yeast, and bovine liver argininosuccinate synthetases yield biphasic plots for ATP regardless of the concentrations of the other substrates. The negative homotropic interactions of argininosuccinate synthetase under conditions of limiting substrate may be valuable in maintaining a steady flow of nitrogen through the urea cycle. At the times when ATP may be limiting in vivo, the allosteric properties of the enzyme would provide an enhanced affinity for ATP in response to the limitation in energy supply.

We could detect no difference between the argininosuccinate synthetases purified from the normal and overproducer lymphoblast lines. Enzymes from the two cell lines had the same subunit molecular weight and tetrameric structure. The two enzymes copurified at each step with similar yields, and the final specific activities were very close. Furthermore, the kinetic parameters of the two enzymes were essentially identical. Finally, antibody produced in response to argininosuccinate synthetase from MGL8D1 reacted with enzyme from MGL8B2 with apparent identity. Thus, the argininosuccinate synthetase produced at high level by the canavanine-resistant line MGL8D1 appears identical in all respects with the enzyme produced by its arginine-repressible parent MGL8B2 as would be expected were MGL8D1 a constitutive mutant synthesizing argininosuccinate synthetase independent of arginine repression. Whether canavanine resistance is due to an increased number of argininosuccinate synthetase gene copies, as recently observed for dihydrofolate reductase in methotrexate-resistant cells (Alt et al., 1978), awaits further study.

Acknowledgments

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Inactivation of 3-(3,4-Dihydroxyphenyl)alanine Decarboxylase by 2-(Fluoromethyl)-3-(3,4-dihydroxyphenyl)alanine†

A. L. Maycock,* S. D. Aster, and A. A. Patchett

ABSTRACT: 2-(Fluoromethyl)-3-(3,4-dihydroxyphenyl)alanine [α-FM-Dopa (I)] causes rapid, time-dependent, stereospecific, and irreversible inhibition of hog kidney aromatic amino acid (Dopa) decarboxylase. The inactivation occurs with loss of both the carboxyl carbon and fluoride from I and results in the stoichimetric formation of a covalent enzyme–inhibitor adduct. The data are consistent with I being a suicide inactivator of the enzyme, and a plausible mechanism for the inactivation process is presented. The inactivation is highly efficient in that there is essentially no enzymatic turnover of I to produce the corresponding amine, 1-(fluoromethyl)-2-(3,4-dihydroxyphenyl)ethylamine [α-FM-dopamine (II)]. Amine II is also a potent inactivator of the enzyme. In vivo compound I is found to inactivate both brain and peripheral (liver) Dopa decarboxylase activity. The possible significance of these data with respect to the known antihypertensive effect of I is discussed.

It was recently reported by workers at Merck (Kollonitsch et al., 1978) and independently by others (Metcalf et al., 1978; Palfreyman et al., 1978) that a number of α-fluoromethyl amino acids are potent, time-dependent inhibitors of α-amino acid decarboxylases. The investigations by the Merck group were prompted by earlier observations that fluoroalanine is bactericidal (Kollonitsch et al., 1973) as a consequence of irreversible inhibition (inactivation) of the pyridoxal phosphate dependent enzyme alanine racemase (Kahan & Kropp, 1975). Although it was only recently verified (Wang & Walsh, 1978) that D-fluoroalanine is acting as a suicide inactivator (Morisaki & Bloch, 1972; Rando, 1974; Abeles & Maycock, 1976; Walsh, 1977; Rando, 1978) of the enzyme, that possibility was recognized earlier by one of us (A.A.P.) and by others (Kollonitsch, 1978; Bey et al., 1977). This inhibitor design principle was considered extendable to other pyridoxal phosphate dependent enzymes. As part of this development, α-fluoromethyl amino acids, that is, 2-substituted fluoroalanines,

† From Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065. *Received July 6, 1979; revised manuscript received November 15, 1979.*

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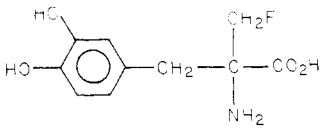
were synthesized and investigated as inhibitors of α-amino acid decarboxylases (Kollonitsch & Patchett, 1977). The biochemical importance of these enzymes in the biosynthesis of biogenic amines is well-known. Although the literature contained numerous reports of inhibitors for these enzymes (Abdel-Monem et al., 1975; Bartholini & Pletcher, 1975; Levine & Noll, 1969; Sourkes, 1966), there had been no report of suicide inactivators for them.

The α-fluoromethyl derivatives of α-amino acids were attractive as potential suicide inactivators of decarboxylases for a number of reasons (Kollonitsch et al., 1978). It was known that α-methyl-α-amino acids are substrates for these enzymes (O'Leary & Herreid, 1978; Yamada & O'Leary, 1978; Weissbach et al., 1960). Fluorine is so close to hydrogen in size that no steric problem was anticipated with respect to α-fluoromethyl amino acids compared to their α-methyl counterparts. Fluorine is a particularly attractive atom to utilize in the suicide inhibitor design because it is hard to displace in S_N2 reactions (Parker, 1963), but it can undergo β-elimination from carbanions quite well (Sakai & Santi, 1973; Stubbe & Abeles, 1977). Thus, α-fluoromethyl amino acids were anticipated to be nonreactive as alkylating agents until fluorine was expelled via enzyme-catalyzed decarboxylative elimination. Highly electrophilic species would result, which would be capable of undergoing addition of fortuitously positioned nucleophilic groups at or near the enzyme active sites to form stable covalent enzyme–inhibitor adducts, thereby inactivating the enzyme (see Scheme I). Bey (1978) has recently reviewed mechanistic principles by which decarboxylases function and has discussed the design of potential suicide inactivators of them.

Here we report a detailed study of the inactivation of one of the decarboxylases, aromatic-amino acid decarboxylase (also called Dopa decarboxylase, EC 4.1.1.28), by the corresponding α-fluoromethyl-substituted substrate analogue α-fluoromethyl-Dopa (α-FM-Dopa, I)[1] and by the corresponding product analogue α-fluoromethyldopamine (α-FM-dopamine, II).



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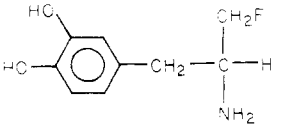


II

Experimental Procedures

Materials

Hog kidneys, obtained within minutes after the animals were killed, were stored at −20 °C until used. Male Wistar rats were maintained before and after treatment on an unrestricted diet.

All unlabeled inhibitors were obtained from Dr. J. Kollonitsch and co-workers of our laboratories. The synthesis of the following compounds has been published (Kollonitsch et al., 1978): (*RS*)-α-FM-Dopa, (*R*)- and (*S*)-α-FM-Dopa, (*RS*)-α-(fluoromethyl)histidine, (*RS*)-α-(fluoromethyl)-*p*-tyrosine, and (*R*)- and (*S*)-α-FM-dopamine. (*RS*)-α-Fluoro-*m*-tyrosine was prepared by a procedure similar to that used for the para compound.[2]

L-Dopa was obtained from Calbiochem and (*RS*)-[^{14}C]Dopa was from New England Nuclear. (*RS*)-[^{3}H]-α-FM-Dopa (specific activity 348 mCi/mmol) and (*R*)-[^{3}H]-α-FM-dopamine (specific activity 476 mCi/mmol) were obtained from Dr. A. Rosegay and M. Walsh of these laboratories, who prepared them by treating the unlabeled materials with 4 N ^{3}HCl at 85–95 °C for 6–8 h. (*RS*)-[^{14}C]-α-FM-Dopa (specific activity 4.95 mCi/mmol) was obtained from Dr. R. Ellsworth of these laboratories, who prepared it from (*RS*)-[^{14}C]Dopa by the pathway used for the preparation of unlabeled material.

(*S*)-α-Methyl-Dopa (ALDOMET) was obtained from Merck & Co. Other compounds and supplies were obtained from Sigma Chemical Co., Calbiochem, Pharmacia, or Amicon.

Methods

Assays and Analytical Procedures. Absorption spectra were obtained on a Beckman ACTA CIII spectrophotometer. Radioactivity was determined in a Packard Tri-Carb scintillation spectrometer, Model 3330, using 10–15 mL of Omnifluor–dioxane scintillator containing 0.5 mL of H_2O. Either [^{3}H]- or [^{14}C]toluene was used as an internal standard. Pyridoxal phosphate was determined by the fluorometric procedure of Adams (1969) using an Aminco-Bowman spectrophotofluorometer, and protein was estimated by the method of Lowry et al. (1951). Fluoride ion was determined with an Orion Model 96-09 combination fluoride electrode on a Beckman Expandomatic IV pH meter. Solutions were diluted with an equal portion of total ionic strength adjustor (Orion 94-09-09), and sodium fluoride (Orion 94-09-06) was used as the standard.

Enzyme activity was measured by monitoring $^{14}CO_2$ evolution from [^{14}C]-L-Dopa as described (Christensen et al., 1970) with the following minor changes in conditions: the incubation period was usually only 10 min and the final mixture was 2.8 mM in L-Dopa and contained 0.1–0.3 μCi of (*RS*)-[^{14}C]Dopa. A unit of enzyme catalyzes the production of 1 nmol of CO_2 per min under these conditions. Assays were usually performed with 2–25 units of enzyme. The assay reaction vessel has been described (Ellenbogen, 1969). K_m values were determined by the method of Lineweaver & Burk (1934), and the least-squares method was used to obtain the best straight line fit of the data. Enzyme was analyzed by electrophoresis on 7.5% polyacrylamide disc gels prepared by the method of Ornstein (1964) and stained with Coomassie Blue and on $NaDodSO_4$–10% polyacrylamide gels prepared by the method of Weber & Osborn (1969) and also stained with Coomassie Blue. Bovine serum albumin, ovalbumin, trypsin, and lysozyme were used as molecular weight standards. Buffer was sodium phosphate unless otherwise indicated.

Enzyme Purification. The procedure of Christensen et al. (1970) was followed. In cases where material was rechromatographed over DEAE-Sephadex A-50, the column (1.5 × 20 cm) was equilibrated with 50 mM buffer, pH 7.2, and 10 mM 2-mercaptoethanol. Protein (0.70–1.3 mg) was applied, and the column was developed with a linear gradient of 0–0.2 mM sodium chloride in 200-mL total volume of the same buffer.

Inhibition Experiments. Two types of inhibition experiments were performed. The first was carried out by running regular enzyme assays in the presence of inhibitor (added last). Compounds were tested at several concentrations from 0.001

[1] Abbreviations used: Dopa, 3-(3,4-dihydroxyphenyl)alanine; [^{14}C]-Dopa, 3-(3,4-dihydroxyphenyl)[1-^{14}C]alanine; α-FM-Dopa, 2-(fluoromethyl)-3-(3,4-dihydroxyphenyl)alanine; [^{3}H]-α-FM-Dopa, 2-(fluoromethyl)-3-(3,4-dihydroxyl[G-^{3}H]phenyl)alanine; [^{14}C]-α-FM-Dopa, 2-(fluoromethyl)-3-(3,4-dihydroxyphenyl)[1-^{14}C]alanine; α-FM-dopamine, 1-(fluoromethyl)-2-(3,4-dihydroxyphenyl)ethylamine; [^{3}H]-α-FM-dopamine, 1-(fluoromethyl)-2-(3,4-dihydroxy[G-^{3}H]phenyl)ethylamine; $NaDodSO_4$, sodium dodecyl sulfate.

[2] J. Kollonitsch and S. Marburg, unpublished results.

to 5 mM. The second, designed to determine by "preincubation" whether a compound would produce time-dependent inhibition, i.e., inactivation, was performed by treating enzyme with inhibitor at room temperature under conditions similar to normal assay conditions (50–100 mM buffer, pH 7.0, 10–20 mM 2-mercaptoethanol, and 0.07 mM pyridoxal phosphate) but in the absence of substrate (Dopa). At time intervals, aliquots of the preincubation solution were removed and diluted (usually 15–300-fold) into normal assay mixtures containing substrate (Dopa), and enzyme activity was determined in the usual fashion.

Inactivated enzyme was dialyzed at 4–6 °C vs. at least 2000 volumes of 10–100 mM buffer, pH 7.0, with 10 mM 2-mercaptoethanol to check for reversibility of inactivation. Periodically, samples were withdrawn and assayed for both enzyme activity and protein content.

The K_i for (*S*)-α-FM-Dopa was estimated by determining the initial rate of decarboxylation of 2.7 mM L-Dopa under normal assay conditions in the presence of varying amounts of (*S*)-α-FM-Dopa. This experiment was carried out by heating the solutions without enzyme at 37 °C for 3 min and then adding enzyme (27 units, specific activity 1620 units/mg) to initiate the reaction. After 1 min[3] the reaction was terminated and CO_2 was collected in the normal fashion. K_m for Dopa was determined concurrently. The data were treated according to the procedure of Dixon (1953) for a competitive inhibitor.

Covalent Labeling of Enzyme by Inactivators. Three samples of enzyme (each 2870 units, specific activity 11 200 units/mg) were preincubated (see above) in a total volume of 300 μL for 30 min at room temperature, one with 102 μM (10.3 μCi) (*RS*)-[^{3}H]-α-FM-Dopa under normal conditions, one with 102 μM (10.3 μCi) (*RS*)-[^{3}H]-α-FM-Dopa in the absence of exogenous pyridoxal phosphate, and one with 127 μM (18.8 μCi) (*R*)-[^{3}H]-α-FM-dopamine. A larger sample (7180 units) of the same enzyme was preincubated with 90 μM (0.52 μCi) (*RS*)-[^{14}C]-α-FM-Dopa. After inactivation, the protein was separated from excess inhibitor by gel filtration on a 0.9 × 20 cm Sephadex G-25 column using 10 mM buffer, pH 7.0, with 10 mM 2-mercaptoethanol as the elution buffer. The pooled protein fractions were assayed for enzyme activity, protein content, and radioactivity and then dialyzed for 40 h at 4 °C against at least 100 volumes of 10 mM buffer, pH 7.0, with 10 mM 2-mercaptoethanol. Protein content and radioactivity were then redetermined.

In a similar experiment designed to determine the extent of incorporation of inhibitor during the rapid phase of inactivation, enzyme (1520 units, specific activity 4610 units/mg) was preincubated in a total volume of 600 μL with 44 μM (8.2 μCi) (*RS*)-[^{3}H]-α-FM-Dopa. After 7 min 300 μL was removed, chilled on ice, assayed, and diluted with 500 μL of 10 mM buffer, pH 7.0, containing 10 mM 2-mercaptoethanol. The remaining preincubation mixture was allowed to stand for a total of 2.5 h (99% inactivation) before being treated in an identical fashion. Each sample was subjected to gel filtration and assayed as described above.

Enzyme (1340 units, specific activity 2890 units/mg) was preincubated with 21 μM (*S*)-α-FM-Dopa for 20 min (99% inactivation) in a total volume of 725 μL in order to determine whether enzyme inactivation was accompanied by fluoride loss from α-FM-Dopa. A sample (335 μL) was added to 165 μL of H_2O for determination of fluoride ion. The blank lacked enzyme. Concurrently, a sample of enzyme (1030 units, specific activity 2890 units/mg) was treated with 49 μM (8.2 μCi) (*RS*)-[^{3}H]-α-FM-Dopa for 15 min (97% inactivation) in a total volume of 530 μL. The material was subjected to gel filtration and assayed as described above.

α-FM-Dopa As a Substrate for Dopa Decarboxylase. α-FM-dopamine Production. Stored enzyme which had lost some activity (348 units, present specific activity 322 units/mg, original activity 2080 units/mg) was preincubated with 12 μM (5.5 μCi) (*RS*)-[^{3}H]-α-FM-Dopa for 120 min (79% inactivation). Unlabeled (*R*)-α-FM-dopamine hydrochloride (0.20 mg, 0.90 μmol) was added, the pH was adjusted to 3–4 with dilute HCl, and the solution was filtered (Amicon ultrafiltration apparatus with UM 10 membrane) to remove protein. The (*R*)-α-FM-dopamine was separated from the residual (*RS*)-α-FM-Dopa by ion-exchange chromatography (Bertler et al., 1958) over a 1 × 6 cm column of Dowex 50 × 4 (100–200 mesh), sodium cycle. The amino acid was eluted with H_2O (monitored by radioactivity in fractions) and the amine was eluted by subsequent elution with 2 N HCl (monitored by UV). The pooled amine fractions were evaporated to dryness, taken up in 1 mL of H_2O and assayed for α-FM-dopamine by UV [λ_{max} (1 N HCl) 280 nm (ϵ 2531)] and for radioactivity. The control experiment was performed identically but with no enzyme.

α-FM-Dopa As a Substrate for Dopa Decarboxylase. CO_2 Production. The following experiment was carried out in the apparatus used for normal enzyme assays. Samples of enzyme (820 units, specific activity 11 200 units/mg) were incubated with 6.9 μM (51 μCi) (*RS*)-[^{14}C]-α-FM-Dopa in 1.50 mL of 80 mM buffer, pH 7.0, with 10 mM 2-mercaptoethanol, 70 μM pyridoxal phosphate, and 200 nmol of $NaHCO_3$ for various lengths of time at room temperature before quenching with 35% trichloroacetic acid. The tubes were then placed in a 37 °C shaker bath and the CO_2 was collected for 30 min as in the normal assay procedure. Blanks lacked enzyme. Concurrently, a preincubation experiment was conducted with 820 units of enzyme and 6.7 μM (*RS*)-[^{14}C]-α-FM-Dopa. At time intervals 5-μL aliquots were withdrawn and diluted into assay mixtures containing Dopa to determine enzyme activity.

In Vivo Effects of (RS)-α-FM-Dopa on Dopa Decarboxylase. Male Wistar rats (135–185 g) were injected intraperitoneally with 0.5 mL of 4.9 mM (*RS*)-α-FM-Dopa in 0.9% saline. Control animals received 0.5 mL of saline. After 4 h the animals were decapitated, and the brains and/or livers were removed immediately. Brains (stored on dry ice for <1 h) were separately weighed, thawed, and homogenized in ~3 volumes of ice-cold 5 mM sodium phosphate, pH 7.0, with a motor-driven glass homogenizer. Livers (stored on ice for <15 min) were separately weighed and homogenized in 20–25 mL of ice-cold 5 mM sodium phosphate, pH 7.0, with a Virtis homogenizer. Aliquots (0.50 mL) of the homogenates were assayed for Dopa decarboxylase activity by the normal procedure.

Results

Purification and Properties of Dopa Decarboxylase. The purification proceeded generally as described (Christensen et al., 1970). The specific activity of purified enzyme varied from preparation to preparation, ranging from 4700 to 11 400 units/mg.

In some preparations two peaks of enzyme activity were recovered from chromatography over DEAE-Sephadex A-50 (Figure 1). The two peaks were indistinguishable with respect to their behavior with inhibitors [α-vinyl-Dopa and α-ethinyl-Dopa produced rapid, incomplete, and largely reversible inhibition (Maycock et al., 1978) whereas α-FM-Dopa pro-

[3] No inactivation was observed under these conditions during the 1-min incubation period.

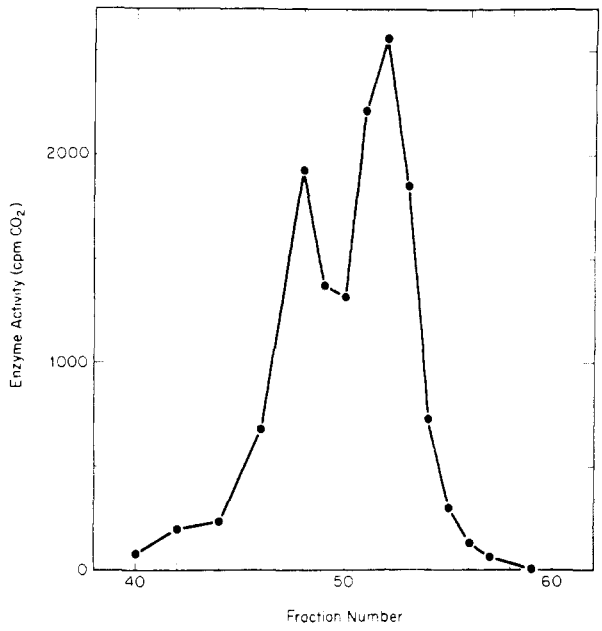


FIGURE 1: Dopa decarboxylase activity recovered from DEAE-Sephadex A-50 chromatography. No enzyme activity was found in earlier or later fractions. In this particular preparation, which had not been heat treated, fractions 47–53 were pooled and further purified, resulting in "purified" enzyme of specific activity 8900 units/mg.

duced rapid, biphasic, and irreversible inhibition], and they exhibited similar K_m values for L-Dopa. The two activities were never resolved during subsequent chromatography of the pooled fractions over hydroxylapatite. There was no apparent correlation between the final specific activity and the number of bands observed on DEAE-Sephadex A-50. The pyridoxal phosphate content of all preparations ranged from 7.7 to 10.2 nmol/mg of protein, which corresponds to 1 mol of coenzyme per 98 000–129 000 g of protein. All preparations gave the same pattern upon $NaDodSO_4$–polyacrylamide disc gel electrophoresis, a single band at 53 000–56 000 daltons. High specific activity purified enzyme always appeared as a single but rather broad band as previously described (Christensen et al., 1970), whereas the lower specific activity purified material usually appeared as two distinct but barely separated bands.

Behavior of Dopa Decarboxylase with α-FM-Dopa. When Dopa decarboxylase was treated with (*RS*)-α-FM-Dopa, a rapid, time-dependent loss of enzyme activity occurred (Figure 2A). If the preincubation period was extended, the loss of enzyme activity went to completion. At all stages of enzyme purification the time course of inactivation was biphasic, most of the activity being lost during the rapid phase. The slower, second phase of inactivation appeared to be approximately pseudo first order. Extrapolation of it back to zero time showed it to correspond to 5–15% of the total enzyme activity.

The inactivation was independent of whether pyridoxal phosphate was included in the reaction mixture. Likewise, the extent to which inactivated enzyme was diluted just prior to assay did not affect the degree of inactivation. These data suggest that inactivation is not caused by a depletion of pyridoxal phosphate or by accumulation of a competitive inhibitor in solution. Neither extensive dialysis nor gel filtration of inactivated enzyme restored activity. These results suggest that inactivation involves chemical modification of the enzyme and/or of the bound coenzyme. The rate of inactivation was retarded in the presence of the known (Lovenberg et al., 1963) competitive inhibitor (S)-α-methyl-Dopa (ALDOMET) (Figure 2B), an observation which suggests that the active site

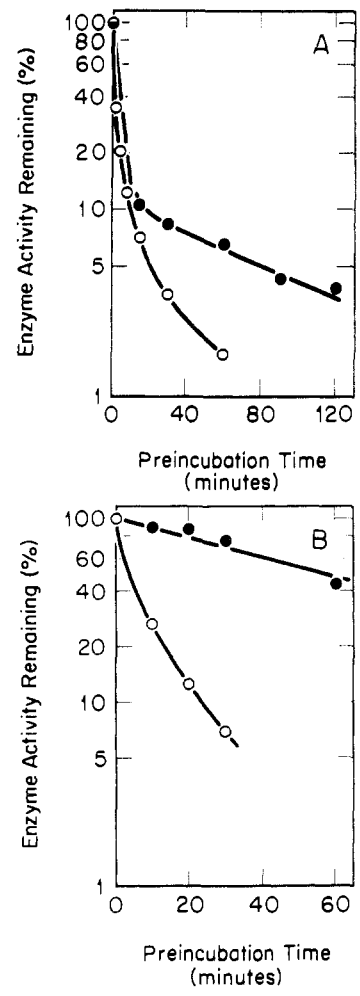


FIGURE 2: Time-dependent inhibition of Dopa decarboxylase caused by preincubating it with (*RS*)-α-FM-Dopa. Controls, which lacked inhibitor, lost <15% activity during the course of the experiments. (A) Different enzyme samples treated with 5 μM (*RS*)-α-FM-Dopa: fully purified enzyme, specific activity 11 200 units/mg (●); enzyme from ammonium sulfate fractionation, specific activity 110 units/mg (○). (B) Effect of preincubation of crude enzyme (specific activity 10 units/mg) with 1 μM (*RS*)-α-FM-Dopa in the presence (●) and absence (○) of 4 mM (*S*)-α-methyl-Dopa.

must be accessible for inactivation to occur.

When enzyme was treated with micromolar concentrations of the resolved isomers of α-FM-Dopa, only the *S* isomer was found to cause rapid inactivation. Slow inactivation was observed with (*R*)-α-FM-Dopa, but only at high (8 mM) concentration. This could have been due to contamination by a small amount of the enantiomer as less than 0.1% of it would account for the observed effect. By assuming that (*S*)-α-FM-Dopa acts as a competitive inhibitor of the decarboxylation of Dopa prior to its causing inactivation, it was possible to estimate the K_i for (*S*)-α-FM-Dopa as 4×10^{-8} M by treating the data of Figure 3 according to the method of Dixon (1953). Control experiments showed that during the course of obtaining the data in Figure 3, no significant inactivation of the enzyme occurred. The fact that α-FM-Dopa is decarboxylated by the enzyme (see below) shows that the compound binds at the active site. This lends credence to the assumption that the compound functions as a competitive inhibitor prior to causing inactivation.

Labeling of Dopa Decarboxylase with (RS)-[^{3}H]-α-FM-Dopa and (RS)-[^{14}C]-α-FM-Dopa. Fluoride Release. Several samples of enzyme were inactivated with (*RS*)-[^{3}H]-α-FM-Dopa, subjected to gel filtration, and analyzed for ^{3}H content.

Table I: Incorporation of Radioactive Inactivators into Dopa Decarboxylase

inactivator	special conditions[a]	sp act. of enzyme[b]	% inactivation after gel filtration	nmol of inhibitor bound per nmol of enzyme[c] before dialysis	nmol of inhibitor bound per nmol of enzyme[c] after dialysis	% inactivation after dialysis
[^{3}H]-α-FM-Dopa		11 200	93	1.15	0.99	95
[^{3}H]-α-FM-Dopa	no exogenous pyridoxal phosphate	11 200	94	1.19	1.1	96
[^{3}H]-α-FM-Dopa	17-min preincubn	4 600	92	*e*	0.41[d]	*e*
[^{3}H]-α-FM-Dopa	150-min preincubn	4 600	99	*e*	0.42[d]	*e*
[^{14}C]-α-FM-Dopa		11 200	96	0.024	0.02	*e*
[^{3}H]-α-FM-dopamine		11 200	88	1.01	0.92	88

[a] Normal conditions are described under Experimental Procedures. [b] Nanomoles of CO_2 evolved per minute per milligram of protein under the usual assay conditions. [c] Based on the enzyme having an M_r of 112 000 and all protein being active enzyme. [d] The enzyme used in this experiment had lost activity on storage so not all protein was active enzyme. [e] Not determined.

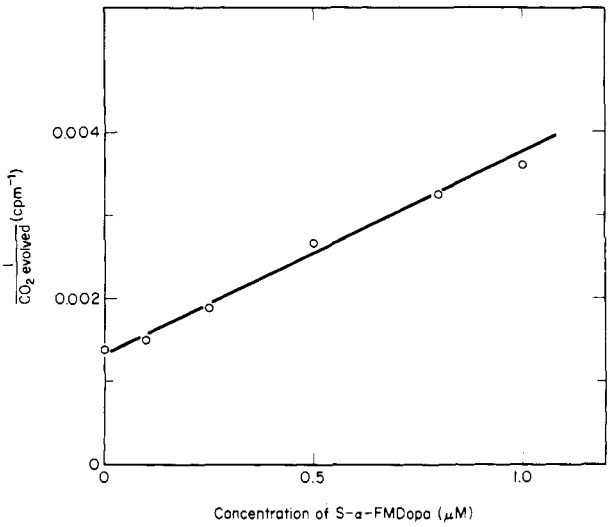


FIGURE 3: Estimation of K_i for (*S*)-α-FM-Dopa as a competitive inhibitor of Dopa decarboxylase (specific activity 1620 units/mg). CO_2 evolution from Dopa was determined as a function of the concentration of (*S*)-α-FM-Dopa. See Experimental Procedures for details. The K_m for Dopa, determined concurrently, was 1.9×10^{-4} M.

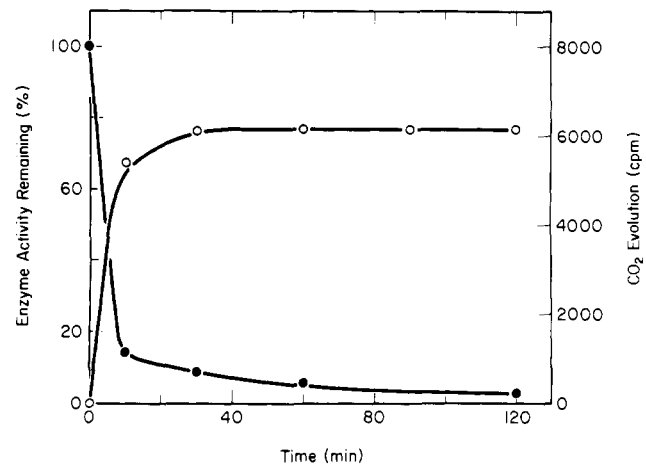


FIGURE 4: Incubation of Dopa decarboxylase (specific activity 9830 units/mg) with (*RS*)-[^{14}C]-α-FM-Dopa: inactivation (●) and $^{14}CO_2$ evolution (○).

The results of several such experiments are collected in Table I. Clearly, ^{3}H was associated with inactivated enzyme. A number of other features are noteworthy. Inactivated enzyme contained approximately stoichiometric amounts of ^{3}H. The same amount of ^{3}H was incorporated whether the inactivation was carried out in the presence or absence of exogenous pyridoxal phosphate. The ^{3}H incorporation observed during the rapid phase of inactivation (to 92% inactivation) was not increased significantly by allowing the inactivation to proceed further (to 99%). Most of the ^{3}H remained associated with the protein after prolonged dialysis. In sharp contrast to the labeling outcome with (*RS*)-[^{3}H]-α-FM-Dopa, when enzyme was inactivated with (*RS*)-[^{14}C]-α-FM-Dopa, essentially no radioactivity became associated with the enzyme. This result shows that the form of the inhibitor which remains bound to the enzyme has lost the carboxyl carbon.

The fluoride release experiment showed that per milligram of protein, 4.7 nmol of fluoride was released from (*S*)-α-FM-Dopa and 4.4 nmol of (*RS*)-[^{3}H]-α-FM-Dopa became irreversibly bound. The data demonstrate that approximately 1 mol of inhibitor becomes associated with protein for each mole of fluoride released.

(RS)-α-FM-Dopa As a Substrate for Dopa Decarboxylase. Using (*RS*)-[^{3}H]-α-FM-Dopa and α-FM-dopamine, we were able to show by reverse isotope dilution techniques that during the course of inhibition, no significant amount of α-FM-dopamine, the product expected from the normal decarboxylation of α-FM-Dopa, was released into solution. Since the recovered α-FM-dopamine from the experiment and from the control (no enzyme) had about the same (small) amount of radioactivity (6.4×10^4 and 7.7×10^4 dpm, respectively) associated with it, we can assume that little α-FM-dopamine was produced as a result of enzymatic activity.[4] A single turnover would have produced 23×10^4 dpm in the recovered α-FM-dopamine. Hence, each enzyme molecule turned over, on the average, far less than one molecule of α-FM-Dopa during the course of the experiment. By contrast, each enzyme molecule would have turnover over $\sim 10^5$ molecules of L-Dopa or ~2500 molecules of (*S*)-α-methyl-Dopa (O'Leary & Baughn, 1977) during the same period. Incubation of Dopa decarboxylase with (*RS*)-[^{14}C]-α-FM-Dopa did, however, produce a rapid evolution of $^{14}CO_2$ which ceased when inactivation was complete (Figure 4). The amount of CO_2 released corresponded to ~1.26 nmol/nmol of enzyme. In a similar experiment (data not shown) 1.0 to 1.1 equiv of CO_2 was released. These results are consistent with both the lack of ^{14}C incorporation into protein during inactivation and lack of normal turnover to produce free α-FM-dopamine.

Inactivation of Dopa Decarboxylase by α-FM-dopamine. When enzyme was treated with (*R*)-α-FM-dopamine, a rapid, time-dependent loss of enzyme activity was observed (Figure 5). The rate of inactivation was not pseudo first order; curves such as those seen in Figure 5 were always obtained. Dialysis or gel filtration did not restore enzyme activity. By contrast,

[4] The small amount of radioactivity recovered in the two samples may be due to the presence of an unknown contaminant.

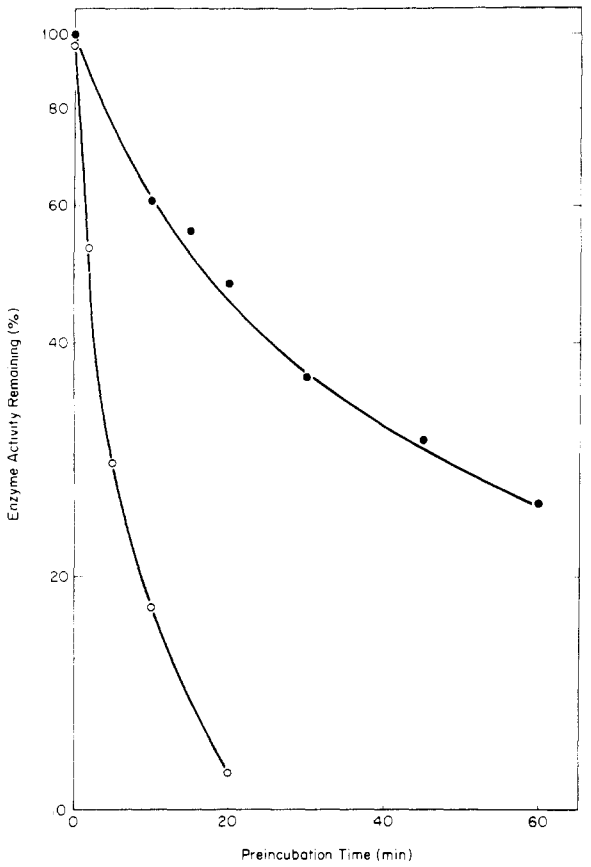


FIGURE 5: Time-dependent inhibition of Dopa decarboxylase (specific activity 4110 units/mg) caused by preincubating it with (*R*)-α-FM-dopamine at 5 μM (●) and at 50 μM (○).

the inhibition caused by the *S* isomer of α-FM-dopamine (30% at 0.4 mM in the presence of 2.8 mM L-Dopa) was abolished by dilution of a preincubation sample, demonstrating that with this isomer no inactivation occurred. The K_i for (*R*)-α-FM-dopamine was not determined, but at a given concentration the rate of inactivation was many fold slower than that caused by (*RS*)-α-FM-Dopa. When the inactivation was carried out with (*R*)-[^{3}H]-α-FM-dopamine, ~1 equiv of ^{3}H became associated with protein (Table I), demonstrating the stoichiometric formation of an enzyme–inhibitor adduct.

In Vivo Experiments with (RS)-α-FM-Dopa. There was a marked reduction in the levels of Dopa decarboxylase observed in homogenates of the brains and of the livers obtained from rats 4 h after treatment with a 4 mg/kg dose of (*RS*)-α-FM-Dopa (Table II).

Table II: Effect of (*RS*)-α-FM-Dopa on Dopa Decarboxylase Levels in Rats[a]

dose (mg/kg)	time (h)	enzyme act. (nmol of CO_2 per min per g of tissue) brain	liver
0	4	53 ± 2 (5)	616 ± 110 (2)
4	4	22 ± 6[b] (2)	35 ± 5[c] (2)

[a] Results are mean ± standard deviation of the mean. Confidence levels are based on the Student's *t* test. Values in parentheses are the number of animals used. [b] $p < 0.01$. [c] $p < 0.02$.

Discussion

Fully purified Dopa decarboxylase appeared as a single band (53 000–56 000 daltons) on NaDodSO$_4$ gels. Coupled with the observed pyridoxal phosphate content, these data are consistent with enzyme being a dimer of two 53 000–56 000-dalton subunits per molecule of coenzyme. These results agree favorably with the reported (Christensen et al., 1970) molecular weight of 112 000 obtained by sedimentation equilibrium but differ from earlier reports of three (Christensen et al., 1970) subunits (57 000, 40 000, and 21 000 daltons) and of two (Lancaster & Sourkes, 1972) subunits (48 000–50 000 and 40 000–44 000 daltons) observed on NaDodSO$_4$ gels. On native polyacrylamide gels purified enzyme usually displayed the previously described (Christensen et al., 1970) single broad band. Occassionally, however, two very closely spaced bands were obtained. In one such preparation "purified" enzyme was rechromatographed over DEAE-Sephadex A-50. Two peaks of activity were obtained and they corresponded to the two bands observed on gels.

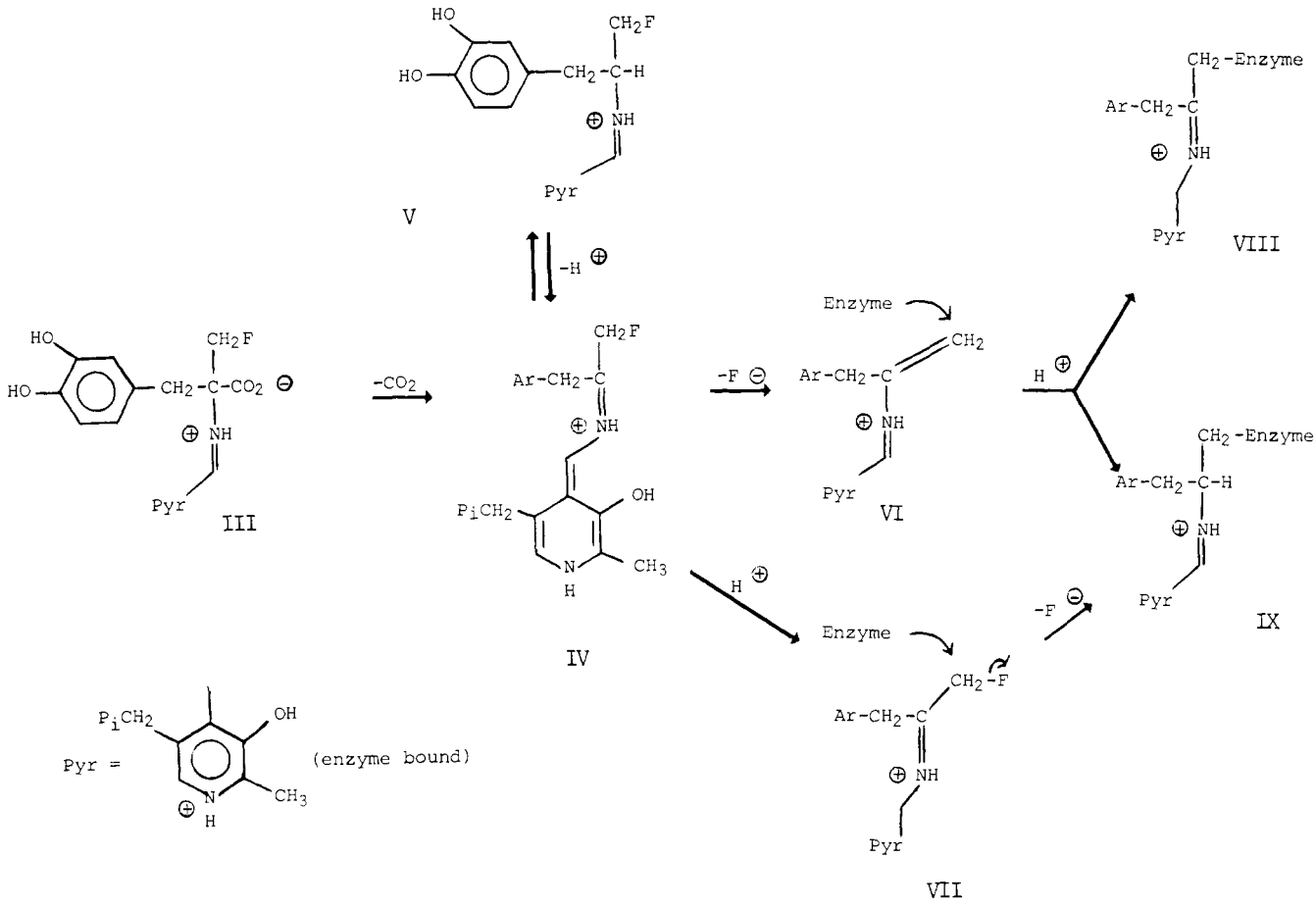
As mentioned earlier, we have occasionally observed two closely spaced peaks of enzyme activity during chromatography of partially purified Dopa decarboxylase over DEAE-Sephadex A-50. Although this appearance of separated activities may be an artifact of purification, it is interesting that there is a longstanding question of whether a given organism or organ contains multiple forms of Dopa decarboxylase with different substrate specificities (Srinivasan & Awapara, 1978; Bender & Coulson, 1977; Sims et al., 1973; Dairman et al., 1973). The two partially purified enzyme activities exhibit almost identical behavior with substrate (Dopa) and various inhibitors. Therefore, as we have been interested primarily in the chemical mechanism of inactivation by α-FM-Dopa and related compounds, we have not yet concerned ourselves with separating and purifying the two proteins.

The biphasic nature of the inhibition caused by (*RS*)-α-FM-Dopa (Figure 2), which has also been observed by other workers (Bey, 1978), is observed at all stages of purification. Although the ratio of the two phases varies somewhat from preparation to preparation, the rapid phase always represents 85–95% of total activity. The reason for this two-phase behavior has not been determined. Perhaps there are multiple enzyme forms which are inactivated at different rates.[5] Although we have no direct evidence to support this possibility, we do know that such forms could not correspond to the two activities sometimes observed during DEAE-Sephadex A-50 chromatography, because those bands behave in the same manner with α-FM-Dopa. Since the incorporation of inactivator is approximately stoichiometric with bound pyridoxal phosphate (Table I), the biphasic inactivation is not due to each enzyme molecule incorporating two molecules of inactivator, the first rapidly (resulting in the loss of 85–95% of the activity) and the second slowly (resulting in the loss of the remaining enzyme activity). The fact that the extent of incorporation is not affected by carrying out the labeling experiment in the absence of exogenous pyridoxal phosphate also eliminates the possibility that the enzyme contains catalytic sites which become functional (and therefore susceptible to being labeled) only in the presence of exogenous coenzyme.

Several recent reports discuss the concept of suicide enzyme inactivation and outline various criteria by which this type of enzyme inhibition can be identified (Seiler et al., 1978; Rando, 1978; Walsh, 1977; Abeles & Maycock, 1976). The central feature of this process is the enzymatic (catalytic) conversion of a relatively nonreactive compound into a reactive species which then interacts with the enzyme in such a manner as to cause irreversible inhibition (inactivation). We believe that our results demonstrate that α-FM-Dopa is a suicide inacti-

[5] Our preliminary results with α-vinyl- and α-ethinyl-Dopa as inhibitors of Dopa decarboxylase also suggest the possibility of multiple forms of the enzyme (Maycock et al., 1978).

Scheme I



vator of Dopa decarboxylase. The data presented earlier show that the inactivation is time dependent, stereospecific,[6] irreversible, independent of the presence of exogenous pyridoxal phosphate, and retarded in the presence of compounds known to bind at the active site. The active (*S*) isomer has the same absolute configuration as the isomers of Dopa (*S*) and α-methyl-Dopa (*S*) which are decarboxylated (Lovenberg et al., 1962). The apparent binding constant ($K_i = 4 \times 10^{-8}$ M) for (*S*)-α-FM-Dopa as a competitive inhibitor is several orders of magnitude lower than K_m for Dopa (2×10^{-4} M). Inactivator K_i's much lower than substrate K_m's have been reported for other suicide inactivators of pyridoxal phosphate dependent enzymes (Rando & Bangerter, 1976; Metcalf et al., 1978). Studies with isotopically labeled samples of α-FM-Dopa show that inactivation is accompanied by the incorporation into enzyme of approximately 1 equiv of tritium from [^{3}H]-α-FM-Dopa, the incorporation into enzyme of no ^{14}C from [^{14}C]-α-FM-Dopa, and the release of approximately 1 equiv of $^{14}CO_2$ from [^{14}C]-α-FM-Dopa. Furthermore, during inactivation approximately 1 equiv of fluoride is liberated. Neither fluoride nor CO_2 is lost from α-FM-Dopa under assay conditions in the absence of enzyme. These data demonstrate that inactivation results in stoichiometric formation of a stable enzyme–inhibitor adduct which contains neither the fluoride nor the carboxyl group of α-FM-Dopa. Since α-FM-Dopa is stable in the absence of enzyme, these transformations must result from interaction of inhibitor with the active site of the enzyme. These data can be accommodated by the mechanism of inactivation depicted in Scheme I.

In this mechanism we propose that α-FM-Dopa forms the normal (Boeker & Snell, 1972) Schiff base (III) with enzyme-bound pyridoxal phosphate and undergoes decarboxylation to give the expected (Boeker & Snell, 1972) quinoid intermediate (IV). In a normal substrate reaction, IV would be protonated to give V which would hydrolyze to afford the corresponding amine product. Our results show that α-FM-dopamine, the expected product from decarboxylation of α-FM-Dopa, is itself an inactivator of the enzyme but that the rate of inactivation is slower than that caused by α-FM-Dopa. Hence, we conclude that inactivation caused by α-FM-Dopa does not proceed through free α-FM-dopamine. This conclusion is supported by the fact that no free α-FM-dopamine can be detected after enzyme is inactivated by α-FM-Dopa.

Schiff base V, which could be generated from either α-FM-Dopa or α-FM-dopamine, is unlikely to be an inactivating species for the following reasons. Alkyl fluorides are very poor alkylating agents (Parker, 1963). α-FM-Dopa and α-FM-dopamine are both stable to loss of fluoride ion in the presence of 2-mercaptoethanol (pH 7.0; room temperature; many hours) and α-FM-Dopa is stable in 1 M sodium hydroxide (room temperature; 24 h). By itself, Schiff base formation with pyridoxal phosphate as in V is not expected to activate the fluoride toward displacement. Hence, V should not be a particularly reactive species. Lastly, if inactivation by α-FM-Dopa proceeded through V, the only way in which inactivation by α-FM-dopamine could be slower than that caused by α-FM-Dopa is if the formation of V from the amine were slow compared to the ensuing inactivation step(s).

[6] Although the *R* isomer does not appear to cause inactivation, it is likely that is has some affinity for the enzyme because the rate of inactivation with the racemic mixture is slower than would be predicted from the rate with the *S* isomer. This observation is consistent with the recent reports that [*R*(D)]-Dopa is an inhibitor of Dopa decarboxylase (Shikimi & Inagaki, 1978; Inagaki & Tanaka, 1978).

Rather than undergoing protonation to form V, intermediate IV could expel fluoride to give intermediate VI or become protonated to give the α-imino fluoride VII. Alternatively, if expulsion of fluoride ion were concerted with loss of CO_2, VI would be produced directly. Intermediate VII corresponds to the "abortive transamination" process which is known to compete with normal protonation, infrequently with normal substrate (Dopa), but more often with α-substituted analogues (O'Leary & Baughn, 1977). Although we have no direct evidence that VII is not formed, we favor VI as the reactive intermediate for the following reasons. (1) Model studies with α-(difluoromethyl)phenylalanine in the presence of *catalytic* amounts of pyridoxal phosphate (Bey, 1978) demonstrate that decarboxylative elimination from α-halomethyl amino acids can be a facile process. (2) Even with α-methyl-Dopa as the substrate, the abortive transamination pathway occurs infrequently compared to the normal pathway (O'Leary & Baughn, 1977). On the basis of steric and inductive effects of fluoromethyl vs. methyl, one would expect α-FM-Dopa to have no greater tendency than α-methyl-Dopa to undergo the abortive transamination reaction. (3) It has recently been demonstrated (Wang & Walsh, 1978) that the inactivation of alanine racemase by a series of α-substituted alanine derivatives, including fluoroalanine, proceeds through an aminoacrylate intermediate similar to VI.

Structure VI is unlikely as the final enzyme–inactivator adduct because inactivator would eventually dissociate from the enzyme upon dialysis, resulting in the loss of the ^3H associated with the protein. Structure VI would be highly electrophilic and could capture an active-site nucleophile to give VIII or IX, either of which could be a stable enzyme–inactivator adduct. We have not determined whether VIII or IX (or the corresponding hydrolysis products) better represents the enzyme–inhibitor adduct.

As shown earlier, α-FM-dopamine is also a potent, time-dependent, irreversible, and stereospecific inhibitor of the enzyme and also leads to formation of a stoichiometric enzyme–inhibitor adduct. Again, the active (*R*) isomer of the amine has the expected absolute configuration based on the stereochemical course of the decarboxylation of α-methyl-Dopa, which gives (*S*)-α-methyldopamine[7] (Ames et al., 1977; Marshall & Costagnoli, 1973). The inactivation is much slower than that caused by an equivalent concentration of α-FM-Dopa, and the loss of enzyme activity is found to be distinctly non-first-order (Figure 5). The reason for this non-first-order behavior has not been investigated. We suggest that inactivation of the enzyme by α-FM-dopamine occurs by formation of V (Scheme I), followed by enzyme-catalyzed proton removal to produce IV (or VI if fluoride ion loss is concerted), which is an intermediate common to inactivation by both α-FM-Dopa and α-FM-dopamine. This scheme seems reasonable since, by the principle of microscopic reversibility, the enzyme must be capable of catalyzing the removal of an α proton from any amine which can be produced by enzyme-catalyzed decarboxylation of the corresponding amino acid. Although attempts to demonstrate this proton-abstracting capability of decarboxylases have been unsuccessful (Yamada & O'Leary, 1977), there are recent reports of enzyme inactivators which function on this basis. The inactivation of ornithine decarboxylase by α-ethinylputrescine (Metcalf et al., 1978) and of glutamate decarboxylase by γ-ethinyl-γ-aminobutyric acid (Jung et al., 1978) proceeds by way of enzyme-catalyzed removal of an α proton from the inactivators.

Scheme I is similar to the mechanisms proposed for the inactivation of several other pyridoxal phosphate enzymes by α-substituted substrate analogues (Seiler et al., 1978). To our knowledge the identity of the key intermediate has been firmly established in only one case. Inactivation of alanine racemase proceeds by way of an aminoacrylate (analogous to VI in Scheme I) rather than a β-substituted imino compound (analogous to VII in Scheme I) (Wang & Walsh, 1978). The formation of the stable enzyme–inhibitor adduct from alanine racemase leaves the coenzyme in the oxidation state of pyridoxamine phosphate, which corresponds to structure VIII in Scheme I.

In assessing the effectiveness of a suicide enzyme inactivator, particularly for possible in vivo utility, one needs to consider the compound's specificity, affinity (noncovalent) for the target enzyme, rate of inactivation, and inactivation efficiency (partitioning ratio). Although the specificity of α-FM-Dopa has not been widely studied, it has been shown to have no inhibitory effect on mammalian histidine decarboxylase in vitro (Kollonitsch et al., 1978). As shown above, (*S*)-α-FM-Dopa has high affinity for Dopa decarboxylase ($K_i = 4 \times 10^{-8}$ M vs. K_m for Dopa of 2×10^{-4} M) and produces inactivation of most of the enzyme activity within a few minutes. The partitioning ratio (Walsh et al., 1978) for a suicide inactivation process is the average number of times the enzyme turns over the inactivator as a substrate before an inactivating event occurs. The lower the number, the more efficient the inactivator. One potential problem with inefficient inactivators (high partitioning ratios) is the release of what could be highly reactive species from the enzyme into the surrounding medium. For an example, see Kaczorowski et al. (1975). Reported values for pyridoxal phosphate dependent enzymes range from ca. zero (every catalytic event leads to inactivation) to several thousand (Walsh et al., 1978). The partitioning ratio during Dopa decarboxylase inhibition by α-FM-Dopa is <0.05 based on α-FM-dopamine and <0.26 based on CO_2. Hence, in addition to having a high affinity for the enzyme and causing rapid inactivation, α-FM-Dopa is essentially 100% efficient.

The in vivo experiments with rats show that an intraperitoneal dose of 4 mg/kg of (*RS*)-α-FM-Dopa is capable of producing inhibition of both central (brain) and peripheral (liver) Dopa decarboxylase activities. Other workers (Ulm et al., 1979; Jung et al., 1979) have shown a marked reduction of catecholamine levels occurs after rats are treated with α-FM-Dopa. In our experiment the inhibition of the liver enzyme was nearly complete. The inhibition of the brain enzyme was not complete, however, probably because the dose and/or time were insufficient. A preliminary experiment carried out for 5 h with a larger dose (17 mg/kg) of (*RS*)-α-FM-Dopa gave brain homogenates which showed no enzyme activity and which contained material capable of inactivating exogenously added enzyme. Both of these experiments show that α-FM-Dopa (or, less likely, a metabolite) is capable of crossing the so-called "blood-brain barrier". These observations, combined with the fact that in vitro we can detect no turnover of α-FM-Dopa to produce α-FM-dopamine, may be of particular significance in view of the fact that in experimental animals α-FM-Dopa has been shown to be antihypertensive (Ulm et al., 1979). A closely related compound, (*S*)-α-methyl-Dopa (ALDOMET), a widely used antihypertensive agent, is believed to function through conversion in the brain, by decarboxylation (to α-methyldopamine), followed

[7] The assignments here may appear confusing. (*R*)-α-FM-dopamine has the same absolute configuration as (*S*)-α-methyldopamine, and (*S*)-α-FM-Dopa has the same absolute configuration as (*S*)-α-methyl-Dopa.

by hydroxylation to the active compound α-methylnorepinephrine (Nickerson & Ruedy, 1975; Day et al., 1973). Although the mechanism of antihypertensive action of α-FM-Dopa has not yet been established, one might expect that it would act analogously, by conversion to α-(fluoromethyl)-norepinephrine. Our results, however, suggest that such a conversion is unlikely because α-FM-Dopa will inactivate the first required enzyme (Dopa decarboxylase) and no free α-FM-dopamine will be produced. Thus, the mechanisms by which (*S*)-α-methyl-Dopa (ALDOMET) and α-FM-Dopa produce hypertensive effects may be fundamentally different.

Although *m*-tyrosine is a good substrate for Dopa decarboxylase (O'Leary & Baughn, 1977), both *p*-tyrosine and histidine are reported to be very poor substrates (Christensen et al., 1970). We find that the inactivation characteristics of the α-fluoromethyl derivatives of these three compounds generally follow the substrate reactivity: α-(fluoromethyl)-*m*-tyrosine produces rapid inactivation, α-(fluoromethyl)-*p*-tyrosine produces very slow inactivation, and α-(fluoromethyl)histidine is noninhibitory.[8]

Previous reports by us (Kollonitsch et al., 1978) and by others (Metcalf et al., 1978; Palfreyman et al., 1978; Bey, 1978) have shown that many α-fluoromethyl analogues of amino acids are inactivators of the respective decarboxylases. Although the present work is the only case to date in which the enzyme-catalyzed chemical transformation of inactivator has been identified, it seems reasonable that mechanisms analogous to Scheme I also account for the inactivation of other decarboxylases caused by α-fluoromethyl amino acids.

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Comparison of the Zinc Binding Domains in the 7S Nerve Growth Factor and the Zinc–Insulin Hexamer†

Michael F. Dunn,* Scott E. Pattison,‡ Michael C. Storm, and Edward Quiel§

ABSTRACT: The chromophoric divalent metal ion chelator 2,2′,2″-terpyridine (Terpy) is used as a kinetic and spectroscopic probe to investigate the zinc binding domains in the mouse 7S nerve growth factor protein (7S NGF_n) and the zinc–insulin hexamer $(In)_6(Zn^{2+})_2$. The stopped-flow rapid-mixing kinetic time courses for the sequestering and removal of zinc ion from both of these zinc metalloprotein hormones are remarkably biphasic. The fast phase of each reaction is second order overall: first order in Terpy and first order in protein-bound zinc ion. Although the spectral changes associated with the slow phase correspond to the uptake of 1 mol of Terpy per g-atom of zinc ion, the slow phase is a first-order process (zero order in Terpy). The concentration dependencies and spectral changes are consistent with a common reaction mechanism for both proteins; the fast phase involves the formation of a mono(Terpy)–Zn(II)–protein complex at each zinc site, and the slow phase involves the rate-limiting dissociation of the Terpy-bound zinc ions from the protein, followed by the rapid coordination of a second Terpy molecule and formation of the bis(Terpy)–Zn(II) complex. In contrast, the reactions of Terpy with carboxypeptidases A and B, carbonic anhydrase, thermolysin, and horse liver alcohol dehydrogenase all were very slow ($t_{1/2}$ of hours to days). In each case, the time course was described by a single exponential. The high-resolution X-ray structure of $(In)_6(Zn^{2+})_2$ [Blundell, T., Dodson, G., Hodgkin, D., & Mercola, D. (1972) *Adv. Protein Chem. 26*, 279] provides a satisfying structural rationale for the biphasic time course. Each of the two zinc ions is hexacoordinate. Three of the ligands are water molecules; the remaining three are imidazolyl moieties of His-B10. Based on this structural information, we conclude that the rapid phase of the reaction involves displacement of the three water molecules by the tridentate Terpy. The striking similarities between the reactions of 7S NGF_n and $(In)_6(Zn^{2+})_2$ with Terpy suggest the existence of structural similarity in the zinc binding domains of 7S NGF_n and $(In)_6(Zn^{2+})_2$.

The distantly related hormones nerve growth factor (NGF)[1] and insulin (In) both exist in dissociative equilibrium with free protomers under physiological conditions (Server & Shooter, 1977; Blundell et al., 1972). Zinc ion strongly shifts the equilibrium for each system toward the oligomeric state (Pattison & Dunn, 1975, 1976a,b; Au & Dunn, 1977; Grant et al., 1971; Goldman & Carpenter, 1974; Jeffrey, 1974; Blundell et al., 1972).

The native NGF oligomer (a 140000 M_r species, designated 7S NGF_n) appears to be a hexamer made up of three different classes of subunits (designated α, β, and γ) and contains two zinc ions. The γ subunit is a trypsin-like protease, and the β subunit contains the growth promoting activity, while the α subunit has no well-defined biological activity. The proteolytic activity of the γ subunit is completely inhibited in the 7S NGF_n species. Removal of zinc ion from the oligomer brings about full activation of the γ-subunit proteolytic activity (Pattison & Dunn, 1975, 1976a,b; Au & Dunn, 1977). The β subunit consists of two identical polypeptide chains made up of 118 amino acids each. The sequence of the β chain has been found to exhibit approximately 20–25% sequence homology with human proinsulin (Frazier et al., 1972; Angeletti & Bradshaw, 1971). Zinc ion binds to the 7S NGF oligomer with an apparent dissociation constant of 10^{-10}–10^{-11} M (Pattison & Dunn, 1976b).

Insulin in the presence of limited amounts of zinc ion forms a hexamer (constructed as a trimer of dimers) containing two zinc ions, hereafter designated as $(In)_6(Zn^{2+})_2$ (Blundell et al., 1972). In the presence of zinc ion, proinsulin also forms a hexamer containing 2 g-atoms of zinc ion (Frank & Veros, 1970; Pekar & Frank, 1972). The X-ray structure of $(In)_6(Zn^{2+})_2$ shows the two zinc ions are related by three twofold symmetry axes, and they are located on a threefold symmetry axis through the molecule. The two zinc ions are separated by an internal, water-filled cavity which forms a core about which the protomers are situated. Each of the two zinc ions is hexacoordinate (Blundell et al., 1972). The six inner sphere ligands are positioned in an octahedral array. Three of the

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‡ Present address: Department of Chemistry, California Polytechnic Institute, Pomona, CA 91768.
§ Present address: School of Medicine, University of California, San Diego, La Jolla, CA 92093.

[1] Abbreviations used: 7S NGF_n and 7S NGF_a, the native and chelator-activated mouse submaxillary nerve growth factor proteins; In and $(In)_6(Zn^{2+})_2$, the insulin monomer and hexamer, respectively; Terpy, 2,2′,2″-terpyridine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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