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2,3-Dihydroxybenzoic acid decarboxylase from *Aspergillus niger*

A novel decarboxylase

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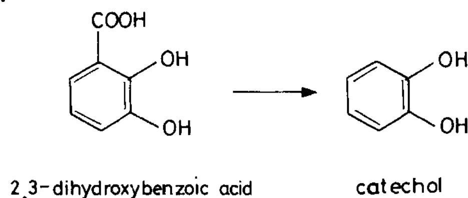
2,3-Dihydroxybenzoic acid decarboxylase, the last enzyme in the fungal metabolism of indole to catechol, catalyzes the non-oxidative decarboxylation of 2,3-dihydroxybenzoic acid to catechol. Unlike most other decarboxylases, this enzyme does not require a cofactor, underlining the importance of active-site residues in the reaction mechanism. Earlier studies from this laboratory [Kamath, A. V., Appaji Rao, N. & Vaidyanathan, C. S. (1989) *Biochem. Biophys. Res. Commun. 165*, 20–26], have shown that the sulfhydryl agent *N*-ethylmaleimide (Mal*N*Et) inactivated the enzyme by modifying a single class of cysteine residues and that this inactivation was prevented in the presence of salicylate, a substrate analogue. In the present study, this essential cysteine residue has been identified by specific labelling with [^{14}C]-Mal*N*Et using the differential labelling technique. The stoichiometry of incorporation of [^{14}C]Mal*N*Et was approximately one/subunit of the homotetrameric protein. The peptide bearing this reactive cysteine residue was isolated by tryptic digestion of the differentially labelled enzyme and subsequent reverse-phase chromatography of the peptide mixture. The sequence of the major radioactive peptide that was identified to be the active-site peptide, was LLGLAETCK. A search for sequences similar to this active-site peptide indicated that this sequence was probably unique to the decarboxylase under study. A partial primary structure map constructed from the sequences of peptides derived from enzymic cleavage of the protein using endoproteinase Glu-C and trypsin did not share any significant sequence similarity with sequences reported in the database, again suggesting the uniqueness of the enzyme. This is the first report on the active-site peptide and the partial primary structure of a non-oxidative decarboxylase catalyzing the removal of a carboxyl group from an aromatic nucleus.

Keywords. Decarboxylase; non-oxidative decarboxylation; aromatic acids; active-site cysteine; primary structure; 2,3-dihydroxybenzoic acid.

2,3-Dihydroxybenzoic acid decarboxylase (DHBD) is the last enzyme in the pathway for the dissimilation of indole by fungi [1]. It catalyzes the non-oxidative decarboxylation of 2,3-dihydroxybenzoic acid to catechol, as shown below:

Scheme 1.



This enzyme does not seem to require the external addition of any cofactor for its activity [2–4]. Enzymic reactions of this kind which involve, apparently, a simple elimination of the COO^- group from the aromatic nucleus, have been reported for substrates such as 4,5-dihydroxyphthalate, gallate, vanillate, orsellinate, 2,4-dihydroxybenzoic acid and 2,6-dihydroxybenzoic acid. Although such reactions have been noticed extensively in microorganisms transforming aromatic compounds, they are equally as important in metabolic pathways as in the biosynthesis of naphthoquinones [5].

Reactions of this kind are designated as non-oxidative decarboxylation reactions, as opposed to the oxidative decarboxylation of salicylate catalyzed by salicylate hydroxylase, a flavoprotein which effects the decarboxylation by concomitant hydroxylation at the relevant carbon atom [6]. In contrast to the well studied reaction of salicylate hydroxylase, mechanistic studies on the enzymic basis of non-oxidative decarboxylation are not available, primarily due to the lack of purified enzyme preparations with the exception of 4,5-dihydroxyphthalate decarboxylase [7] and DHBD [4]. This communication contributes to this little known subject by describing the identification of an essential cysteine as part of the primary structure of the protein. These results, along with the partial primary structure detailed for the enzyme, constitute, to our knowledge, the first report on the structure and function of a novel non-oxidative aromatic acid decarboxylase.

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Abbreviations. DHBD, 2,3-dihydroxybenzoic acid decarboxylase; RP, reverse phase; Mal*N*Et, *N*-ethylmaleimide.

Enzymes. 2,3-Dihydroxybenzoic acid decarboxylase (EC 4.1.1.46); salicylate hydroxylase (EC 1.14.13.1); orotidine 5′-monophosphate decarboxylase (EC 4.1.1.23); 4,5-dihydroxyphthalate decarboxylase (EC 4.1.1.55).

Note. The novel amino acid sequences reported in this study have been deposited in the SWISS-PROT protein sequence databank and are available under accession number P80346.

EXPERIMENTAL PROCEDURES

Materials. Ultrapure urea was obtained from Schwarz-Mann. HPLC-grade solvents were obtained from Spectrochem or E. Merck. Trifluoroacetic acid and all reagents for gas-phase sequencing were obtained from Wako Pure Chemical Co. Tokyo, Japan. ^{14}C-labeled *N*-ethylmaleimide (Mal*N*Et; 7.4 Ci/mol) was purchased from Amersham International plc. All other fine chemicals, including sequencing-grade proteases, were purchased from Sigma Chemical Co. Centricon-30 was obtained from Amicon (W. R. Grace and Co.).

Assay of DHBD. The enzyme was assayed by the disappearance of 2,3-dihydroxybenzoic acid, as described by Kamath et al. [4]. Protein was estimated by the method of Lowry et al. [8] using bovine serum albumin as the standard.

Purification of DHBD. Large-scale purification of DHBD was effected by substantial modification of the earlier protocol described from our laboratory [4]. Briefly, the steps include the preparation of a crude extract from mycelia grown in a medium containing anthranilate as inducer of the enzyme, ammonium sulphate fractionation (0–50% saturation), ion-exchange chromatography on DEAE-Sephacel and affinity chromatography on a matrix containing salicylate. The modifications involve the inclusion of protease inhibitors pepstatin (10 µg/ml), leupeptin (10 µg/ml), phenylmethanesulfonyl fluoride (1 mM), 1,10-phenanthroline (5 mM) and EDTA (1 mM) during the first three steps, salt washes to eliminate non-specific binding on the affinity matrix and the use of salicylate rather than 2,3-dihydroxybenzoic acid for affinity elution to avoid the formation of the quinone of 2,3-dihydroxybenzoic acid at pH 7.0 of the buffer. The preparation was homogeneous as assessed by SDS/PAGE, analytical ultracentrifugation and N-terminal analysis (unpublished results).

Specific labelling of the active-site cysteine residue. The technique of differential labelling [9] was used to modify the active-site cysteine residue of DHBD. In the first step, non-essential cysteine residues on the enzyme were modified with unlabeled Mal*N*Et with the essential cysteine residue at the active site protected by salicylate, a substrate analogue and competitive inhibitor of DHBD [4]. In the next step, salicylate and Mal*N*Et were dialyzed out and the essential cysteine residue, now free, was labelled using [^{14}C]Mal*N*Et.

[^{14}C]Mal*N*Et (solution in pentane, 7.4 Ci/mol, 500 µl, 13.5 mM) was layered over 250 µl water contained in a conical tube. The tube was left open in the fume hood and the pentane allowed to evaporate (approximately 2 h). The aqueous solution of Mal*N*Et thus obtained (27 mM) was frozen at −20°C until use.

Approximately 300 µg (2 nmol) protein in 50 µl 50 mM sodium phosphate, pH 6.8, was incubated with 6 mM salicylate for 1 h. An incubation time of 10 min was adequate to protect the active site. However, experiments described here were part of other protection studies not described in this paper, in which the incubation time was 1 h. Unlabelled Mal*N*Et was added to the reaction mixture at a final concentration of 10 mM to modify the non-essential cysteine residues. The reaction was carried out for 1 h, after which time the unreacted Mal*N*Et was inactivated by the addition of 10 mM 2-mercaptoethanol. The enzyme was dialyzed against 50 mM sodium phosphate, pH 6.8, in order to remove salicylate and unreacted Mal*N*Et, and concentrated to a volume of 50 µl in a Centricon-30 microconcentrator.

[^{14}C]Mal*N*Et (27 mM), was added to the enzyme to a final concentration of 2 mM. An aliquot (1 µl) of this mixture was withdrawn at defined time intervals and assayed for residual enzyme activity. A similar aliquot was simultaneously spotted onto a glass-fibre-coated disc. Individual filters containing aliquots of the reaction at different time points, were processed by the trichloroacetic-acid-precipitation procedure to determine the extent of incorporation of [^{14}C]Mal*N*Et into the protein. For this, the filters were first washed extensively in cold 20% mass/volume trichloroacetic acid (10 min, five times) to precipitate the protein, then successively with acetone, 95% ethanol and ether (10 min, twice each). The filters were air dried and radioactivity determined in a toluene-based scintillation fluid. The stoichiometry of incorporation ([^{14}C]Mal*N*Et/protein) at each time point was calculated from an estimate of the protein spotted onto each filter and a specific activity of 7.4 Ci/mol for Mal*N*Et.

In experiments where the protein was used for subsequent peptide mapping, the specific labelling was carried out for 15 min using 10 mM [^{14}C]Mal*N*Et, at the end of which time the enzyme was completely inactive. The enzyme was dialyzed against 0.1 M NH_4HCO_3, pH 8.0, to remove the unreacted Mal*N*Et.

Peptide mapping. The specifically labelled protein (400 µg) was digested with freshly prepared tosylamidophenylethylchloromethane-trypsin at a protease/substrate ratio of 1:25 (by mass) for a period of 3 h at 37°C in 0.1 M NH_4HCO_3, pH 8.0, containing 1 M urea. The digest was dried and reconstituted in 100 µl 6 M guanidine hydrochloride in 0.1% trifluoroacetic acid. Peptides were separated by reverse-phase (RP) HPLC on a C_8 column using procedures described subsequently. Individual peaks were collected separately and 20% vol. each peak used for measuring radioactivity in 3 ml Aquasol [10]. Peptides containing appreciable radioactivity (>6000 cpm) were rechromatographed on a C_{18} column as detailed later. Purified peptides containing radioactivity were sequenced and the radioactivity associated with each cycle of Edman degradation determined by measuring the radioactivity of the fractions collected in the fraction collector of the sequenator, using Aquasol as the scintillation fluid.

Amino acid analysis. *In situ* performic acid oxidation of the protein (700 pmol) and hydrolysis using 6 M HCl in the vapour phase for 24 h at 110°C were carried out by established procedures. The hydrolysate was dried and the amino acids analyzed on the Waters PICOTAG work station [11].

Reduction and alkylation of DHBD. DHBD was reduced with dithiothreitol and the cysteine residues carboxymethylated using iodoacetic acid [12]. Alternately, alkylation was carried out using 4-vinylpyridine [13].

Enzymic cleavage of DHBD. *Endoproteinase Glu-C (V8 protease).* Reduced and carboxymethylated protein (2 mg), dissolved in 50 mM NH_4OAc, pH 4.2, containing 1 M urea at a final concentration of 5 mg/ml, was digested with Glu-C at a protease/substrate ratio of 1:100 (by mass). The digestion was carried out at 37°C for 18 h [14, 15]. The digestion mixture was acidified to pH 2.0 to stop the reaction.

Trypsin. A suspension of the reduced and carboxymethylated protein (1 mg) in 100 µl 0.1 M NH_4HCO_3, pH 8.2, containing 1 M Gdn/HCl was digested with tosylamidophenylethylchloromethane-trypsin at a protease/substrate ratio of 1:100 (by mass) at 37°C for 24 h with constant stirring [16]. The digestion mixture included 1 mM Ca^{2+} as $CaCl_2$ to prevent autodigestion [17].

Separation of peptides by HPLC. HPLC was performed on a Shimadzu LC-6A system (Shimadzu Corporation) using the reverse-phase column CLC-C8 (15 cm×4.6 mm, 6 nm pore size and 5 µm particle size) for the preliminary separation of peptides and CLC-ODS (C_{18}) column (15 cm×4.6 mm, 6 nm pore size, 5 µm particle size) for further resolution and purification of peptides. The solvent system used throughout this study consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.08% trifluoroacetic acid in 70% acetonitrile (solvent B) [18].

The tryptic or V8 digests, at the end of the specified time, were centrifuged to remove insoluble material. The soluble fraction was dried, dissolved in 0.1% trifluoroacetic acid containing 6 M guanidine hydrochloride and subjected to RP-HPLC on a C_8 column equilibrated with solvent A. After an initial wash of 20 min with solvent A, bound peptides were separated using a linear gradient of solvents A and B at a rise of 1% B/min and a flow rate of 0.5 ml/min. The absorbance of the eluate was monitored at 225 nm.

Each peak fraction of the primary run was collected manually and constituent peptides resolved further on a CLC-ODS column by manipulations of the gradient employing solvents A and B. Essentially, the gradient was a shallow rise of 0.5% B/min, unlike the increase of 1% B/min for primary runs.

Nomenclature of peptides. Peptide fractions were labelled T or V according to whether or not they were derived from a tryptic or Glu-C digest, respectively, and numbered according to their order of elution. The label TN was used for peptides from the tryptic digest of the Mal*N*Et-labelled protein.

Sequence determination. Amino acid sequence determination of intact protein and peptides was carried out on the automated gas-phase protein sequenator, Shimadzu PSQ-1. Samples for sequencing were dissolved in 10% formic acid.

Analysis of sequences. Sequence comparisons were carried out at the Bioinformatics Centre, Indian Institute of Science, Bangalore, India. The FASTA program [19] was used to identify homologies of the peptides sequenced in this study with sequences reported in the SWISS-PROT protein sequence database (December 1993, release; [20]).

RESULTS AND DISCUSSION

Incorporation of Mal*N*Et at the active site of the enzyme. Our earlier work on the enzyme indicated that DHBD was inactivated by Mal*N*Et, a reagent that is specific for cysteine residues. The inactivation followed pseudo-first-order kinetics and suggested the presence of a single class of essential cysteine residues [21]. In this study, we have established the stoichiometry of this modification reaction using the differential labelling technique. DHBD that had been modified at the non-essential cysteine residues using unlabelled Mal*N*Et in the presence of salicylate, retained greater than 80% of the initial activity. Such a modified enzyme was inactivated by 2 mM [^{14}C]Mal*N*Et in the absence of salicylate. There was a direct correlation between the loss of activity and the incorporation of labelled Mal*N*Et (Fig. 1). At the end of 90 min, when the enzyme had lost 80% of the initial activity, the stoichiometric incorporation of [^{14}C]Mal*N*Et was 3 mol/mol protein. This is equivalent to an incorporation of 0.75 mol/subunit, since DHBD is a homotetramer [4]. In a separate experiment, we noted that Mal*N*Et modified 9.5 mol cysteine/mol protein in the absence of salicylate and 4.2 mol/mol protein in the presence of salicylate. This corresponds to 1.2 mol cysteine/subunit protected by salicylate. We therefore suggest that this represents a single reactive cysteine residue/subunit enzyme.

The active-site cysteine-containing peptide. To identify the essential cysteine residue as part of the primary structure of the protein, the differentially labelled protein was digested with trypsin and the resulting peptides separated by RP-HPLC on a C_8 column (Fig. 2A). The radioactivity associated with each peak was measured (Fig. 2B). Two peaks, TN19 and TN21, each with approximately 12000 cpm and 8000 cpm, respectively, contained the maximum radioactivity. These peaks were individually rechromatographed on a C_{18} column in order to obtain pure

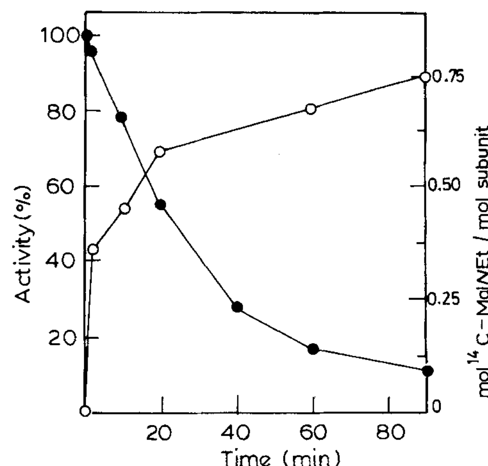


Fig. 1. Incorporation of [^{14}C]Mal*N*Et at the active site of DHBD. DHBD (2 nmol) was modified at the non-essential cysteine residues with unlabelled Mal*N*Et by treating the enzyme with Mal*N*Et (10 mM) in the presence of salicylate, the substrate analogue. The enzyme was dialyzed and inactivated with [^{14}C]Mal*N*Et (2 mM). Aliquots, withdrawn at indicated time intervals were assayed for residual enzyme activity (●) and trichloroacetic-acid-precipitable radioactivity. Incorporation (○) was calculated as [^{14}C]Mal*N*Et/subunit for the tetrameric DHBD from an estimate of the protein and radioactivity aliquoted at each time point.

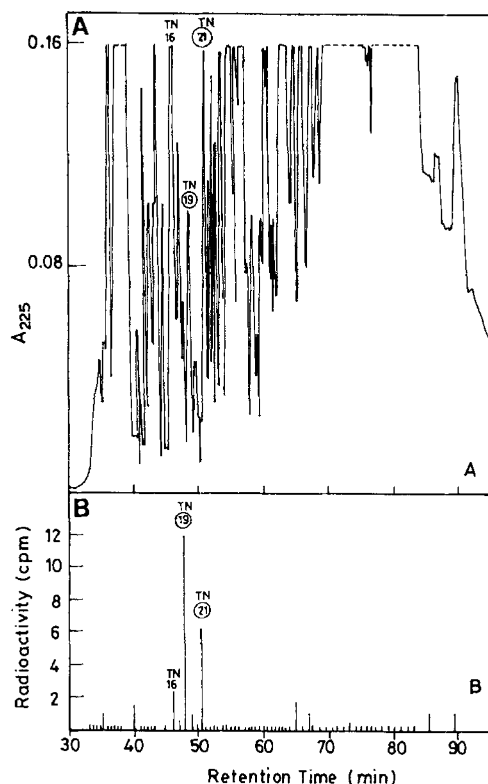


Fig. 2. Peptide mapping of differentially labeled protein. (A) Separation of peptides on a C_8 column. Specifically labelled protein (400 µg) was digested with trypsin at a protease/substrate ratio of 1:25 (by mass) in 0.1 M NH_4HCO_3, pH 8.0, containing 1 M urea. Peptides were separated by RP-HPLC on a C_8 column using a linear gradient of solvent A (0.1% trifluoroacetic acid) and solvent B (0.08% trifluoroacetic acid in 70% acetonitrile). The eluate was monitored at 225 nm. (B) Individual peaks were monitored for radioactivity by counting 20% of the volume of each peak in Aquasol. The total radioactivity in each peak is shown. Peaks containing maximum radioactivity, TN19 and TN21, were rechromatographed and sequenced.

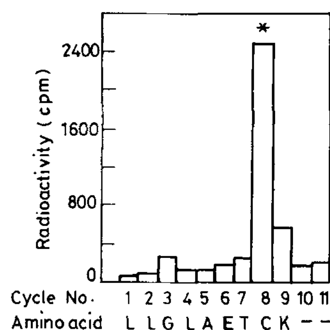


Fig. 3. Sequencing of peptide TN19. Peptide TN19 was rechromatographed to purity and sequenced on an automated gas-phase sequencer. The total radioactivity of each aliquot from each cycle of degradation, which collected in the fraction collector of the sequenator, was determined using Aquasol. The radioactivity depicted is the total radioactivity in each aliquot.

radioactive peptides for sequencing. On rechromatography, peaks TN19 and TN21 each yielded a single major radioactive peptide, the retention times of which were similar. The amount of radioactivity recovered in these peaks, 11500 cpm and 7000 cpm, respectively, was quantitative, indicating the covalent nature of the Mal*N*Et adduct with the peptide. These peptides were sequenced and the radioactivity associated with each cycle of sequencing estimated. The sequence of both peptides was LLGLAETCK, with maximum radioactivity being recovered in cycle 8. The data for TN19 is shown in Fig. 3. The elution of the same peptide at two different points on the gradient of the primary chromatogram could be due to the different nature of contaminating peptides present in these primary peptide fractions, which were eliminated in subsequent rounds of chromatography.

The location of cysteine in the eighth position of the peptide was identified from the recovery of maximum radioactivity in this cycle. Implicit in this argument is the well documented stability of the phenylthiohydantoin derivative of the Mal*N*Et-Cys adduct to reaction conditions of Edman chemistry and automated sequencing [22]. Coinciding with the recovery of radioactivity in this cycle, was the occurrence of a distinct phenylthiohydantoin derivative formed during this cycle of sequencing. This phenylthiohydantoin derivative eluted as a doublet rather than a single sharp peak. The doublet could be attributed to the presence of both diastereomers of the Mal*N*Et-Cys adduct [22, 23]. Similar doublets have been documented during the sequencing of Mal*N*Et-labelled peptides of isocitrate dehydrogenase [24] and proteosome [25]. This was also confirmed independently from the sequence of the peptide PT13 from the pyridethylated protein whose sequence was LLGLAETCK, with pyridethylated cysteine eluting in the eighth cycle (data not shown).

Since a major portion of the radioactivity was detected in two peptides which had identical sequences, LLGLAETCK, and since the loss of activity was associated with the incorporation of label, we suggest that this peptide contains an essential cysteine residue and forms part of the active site protected by salicylate. Similar studies carried out on the enzyme from *A. spergillus oryzae* also identified an essential cysteine residue that was labelled with [^{14}C]Mal*N*Et with a stoichiometry of 0.8 mol/mol subunit, and the peptide bearing the label was identified as LLGLAETCK (data not shown). The identical sequence of this peptide in the two enzymes, apart from indicating a similar active-site structure, testifies to the functional significance of the peptide. Sequence comparison of this peptide with sequences in the SWISS-PROT database revealed that there was no sequence, reported so far, that was similar to the active-site peptide.

Table 1. Amino acid composition of DHBD. The amino acid composition was determined by the PICOTAG method. The number of amino acids/subunit was calculated using a relative molecular mass of 38000 Da, determined by SDS/PAGE, and a mean residue mass of 110 Da. Cysteine was determined as cysteic acid. Tryptophan was not determined.

Amino acid	No. of residues *A. niger*	Global average no. residues
	(mol/100 mol)	
Cys	5 (1.4)	(1.9)
Asp + Asn	44 (12.7)	(10.4)
Glu + Gln	37 (10.7)	(9.4)
Ser	14 (4.0)	(6.8)
Gly	24 (6.9)	(7.2)
His	10 (2.9)	(2.3)
Arg	21 (6.1)	(5.1)
Thr	21 (6.1)	(5.9)
Ala	31 (9.0)	(7.8)
Pro	16 (4.6)	(5.9)
Val	20 (5.8)	(6.6)
Met	8 (2.3)	(2.3)
Ile	19 (5.5)	(5.3)
Phe	30 (8.7)	(3.9)
Leu	22 (6.4)	(9.1)
Lys	18 (5.2)	(5.9)
Tyr	17 (4.9)	(3.2)

Partial primary structure of DHBD. The identification of a unique active-site peptide for DHBD prompted us to elucidate its primary structure. As a first step, the amino acid composition of the protein was determined by the PICOTAG method (Table 1). A comparison of the amount of each residue with the average composition computed for proteins [26] indicated that DHBD had a relatively high content of phenylalanine, which was approximately twice that of an average protein. Other amino acids, however, were present in the same proportions as for the average protein.

The sequencing of reduced and carboxymethylated DHBD yielded the following N-terminal sequence: MLGKIALEEAFALPRFEEKTR. The unambiguous identification of a single amino acid in each cycle testified to the purity of the preparation.

DHBD was sequenced using protein sequencing methods. Enzymic cleavage of DHBD was used to generate peptides for sequencing. Endoproteinase Glu-C, noted for its high specificity, was used in an NH_4^+-based buffer in order to limit the specificity of the enzyme to glutamate residues. A pH of 4.2 was chosen, keeping in mind the easy solubility of the denatured protein at acidic rather than alkaline pH. Digestion with endoproteinase Glu-C provided the maximum number of purified peptides that could be sequenced. A total of 16 peaks (V1–V16) were obtained on RP-HPLC of the Glu-C digest (Fig. 4). Individual rechromatography of these peak fractions on a C_{18} column gave a total of 34 pure peptides. The tryptic digest of reduced and carboxymethylated or pyridethylated protein could be separated by RP-HPLC into about 41 primary peptide fractions (Fig. 5). These were resolved further by chromatography on a C_{18} column. The sequences of some of these were used singly (PT41, Table 2) or in conjunction with peptide sequences from the Glu-C digest to obtain longer stretches of sequence information. The final picture of the sequence information obtained for DHBD is indicated in Table 2.

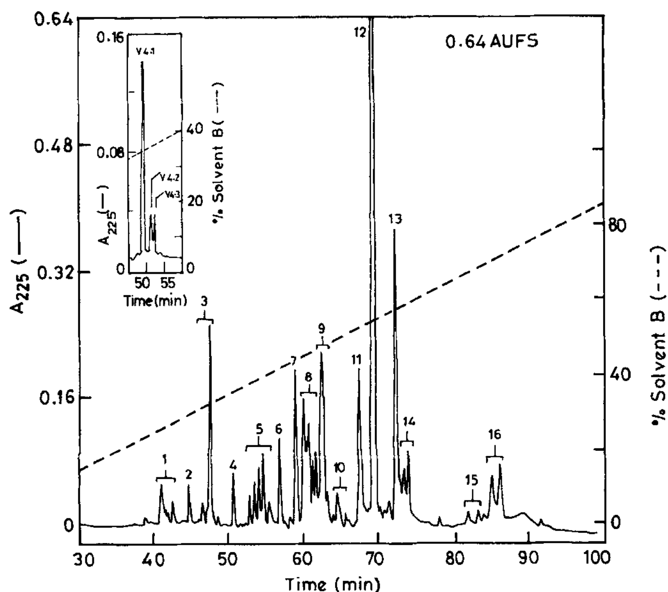


Fig. 4. Separation of peptides from the Glu-C digest of DHBD. Peptides were separated by RP-HPLC on a C_8 column using the acetonitrile solvent system in a gradient application with a rise of 1% B/min between solvent A (0.1% trifluoroacetic acid) and solvent B (0.08% trifluoroacetic acid in 70% acetonitrile). The eluate was monitored at 225 nm and individual peaks numbered V1–V16 were collected separately and rechromatographed on a C_{18} column using the same solvent system with a gradient rise of 0.5% B/min. Inset, the rechromatography of V4 is shown as a representative example. AUFS, absorbance units full scale.

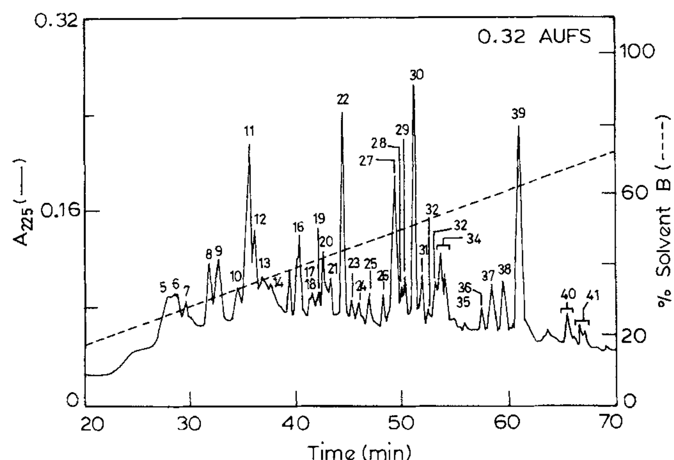


Fig. 5. Separation of peptides from the tryptic digest of DHBD. The separation was carried out on a C_8 column using the acetonitrile solvent system with a gradient of 1% B/min. Each peak was collected manually and rechromatographed.

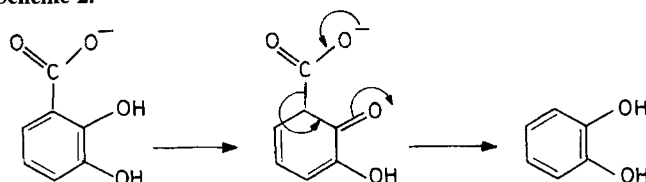
The unambiguous sequence information covered 292 of the expected 345 residues of the protein, although these residues are not continuous. A major limitation in obtaining more sequence information has been the limited solubility of the denatured protein at alkaline pH, the optimal pH value at which most proteases are active. The complete sequence of this protein can be obtained using other strategies for cleavage and separation of peptides or by using the sequence information presented here to design oligonucleotides for a nucleic-acid-based approach, both of which are currently under way in our laboratory. The availability of the complete sequence of this unique decarboxylase would form the basis of intensive structure/function studies of this enzyme.

Comparison of the sequences of peptides with other protein sequences. An estimate of the similarity of the sequence of a protein with the sequence data in databases often provides insight into the evolutionary relationships of the protein and provides clues to its function or possible mechanism of action. A recent example is the identification of the cofactor requirement for 4-chlorobenzoate dehalogenase from an inspection of the sequence relationships of the enzyme [27]. It was, therefore, of interest to determine whether the sequenced stretches of DHBD had sequence similarity to the sequences reported in the database.

Each of the sequences listed in Table 2 was compared with the SWISS-PROT protein sequence database using the software FASTA. The 20 best scorers in each search were a random set of proteins with no obvious structural or mechanistic similarities to DHBD. Furthermore, no single protein was consistently identified, even with a low degree of similarity, in these searches. The lack of significant similarity of any of the sequences obtained for DHBD, including the active-site sequence, with any other protein, could suggest that the sequence information was exclusive to DHBD and could account for the uniqueness of the decarboxylation it catalyzes.

Conclusion. We have earlier proposed a mechanism that accounts for our evidence for the requirement of essential histidine, cysteine and tryptophan residues for the DHBD reaction [21]. The mechanism invokes an acid/base-catalyzed deprotonation of the C(2)-OH to form an intermediate quinone. Decarboxylation is facilitated from this resonance-destabilized intermediate. The intermediate is transformed to the product catechol in a subsequent reprotonation step. The participation of the histidine residue in the first step and a cysteine residue in the last step, are probable with tryptophan anchoring the aromatic substrate:

Scheme 2.



A recent report [28] on the decarboxylation of vanillate carried out by resting cells of *Rhodotorula* in a deuterium-based medium, seems to support our hypothesis. However, structure/function studies on purified enzyme preparations such as that described here would be essential to understand the details of such reactions. For example, the extremely hydrophobic nature of the active-site peptide reported in this study could play a role in shielding the reactive quinone intermediate and prevent unwanted side reactions.

The sequences of several decarboxylases have been reported in the database. All of these decarboxylases, with the exception of orotidine 5′-monophosphate decarboxylase [29], catalyze the decarboxylation from aliphatic substrates, including amino acids. Orotidine 5′-monophosphate too, is a heterocyclic substrate where the ring nitrogen functions to stabilize the incipient carbanion, a key feature of all decarboxylation reactions [30]. 2,3-Dihydroxybenzoic acid, unlike orotidine 5′-monophosphate, is a benzenoid substrate where such decarboxylation strategies are not immediately obvious. Against this background of scant information on the non-oxidative decarboxylases, the information presented in this paper is the first report of the active-site cysteine residue and the partial primary structure of a non-oxidative decarboxylase catalyzing the decarboxylation from an aro-

Table 2. The sequence information of DHBD. Peptides designated Sant. 1 to Sant. 9 were compiled from sequences of Glu-C and tryptic peptides by the overlap method. Sant.10. to Sant.19 represent sequences of individual peptides from the tryptic or Glu-C digests, which have not been placed in the context of other peptides. TN16 is a minor radioactive peptide from Fig. 2. Sant. 18 is the active-site peptide in Fig. 3 whose sequence was confirmed from the sequence of a tryptic peptide PT13.

Peptide	Sequence	Description	No. amino acids
Sant. 1	MLGKIALEEAFALPRFEEKTRWWALSFSVDPE	N-terminus	32
Sant. 2	IEHADKYGVGYQILSYTAPGVQDIWDPVEAQAGE		34
Sant. 3	VGVDRILSIDYPFETFEDAAVV		22
Sant. 4	LRRDVQTYGFIGALVNDTQRTGPMGNNQEEAYN		33
Sant. 5	INDYIAEQIRDKPDRFGAFTLSMHNPQEA		29
Sant. 6	GRDNAARLFE		10
Sant. 7	RNPTGTIYEK		10
Sant. 8	LGAFRDYDAKVKA		13
Sant. 9	EITDINKLRIE		11
Sant. 10	NASWDIFWQTDTE	V11.3	13
Sant. 11	AQALAVE	V2.1	7
Sant. 12	DADVWFDGAE	V7.1	10
Sant. 13	FYDNA	T38.6	5
Sant. 14	AMQYVIA	V1.4	7
Sant. 15	YGAK	V1.4	4
Sant. 16	QADIYGP/G	V 1.7	7
Sant. 17	INH/GWFEDR	TN16	8
Sant. 18	LLGLAETCK	PT13, active site peptide	9
Sant. 19	WLVGPDLSFAHGVSLHVLGMTVNGVFDR	PT41	28
Total no. amino acids sequenced			292/345

matic nucleus. These studies pave the way for in-depth studies to unravel a mechanistically interesting class of reactions.

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