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ELEVATED AROMATIC-L-AMINO ACID DECARBOXYLASE IN HUMAN CARCINOID TUMORS

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Abstract—The carcinoid neoplasm is marked by excessive serotonin, synthesized by the conversion of tryptophan (Trp) to 5-hydroxytryptophan by tryptophan hydroxylase (TPH) (EC 1.14.16.4) and decarboxylation of 5-hydroxytryptophan by aromatic-L-amino acid decarboxylase (AAAD) (EC 4.1.1.28). Because almost no biochemical data were available on human carcinoid TPH and AAAD, we have characterized these enzymes as a preliminary step to developing mechanism-based agents selective against carcinoid tumors. TPH was detected in all fourteen carcinoids analyzed [K_m = 185 ± 17 μM (mean ± SEM); V_{max} = 2.4 ± 1.2 nmol/hr/mg protein]. AAAD was detected in thirteen tumors (K_m = 45 ± 6.7 μM; V_{max} = 11 ± 2.0 nmol/min/mg protein). In a subset of hepatic metastatic tumors obtained with adjacent normal liver, the K_m and V_{max} of TPH (N = 6) and the K_m of AAAD (N = 7) were comparable in both tissues. However, the V_{max} of carcinoid AAAD was 50-fold higher ($P < 0.002$) than that in normal liver (13 ± 3.1 vs 0.26 ± 0.04 nmol/min/mg protein). Western immunoblot analysis indicated that AAAD polypeptide content of carcinoid tumor was >20-fold higher than in adjacent normal liver. These results suggest that AAAD might be an appropriate target for enzyme-activated cytotoxic agents for carcinoid tumors.

Key words: carcinoid; hydroxylase; decarboxylase; kinetics; tumor

When carcinoid tumors arise in the small intestine, the indolent nature of the primary lesion causes most patients to present with unresectable hepatic metastases. These patients often exhibit the "malignant carcinoid syndrome," the occurrence and severity of which are related to tumor bulk draining into the systemic circulation. Flushing is a hallmark symptom, diarrhea is frequent, while carcinoid heart disease is a late complication in patients with high circulating 5-HT† levels for several years. Because the average duration of small intestinal carcinoid disease is over 9 years, with a range to 41 years [1], many patients are alive with advanced disease for which there is little, if any, effective treatment.

A primary characteristic of the carcinoid tumor is production and release of large amounts of 5-HT. Following active uptake, Trp is converted to 5-OH-Trp by TPH (EC 1.14.16.4) and 5-OH-Trp is decarboxylated by AAAD (EC 4.1.1.28). In normal individuals, about 1% of dietary Trp is metabolized to 5-HT. In patients with carcinoid syndrome, up to 60% is converted by excessive 5-HT synthesis in the tumor [2]. The highly active serotonergic biosynthetic pathway in carcinoid tumors is an attractive target for mechanism-based agents directed against these tumors. However, almost no data were available on the characteristics of carcinoid TPH and AAAD. Hosada *et al.* [3] published an apparent K_m of 13 μM (no V_{max}) for TPH partially purified from one hepatic carcinoid metastasis after storage for 4 years. Lovenberg *et al.* [4] described TPH activity (no K_m or V_{max}) in one hepatic carcinoid metastasis. Giarman *et al.* [5] reported that AAAD activity in one appendiceal carcinoid tumor was greater than that in normal appendix (no K_m or V_{max}). We therefore characterized TPH and AAAD, and determined the variation of these enzymes in individual carcinoid tumors and, whenever possible, in adjacent normal liver. Western immunoblotting was employed to quantitate AAAD, which had elevated activity in carcinoid tumors.

MATERIALS AND METHODS

Materials

Dithiothreitol, 5-HT, L-5-OH-Trp, and Tween-20 were purchased from the Sigma Chemical Co. (St. Louis, MO). *N*-Methyl-*N*-propargylbenzylamine HCl, ferrous ammonium sulfate, and L-Trp were obtained from the Aldrich Chemical Co. (Milwaukee, WI), and pyridoxal-5-phosphate and 2-amino-4-hydroxy-6-methyltetrahydropteridine HCl were from the Calbiochem Corp. (San Diego, CA). Catalase was obtained from Boehringer Mannheim (Indianapolis, IN), and HPLC grade methanol was purchased from EM Industries (Gibbstown, NJ). Electrophoretic grade Tris, glycine, SDS, bromophenol blue, and *N,N,N′,N′*-tetramethylethylenediamine were from Amresco (Solon, OH); and Fast Green, ammonium persulfate, preweighed acrylamide/bis 37.5:1, and 2-mercaptoethanol were supplied by Bio-Rad (Hercules, CA). New England Nuclear Research Products (Boston, MA) supplied L-[side-chain-3-^{14}C]Trp. All other chemicals used were reagent grade.

Crude enzyme preparations from control tissues

Crude AAAD was prepared by the method of Voltattorni *et al.* [6]. Livers from 4-5 CD2F1 mice (National

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† Abbreviations: 5-HT, 5-hydroxytryptamine or serotonin; Trp, tryptophan; 5-OH-Trp, 5-hydroxytryptophan; TPH, tryptophan hydroxylase; AAAD, aromatic-L-amino acid decarboxylase; carbidopa, α-methyldopahydrazine; and TBS, 10 mM Tris, 150 mM NaCl, pH 7.4.

Cancer Institute, Bethesda, MD) were removed following cervical dislocation, rinsed in ice-cold 0.1 M potassium phosphate (pH 7.5), pooled, and minced in 2 vol. of buffer. The preparation was homogenized with ten strokes in a pre-chilled, power-assisted, teflon pestle tissue grinder (Thomas, Swedesboro, NJ) and centrifuged (model J2-21, Beckman, Palo Alto, CA) at 10,000 *g* for 30 min at 4°. The supernatant was made 0.1 mM in dithiothreitol before division into aliquots and storage at −70°.

P815 murine mastocytoma cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) with 10% fetal bovine serum (Gibco BRL). Cells were incubated at 37° in a humidified environment of 95%:5% air:CO_2 (Wedco, St. Augustine, FL). Crude TPH was prepared at 4° on the day of use by washing 2 × 10^8 cells twice with Locke's solution with 5% glucose, adding a volume of 0.3 M Tris-acetate (pH 7.6), and probe-sonicating (Braun-sonic 1510, B. Braun, Melsungen, AG) at 100 W for 1.5 min.

Homogenates of human tissues

Sections of human carcinoid tumors (one small bowel primary, one spleen metastasis, twelve liver metastases) and, in some cases, adjacent normal tissue were snap-frozen following excision and stored at −70° for 2–14 months. Homogenates were prepared at 4° by mincing frozen tissue, adding a volume of 0.3 M Tris-acetate, 5 mM dithiothreitol (pH 7.6), and homogenizing. Following centrifugation (Beckman TL-100 Ultracentrifuge) at 100,000 *g* for 1 hr, the supernatant was divided into aliquots and stored at −70°. Aliquots were assayed directly for AAAD or, for some carcinoids, concentrated by centrifugation (Centricon-30; Amicon, Beverly, MA) for 1 hr at 3000 *g* before use in the TPH assay.

HPLC analysis

Simultaneous separation of Trp, 5-OH-Trp, and 5-HT was afforded by modifying the method of Yamaguchi *et al.* [7]. Analyses were performed with a Shimadzu system (Kyoto, Japan) consisting of an LC-600 pump, an SIL-9A Auto Injector, and a C-R4AX Chromatopac data processor. The analytical column was a 5 μm octadecyl column (0.45 × 25 cm, I.I.I. Supplies Co., Meriden, CT; or 0.46 × 25 cm, Jones Chromatography, Lakewood, CO) with a guard column of 7 μm Newguard RP-18 (0.32 × 1.5 cm; Brownlee Labs, Santa Clara, CA). The mobile phase was 20% methanol, 10 mM potassium phosphate (pH 5.4) at a flow rate of 1 mL/min (retention times of 5-OH-Trp and 5-HT were 4.4 and 7 min, respectively); 10% methanol was employed when HPLC fractions were collected (retention times of 5-OH-Trp and Trp were 7 and 13 min, respectively), and 20 mM potassium phosphate was used with the Jones column for AAAD assay analyses.

TPH assay

Assay conditions were modified from those of Fujisawa and Nakata [8]. Incubation mixtures contained 0.29 to 0.42 mg protein of crude P815 preparation, 0.37 to 2.1 mg protein of human carcinoid homogenate, or 0.61 to 3.7 mg protein of normal human liver homogenate, and final concentrations of 60 mM Tris-acetate, pH 7.6; 2 mg/mL catalase; 25 mM dithiothreitol; 0.1 mM ferrous ammonium sulfate; 0.2 mM 2-amino-4-hydroxy-6-methyltetrahydropteridine HCl; and 0.05 to 1.00 mM Trp, in a final volume of 100 μL. Reactions at 37° were started by addition of substrate following 10 min of preincubation and were terminated after 5 min by addition of 10 μL of 60% perchloric acid. Following incubation on ice for 5 min, protein was removed by centrifugation (Microfuge 12, Beckman) at 10,000 *g* for 5 min. Supernatants were analyzed by HPLC with fluorescence detection (Fluorichrom, Varian Associates, Inc., Palo Alto, CA; excitation 280 nm, emission 300–400 nm). With non-radioactive Trp as substrate, concentrations of product were determined from a 5-OH-Trp standard curve.

For assaying homogenates of human tissues, 50 μM carbidopa (a gift from Merck Sharp & Dohme, Rahway, NJ) was included as an AAAD inhibitor; TPH activity is not affected by a concentration of carbidopa as high as 0.1 mM [9]. L-[side-chain-3-^{14}C]Trp was employed as substrate, and incubation length was 2.25 hr. One-minute HPLC fractions were collected (2070 Ultrorac II, LKB, Bromma, Sweden) and subjected to scintillation counting (Beckman LS-5801) after addition of 6 mL of scintillation fluid (Ultima Gold, Packard Instrument Co., Meriden, CT). Due to the limited quantities of human tissues available and the relatively large amount of protein required, TPH assays were performed in duplicate rather than in triplicate.

AAAD assay

Assay conditions were modified from those of Sourkes [10]. Incubation mixtures contained 0.07 to 0.11 mg protein of crude murine liver AAAD, 0.007 to 0.32 mg protein of human carcinoid homogenate, or 0.49 to 0.63 mg protein of normal human liver homogenate, and final concentrations of 60 mM Tris-acetate, pH 7.6; 0.1 mM pyridoxal-5-phosphate; 1 mM *N*-methyl-*N*-propargylbenzylamine HCl to inhibit any residual monoamine oxidase A and B; and 0.015 to 0.40 mM 5-OH-Trp, in a final volume of 100 μL. Reactions at 37° were started by addition of substrate following 5 min of preincubation and were terminated after 10 min by addition of 10 μL of 60% perchloric acid. Following incubation on ice for 5 min, protein was removed by centrifugation at 10,000 *g* for 5 min. Supernatants were analyzed by HPLC with fluorescence detection, and concentrations of product were determined from 5-HT standard curves.

Western immunoblot analysis of AAAD

Homogenates of human carcinoid tumor and adjacent normal liver (2.5 to 75 μg protein; five concentrations of each sample per gel) were subjected to Laemmli SDS–PAGE (10% polyacrylamide separating gels; Protean II xi Slab Cell; Bio-Rad) [11] followed by electrophoretic transfer (Trans-Blot Electrophoretic Transfer Cell, Bio-Rad) to Hybond-ECL nitrocellulose membranes (Amersham, Arlington Heights, IL) by the method of Otter *et al.* [12]. Membranes were stained (5 min) with 0.1% Fast Green in ethanol:glacial acetic acid:H_2O (25:10:65) and destained in ethanol:glacial acetic acid:H_2O (25:10:65 and 10:10:80; 35 min, total). After equilibration in TBS, membranes were incubated at 24° for 1 hr in TBS containing 10% dry milk and then with rabbit anti-AAAD antibody (diluted 1:2000 in TBS with 3% dry milk; Eugene Tech International, Inc., Ridgefield Park, NJ). Following three washes in TBS with 0.05% Tween-20, blots were reacted with affinity-purified, goat anti-rabbit antibody conjugated to horseradish peroxidase (diluted 1:

5000 in TBS with 3% dry milk; Pierce, Rockford, IL). Antibody complexes were detected using the enhanced chemiluminescence (ECL) kit and Hyperfilm-ECL (Amersham) and quantitated on a Shimadzu densitometer (CS9000U). All readings were zeroed against background and expressed as area. Dilution of the tumor homogenate prior to SDS–PAGE was required to simulate the AAAD protein quantities detected in the normal liver.

Protein assay

Protein was quantitated with the BCA protein assay (Pierce).

RESULTS

To study TPH and AAAD in human carcinoid tumors and adjacent normal tissues, HPLC assays were established to quantitate products. Tissues rich in these enzymes (P815 murine mastocytoma cells and CD2F1 murine liver, respectively) were employed for assay development. Time course and cofactor dependency studies for both enzymes confirmed the appropriateness of the assay conditions for human tissue homogenates (data not shown).

TPH kinetics

Cultured P815 murine mastocytoma cells served as the control source of TPH. The K_m for P815 TPH (135 ± 1 μM; N = 3) in our assay was 3- to 4-fold higher than published K_m values (31–45 μM) [13–15]. Our P815 cells were cultured *in vitro* rather than being cultured intraperitoneally in mice and harvested from ascitic fluid before use. The one reported V_{max} value [14] for P815 TPH, 2.81 nmol/mg/min, was similar to our value (1.5 ± 0.3 nmol/min/mg protein; N = 3).

When human tissues containing both enzymes were analyzed, carbidopa, a tight-binding AAAD inhibitor [16], prevented conversion of [^{14}C]5-OH-Trp to [^{14}C]5-HT by human AAAD. TPH activity was detected in all fourteen carcinoid tumors (Tables 1 and 2). Figure 1A illustrates a typical substrate versus velocity plot of carcinoid TPH activity. The K_m of TPH varied within a 3-fold range, while the V_{max} varied 70-fold.

TPH kinetics in carcinoid tumor compared with adjacent normal tissue

Six of the seven carcinoid tumors obtained with adjacent normal liver were of sufficient quantity for TPH analysis. The average K_m in the six tumors (2-fold range in individual values) was comparable to that in the corresponding normal liver samples (3-fold range). Similarly, the average V_{max} for carcinoid TPH (36-fold range) was similar to that in the normal samples (2-fold range).

AAAD kinetics

The AAAD assay was developed using crude enzyme from rodent liver. While there were no publications on

Table 1. Kinetic parameters of tryptophan hydroxylase (TPH) and aromatic-L-amino acid decarboxylase (AAAD) in human carcinoid tumors and normal tissues

		TPH*		AAAD†	
Patient no.	Site	K_m (μM)	V_{max} (nmol/hr/mg protein)	K_m (μM)	V_{max} (nmol/min/mg protein)
A. Carcinoid tumors					
1	Primary in small bowel	197	0.44	37 ± 12	3.3 ± 0.3
2	Metastasis to spleen	133	4.8	43 ± 0.9	14 ± 2
3	Metastasis to liver	335	0.25	Not detectable	
4	Metastasis to liver	151	0.68	78 ± 14	7.0 ± 0.6
5	Metastasis to liver	284	1.1	61 ± 18	0.97 ± 0.27
6	Metastasis to liver	194	1.2	106 ± 8	12 ± 1
7	Metastasis to liver	194	0.98	21 ± 3	10 ± 0.7
B. Carcinoid tumors with adjacent normal tissue					
8	Metastasis to liver	170	17	36 ± 1	6.3 ± 0.3
8a	Normal liver	201	4.5	35 ± 4	0.07 ± 0.004
9	Metastasis to liver	146	1.0	28 ± 5	11 ± 1
9a	Normal liver	138	6.9	57 ± 12	0.23 ± 0.03
10	Metastasis to liver	131	0.49	45 ± 2	12 ± 0
10a	Normal liver	202	4.5	24 ± 7	0.33 ± 0.03
11	Metastasis to liver	109	1.2	27 ± 5	14 ± 0.9
11a	Normal liver	142	4.8	53 ± 9	0.38 ± 0.003
12	Metastasis to liver	199	0.87	38 ± 10	8 ± 1
12a	Normal liver	146	5.9	44 ± 14	0.15 ± 0
13	Metastasis to liver	231‡	1.1‡	48 ± 9	10 ± 1
13a	Normal liver	ND§	ND§	98*	0.30*
14	Metastasis to liver	120	1.6	21 ± 0.5	31 ± 0.8
14a	Normal liver	354‡	3.7‡	39*	0.33*

* Values are the averages of two determinations.
† Values are means ± SEM, N = 3 or 4.
‡ Value represents a single determination.
§ Not determined.

Table 2. Summary of kinetic data of tryptophan hydroxylase (TPH) and aromatic-L-amino acid decarboxylase (AAAD) in human carcinoid tumors and normal tissues

	TPH		AAAD	
	K_m (μM)	V_{max} (nmol/hr/mg protein)	K_m (μM)	V_{max} (nmol/min/mg protein)
A. Carcinoid tumors	185 ± 17 (14)	2.4 ± 1.2 (14)	45 ± 6.7 (13)	11 ± 2.0 (13)
B. Carcinoid tumors with adjacent normal tissue*				
Carcinoid tumor	146 ± 14 (6)	3.8 ± 2.7 (6)	35 ± 3.7 (7)	13 ± 3.1 (7)
Normal liver	197 ± 34 (6)	5.0 ± 0.47 (6)	50 ± 9.0 (7)	0.26 ± 0.04† (7)

Values are means ± SEM; numbers in parentheses are the number of patient samples.

* Matched carcinoid hepatic metastases and adjacent normal liver samples. The tumor samples are a subset of those in part A.

† Statistically significant difference [$P < 0.002$, as determined by a paired, two-tailed t-test employing transformed data (log V_{max}), as the kinetic parameters obtained in this study did not demonstrate Gaussian distributions].

the kinetics of murine, hepatic AAAD, our K_m (15 ± 3 μM; N = 3) compared relatively well to values reported for rat hepatic AAAD (38–45 μM) [18–20]. Literature V_{max} values, when reported, were difficult to compare with our value (1.9 ± 0.1 nmol/min/mg protein; N = 3) due to differences in assay methodologies.

AAAD activity was detected in all but one of fourteen carcinoid tumors (Tables 1 and 2). Figure 1B illustrates a typical substrate versus velocity plot of carcinoid AAAD activity. The K_m of AAAD in the thirteen tumors with measurable activity varied within a 5-fold range, while the V_{max} varied 32-fold.

AAAD kinetics in carcinoid tumor compared with adjacent normal tissue

AAAD assays were performed on seven carcinoid tumors obtained with adjacent normal liver, as sample quantity allowed (Tables 1 and 2). The average K_m for the seven tumors (2-fold range) was comparable to that for the corresponding normal liver samples (4-fold range). However, the average V_{max} for carcinoid AAAD (5-fold range) was 50-fold higher than that in the normal samples (5-fold range). The V_{max} for AAAD in the individual tumors was 33- to 94-fold higher than that of the matched normal liver samples.

Western immunoblot analysis of AAAD

Due to the very large difference in AAAD V_{max} values in carcinoid tumor versus normal liver, western immunoblotting was performed to quantitate AAAD polypeptide content in two sets of carcinoid tumor and adjacent normal liver. A single band with a molecular mass intermediate to the 46 and 69 kDa standards was detected in carcinoid tumors and matched normal liver samples. We believe this to be AAAD based on the previously reported molecular mass of 50 kDa for human pheochromocytoma AAAD [21]. When AAAD bands (Fig. 2) were quantitated and corrected for sample protein concentration, the densitometric reading of the AAAD band from carcinoid tumor was >20-fold higher than that of adjacent normal liver.

DISCUSSION

Excessive 5-HT production by many carcinoid tumors provides a biochemical target for mechanism-based agents. We are preparing Trp analogs designed for active uptake into carcinoid tumors and subsequent irreversible interaction with TPH and/or AAAD. These analogs could reduce 5-HT synthesis, severe as imaging agents, and/or be effective cytotoxic agents. This mechanism-based approach of developing irreversible inhibitors of TPH and/or AAAD assumes that the target enzymes in the carcinoid tumor are similar with regard to substrate affinity and catalytic mechanism to their counterparts in normal tissues. However, there was a paucity of biochemical data on carcinoid TPH and AAAD with which to verify this hypothesis. Our report represents the first study of TPH and AAAD kinetics in a series of human carcinoid tumors, the first comparison of TPH and AAAD kinetics in carcinoid tumors with those in adjacent normal (liver) tissues, and the first quantitation of AAAD polypeptide in carcinoid and adjacent liver tissues.

TPH was detected in all fourteen human carcinoid tumors analyzed. The average K_m (185 μM) was similar to that in human raphe nucleus (142 μM) [7] and adjacent normal human liver (197 μM, as determined in this study), but higher than the value (13 μM) reported for TPH in a single human carcinoid after long storage [3]. No published V_{max} values were available for TPH in normal human tissues for comparison. While there was substantial variability in TPH activity in these tumors (70-fold V_{max} range), the average V_{max} in a subset of six hepatic metastatic lesions was similar to that in adjacent normal liver samples. These data suggest that TPH in human carcinoid tumors is functionally similar to that in normal human tissues, and that the activity of carcinoid TPH, while varying from lesion to lesion, is, on average, comparable to that in normal liver.

Thirteen of the fourteen carcinoid tumors also had measurable AAAD activity. The average K_m of AAAD activity in human carcinoid (45 μM) was similar to that in rodent liver (15–45 μM) (this study and others, [18–20]) and adjacent normal human liver (50 μM, as determined in this study). No previously published kinetic data were available for AAAD in normal human tissues for comparison. These K_m data are consistent with the assumption that carcinoid AAAD is functionally similar to AAAD from normal mammalian tissues. In addition, human carcinoid AAAD was inactivated in a similar manner to murine hepatic AAAD by a mechanism-based AAAD inhibitor prepared in our laboratory (preliminary data). Data from seven matched sets of hepatic carcinoid

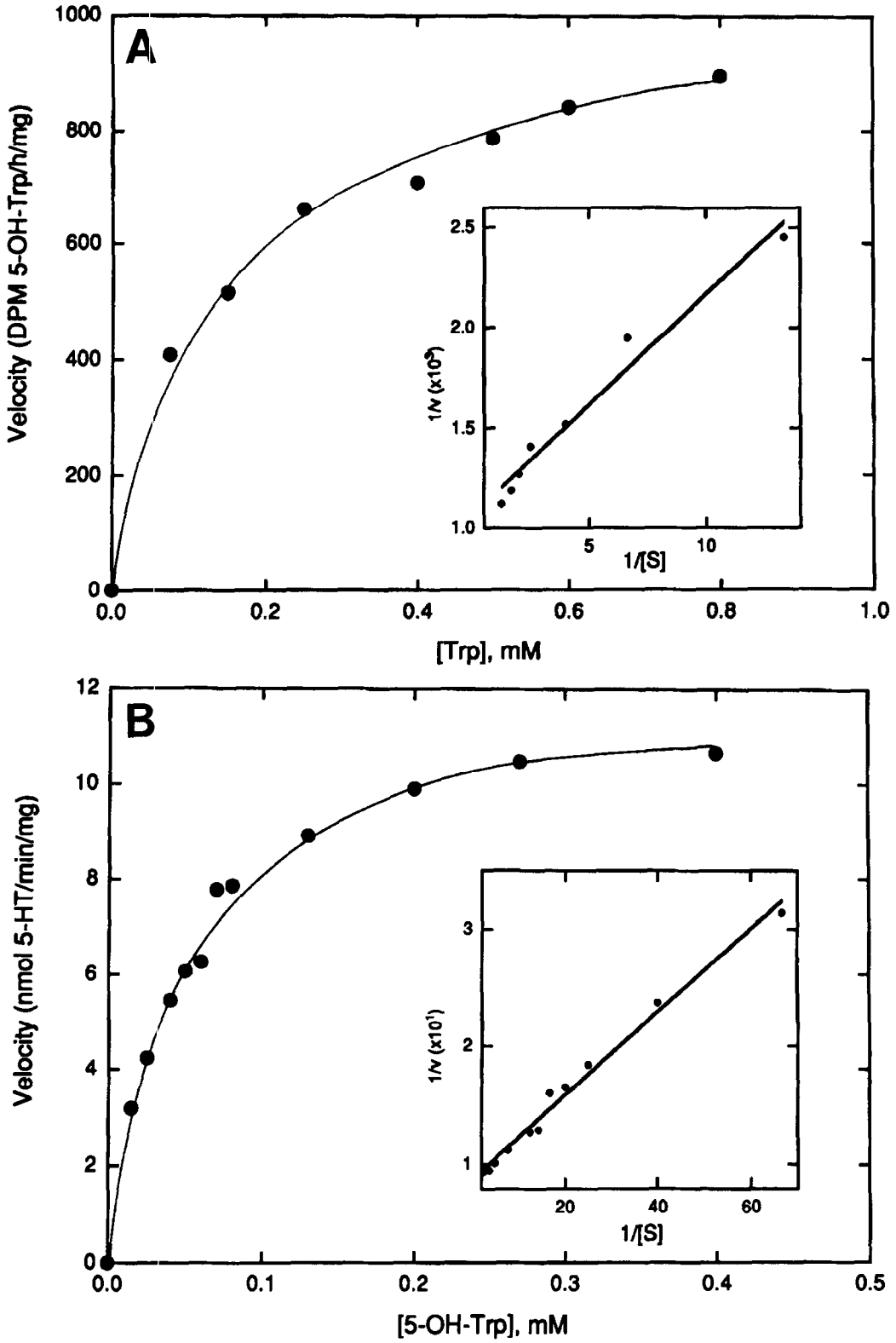

Fig. 1. Effect of substrate concentration on the rate of product formation by 5-HT biosynthetic enzymes in carcinoid homogenate. A crude homogenate from one carcinoid tumor (a metastasis to the liver) was prepared and enzyme kinetics were performed as described in Materials and Methods. (A) A typical substrate versus velocity plot of TPH activity. Inset: A double-reciprocal plot of the data, typically corresponding to substrate (L-[side-chain-3-^{14}C]Trp) concentrations in the range of 0.5 K_m–5 K_m. (B) A typical substrate versus velocity plot of AAAD activity. Inset: A double-reciprocal plot of the data, typically corresponding to substrate (5-OH-Trp) concentrations of 400 μM or less due to substrate inhibition seen at higher concentrations. Experimental data for both enzymes were analyzed by the computer program of Cleland [17] to provide values for K_m and V_{max}.

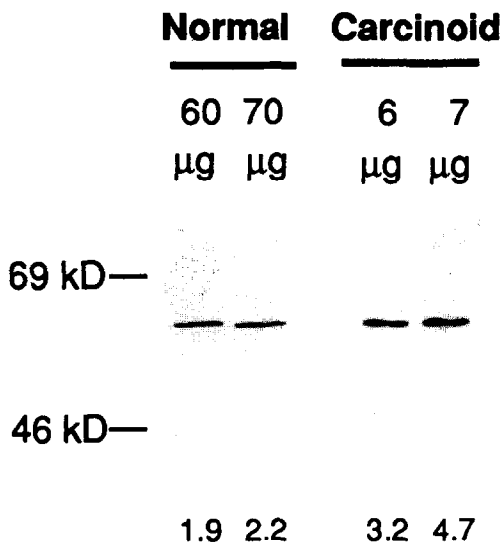

Fig. 2. Western immunoblot analysis of AAAD in carcinoid tumor and adjacent normal liver tissue. Crude homogenates of carcinoid tumor and adjacent normal liver from one patient were analyzed by western immunoblotting as described in Materials and Methods. Numbers at the top of the figure indicate the quantity of protein per sample (μg); numbers at the bottom of the figure provide the densitometric readings of the respective bands (area × 10^{-4}). This western blot was representative of three replicate experiments from one (Patient No. 8 and No. 8a in Table 1) of two matched sets of carcinoid and normal liver tissue analyzed.

tumors and adjacent normal liver tissues demonstrated that the average V_{max} for carcinoid AAAD was 50-fold greater than that in normal human liver. Western immunoblot analysis confirmed that this high V_{max} ratio was reflective of much greater AAAD polypeptide content in carcinoid tumor than in surrounding normal liver. This tissue-specific difference in AAAD content provides an important, exploitable biochemical feature for cytotoxic Trp analogs targeted at carcinoid tumors.

Whether the described biochemical properties of carcinoid TPH and AAAD, especially the large quantities of carcinoid AAAD, are totally responsible for excessive serotonin production in carcinoid tumors is not certain. Other factors may play important roles, such as activation of TPH (resulting in an increased affinity for pterin cofactor) or decreased monoamine oxidase activity. Our results suggest the rational design of irreversible, mechanism-based inhibitors of AAAD as cytotoxic agents targeted towards the carcinoid tumor. The elevated content of AAAD protein in carcinoid tumors will be important for the tissue selectivity and concentration-dependent cytotoxicity of the proposed AAAD-activated antitumor drugs. We are continuing our biochemical characterization of enzymes in the serotonergic biosynthetic pathway of human carcinoid tumors as well as the development of agents useful in the diagnosis and treatment of this disease.

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