. The inactive enzyme was then treated with 1 M hydroxylamine (adjusted to pH 7.0 with KOH) at a final concentration of 0.1 M. The reaction mixture was kept at 4^{o}C overnight and dialysed extensively for 8 hr. in cold. An aliquote was used for assaying the residual activity. In the control set, the enzyme was not treated with DEPC, but only with NH_2OH, and processed in a similar manner.

RESULTS

Inactivation of the enzyme by DEPC:- From the inactivation curves obtained at different concentrations of DEPC (0-4 mM), a first order plot was constructed (Fig.1). The log of the pseudo-first order rate constant (k_{app}) value when plotted vs log [DEPC], gave a slope of 0.95 (Fig.1 inset), suggesting that reaction with one molecule of DEPC per active site was sufficient to inactivate the enzyme. To examine the extent of protection afforded by substrate analogs and to know about the requirements of the side group(s) to interact with the enzyme, the decarboxylase was preincubated with the analogs (2 mM) prior to DEPC (4 mM) inactivation. Many of these analogs were strong competitive inhibitors(8,9) and they protected the enzyme against inactivation(Table1). Compounds containing a substitution at *ortho*-position to the -COOH group on the benzene ring (eg. salicylate, anthranilate, 2-chlorobenzoate) afforded very significant protection. 3-Hydroxybenzoate, 3,4-dihydroxybenzoate were neither inhibitors nor protected the enzyme against inactivation.

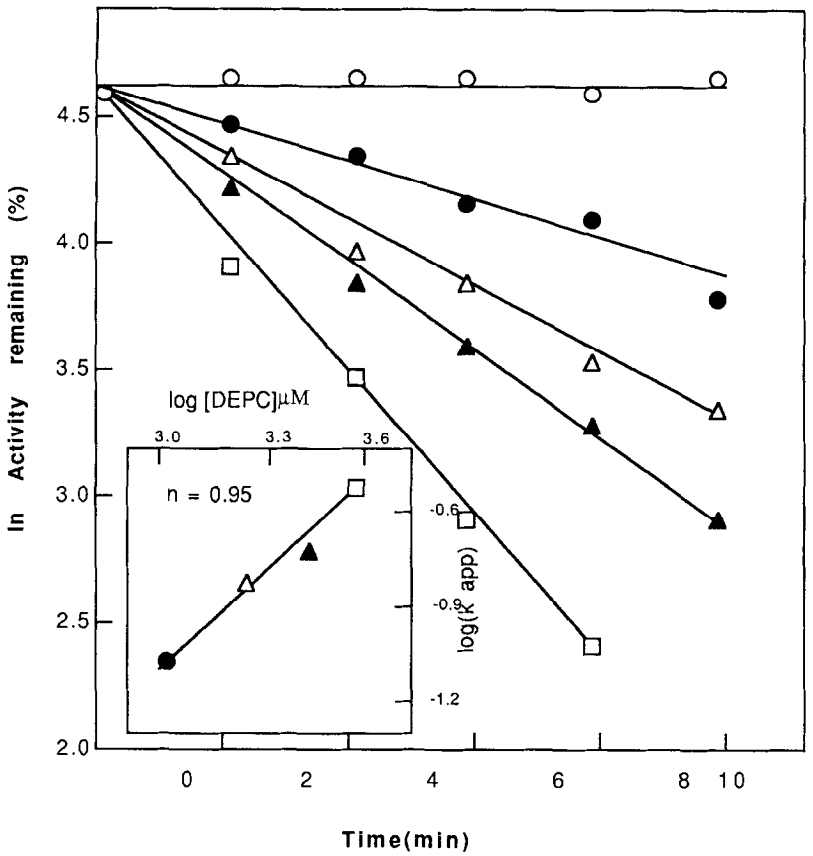

Fig.1. First order plots for the inactivation of DHBA decarboxylase by DEPC. Inactivation of the enzyme was carried out at different concentrations (○, 0 mM;●, 1 mM;△, 2 mM;▲, 3 mM; □, 4 mM) of DEPC. Aliquotes were withdrawn at different time intervals and assayed for residual activity (for details see the text). Inset: second order plot of the pseudo-first order rates(k_{app}) of the inactivation at different concentrations of DEPC.

It has been reported that 1:1 DEPC:histidine complex can be cleaved by reaction with a strong nucleophilic reagent such as, hydroxylamine(15). When the DEPC-inactivated enzyme was treated with NH_2OH, more than 90% of the enzyme activity was regained (Table 2) suggesting that DEPC modified a histidine residue which was essential for the activity.

Time course of inactivation of the enzyme by NBS and NEM :- The rates of inactivation of DHBA decarboxylase at different concentrations(7.5,10,15,20 and 25 μM) of NBS is shown in Fig. 2. The apparent rate constants at these concentrations of NBS were 0.027 min^{-1}, 0.033 min^{-1}, 0.047 min^{-1},0.059 $min^{-1}$and 0.079 min^{-1}respectively. From a plot of log k_{app} vs log [NBS], a slope of 0.9 was obtained suggesting that atleast one tryptophan residue was modified per active site(Fig.2 inset). NEM at concentrations of 1-5 mM were used to inactivate the enzyme. A slope of 0.78 was obtained (data not presented) when results were plotted as described for the other two reagents. This suggested that modification of atleast one cysteine/active site led to the loss of activity.

Table 1. Pseudo-first order rate constant (k_{app}) values for the inactivation of DHBA decarboxylase by DEPC, NBS and NEM in the presence of substrate analogs

Compound (2 mM)	$k_{app}(min^{-1})$		
	DEPC (4 mM)	NBS (20 μM)	NEM (4 mM)
None	0.300	0.065	0.062
Salicylate	0.015	0.030	0.015
Anthranilate	0.020	0.022	0.015
2,3-Dihydroxybenzaldehyde	0.020	0.010	0.010
2-Chlorobenzoate	0.020	0.022	0.017
Catechol	0.010	0.012	0.002
3-Hydroxybenzoate	0.175	0.050	0.045
3,4-Dihydroxybenzoate	0.150	ND	0.122
Benzoate	ND	0.060	ND

The concentrations of the compounds and reagents added are indicated in parentheses.
ND, Not determined.

Various substrate analogs and the product(catechol) protected the enzyme against inactivation by NBS and NEM. As shown in Table 1, most of the inhibitors with a substitution in the *ortho*-position to the -COOH group afforded significant protection; where as, benzoate, 3-hydroxybenzoate, 3,4-dihydroxybenzoate failed to protect against inactivation.

DISCUSSION

This is the first report on the identification of the amino acid residues at the active site of a decarboxylase catalysing non-oxidative decarboxylation of an aromatic acid. DHBA decarboxylase and other decarboxylases of this group (3,4,6,7,13) do not require pyridoxal phosphate, thiamine pyrophosphate or a pyruvoyl group (16) for catalysis. Earlier workers (3,10) proposed the possible involvement of a basic amino acid residue at the active site of these

Table 2. Reversal of DEPC-inactivation of DHBA decarboxylase by treatment with hydroxylamine

Treatment	Activity relative to control (%)
Control	100
E+ DEPC (4 mM)	17
E+ DEPC (4 mM) + NH_2OH (0.1 M)	90

Experimental details are given in the text.

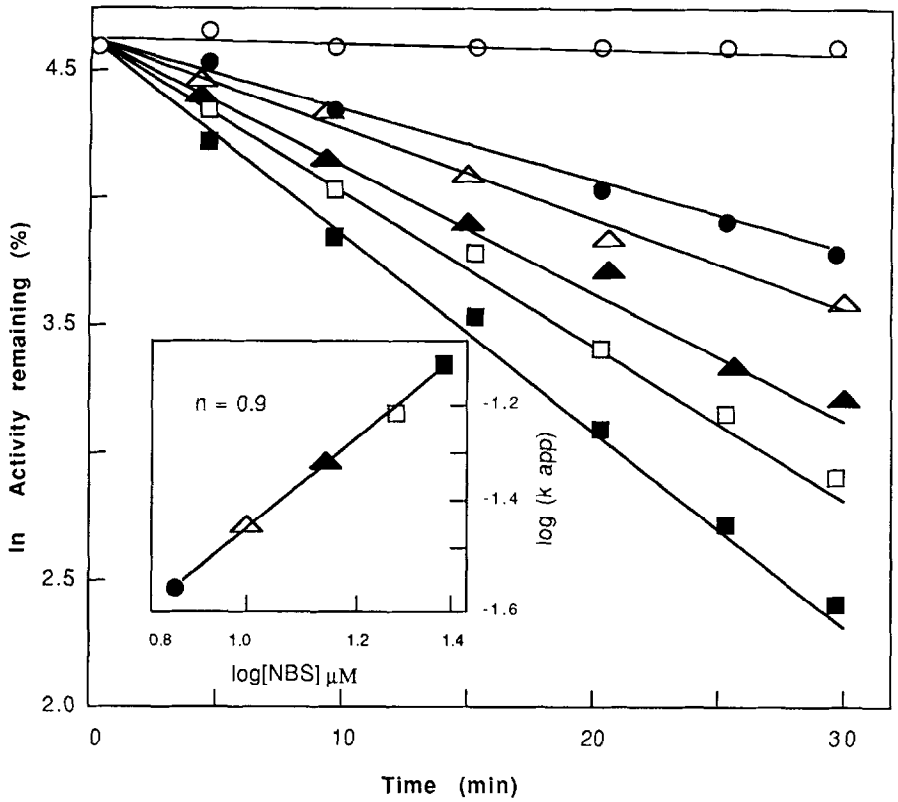

Fig.2. First order plots for the inactivation of DHBA decarboxylase by NBS. Inactivation of the enzyme was carried out at different concentrations (○, 0 μM; ●, 7.5 μM; △,10 μM; ▲,15 μM; □,20 μM; ■,25 μM) of NBS. Aliquotes were withdrawn at different time intervals and assayed for residual activity (for details see the text). Inset: replot of log(k_{app}) vs log[NBS].

enzymes. In our studies, DHBA decarboxylase was not inactivated by reagents specific for arginine and lysine residues. Previously we reported that the pKa value of the amino acid residue involved in the catalysis was 6.2 (9), which well corresponded to that of histidine residue, suggesting its role in catalysis. Following observations clearly implicate the modification of histidine residue at or near the active site. These are, (i) the inactivation of the enzyme by DEPC followed a pseudo-first order kinetics as in many enzyme inactivations (17-19) (ii) the inactivation was carried out at pH 7.0 at which histidine residue was specifically modified (15,17,19), (iii) the enzyme was protected against inactivation by competitive inhibitors (Table 1), suggesting the modification of the enzyme at or near the active site (15,17-19), (iv) hydroxylamine very significantly (>90%) reactivated the inactive enzyme (Table 2) indicating the modification of histidine by DEPC(17,19) ; it also suggested that the loss of activity was not due to modification of any other groups, and (v) in a difference spectrum (data not presented) the modified enzyme showed a peak at 240 nm representing the carbethoxy derivative of histidine residue.

Tryptophan residues have been reported at the hydrophobic substrate or cofactor binding sites in enzymes (20-22). The substrate and competitive inhibitors significantly quenched the

intrinsic flourescence of DHBA decarboxylase. The near UV circular dichroism spectrum of the enzyme distinctly changed when treated with 2-flourobenzoate , a competitive inhibitor (results not presented). These studies suggested the possible involvement of tryptophan residues at the active site of DHBA decarboxylase. The enzyme was inactivated at very low concentrations of NBS at pH 5.2. It may be speculated that the tryptophan residue might be involved in a hydrophobic interaction with DHBA. Cysteine residues have been identified at the active site of both pyruvoyl-dependent (23) and other decarboxylases (cited in 24). Like many other non-oxidative decarboxylases (3,4), DHBA decarboxylase was also inhibited by heavy metal ions and thiol reagents such as, p-hydroxymercuribenzoate, dithionitrobenzoate and NEM (8).

Many of the substrate analogs used in the protection experiments were potent inhibitors of the enzyme (8,9). In these compounds one or more of the functional groups were either missing or replaced by different substituents. Use of these compounds was expected to yield important information regarding the group(s) that interact with the enzyme. Salicylate, anthranilate, 2-chlorobenzoate, 2,3-dihydroxybenzaldehyde protected the enzyme significantly suggesting that the group in the *ortho*-position to the carboxyl group is essential for interaction. Compounds containing a -OH group on C-3 (3-hydroxybenzoate and 3,4-dihydroxybenzoate) were neither inhibitors (8,9) nor afforded protection against inactivations, suggesting that this hydroxyl group might not be required for binding. Similar observations were made for other enzymes of this class (3,6,10).

In conclusion, using chemical modification studies it was shown that in DHBA decarboxylase, histidine, tryptophan and cysteine residues are present at or near the active site. It may be conjectured that the tryptophan residue might 'hold' the substrate by means of hydrophobic interaction, while the histidine residue interact with either -COOH or -OH group to initiate the decarboxylation reaction.

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