Gratton, E., Jameson, D. M., & Hall, R. D. (1984) *Annu. Rev. Biophys. Bioeng. 13*, 105-125.
Jameson, D. M., & Weber, G. (1981) *J. Phys. Chem. 85*, 953-958.
Jameson, D. M., & Gratton, E. (1983) in *New Directions in Molecular Luminescence* (Eastwood, D., & Cline-Love, L., Eds.) pp 67-81, Phil. ASTM.
Jameson, D. M., Gratton, E., & Hall, R. D. (1984) *Appl. Spectrosc. Rev.* (in press).
Johnson, M. L., Halvorson, H. R., & Ackers, G. K. (1976) *Biochemistry 15*, 5363-5371.
Johnson, M. L., Correia, J. J., Yphantis, D. A., & Halvorson, H. H. (1981) *Biophys. J. 36*, 575-588.
Klausner, R. D., Kleinfeld, A. M., Hoover, R. L., & Karnovsky, M. J. (1980) *J. Biol. Chem. 255*, 1286-1295.
Knutson, J. R., Walbridge, D. G., & Brand, L. (1982) *Biochemistry 21*, 4671-4679.
Knutson, J. R., Beechem, J., & Brand, L. (1983) *Chem. Phys. Lett. 102*, 501-507.
Lakowicz, J. R., & Cherek, H. (1980) *J. Biol. Chem. 255*, 831-834.
Lakowicz, J. R., & Cherek, H. (1981) *J. Biol. Chem. 256*, 6348-6353.
Laws, W. R., & Brand, L. (1979) *J. Phys. Chem. 83*, 795-802.
Lentz, B. R., Alford, A. R., Hoechli, M., & Dombrose, F. A. (1982) *Biochemistry 21*, 4212-4219.
Matayoshi, E. D., & Kleinfeld, A. M. (1981) *Biophys. J. 35*, 215-235.
Ross, J. B. A., Schmidt, C. J., & Brand, L. (1981) *Biochemistry 20*, 4369-4377.
Turner, B. W., Pettigrew, D. W., & Ackers, G. K. (1981) *Methods Enzymol. 76*, 596-628.
Weber, G. (1981) *J. Phys. Chem. 85*, 949.

Use of Radiolabeled Monofluoromethyl-Dopa To Define the Subunit Structure of Human L-Dopa Decarboxylase†

Rhoda Maneckjee and Stephen B. Baylin*

ABSTRACT: Human L-Dopa decarboxylase (L-aromatic amino acid decarboxylase, DDC) has been purified from pheochromocytoma tissue, a benign tumor of the catecholamine-synthesizing cells of the adrenal medulla. The binding characteristics of a new radiolabeled enzyme-activated suicide inhibitor of DDC ([^{3}H]monofluoromethyl-Dopa, [^{3}H]MFMD) have been established, and the covalent linkage of the inhibitor to the enzyme has been used to identify that human DDC exists as a dimer of a 50-kDa subunit. An antibody to human DDC identically precipitates the enzyme activity from different human, rat, and mouse tissues. Our data demonstrate the value of [^{3}H]MFMD for probing the structure of DDC and facilitating the purification of this enzyme, and further emphasize the high degree of conservation of the DDC molecule over a wide variety of species.

This paper describes the first use of [^{3}H]monofluoromethyl-Dopa ([^{3}H]MFMD), a new radiolabeled form of this enzyme-activated, irreversible inhibitor (Palfreyman et al., 1978; Jung et al., 1979; Sjoerdsma, 1981), for defining the subunit structure of human L-Dopa decarboxylase (DDC)[1] (L-aromatic amino acid decarboxylase, EC 4.1.1.28). DDC is a pyridoxal-containing enzyme that catalyzes the decarboxylation of certain aromatic L-amino acids (Lovenberg et al., 1962; Christenson et al., 1970, 1972). This protein has long been of interest to a diverse body of investigators for the following reasons: (a) in the central and peripheral nervous system of most species, it catalyzes the middle step in the synthesis of the monoamine neurotransmitters serotonin, dopamine, epinephrine, and norepinephrine; (b) some neurons, which do not contain the above monoamines, contain only DDC among the catecholamine-synthesizing steps and may synthesize important and yet to be characterized neurotransmitters directly from amino acid substrates such as tyrosine, phenylalanine, and tryptophan (Jaeger et al., 1983); (c) in insect species such as the drosophila, DDC is a temperature-regulated and possibly hormonally regulated enzymatic step in the synthesis of amine products that are necessary for proper formation of the cuticle (Kraminsky et al., 1980; Marsh & Wright 1980; Hirsh & Davidson, 1981); (d) DDC is an important phenotypic expression of widely dispersed endocrine cells that synthesize and secrete small polypeptide hormones (Pearse, 1969). In this latter regard, DDC is particularly important in humans because several types of cancer contain such DDC-rich endocrine cells [for review, see Pearse (1969) and Baylin & Mendelsohn (1980)]. The most common such tumor is the human small cell carcinoma of the lung, a virulent neoplasm that, at different stages of differentiation, manifests high DDC activity both in vivo (Berger et al., 1981) and especially in cell culture (Baylin et al., 1980; Goodwin & Baylin, 1982).

In order to learn more about the function of DDC in various cell types, including normal and neoplastic human endocrine cells, it is of interest to isolate and physicochemically characterize this human enzyme. To these ends, we now define the binding characteristics of a recently developed labeled preparation of the selective inhibitor of DDC, monofluoro-

† From the Oncology Center and the Department of Medicine, The John Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received September 28, 1983.* This work was supported by Grant CA-18404 from the National Cancer Institute, Grant PDT-108 from the American Cancer Society, and a gift from the W. W. Smith Foundation

[1] Abbreviations: BSOCOES, bis[2-[[(succinimidooxy)carbonyl]-oxy]ethyl] sulfone; DDC, L-Dopa decarboxylase; Dopa, dihydroxyphenylalanine; MFMD, monofluoromethyl-Dopa; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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methyl-Dopa (MFMD), and we combine these studies with classic methods of enzyme purification to define the subunit structure of human DDC.

Experimental Procedures

Tissues. Samples of pheochromocytoma, a benign tumor of the catecholamine-synthesizing cells of the adrenal medulla, were obtained at surgery. The tissues were stored frozen at −70 °C until used.

Chemicals. [1-^{14}C]-L-Dopa (sp act. 7.9 mCi/mmol) was purchased from the Radiochemical Center, Amersham. Monofluoromethyl-Dopa (MFMD) was initially synthesized by Merrill-Dow Research Center (1-3); [^{3}H]MFMD, 27.2 Ci/mmol, was a generous gift from Dr. Peter McCann of Merrell-Dow Research Center and is made by New England Nuclear. L-Dopa, pyridoxal phosphate, dithiothreitol, 2-mercaptoethanol, ammonium sulfate, alumina Cy gel, and aromatic amino acids were obtained from Sigma and DEAE-Sephadex A-50 and Sephacryl S-300 from Pharmacia. Hydroxylapatite (Bio-Gel HTP) and materials for polyacrylamide tube and slab gel electrophoresis, including sodium dodecyl sulfate (SDS), electrophoresis grade urea, *N,N,N′,N′*-tetramethylethylenediamine, ammonium persulfate, and Coomassie Brilliant Blue R-250, were obtained from Bio-Rad. Silver nitrate was purchased from Fisher. Marker proteins used for molecular weight calibrations and ampholytes for isoelectric focusing were purchased from Pharmacia. High specific activity Na^{125}I was obtained from Amersham, Ionagar No. 2 for immunodiffusion studies from Consolidated Labs, Freund's complete and incomplete adjuvant from Calbiochem, and goat anti-rabbit γ-globulin from Cappel Laboratories.

Enzyme Purification Procedure. The human DDC was purified from human pheochromocytoma by modifications of the method of Christenson et al. (1970). All buffers throughout the procedure contained 0.01 M 2-mercaptoethanol.

(*A*) *Preparation of Crude Extract.* Frozen tissue was homogenized with a Polytron (Brinkmann) homogenizer in 3 volumes of ice-cold 0.05 M sodium phosphate buffer, pH 7.2, and centrifuged at 100000*g* for 60 min.

(*B*) *Ammonium Sulfate Precipitation.* The supernatant fluid (crude extract) was then precipitated with 30–60% saturated ammonium sulfate, centrifuged at 25000*g* for 30 min, dissolved in 0.005 M sodium phosphate buffer, pH 7.2, and dialyzed for 24 h against several changes of the same buffer.

(*C*) *Alumina Cy Gel Adsorption.* The dialyzed ammonium sulfate pellet was treated with a 3% aqueous suspension of alumina to a final concentration of 0.4 mg dry weight of gel per optical density unit. The mixture was stirred for 20 min and centrifuged, and the gel was washed with 0.01 M 2-mercaptoethanol and resuspended in 0.05 M phosphate buffer, pH 7.2. After being stirred for 20 min, followed by centrifugation, the clear yellow eluate containing the enzyme was retained.

(*D*) *DEAE-Sephadex A-50 Chromatography.* The alumina Cy gel eluate was applied to a column (2.5 × 30 cm) of DEAE-Sephadex A-50, preequilibrated with 0.05 M phosphate buffer, pH 7.2. Fractions (10 mL) were eluted with 1 L of a linear gradient of 0–0.5 M NaCl in the same buffer, and those with the highest enzyme activity (0.05–0.1 M NaCl) were pooled, concentrated to 1 mL in collodion bags, and dialyzed overnight against two changes of 1 L each of 0.05 M phosphate buffer, pH 7.2.

(*E*) *Gel Filtration on Sephacryl S-300.* The dialyzed DEAE-Sephadex concentrate was chromatographed on a Sephacryl S-300 column (2.5 × 85 cm) in 0.05 M phosphate buffer, pH 7.4, containing 0.15 M NaCl at a flow rate of 10 mL/h. Fractions (2 mL) containing DDC were pooled, concentrated to about 10 mL in collodion bags, and dialyzed overnight against 0.01 M phosphate buffer, pH 7.2.

(*F*) *Hydroxylapatite Column Chromatography.* The dialyzed Sephacryl concentrate was applied to a freshly prepared hydroxylapatite column (0.9 × 15 cm), preequilibrated for at least 20 h with 0.01 M sodium phosphate buffer, pH 7.2, and eluted with about 500 mL of a linear gradient of 0.01–0.3 M sodium phosphate buffer, pH 7.2. Fractions (5 mL) with the highest enzyme activity (usually 0.1 M concentrations of phosphate buffer) were pooled and concentrated to about 1 mL with collodion bags.

Enzymatic Assay. DDC activity was determined by a modification (Baylin et al., 1978) of the method of Beaven et al. (1978). Specific activity is expressed as units per milligram of protein, where 1 unit = 1 nmol of $^{14}CO_2$ released from decarboxylated [1-^{14}C]-L-Dopa per hour of incubation.

Protein Determination. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as the standard or were monitored in column eluates by measuring absorbance at 280 nm.

Polyacrylamide Gel Electrophoresis. Nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Davis (1964) on 4% gels (Bio-Rad) at a constant current of 2 mA/tube over 3–4 h with an ice water cooled jacket. Gels were stained with Coomassie Blue and scanned in a Gilford recording spectrophotometer at 600 nm. DDC activity was located by sectioning unstained gels (3 mm) with a scalpel and incubating slices for 24 h at 4 °C in 0.05 M sodium phosphate buffer, pH 7.2 (250 μL), prior to assaying 80-μL aliquots.

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was carried out by the method of Weber & Osborn (1969) or Laemmli (1970) at room temperature. Also, two types of two-dimensional PAGE were used. In one, the first-phase electrophoresis, without SDS, was carried out on 4% polyacrylamide tube gels, which were then stored at −70 °C in O'Farrell's equilibration buffer (O'Farrell, 1975). Prior to being run in the second dimension, the gels were equilibrated with O'Farrell's equilibration buffer for 1 h at 37 °C, embedded in agarose on top of 5–15% acrylamide slab gels prepared as per Laemmli (1970), and electrophoresed at 10 mA/slab, and the second-dimension gels were then stained with silver stain (Merril et al., 1981). In the second type of two-dimensional gels, purified preparations were examined exactly as outlined by O'Farrell (1975) with the first-dimension tube gels prepared by isoelectric focusing.

Chemical Cross-Linking with BSOCOES. Chemical cross-linking of purified DDC, using a bifunctional base-cleavable reagent, BSOCOES [bis[2-[[(succinimidooxy)-carbonyl]oxy]ethyl] sulfone, Pierce Chemicals], was carried out according to the method of Zarling et al. (1980). Purified enzyme was first radioiodinated with ^{125}I by using chloramine-T (Hunter & Greenwood, 1962). The iodinated enzyme preparation was analyzed by non-SDS-PAGE, and the enzyme activity on companion gels was located by assaying for DDC activity in 3-mm slices. Iodinated enzyme was eluted in phosphate-buffered saline (PBS) for 24 h from gel sections in the region of enzyme activity. Pooled eluates from three gels were concentrated in collodion bags and incubated in PBS (200 μL) containing a final concentration of 0.5% Me_2SO and 1 mM BSOCOES for 1 h at 0 °C. Following addition of arginine (1 M stock, pH 7.5; final concentration = 0.1 M) and

Table I: Typical Purification of DDC from Human Pheochromocytoma

step	total protein (mg)	total enzyme act. (nmol)	sp act. (units/mg of protein)	purification (x-fold)	yield of enzyme (%)
crude homogenate	2160	46 260	21.4		100
ultracentrifugation (100000g)	1815	48 800	26.8	1.2	100
ammonium sulfate fractionation (30–60%)	800	21 840	27.3	1.3	47
alumina Cy gel	114	4 560	40.0	1.9	10
DEAE-Sephadex A-50	54	3 909	72.0	3.4	8.4
Sephacryl S-300	12	3 456	288.0	13.5	7.5
hydroxylapatite	6	5 491	9151	428	0.2

20-min incubation at 0 °C, the cross-linked enzyme preparation was analyzed by gradient 5–15% SDS gels. Non-cross-linked, 0.5% Me_2SO-treated enzyme was used as the control. The slab gels were stained with Coomassie Blue, dried under vacuum, and exposed to Kodak X-Omat AR film in a Kodak X-Omatic cassette for 4 days at −70 °C. Cleavage of cross-linked subunits was achieved by incubating gel sections containing cross-linked proteins at pH 11.6 (NaOH) for 2 h at 37 °C and neutralizing the gel to pH 7.2 before the products were again separated on gradient reducing SDS–PAGE.

Preparation of Antiserum. Antibodies to purified DDC were raised in 8-week-old female white New Zealand rabbits by initial injection of 50 μg of DDC, purified to homogeneity, and emulsified in 0.5 mL of 0.15 M NaCl plus 0.5 mL of Freund's complete adjuvant, subcutaneously at multiple sites along the back. Serum was obtained at 2-week intervals. Six weeks after the first injection, rabbits received a second injection of 25 μg of pure DDC, followed by periodic booster injections of 25 μg of a partially purified enzyme preparation.

Sera were analyzed by Ouchterlony immunodiffusion analysis (Ouchterlony, 1958) and by immunoelectrophoresis (Scheidegger, 1955). Immunochemical titrations were performed by incubating increasing amounts of antiserum with fixed aliquots of active DDC in homogenates of various tissues for 1 h at 37 °C. The enzyme–antibody complexes were then precipitated with a 20:1 ratio of goat antiserum to rabbit globulin after overnight incubation at 4 °C, suspended in 30-μL aliquots of pH 7.2, 0.05 M phosphate buffer, and assayed for DDC activity.

Results

Purification of Human DDC. In eight separate experiments, DDC was purified from human pheochromocytomas by the modified procedure of Christenson et al. (1970). On the average, we acheived a range of 200–600-fold purification, similar to values found for purification of nonhuman DDC (Christenson et al., 1970), with recoveries of 1% or less (Table I). In one instance only were we able to obtain a preparation that appeared to be homogeneous with a purification of approximately 400-fold. The apparent low-fold purification results from the fact that, despite addition of fresh 2-mercaptoethanol at all steps, enzyme activity decreases rapidly throughout the purification procedure (50% loss per 3–6 days during a 10-day purification). The behavior of the protein from this purification is outlined in Figure 1. In this preparation, a single band was visualized by Coomassie Blue staining (which coincided with a single peak of enzymatic activity) (Figure 1A), and SDS gels yielded a single band with an approximate molecular weight of 50 000 (Figure 1B). In most purifications, however, SDS gels consistently revealed three bands of 62, 50, and 44 kDa (Figure 1C). Two of these bands (50 and 44 kDa) were seen in the area that coincided with DDC activity when the native gels were electrophoresed on a second-dimension, SDS–polyacrylamide gel (Figure 1F).

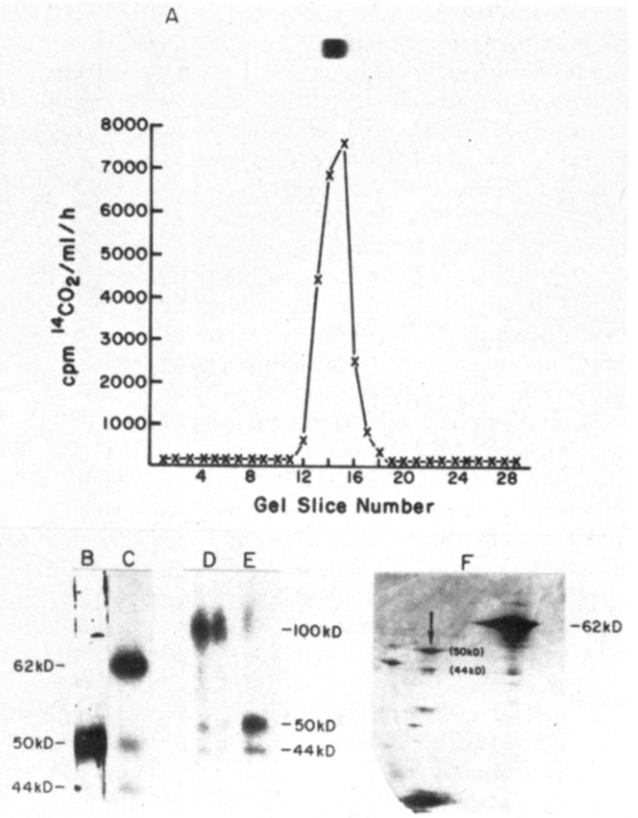

FIGURE 1: Polyacrylamide gel electrophoresis analysis of DDC purified from human pheochromocytoma. (A) A 4% non-SDS gel of DDC from one purification which yielded apparently homogeneous enzyme; approximately 25 μg was applied to each of two gels, one of which was stained with Coomassie Brilliant Blue (upper part) and the other assayed, as outlined under Experimental Procedures, for DDC activity. (B) A 10% SDS gel of the same preparation shown in panel A; approximately 25 μg was applied to the gel. (C) Radioautograph of a 5–15% SDS gel of an iodinated preparation of partially purified DDC. (D) Radioautograph of an iodinated DDC preparation which was cross-linked, as outlined under Experimental Procedures, with BSOCOES. The 100-kDa band was identified on an initial 5–15% SDS gel, excised, and reelectrophoresed on the 5–15% gel shown. (E) The same experiment as shown in (D) except that the gel slice containing the 100-kDa material was first exposed to a pH of 11.6 (Experimental Procedures) to cleave the cross-linked oligomer prior to reelectrophoresis of the iodinated material on the 5–15% SDS gel shown. (F) Second-dimension 5–15% SDS gel of 25 μg of partially purified DDC which was first electrophoresed on a 4% non-SDS gel. The arrow marks the position of enzyme activity on the first-dimension gel, and the protein was visualized by silver staining (Merril et al., 1981).

Upon storage of the purified preparation, as enzyme activity decreased, the 44-kDa band increased while the 50-kDa band decreased. We thus concluded preliminarily that (a) human DDC has a monomer subunit of 50 kDa, (b) the 44-kDa band was an enzymatically inactive proteolytic cleavage product of the 50-kDa band, and (c) the 62-kDa band was a contaminant.

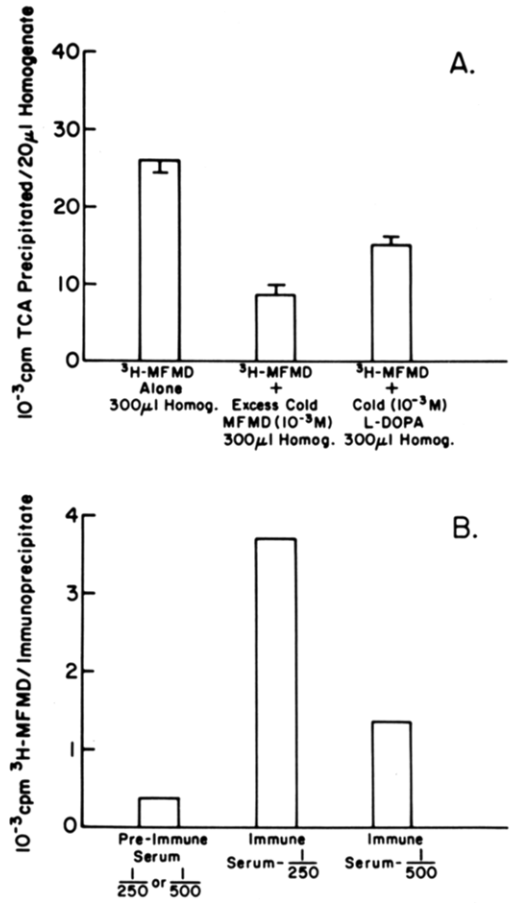

FIGURE 2: (A) TCA precipitates of homogenates (300 μL, 1:2 wt/vol) of human pheochromocytoma tissue incubated with 3×10^5 cpm of [^{3}H]MFMD. The homogenates were first determined to have high DDC activity by radioassay. Following 1 h of incubation with [^{3}H]MFMD, 20 μL of the homogenate was added to 20 μL of 10 mg/mL carrier BSA and 30 μL of 50% TCA. After 30 min, precipitates were collected by centrifugation at top speed in a Brinkmann microfuge and washed twice in 500 μL of 25% TCA. The precipitates were then solubilized overnight in 50 μL of 2 N NaOH and counted in 7.0 mL of Aquasol. The left bar represents binding for 300 μL of homogenate minus the counts in the second bar. This second bar represents displacement of [^{3}H]MFMD by excess cold MFMD (10^{-3} M cold MFMD, the K_i for MFMD is 10^{-6} M; Palfreyman et al., 1978; Jung et al., 1979; Sjoerdsma, 1981), which we assume to represent residual nonspecific binding. The first bar value represents essentially 100% of the added cpm, indicating full binding of the label to enzyme under these conditions. The third bar shows competition of [^{3}H]-MFMD binding by 10^{-3} M cold L-Dopa, a natural substrate for the enzyme ($K_m = 2 \times 10^{-4}$ M). The values for each bar are from two separate experiments, and the brackets indicate the *range* of values obtained. (B) Immunoprecipitation of [^{3}H]MFMD from the human pheochromocytoma homogenates detailed in (A). Five microliters of homogenate plus 50 μL of pH 7.5, 0.1 M phosphate buffer was incubated 1 h with 10 μL of 1:50 or 1:100 preimmune serum (obtained from rabbit prior to three immunizations with purified human DDC) or the same dilutions of postimmunization serum. This serum, as detailed under Experimental Procedures, immunoprecipitates enzymatically active DDC from various sources. After this first incubation, 200 μL of goat anti-rabbit γ-globulin serum was added, and a second incubation was carried out at 4 °C overnight. Precipitates were collected by 3 min of centrifugation at top speed in a Brinkmann microfuge and washed twice with 200 μL of the 0.1 M phosphate buffer. The pellets were then suspended in 50 μL of 5% SDS and counted in 7.0 mL of Aquasol. For the 1:250 final dilution of post-immune serum, the counts precipitated represent essentially 100% of the total TCA-precipitable cpm added to the immunoprecipitation.

However, the finding of multiple bands in our SDS gels and similar previous results from other investigators (Christenson et al., 1970; Clark et al., 1978) could suggest that DDC is constructed as an oligomer of unequal size subunits. We thus

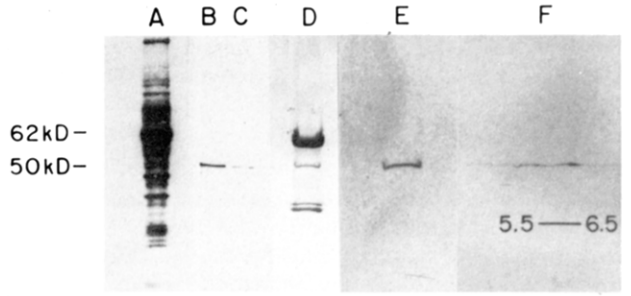

FIGURE 3: Five to fifteen percent polyacrylamide gradient SDS gels of human pheochromocytoma homogenates preincubated with [^{3}H]MFMD. (A) Coomassie Blue stain of 20 μL of the crude pheochromocytoma homogenate detailed in Figure 2A. (B) Radioautograph of the same gel shown in panel A. The gel was treated with Enhance (New England Nuclear) as per the manufacturer's specifications prior to drying and exposure to the X-ray film. (C) The same experiment shown in panels A and B except that unlabeled L-Dopa (see Figure 2A) was incubated with the homogenate and [^{3}H]MFMD. (D) Coomassie Blue stain of 50 μg of partially purified DDC from a pheochromocytoma homogenate preincubated with 400 μCi of [^{3}H]MFMD. (E) Radioautograph of the same gel shown in panel D. (F) Second-dimension 5–15% gel of an O'Farrell two-dimensional analysis of the same preparation used in panels D and E. Only the pertinent pH and molecular weight region of the gel is shown.

analyzed, further, the multiple bands obtained in our preparations.

Cross-Linking Studies with BSOCOES. Partially purified preparations of DDC were iodinated and electrophoresed, and the usual three bands were seen on radioautographs of 5–15% gradient SDS gels (Figure 1C). When iodinated material was cross-linked with BSOCOES, as outlined under Experimental Procedures, a 100-kDa band appeared (Figure 1D). When this oligomer was excised, the cross-links cleaved by raising the pH to 11.6, and the material reelectrophoresed on the gradient gels, the 50- and 44-kDa bands were regenerated (Figure 1E). We concluded, preliminarily, from these cross-linking studies that human DDC is a dimer of a 50-kDa subunit and that the 44-kDa proteolytic product could also be cross-linked to this subunit or with itself. These conclusions fit well with a Stokes radius estimation of approximately 100 kDa found for the DDC activity in the Sephacryl gel filtration step of the purification. The cross-linking studies also further confirmed the 62-kDa band as a contaminant that did not participate in the cross-linking process.

Confirmation of the Subunit Structure of Human DDC Using Tritiated MFMD. High specific activity radiolabeled MFMD, a potent and selective suicide inhibitor of DDC (Palfreyman et al., 1978; Jung et al., 1979; Sjoerdsma, 1981), was used to document the fact that the active site for DDC is on the 50-kDa subunit demonstrated in our purification studies. When the labeled inhibitor was incubated with crude homogenates of human pheochromocytoma (Figure 2A), we obtained high amounts of trichloroacetic acid (TCA) precipitable radioactivity, which were not present when the inhibitor was incubated with albumin controls and which could be markedly diminished by coincubation of the inhibitor and the homogenate with excess amounts of either unlabeled MFMD (Figure 2A) or the competing enzyme substrate L-Dopa (Figure 2A). Furthermore, when the homogenates plus the labeled inhibitor were incubated with an antiserum prepared against our purest preparations of human pheochromocytoma DDC, specific immunoprecipitation of labeled material could be demonstrated (Figure 2B).

The incubations of pheochromocytoma homogenates plus [^{3}H]MFMD were analyzed by 5–15% SDS–polyacrylamide gel electrophoresis (Figure 3). Even in such crude prepara-

tions, radioautographs demonstrated a single labeled band in the position of the 50-kDa subunit (Figure 3A,B). In the experiment where the [^{3}H]MFMD was coincubated with excess L-Dopa (Figure 2A), label was displaced from the 50-kDa band (Figure 3C).

We used [^{3}H]MFMD to follow one partial purification of DDC from human pheochromocytoma. The labeled inhibitor (400 μCi) was preincubated for 2 h with 2 mL of a 1:3 crude homogenate. At this time point, the labeled compound inhibited the DDC by 98%. The ammonium sulfate precipitation, DEAE-Sephadex, Sephacryl, and hydroxylapatite purification steps outlined in Table I were then performed. A last Affi-Gel blue column step was added, which partially removed some of the 62-kDa contaminant from this particular preparation. The dialysis of the ammonium sulfate precipitated material completely removed nonbound [^{3}H]MFMD, leaving behind an amount of radioactivity that was identical with TCA precipitations of both the original homogenate and the solubilized ammonium sulfate precipitated material. From calculations of the specific activity of TCA precipitation and/or postdialyzed labeled protein in the initial homogenate and from determination of the milligram concentration of DDC in the preparation from the millimoles of bound tritiated MFMD required to give 100% inhibition of enzyme, we calculated that the active pheochromocytoma DDC was approximately 0.1% of the total protein. Thus, isolation of DDC to theoretical homogeneity should represent a 1000-fold purification. This fits well with the data in Table I considering the previously mentioned loss of enzyme activity during the purification. At all steps of the purification, [^{3}H]MFMD eluted from columns in the exact positions observed for enzyme activity in the previous purification experiments. As shown in Figure 3D, the final purified preparation contained 62- and 50-kDa bands plus some lower molecular weight material. The radioautographs revealed a single band of radioactivity that, again, calculated to 50 kDa (Figure 3E). Two-dimensional gel analysis of this purification by the method of O'Farrell (1975) revealed a single, long, radiolabeled band of 50 kDa which spanned a pH range of 5.5–6.5 (Figure 3F).

Comparison of DDC from Different Human Tissues and Different Species. The kinetic properties of purified human DDC were similar to those reported for the enzyme from other species (Lovenberg et al., 1962; Christenson et al., 1970; Clark et al., 1978; Borri Voltattorni et al., 1979). The K_m for L-Dopa as substrate was 1.7×10^{-4} M with a pH optimum of 7.0, and the order of substrate specificities was L-Dopa > 5-hydroxytryptophan > tyrosine and tryptophan. Pyridoxal phosphate, 10^{-5} M, increased activity of the purified DDC by 3.5-fold, and addition of fresh dithiothreitol (5×10^{-3} M) plus the pyridoxal prior to assay increased activity 6-fold.

We investigated the antigenicity of DDC in different tissues. The antibody used, generated as per Experimental Procedures, gave a single precipitin arc on immunoelectrophoresis and immunodiffusion, even when tested against crude preparations of pheochromocytomas. In the immunoelectrophoresis studies, enzyme activity was found in the region of the arc (data not shown). After subtraction of nonspecific binding for preimmune serum at each point, increasing amounts of enzyme activity were found in immunoprecipitation pellets from incubations of pheochromocytoma homogenates with increasing amounts of rabbit sera; 20 μL of a 1:4000 diluted antiserum precipitated approximately 50% of active enzyme from tissue homogenates. By use of the above titration technique with 20 μL of the 1:4000 diluted antiserum, virtually identical cross-reaction was found for DDC activity from human tissues, including pheochromocytoma, neuroblastoma (whole tissue and cells in culture), ganglioneuroma, kidney, small cell carcinoma of the lung (whole tissue and cells in culture), and medullary thyroid carcinoma, and from nonhuman tissues, including adrenals and liver and tumor tissues (medullary thyroid carcinoma) of the rat and mouse.

Discussion

We have combined standard protein purification techniques, using a new radiolabeled, enzymatic-activated, irreversible inhibitor, to define the subunit structure of human DDC. The active site is contained on a 50-kDa monomer subunit. From our gel filtration data on Sephacryl S-300, cross-linking studies with BSOCOES, and the behavior of [^{3}H]MFMD-conjugated DDC during purification, the native human enzyme behaves as a dimer of this monomer subunit. A similar oligomer size has been proposed for DDC from drosophila (Clark et al., 1978) and pig kidney tissue (Christenson et al., 1970; Borri Voltattorni et al., 1979). Although there has been controversy about the structure of DDC in different species, with some investigators concluding that the enzyme is an oligomer of nonidentical subunits (Fragoulis & Sekeris, 1975), it is probable that these previous studies were interpreting either contaminating proteins or degradation products as part of the active DDC molecule. Our own purifications consistently yielded three monomer proteins under denaturing gel conditions. One of these peptides (62 kDa) is a major contaminant, and another (44 kDa), a size also seen in addition to a 50-kDa band during purification of DDC from blowfly larvae (Fragoulis & Sekeris, 1975), is probably an inactive degradation product of the DDC molecule.

Our current data emphasize further the apparent high degree of conservation of the DDC molecule across a wide range of species. First, the subunit structure for human DDC is virtually identical with that reported for the drosophila (Clark et al., 1978) enzyme. The latter exists as a dimer of a 54-kDa peptide (Clark et al., 1978), which migrates with a similar pH range and pattern on O'Farrell's two-dimensional polyacrylamide gels (Hirsh & Davidson, 1981) to those we have now shown for human DDC. Second, our antibody studies revealed high cross-reactivity between DDC from human, rat, and mouse tissues; Christenson et al. (1972) found a similar sharing of antigenic determinants between porcine, rabbit, and rat DDC.

Our current demonstration that [^{3}H]MFMD is covalently linked to only one protein, even in crude homogenates of DDC-containing tissues, emphasizes the broad spectrum of uses for this labeled inhibitor. Recently, another enzyme-activated, irreversible inhibitor of an important decarboxylation enzyme, ornithine decarboxylase, has proven similarly useful for substantiating the subunit structure of this enzyme (Pritchard et al., 1981; Seely et al., 1982). It should now be possible to characterize DDC in a wide variety of tissues by simple incubation with [^{3}H]MFMD and subsequent analysis of the enzyme on native and denatured polyacrylamide gels. Furthermore, the specificity of the binding demonstrated in our studies should rapidly facilitate the use of [^{3}H]MFMD as a tool for histochemical analysis of the cellular distribution of DDC. Again, the use of [^{3}H](difluoromethyl)ornithine has proven particularly useful for such tissue studies of ornithine decarboxylase (Pegg et al., 1982). Specific tracing of the cellular distribution of DDC may help define the temporal appearance of the enzyme during embryologic development of neural tissue and cells with neuroendocrine phenotype. Also, in important human tumors, the cell populations responsible for the high DDC activity might be outlined. Such studies

should enhance our knowledge about the role of DDC during differentiation of neural and neuroendocrine tissues and in the evolution of important cell populations within human neoplasms.

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References

Baylin, S. B., & Mendelsohn, G. (1980) *Endocr. Rev. 1*, 45–77.

Baylin, S. B., Stevens, S. A., & Shakir, K. M. M. (1978) *Biochim. Biophys. Acta 541*, 415–419.

Baylin, S. B., Abeloff, M. D., Goodwin, G., Carney, D. N., & Gazdar, A. F. (1980) *Cancer Res. 40*, 1990–1994.

Beaven, M. A., Wilcox, G., & Terpstra, G. K. (1978) *Anal. Biochem. 84*, 638.

Berger, C. L., Goodwin, G., Mendelsohn, G., Eggleston, J. C., Abeloff, M. D., Aisner, S., & Baylin, S. B. (1981) *J. Clin. Endocrinol. Metab. 53*, 422–428.

Borri Voltattorni, C. B., Minelli, A., Vecchini, P., Fiori, A., & Turano, C. (1979) *Eur. J. Biochem. 93*, 181–188.

Christenson, J. G., Dairman, W., & Udenfriend, S. (1970) *Arch. Biochem. Biophys. 141*, 356–367.

Christenson, J. G., Dairman, W., & Udenfriend, S. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 343–347.

Clark, W. C., Pass, P. S., Vankataranar, B., & Hodgetts, R. B. (1978) *Mol. Genet. 162*, 287–297.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Fragoulis, E. G., & Sekeris, C. E. (1975) *Arch. Biochem. Biophys. 168*, 15–25.

Goodwin, G., & Baylin, S. B. (1982) *Cancer Res. 42*, 1361–1367.

Hirsh, J., & Davidson, N. (1981) *Mol. Cell. Biol. 1*, 475–485.

Hunter, W. M., & Greenwood, F. C. (1962) *Nature (London) 194*, 495–496.

Jaeger, C. B., Teitelman, G., Joh, T. H., Albert, V. R., Park, D. H., & Reis, D. J. (1983) *Science (Washington, D.C.) 219*, 1233–1235.

Jung, M. J., Palfreyman, J., Wagner, J., Bey, P., Ribereau-Gayon, G., Zraika, M., & Koch-Weser, J. (1979) *Life Sci. 24*, 1037–1042.

Kraminsky, G. P., Clark, W. C., Estelle, M. A., Gietz, R. D., Sage, B. A., O'Connor, J. D., & Hodgetts, R. B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4175–4179.

Laemmli, U. (1970) *Nature (London) 227*, 680–685.

Lovenberg, W., Weisbach, H., & Udenfriend, S. (1962) *J. Biol. Chem. 237*, 89–92.

Lowry, O. H., Rosebrough, H. J., Farr, A. J., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Marsh, J. L., & Wright, T. R. F. (1980) *Dev. Biol. 80*, 379–387.

Merril, C. R., Goldman, D., Sedman, S. A., & Eberi, M. H. (1981) *Science (Washington, D.C.) 211*, 1437–1438.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.

Ouchterlony, O. (1958) *Prog. Allergy 5*, 1–78.

Palfreyman, M. G., Danzin, C., Jung, M. J., Fozard, J. R., Wagner, J., Woodward, J. K., Aubry, M., Dage, R. C., & Koch-Weser, J. (1978) in *Enzyme-Activated Irreversible Inhibitors* (Seiler, N., Jung, M. J., & Koch-Weser, J., Eds.) pp 221–223, Elsevier/North-Holland, Amsterdam.

Pearse, A. G. E. (1969) *J. Histochem. Cytochem. 17*, 303–313.

Pegg, A. E., Seely, J. E., & Zagon, I. S. (1982) *Science (Washington, D.C.) 217*, 68–70.

Pritchard, M. L., Seely, J. E., Poso, H., Jefferson, L. S., & Pegg, A. E. (1981) *Biochem. Biophys. Res. Commun. 100*, 1597–1603.

Scheidegger, J. J. (1955) *Int. Arch. Allergy Appl. Immunol. 7*, 103–110.

Seely, J. E., Poso, H., & Pegg, A. E. (1982) *J. Biol. Chem. 257*, 7549–7553.

Sjoerdsma, A. (1981) *Clin. Pharmacol. Ther. 30*, 3–22.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Zarling, D. A., Watson, A., & Bach, F. H. (1980) *J. Immunol. 124*, 913–920.