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KINETIC PROPERTIES AND INHIBITION OF *ACINETOBACTER* GLUTAMINASE-ASPARAGINASE

JOPH STECKEL
Harvard College, Cambridge, MA 02138, U.S.A.
and
JOSEPH ROBERTS, FREDERICK S. PHILIPS and TING-CHAO CHOU*
Laboratories of Experimental Enzyme Therapy and Pharmacology, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, U.S.A.

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Abstract—Kinetic parameters, substrate specificity and exclusivity of ligands at binding sites of L-glutaminase-L-asparaginase purified from *Acinetobacter glutaminasificans* were studied in order to gain knowledge about the dual activities of this enzyme and its inhibition by structural analogs. Both L-glutamine and L-asparagine, which showed similar K_m ($4 \sim 7 \times 10^{-5}$ M) and V_{max} (molecular activity 1.0 min^{-1}) values, were competitive with each other for the substrate binding site. The products, L-glutamic acid and L-aspartic acid, showed competitive inhibition with respect to either L-glutamine or L-asparagine as substrates. Multiple inhibition of the glutaminase activity by L-glutamic acid and L-aspartic acid indicated that these ligands are mutually exclusive at the product-releasing site. The initial rates of both of the enzyme's activities were competitively inhibited by the following inhibitors (in decreasing order of activity): 6-diazo-5-oxo-L-norleucine (DON), L-methionine sulfoximine, azaserine, and Acivicin. DON and azaserine inhibited both the asparaginase and glutaminase activities in a time-dependent and irreversible manner. The kinetic data suggest an ordered mechanism with glutamine or asparagine as the first substrate and glutamic acid or aspartic acid, respectively, as the last product. These results also suggest that a single mechanism and a single set of binding sites are responsible for catalyzing both of the enzyme's activities. The data also showed that succinylated enzyme, which has a 10-fold increase of plasma half-life in animals and humans and, thus, has benefit as a cancer chemotherapeutic agent, retained its catalytic activity and maintained K_m and V_{max} values similar to the native enzyme.

L-Asparaginase of *Escherichia coli* and some other sources, which have limited therapeutic effectiveness against solid tumors in man, is an effective agent for the treatment of acute lymphocytic leukemia [1]. This result has led to the use of other amino acid-degrading enzymes for cancer treatment, since tumor cells may lack those enzymes which catalyze the production of nonessential amino acids [2]. In tissue culture, glutaminase-asparaginase, which has been purified from *Acinetobacter glutaminasificans* selectively kills human leukemic leukocytes at approximately one-hundredth the effective concentration of *E. coli* asparaginase [3]. Specificity of action of these enzymes and their physico-chemical properties may, in part, dictate their selective cytotoxic effects.

Glutaminase-asparaginase from *A. glutaminasificans* (AGA)† has been isolated and crystallized and its physico-chemical properties have been characterized [4, 5]. AGA, which utilizes both L-glutamine and L-asparagine as substrates, is composed of four identical subunits. The tetramer has a molecular weight of 138,000 g/mole and undergoes reversible dissociation to an inactive dimer during gel filtration and ultracentrifugation [5].

Antitumor activity of AGA and other purified glutaminase-asparaginases (i.e. *Pseudomonas* 7A glutaminase-asparaginase) is also related to their plasma half-lives [1]. Succinylation and glycosylation of AGA have been shown to increase its half-life about 10-fold [6]. Succinylation protects the enzyme from trypsin digestion. Glycosylated enzyme preparations exhibit less heat inactivation than does native or succinylated enzyme [6].

The purpose of this investigation is to explore the ligand bindings of AGA and examine the kinetic mechanism and inhibition of both activities of this enzyme. The kinetic parameters of succinylate AGA were also determined.

MATERIALS AND METHODS

AGA was purified to homogeneity from *A. glutaminasificans* (ATTCC 27197) with the method described by Roberts *et al.* [4]. The enzyme was stored as a lyophilized powder at 4°. The enzyme used had a specific activity of 150 I.U./mg protein. One I.U. of activity is defined as that amount of enzyme which catalyzes the formation of 1 μmole ammonia/min as measured by nesslerization [4] or by the assay of glutamic acid formation as described

* To whom reprints requests should be sent. Address: Dr. Ting-Chao Chou, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, U.S.A.

† Abbreviations: AGA, *Acinetobacter* glutaminase-asparaginase; SAGA, succinylated *Acinetobacter* glutaminase-asparaginase; DON, 6-diazo-5-oxo-L-norleucine; azaserine, *O*-diazoacetyl-L-serine; and AT-125, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (Acivicin).

previously [7]. Protein concentration was determined by the method of Lowry *et al.* [8]. AGA solution was freshly prepared in 5 mM Tris–HCl buffer, pH 7.5, containing 5 mg/ml of crystalline bovine serum albumin. Succinylation of AGA with succinic anhydride was performed by a method described previously [7]. L-[^{14}C-U]Glutamine, 200 mCi/mmole, L-[^{14}C-U]glutamic acid, 250 mCi/mmole, L-[^{14}C-U]asparagine, 150 mCi/mmole, and L-[^{14}C-U]aspartic acid, 200 mCi/mmole, were obtained from the New England Nuclear Corp., Boston, MA. L-Glutamic acid, L-asparagine and L-aspartic acid were purchased from the Sigma Chemical Co., St. Louis, MO. DON and azaserine were obtained from the Parke Davis Co., Ann Arbor, MI. DON was purified on a charcoal:Celite column [9] and was judged homogenous by thin-layer chromatography with several solvent systems as visualized by ninhydrin. The purified DON was white to faintly yellow, with $E^{1\%}_{1cm}$ = 600 at 275 nm. L-Methionine sulfoximine was obtained from the Calbiochem-Behring Corp., La Jolla, CA. Acivicin (AT-125) was obtained from the Natural Product Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. AG 1 × 8 anionic exchange resin, 200–400 mesh in chloride form, was purchased from the Bio-Rad Laboratories, Richmond, CA. It was suspended in 2 parts of 5 M acetic acid for 15 min to convert it into the acetate form. It was then washed with distilled water until a neutral pH was obtained.

Assay for L-*glutaminase and* L-*asparaginase.* The standard reaction mixture (0.5 ml) contained 0.1 ml of 0.3 M Tris–HCl buffer, pH 7.5, 0.2 ml [^{14}C]glutamine or [^{14}C]asparagine (1 μCi/ml), 0.1 ml AGA (0.0025 I.U.) and 0.1 ml of unlabeled glutamine or asparagine, to yield final substrate concentrations ranging from 5 to 100 μM. With the exception of time–course studies on DON and azaserine, all kinetic inhibition studies were carried out in the simultaneous presence of substrate and inhibitor, with enzyme being the last ingredient added to start the reaction. All reaction mixtures were incubated for 15 min at 37°. The reaction was stopped by placing the tubes in an ice bath at 4° while adding 0.5 ml of ice-cold 0.1 M glutamine or asparagine. The contents of the tube were then placed on the AG 1 × 8 anionic exchange column (1 cm × 5 cm). Unreacted [^{14}C]glutamine or [^{14}C]asparagine was washed off three times with 6 ml of ice-cold distilled water. [^{14}C]Glutamic acid or [^{14}C]aspartic acid, which was retained on the column, was eluted with 6 ml of 1 N HCl. In initial experiments, the separation of [^{14}C]glutamic acid and [^{14}C]glutamine and [^{14}C]aspartic acid and [^{14}C]asparagine was also confirmed with a Beckman 119 amino acid analyzer, using lithium citrate buffer [10, 11]. Eluate (2 ml) from the AG 1 × 8 column was mixed with 18 ml of Hydrofluor Scintillation Fluid (National Diagnostics, Somerville, NJ). Samples were counted for radioactivity in the Packard Tri-Carb model 3775 liquid scintillation spectrometer.

Analysis of exclusivity of ligand binding. The exclusivity of glutamic acid and aspartic acid at product-releasing site(s) was analyzed by product inhibition using glutamic acid or aspartic acid, both individually and simultaneously in combination. The inhibitory effect in terms of fractional inhibition (f_i) was related to the concentration of inhibitor (I) by the generalized median-effect equation derived by Chou [12]:

$$\log [(f_i)^{-1} - 1]^{-1} = m \log (\mathrm{I}) - m \log (\mathrm{I}_{50}) \quad (1)$$

where m is the Hill-type coefficient and I_{50} is the median-effect concentration that is required to inhibit 50%. A plot of y = log $[(f_i)^{-1} - 1]^{-1}$ vs x = log (I) gives a slope m, and the intercept of the plot at y = 0 gives the log (I_{50}) value. When m = 1, the inhibition follows a simple mass-action law where Michaelis–Menten type of kinetics is indicated.

The median-effect plot can also be readily applied to the multiple inhibition where inhibitor 1, $(\mathrm{I})_1$, and inhibitor 2, $(\mathrm{I})_2$, are simultaneously present and are varied at a constant ratio. The derived general equation for mutually exclusive inhibitor is [13]:

$$\left[\frac{(f_i)_{1,2}}{(f_v)_{1,2}}\right]^{1/m} = \left[\frac{(f_i)_1}{(f_v)_2}\right]^{1/m} + \left[\frac{(f_i)_2}{(f_v)_2}\right]^{1/m} = \frac{(\mathrm{I})_1}{(\mathrm{I}_{50})_1} + \frac{(\mathrm{I})_2}{(\mathrm{I}_{50})_2} \quad (2)$$

For mutually non-exclusive inhibitors, the derived general equation becomes [13]:

$$\left[\frac{(f_i)_{1,2}}{(f_v)_{1,2}}\right]^{1/m} = \left[\frac{(f_i)_1}{(f_v)_1}\right]^{1/m} + \left[\frac{(f_i)_2}{(f_v)_2}\right]^{1/m} + \left[\frac{(f_i)_1(f_i)_2}{(f_v)_1(f_v)_2}\right]^{1/m} = \frac{(\mathrm{I})_1}{(\mathrm{I}_{50})_1} + \frac{(\mathrm{I})_2}{(\mathrm{I}_{50})_2} + \frac{(\mathrm{I})_1(\mathrm{I})_2}{(\mathrm{I}_{50})_1(\mathrm{I}_{50})_2} \quad (3)$$

where $(f_i)_{1,2}$ and $(f_v)_{1,2}$ represent fractional inhibition and fractional velocity, respectively, in the presence of both I_1 and I_2 in which $(f_i)_{1,2} = 1 - (f_v)_{1,2}$.

For mutually exclusive inhibitors (Equation 2), the median-effect plot gives a parallel plot with a slope, m, for I_1, I_2 and $\mathrm{I}_1 + \mathrm{I}_2$.

For mutually non-exclusive inhibitors (Equation 3), the median-effect plot gives a parallel line for I_1 and I_2, but $\mathrm{I}_1 + \mathrm{I}_2$ gives a concave upward curve [13].

RESULTS

When the concentration of ^{14}C-labeled glutamine was varied for measuring the initial reaction rates of AGA, the saturation curves for the deamidase showed Michaelis–Menten kinetics with a K_m = 4.1×10^{-5} M for glutamine. This K_m value is somewhat higher than that previously reported [4], which is probably due to the difference in incubation conditions and assay procedure. Glutamic acid, a product of the reaction, appeared to be a competitive inhibitor with respect to glutaminase activity with $K_i = 5.0 \times 10^{-3}$ M (Fig. 1A). Aspartic acid, which is a product of the alternate catalytic activity of AGA, namely, its asparaginase activity, appeared to be a more potent competitive inhibitor with respect to glutamine deamidation with K_i = 1.6×10^{-4} M (Fig. 1B). The most potent competitive inhibition of the glutaminase activity was observed

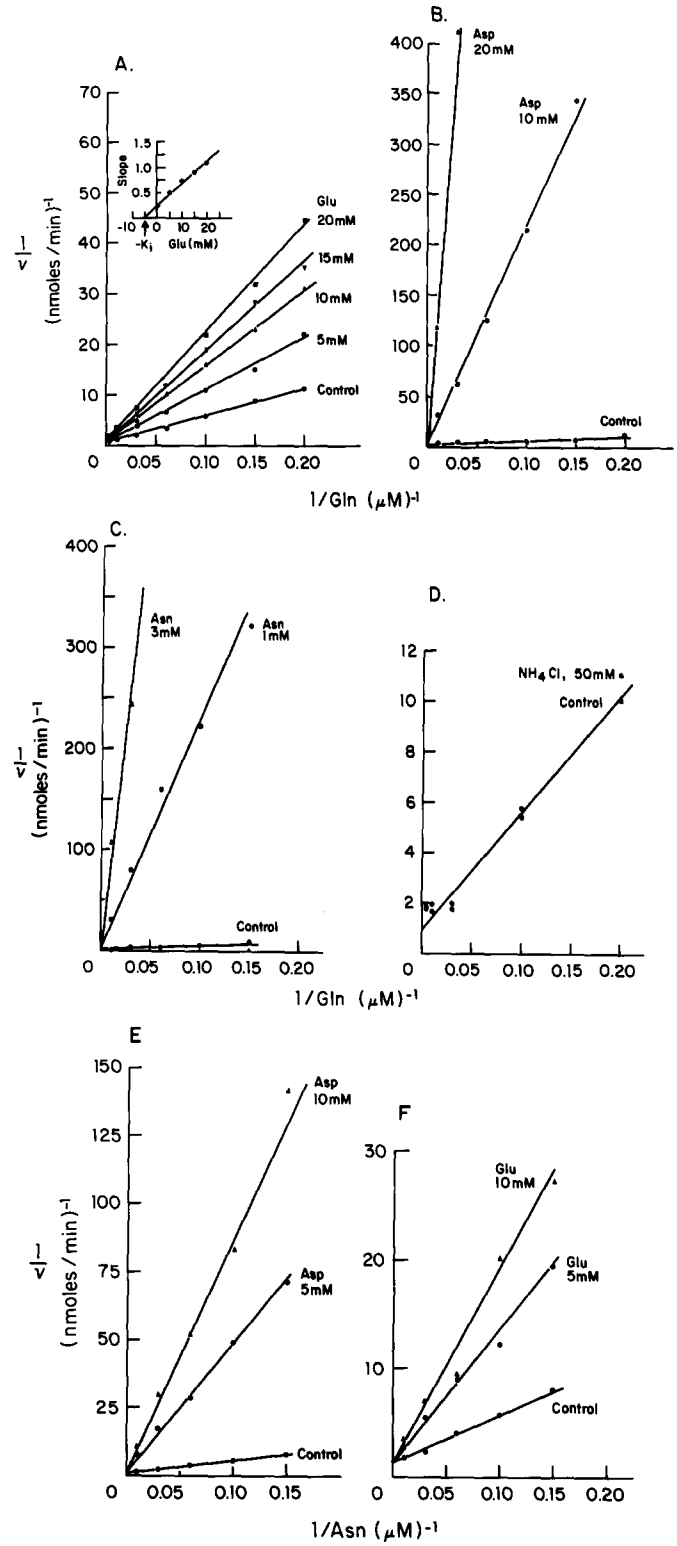

Fig. 1. Inhibition of glutaminase and asparaginase activity by products or alternate products. Inhibitor concentrations are indicated on the figure. Panels A–D: glutamine as substrate and glutamic acid, aspartic acid, asparagine, and ammonium chloride, respectively, as inhibitors. Panels E and F: asparagine as substrate and aspartic acid and glutamic acid, respectively, as inhibitors. Insert in panel A depicts a replot of slope vs inhibitor concentration, showing simple competitive inhibition with a K_i for L-glutamic acid of 5.0×10^{-3} M. The V_{max} values for A–F are 1.1, 0.4, 0.4, 1.0, 1.0 and 0.7 nmole/min, respectively, and the K_m values for A–F are 6.7×10^{-5}, 2.0×10^{-5}, 2.0×10^{-5}, 5.0×10^{-5}, 5.0×10^{-5} and 3.5×10^{-5} M respectively.

with the alternate substrate, asparagine, which showed a $K_i = 2.0 \times 10^{-5}$ M (Fig. 1C). Ammonia, another product (substituted by ammonium chloride, 50 mM, in these studies) showed weak uncompetitive inhibition on the glutaminase activity (Fig. 1D). Aspartic acid exhibited product competitive inhibition of the asparaginase activity with a $K_i = 5.3 \times 10^{-4}$ M (Fig. 1E), and glutamic acid exhibited alternate product competitive inhibition of the asparaginase activity with a $K_i = 3.0 \times 10^{-3}$ M (Fig. 1F).

The initial rates of both activities of AGA were competitively inhibited by the following inhibitors (in decreasing order of activity): 6-diazo-5-oxo-L-norleucine (DON), L-methionine sulfoximine, azaserine, and Acivicin (Fig. 2, A and B). The inhibition constants are summarized in Table 1. DON and azaserine inhibited both the glutaminase (Fig. 3A) and asparaginase (Fig. 3B) activities in a time-dependent manner, and the inhibition was not reversible after extensive dialysis.

Individual and concomitant multiple inhibition of the glutaminase activity by aspartic acid and/or glutamic acid is given in Table 2 and is illustrated in the concentration–effect relationships (Fig. 4A). Linearity is observed in the median-effect plot [12, 13] of the inhibition by aspartic acid and/or glutamic acid which were used alone and in combination (Fig. 4B), suggesting that the dose–effect relationships of these inhibitors follow the simple mass-action law. The plots with slopes (m values) near unity are indicative of Michaelis–Menten type of kinetics. In addition, the parallelism of the plots for each inhibitor individually and both inhibitors in combination indicates that aspartic acid and glutamic acid were mutually exclusive at the binding site (i.e the product-releasing site). The normalized median-effect plot [13], in which the inhibitor concentrations were normalized by their respective I_{50} values, also showed linearity with a slope near unity (Fig. 4C), which further supports the above conclusions derived from the application of the median-effect equation for data analysis.

Succinylated *Acinetobacter* glutaminase-asparaginase (SAGA) showed a similar initial rate of glutaminase activity to the native enzyme (AGA). The K_m for the glutaminase activity of SAGA was 8.3×10^{-5} M (Fig. 5A), and this activity was also inhibited by 0.1 mM DON, in a time-dependent fashion (Fig. 5B).

DISCUSSION

The observation that glutamic acid and aspartic acid are competitive inhibitors with respect to glutamine and asparagine when considering the dual activities of AGA, respectively, is consistent with a reaction in which glutamine or asparagine is the first substrate and glutamic acid or aspartic acid, respectively, is the last product. The second substrate, water, in the assay condition is 55.5 M, and we made no attempt to vary water concentration for initial rate studies. Inhibition by ammonium chloride appeared to be uncompetitive in the presence of a high concentration of water (Fig. 1D). These data suggest an ordered mechanism in which glutamine or asparagine is the first substrate to interact with

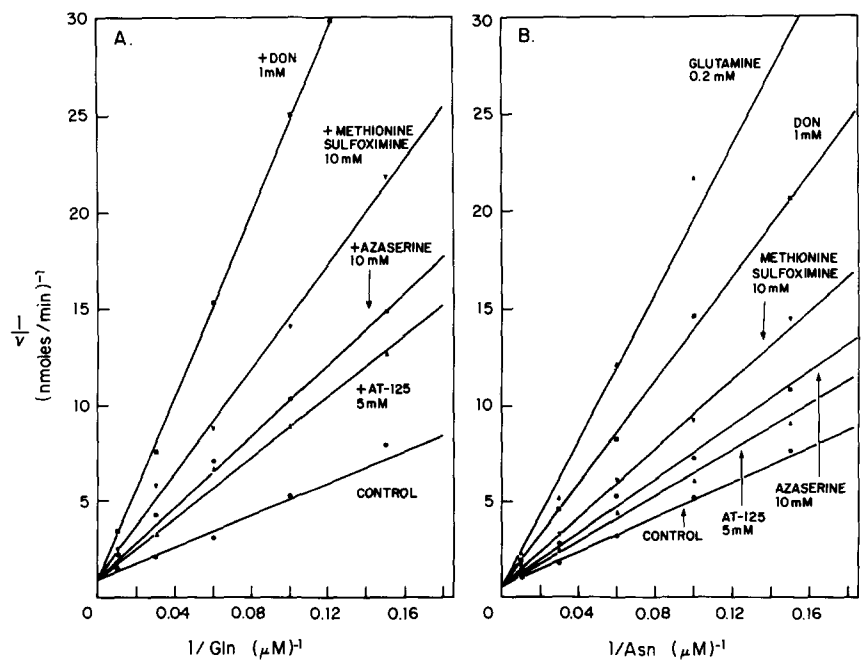

Fig. 2. Dead-end inhibition of glutaminase and asparaginase activity by DON, methionine sulfoximine, azaserine, and Acivicin (AT-125). The K_m values for Gln and Asn are 4.1×10^{-5} and 7.4×10^{-5} M respectively. The V_{max} values for A and B are 1.0 and 1.53 nmoles/min respectively. Other kinetic parameters are summarized in Table 1. In B, glutamine is also used as an inhibitor (an alternate substrate) for asparaginase activity.

Table 1. Kinetic constants of *Acinetobacter* L-glutaminase-L-asparaginase (AGA)*

	L-Glutaminase activity			L-Asparaginase activity		
	K_m (Gln, M)	V_{max} (nmoles/min)	K_i (M)	K_m (Asn, M)	V_{max} (nmoles/min)	K_i (M)
AGA	$4.1 \pm 0.3 \times 10^{-5}$ (N = 8)	0.94 ± 0.09 (N = 8)	NA†	$5.5 \pm 0.8 \times 10^{-5}$ (N = 4)	1.2 ± 0.1 (N = 4)	NA
+Glutamic acid			5.0×10^{-3}			3.1×10^{-3}
+Aspartic acid			4.0×10^{-4}			5.6×10^{-4}
+Asparagine			1.3×10^{-5}			NA
+Ammonium chloride			>1			>1
+6-Diazo-5-oxo-L-norleucine			2.0×10^{-4}			5.0×10^{-4}
+Azaserine			7.3×10^{-3}			1.9×10^{-2}
+AT-125			4.8×10^{-3}			1.6×10^{-2}
+Glutamine			NA			6.0×10^{-5}
SAGA	$8.3 \pm 0.2 \times 10^{-5}$ (N = 3)	1.6 ± 0.0 (N = 2)	NA			

* All inhibition was competitive except for ammonium chloride in which the inhibition appeared to be uncompetitive. Data from four or more experiments are given in means ± S.E., whereas data from two experiments are given in means ± actual range of variation.

† Not applicable.

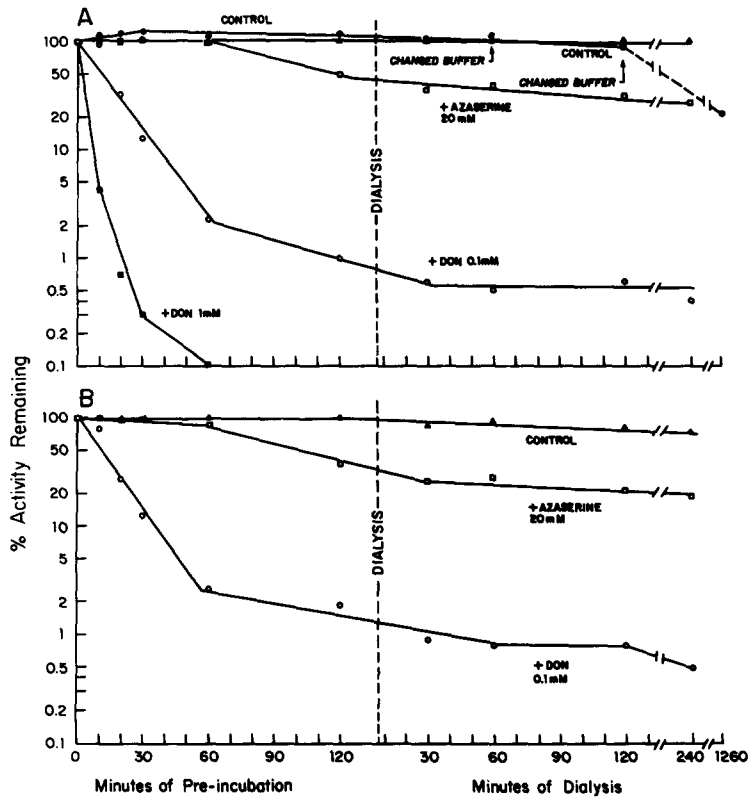

Fig. 3. Time-course of inhibition by DON and azaserine and the reversibility of inhibition by dialysis. Key: (A) glutaminase activity and (B) asparaginase activity. AGA and inhibitor were pre-incubated for the time period indicated. An aliquot was then removed for the assay of enzyme activity by adding labeled substrate and incubating for 15 min. The substrate concentration (i.e. glutamine or asparagine) was fixed at 1.0×10^{-3} M.

Table 2. Inhibition of AGA by aspartate (I_1) and glutamate (I_2)*

Aspartate (mM)	Fractional inhibition (f_i) at glutamate of:					
	0	5 mM	10 mM	15 mM	20 mM	25 mM
0	0	0.682	0.787	0.837	0.871	0.903
0.5	0.594	0.779				
1.0	0.730		0.867			
1.5	0.792			0.905		
2.0	0.849				0.927	
2.5	0.858					0.940

* The theoretical analysis of these data is given in Fig. 4.

AGA and water is the second substrate to react with the enzyme–glutamine or –asparagine complex to form a tertiary complex. Subsequently, ammonia is released as the first product and glutamic acid or aspartic acid as the second product. This scheme is constructed according to the notations and rules of Cleland [14–16], where both glutamic acid and aspartic acid compete with either glutamine or asparagine for the same free enzyme form. Input of water and output of ammonia are connected by a reversible step; however, the high concentration of water (55.5 M) makes the equilibrium overwhelmingly right-sided which may render ammonia as a relatively weak inhibitor.

The observations that glutamate, aspartate, asparagine and glutamine analogs (DON, methionine sulfoximine, azaserine and Acivicin) competitively inhibited glutaminase activity and that aspartate, glutamate, and glutamine analogs competitively inhibited asparaginase activity suggest that all these inhibitors bind with the free enzyme form. Competition of dead-end or product inhibitors with substrate or alternative substrate for the same enzyme form does not necessarily prove that the binding at the enzyme site(s) is mutually exclusive. For instance, Yonetani and Theorell [17] studied inhibition of horse liver alcohol dehydrogenase (EC 1.1.1.1) by *o*-phenanthroline and ADP. These investigators showed that both *o*-phenanthroline and ADP are competitive inhibitors with respect to NAD, but that their bindings were mutually nonexclusive.

The present studies have used the median-effect equation [12, 13] to analyze the data. The linearity and parallelism of the median-effect plot (Fig. 4B) indicated that aspartic acid and glutamic acid are mutually exclusive at the binding site. Michaelis–Menten kinetics was observed for both of these inhibitors since individually and in combination they gave a slope of near unity in the median-effect plots.

The above results indicate that a single ordered bi-bi mechanism and a single set of binding sites are responsible for catalyzing both the L-glutaminase and L-asparaginase reactions of the enzyme, AGA.

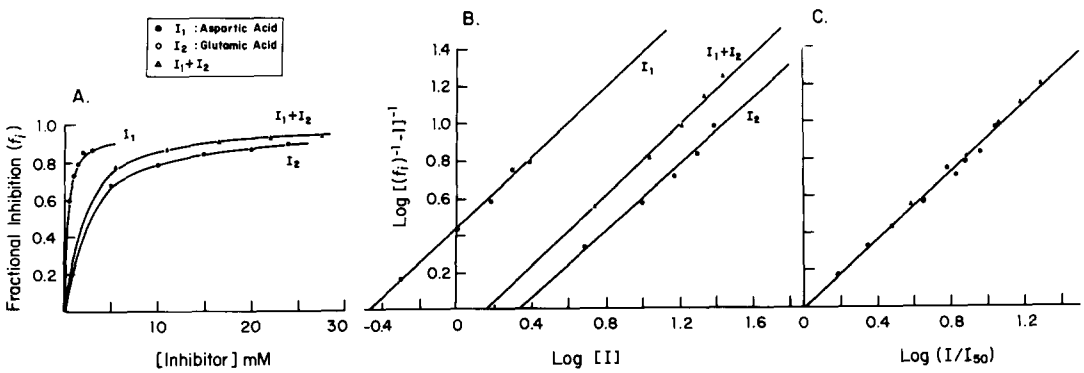

Fig. 4. Inhibition of glutaminase activity by aspartic acid and/or glutamic acid. Key: I_1, aspartic acid (●); I_2, glutamic acid (○); and the combined (total) concentration of I_1 and I_2 (△) in which the concentration of the I_1 + I_2 mixture (in the I_1 and I_2 ratio of 1:10) was varied (see Table 2 for experimental design). The concentration of substrate (glutamine) was fixed at 1.0×10^{-3} M (A) Concentration–effect relationships with each inhibitor alone and in combination. (B) The median-effect plot of the concentration–effect relationship where f_i is the fractional inhibition. For I_1, regression coefficient is 0.996, slope (m_1) is 0.914, and median-effect concentration (I_{50}) is 0.333 mM. For I_2, regression coefficient is 0.993, slope (m_2) is 0.887, and median-effect concentration (I_{50}) is 2.218 mM. For I_1 + I_2, regression coefficient is 0.999, slope ($m_{1,2}$) is 0.930, and median-effect concentration (I_{50})$_{1,2}$ is 1.438 mM. (C) The normalized median-effect plot in which the inhibitor concentration has been normalized by its (or their) I_{50} values. I_{50} is the concentration of inhibitor(s) that is required for 50% inhibition which can be readily obtained by calculating the intercept of the linear regression line with the median-effect axis (i.e. log $[(f_i)^{-1} - 1]^{-1} = 0$). The regression coefficient for the plot is 0.996 and the slope for all data is 0.925.

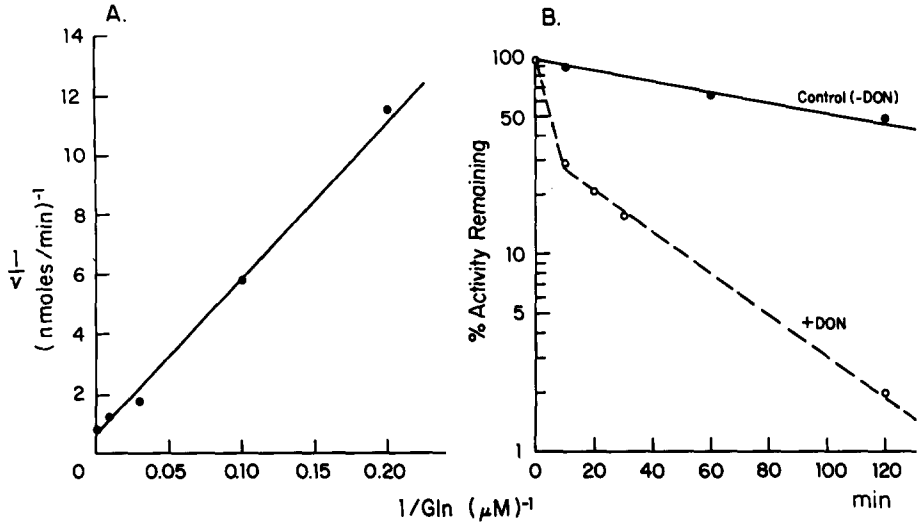

Fig 5. Kinetic properties of succinylated *Acinetobacter* glutaminase-asparaginase (SAGA). (A) The initial rate of glutaminase activity as a function of glutamine concentration. The K_m is 8.2×10^{-5} M and the V_{max} is 1.6 nmoles/min. (B) The time–course of inhibition of glutaminase activity of SAGA by 0.1 mM DON. Enzyme and DON were pre-incubated for the time period indicated. An aliquot was then removed for the assay of glutaminase activity by adding labeled glutamine and incubating for 15 min. The glutamine concentration was fixed at 1.0×10^{-3} M.

The antineoplastic effect of glutaminase-asparaginase *in vivo* is attributed to the reduction of extracellular glutamine which indirectly reduces or depletes intracellular glutamine [7]. A rational approach to enhance the cancer chemotherapeutic effect of AGA (or SAGA) would be the combined use of a glutamine antagonist such as DON which has free access to intracellular sites and is a potent agent that blocks glutamine utilization [18]. The present study shows that DON inhibited AGA or SAGA in a time-dependent and irreversible manner; thus, the selection of the dosage and manipulating the schedule of regimen become major factors in planning combination chemotherapy.

The substrate specificity of AGA has been studied previously at fixed concentration of substrate, and inhibition was carried out at fixed concentrations of substrate and inhibitor [4]. The present paper extends these studies by varying substrate and inhibitor concentrations and calculating kinetic constants. The irreversible inhibition of AGA by [^{14}C]DON was reported previously [9] and showed that the labeling occurred at threonine at position 12 of the molecule. The present paper reports the time–course of inhibition of AGA by DON and azaserine (Fig. 3). The time–course of inhibiting SAGA by DON was also studied (Fig. 5). This information may be useful for combination therapy of SAGA and DON.

Clinical evaluation of SAGA in this Cancer Center showed that the plasma level of glutamate after repeated daily doses of 160–640 I.U./m^2 of body surface reached as high as 2.5 mM [7, 19]. This value is of a magnitude similar to the K_i values of glutamate for SAGA (i.e. 5 mM for L-glutaminase activity and 3.1 mM for L-asparaginase activity). Thus, product inhibition of glutaminase-asparaginase by glutamate is of significance in clinical situations.

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