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INACTIVATION BY ACIVICIN OF CARBAMOYL-PHOSPHATE SYNTHETASE II OF HUMAN COLON CARCINOMA*

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Abstract—The effect of the anti-tumor, anti-glutamine drug acivicin, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, was determined on the activity of the rate-limiting enzyme of *de novo* pyrimidine biosynthesis, carbamoyl-phosphate synthetase II (glutamine-hydrolyzing) (EC 6.3.5.5), in human colon carcinoma. The synthetase II activity in human colon carcinoma was elevated 2- to 3-fold over values of the normal colon mucosa, and the substrate kinetic constants were similar for the enzyme in normal and neoplastic colon. The K_m for glutamine was 17 μM (colon carcinoma) and 23 μM (normal mucosa), whereas the K_m for ATP was 2.1 and 1.7 mM in tumor and mucosa respectively. The synthetase II activity in colon carcinoma was inhibited to a similar extent by UMP, UDP and UTP (36–41%). The three uracil nucleotides were also equally effective in inhibiting the enzyme from normal mucosa (39–46%). Both enzymes were activated by PRPP (63 and 57%) in mucosa and carcinoma respectively. Acivicin *in vitro* selectively inactivated the glutamine-dependent synthetase II from human colon carcinoma, and it did not affect the ammonia-dependent activity. The acivicin inactivation constant (K_{inact}) was 100 μM, and the minimum inactivation half-time (T) was 0.7 min. Acivicin most likely exerts its effect against human colon synthetase II by acting as an active site directed affinity analogue of L-glutamine.

There is a need for the development of effective chemotherapy against colon carcinoma, since this tumor is refractory to current clinical treatment. Previous investigations in this laboratory elucidated aspects of the enzymic imbalance that characterizes human colon tumors and pointed to potential targets for selective anti-cancer chemotherapy [1, 2]. Since increased activities have been observed for all the glutamine-utilizing enzymes of *de novo* purine and pyrimidine biosynthesis in colon tumors, it was of interest to determine their sensitivity to the glutamine antagonist, acivicin§ (NSC 163501), an antibiotic known to exhibit antitumor action [3]. We have focused on the behavior of glutamine-hydrolyzing carbamoyl-phosphate synthetase II (synthetase II; EC 6.3.5.5) activity in human colon carcinomas, because it is the first and the rate-limiting enzyme of *de novo* uridylate biosynthesis [4, 5]. We report here that synthetase II in human colon carcinoma closely resembles the enzyme from rat hepatoma in its kinetic and regulatory properties, and it is rapidly inactivated by acivicin.

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§ Abbreviations: acivicin, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; carbamoyl-P, carbamoyl-phosphate; synthetase II, carbamoyl-phosphate synthetase (glutamine-hydrolyzing); PRPP, 5-phosphoribosyl 1-pyrophosphate; and Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

MATERIALS AND METHODS

Materials. Human colons and carcinomas were obtained from patients who underwent resection at the Indiana University School of Medicine hospitals. Colon mucosa and neoplastic tissue were processed as described [2]. Homogenates (20%, w/v) were prepared immediately in a solution, pH 7.0, containing 30% (v/v) dimethyl sulfoxide, 5% (w/v) glycerol, 2 mM potassium phosphate, and 1 mM dithio threitol. Homogenates were centrifuged at 105,000 *g* for 40 min. For kinetic and regulatory studies of synthetase II, the crude supernatant fractions were desalted on Sephadex G-25 as previously reported [4]. Acivicin was obtained from the Upjohn Co., Kalamazoo, MI.

Biochemical assays. Glutamine-dependent synthetase II activity was measured by following the formation of L-[*carbamoyl*-^{14}C]citrulline as previously described [4]. One millimolar L-glutamine was replaced by 10 mM NH_4Cl in the standard reaction mixture when assaying NH_3-dependent activity. Aspartate carbamoyltransferase activity was determined by the procedure previously reported [6]. Protein was measured by the method of Lowry *et al.* [7].

Enzyme inactivation. In vitro inactivation by acivicin of synthetase II was performed at 22° in a mixture containing 50 mM potassium Hepes buffer (pH 7.0), 10 mM ATP, 15 mM $MgCl_2$, 16.7 mM

$KHCO_3$, 7.5% (v/v) dimethyl sulfoxide, 2.5% (w/v) glycerol, 1 mM dithiothreitol, and acivicin [8]. Enzyme was added to initiate inactivation and, at specified intervals, the remaining synthetase II activity was determined.

RESULTS AND DISCUSSION

Increased activity of synthetase II in colon carcinoma. In five colon carcinomas (three male and two female), synthetase II activity was elevated an average of 2.4-fold relative to normal colon mucosa: 8.5 ± 1.4 vs 3.6 ± 0.3 nmoles/hr/mg protein. Likewise, the activity of aspartate carbamoyltransferase, which is present as a multifunctional protein together with synthetase II, was increased 2.2-fold. In Table 1 are representative data obtained from one of the colon carcinomas (85-year-old male patient). This tumor, which had a 2.6-fold higher synthetase II activity than that of the normal mucosa, served as the enzyme source for the studies described below.

Kinetic properties of synthetase II in human colon mucosa and colon carcinoma. To establish optimum assay conditions for synthetase II in human colon mucosa and carcinoma, kinetic properties of the enzyme were compared in desalted enzyme fractions from the normal and neoplastic tissues. Increasing concentrations of glutamine provided data points that generated hyperbolic curves; the apparent K_m values were 23 and 17 μM for normal mucosa and colon carcinoma respectively (Fig. 1). With increasing Mg^{2+}-ATP concentrations, the velocity curves were also hyperbolic, and the apparent K_m values were 2.1 and 1.7 mM for mucosa and carcinoma respectively (Fig. 2). For these substrates the apparent K_m values in rat liver and hepatomas were similar: 19 and 16 μM glutamine, respectively, and 2.3 mM ATP for both enzymes [9]. Further studies showed that, in the standard assay system, both mucosa and carcinoma synthetase II reactions were linear with respect to enzyme amount and incubation times.

Regulatory properties of synthetase II in colon mucosa and colon carcinoma. The effects of different nucleotides and PRPP on synthetase II activity in the control mucosa and tumor tissues are shown in Table 2. Both enzymes were inhibited to a similar extent by all three uracil nucleotides, UMP, UDP and UTP (39–46% inhibition). None of the other nucleotides tested showed significant effects. In contrast, UMP has no effect on synthetase II in rat liver and hepatoma, whereas UTP results in 80% inhibition [9]. PRPP activated the mucosa and carcinoma enzymes by 63 and 57% respectively. This observation is in line with the role of PRPP as an allosteric activator of synthetase II as reported for the enzyme from rat liver and hepatoma [9].

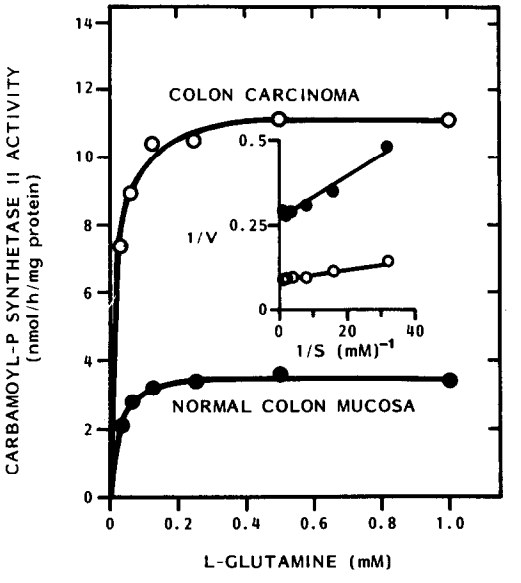

Fig. 1. Effect of L-glutamine concentration on synthetase II activity in normal colon mucosa and colon carcinoma. Activity was measured in the 100,000 *g* supernatant fraction desalted on Sephadex G-25 [4] in the standard assay system [9], except that the glutamine concentration was varied. Inset shows reciprocal plots.

Competitive inhibition of synthetase II in vitro *by acivicin.* Shown in Fig. 3 are double-reciprocal plots of saturation kinetics with L-glutamine for human colon mucosa and colon carcinoma synthetase II in the presence of various acivicin concentrations. Competitive inhibition by acivicin with respect to L-glutamine was observed in each case. The apparent K_i was 4 μM, which agrees well with the previous

Table 1. Carbamoyl-phosphate synthetase II and aspartate carbamoyltransferase activities in a human colon carcinoma*

Tissue	Protein (mg/g tissue)	Specific activity (nmoles/hr/mg protein)	
		Carbamoyl-P synthetase II	Aspartate carbamoyltransferase
Normal colon mucosa	46.8 ± 0.6 (100)	3.7 ± 0.1 (100)	225 ± 3 (100)
Colon carcinoma	45.5 ± 1.1 (97)	9.7 ± 0.3† (262)	585 ± 10† (260)

* Results are expressed as mean ± S.E. of three determinations. Values in parentheses refer to percent control.

† Significantly different from control ($P < 0.05$).

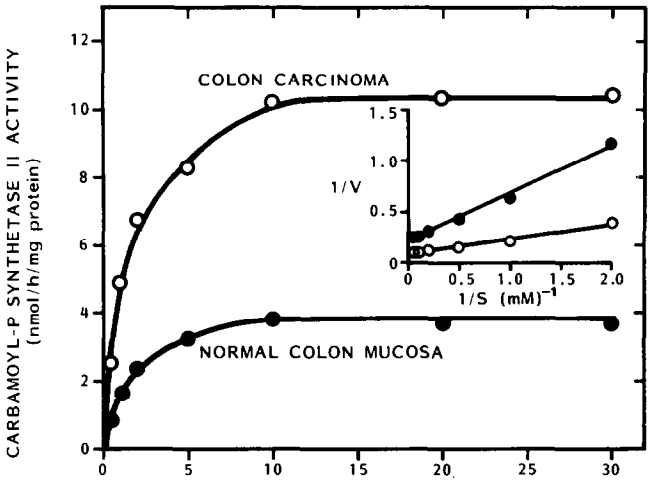

Fig. 2. Effect of Mg^{2+}-ATP concentration on synthetase II activity in normal colon mucosa and colon carcinoma. Activity was measured under the standard conditions except that Mg^{2+}-ATP concentration was varied and free Mg^{2+} was present at 5 mM. Inset shows reciprocal plots.

value of 7 μM observed for the rat liver and hepatoma enzymes [8]. The NH_3-dependent activity of synthetase II in human colon tumor was not inhibited by acivicin (up to 100 μM).

Inactivation of synthetase II in vitro *by acivicin.* Colon tumor synthetase II was preincubated in the presence of various concentrations of acivicin as described in Materials and Methods. The time-course of inactivation of the enzyme demonstrated first-order kinetics up to 90% inactivation (Fig. 4A). A linear relationship emerged when inactivation half-time was plotted as a function of 1/(acivicin concentration) (Fig. 4B), which indicates saturation kinetics. This result is consistent with the formation of a reversible intermediate preceding irreversible inactivation [10, 11]. Based on the following equation of Meloche [10], the linear plot shown in Fig. 4B was used to calculate kinetic parameters for acivicin inactivation:

$$\text{Inactivation half-time} = TK_{inact}/[I] + T,$$

where T is the minimum inactivation half-time at infinite concentration of inactivator and is calculated from the intercept of the ordinate, K_{inact} is the inactivation constant, and $[I]$ is the inhibitor concentration. The inactivation constant in the above experiment was 100 μM, and the minimum inactivation half-time was 0.7 min. These values are virtually the same as those observed for inactivation of the rat hepatoma enzyme [8]. The mechanism of action of acivicin against human colon carcinoma synthetase II may be due to its role as an active-site directed affinity analogue of L-glutamine. Alkylation of a cysteine residue of the glutamine-binding site by

Table 2. Effects of nucleotides and PRPP on carbamoyl-phosphate synthetase II activity in human colon mucosa and colon carcinoma*

	Synthetase II activity			
	Normal mucosa		Carcinoma	
Compound added	nmoles/hr/mg protein	% of Control	nmoles/hr/mg protein	% of Control
None, control	3.7	100	9.7	100
AMP	3.6	97	11.1	114
ADP	3.6	97	11.3	116
CTP	4.0	107	10.0	103
GTP	4.8	130	9.3	96
UMP	2.1	56†	5.7	59†
UDP	2.3	61†	6.0	62†
UTP	2.0	54†	6.2	64†
PRPP	6.0	163†	15.2	157†

* The standard reaction mixture described in the text was modified by reducing ATP and $MgCl_2$ concentrations to 2 and 7 mM respectively. Additions of ribonucleotides and PRPP were at concentrations of 0.5 and 0.1 mM respectively.

† Significantly different from control ($P < 0.05$).

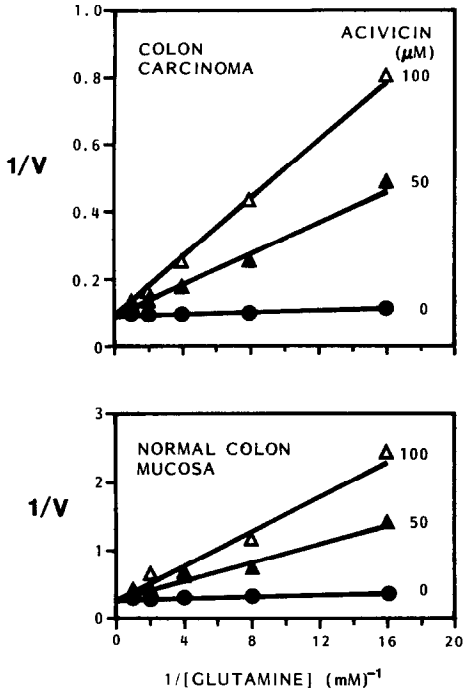

Fig. 3. Inhibition of synthetase II activity by acivicin. Activities were determined in the standard assay system with the addition of acivicin and varied concentrations of L-glutamine. Velocity is expressed as nmoles/hr/mg protein.

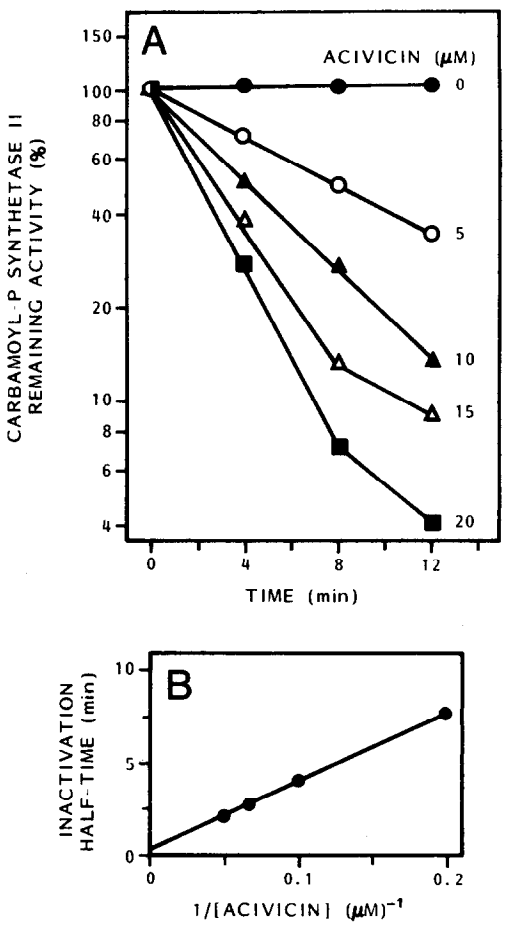

Fig. 4. Effect of acivicin concentration on inactivation of synthetase II. Inactivation of the tumor enzyme was performed as described in Materials and Methods. Key: (A) Time-course of inactivation by different concentrations of acivicin; and (B) Plot of inactivation half-time vs 1/[acivicin].

acivicin was postulated to occur in the rapid inactivation of bacterial glutamine amidotransferases [11]. Inactivation of the glutamine-dependent synthetase II from human colon carcinoma was selective, since NH_3-dependent activity was not affected by up to 50 μM acivicin (data not shown).

Acivicin is currently in Phase II trials. The possible efficacy of acivicin against slowly growing tumors commonly refractory to chemotherapy, as exemplified by colon carcinoma, is of interest. We have shown here that synthetase II from a human colon carcinoma closely resembles the enzyme from rat hepatoma 3924A with regard to its affinity for glutamine and acivicin. In the case of rat hepatoma, we have achieved effective combination chemotherapy with this glutamine antagonist [12]. The susceptibility of a given tissue to acivicin treatment is a function, at least in part, of its L-glutamine content. From our earlier results, we know that synthetase II in a rapidly growing rat hepatoma is much more susceptible to acivicin *in vivo* than the host liver enzyme [13] although both enzymes have the same affinity (K_i) for the drug [8]. This observation is attributed to the low glutamine content in the rapidly growing hepatoma [14]. The concentration of glutamine in human colon mucosa and in colon carcinomas is currently under investigation.

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