THE ACTIVE SITE OF L-ASPARAGINASE: DIMETHYLSULFOXIDE EFFECT OF 5-DIAZO-4-OXO-L-NORVALINE INTERACTIONS

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Summary

The asparagine analog, 5-diazo-4-oxo-L-norvaline is a substrate and an irreversible inhibitor of L-asparaginase. Covalent attachment occurs at an increased rate at concentrations of dimethylsulfoxide which reduce the catalytic decomposition of diazo-oxo-norvaline. In 55% dimethylsulfoxide asparaginase is inactivated by diazo-oxo-norvaline (0.05 M) with a $t_{1/2}$ of twelve seconds. In aqueous buffer the rate of diazo-oxo-norvaline decomposition is increased three-fold in the presence of the nucleophile hydroxylamine; this nucleophile also protects the enzyme against inactivation by diazo-oxo-norvaline in the presence of dimethylsulfoxide.

Introduction

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) possesses a tetrameric structure and is composed of four identical subunits of 35,000 molecular weight, each containing an active site for asparagine. The nature of this active site has been explored using stereochemical analogs of asparagine (1,2,3) including 5-diazo-4-oxo-L-norvaline (DONV)[1] which is bound covalently in the region of the active site (4) to a specific peptide of known sequence (5). The primary sequence of this enzyme has been established (6) and confirms the previously determined sequence of cyanogen bromide peptides (7). Ehrman et al., 1971 (8) demonstrated that in the presence of hydroxylamine, asparaginase catalyzed the formation of β-aspartyl hydroxamate from asparagine. In the current report, dimethylsulfoxide (DMSO)[1] has been used to facilitate active-site labelling and the kinetic properties of asparagine and DONV interaction under these conditions are described.

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[1] DONV = 5-diazo-4-oxo-L-norvaline, DMSO = dimethylsulfoxide.

Materials and Methods

L-Asparaginase from E. coli was prepared by Merck, Sharp and Dome (Lyovac; Lot C7941) and supplied as a lyophilized powder of specific activity 300 units per mg. DONV was prepared according to the procedure of Handschumacher, et al., (9). Asparaginase activity was measured in two types of assays: a spectrophotometric assay based on the decrease in diazo ketone absorbance of DONV as described by Jackson and Handschumacher (7), and a coupled enzyme assay using asparagine as substrate as described by Cooney et al., (10). The basis of the coupled enzyme assay is conversion of aspartic acid, resulting from hydrolysis of asparagine, to oxaloacetic acid by glutamate-oxaloacetate transaminase (GOT) and reduction of the oxaloacetate by malic dehydrogenase (MDH). NADH is oxidized by MDH and the decrease in absorbance per minute at 340 nm is measured. The hydrolysis of glutamine and asparagine was monitored by NH_3 release using a modification of the Nessler assay (11). L-Asparagine, L-glutamine and α-ketoglutarate were purchased from Calbiochem, DMSO and hydroxylamine from Fischer Chemical Co.

For studies of rapid rates of inactivation (> 40% DMSO), two five ml glass syringes containing enzyme (0.16 mg/ml) and DONV (0.05 M) each prepared in the same concentration of DMSO were mounted in a dual infusion pump (Harvard Apparatus). The syringes were connected by polyethylene tubing to a 20 guage T-joint and the mixed stream passed through a stainless steel tube (0.058 cm X 19.2) into a cuvette containing the GOT assay system. The contents of the cuvette were rapidly mixed by a small magnetic stirrer and the decrease in absorbance measured after 0.026 ml of reaction mixture had been introduced. In the cuvette the rate of inactivation is reduced at lease 100-fold by dilution with aqueous buffers. The time the reactants are in contact is measured by the velocity with which the mixture passes through the 19.2 cm tube. Using various pump settings, the number of seconds the solution should be collected in the cuvette was determined so that all samples were of the same volume.

Results

The hydrolysis of L-asparagine and L-glutamine by asparaginase in solutions of increasing DMSO concentrations was determined by the Nessler assay (11). Similarly, the hydrolysis of DONV by asparaginase in the presence of increasing DMSO concentration (7) was monitored by observing the change in the A_{274}. In Figure I, the catalytic activity E. coli L-asparaginase with several substrates is expressed as a percentage of the rate in the absence of DMSO. Glutaminase activity is reduced nearly 50% in 20% DMSO, a concentration in which asparagine hydrolysis is greater than 85% of control values. The simultaneous reduction of asparagine and DONV decomposition between 40 and 60 percent DMSO coincides with the sharp increase in covalent attachment of DONV.

The rate of inactivation of asparaginase by DONV in increasing concentrations of DMSO was measured using the coupled assay system. This assay

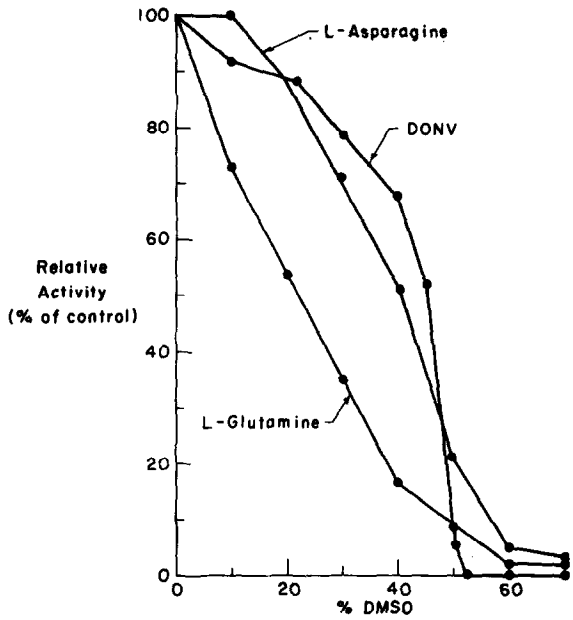

Figure I: The effect of DMSO concentration on hydrolysis of L-asparagine, L-glutamine and DONV decomposition expressed as percent of the activity in the absence of DMSO. All reactions used 0.05 M Na phosphate buffer (pH 7.5) for the aqueous phase; asparagine and glutamine were 3 x 10^{-4} M and DONV was 1 x 10^{-4} M.

measures hydrolysis of asparagine, the preferred substrate, and thus the loss of true enzymatic activity by DONV inactivation is being determined. Samples of asparaginase incubated in the absence of DONV recovered full activity after removal from 30 minute exposure to 55% DMSO by dialysis. To facilitate measurements of the rapid inactivation of asparaginase by DONV in solutions of increasing percent DMSO (30-50%) a "stop-flow" apparatus was used as described in the Materials section. The percent of remaining asparaginase activity as a function of time is shown in Figure II. The rate of inactivation by DONV is a nonlinear function of DMSO concentration, with a $t_{1/2}$ of twelve seconds in 55% DMSO.

The kinetic properties of the enzyme were compared in aqueous and 50% DMSO buffers (Table 1). Although DMSO did not change the Km for inactivation, the Vmax increased 400-fold. Evidence for retention of the catalytic struc-

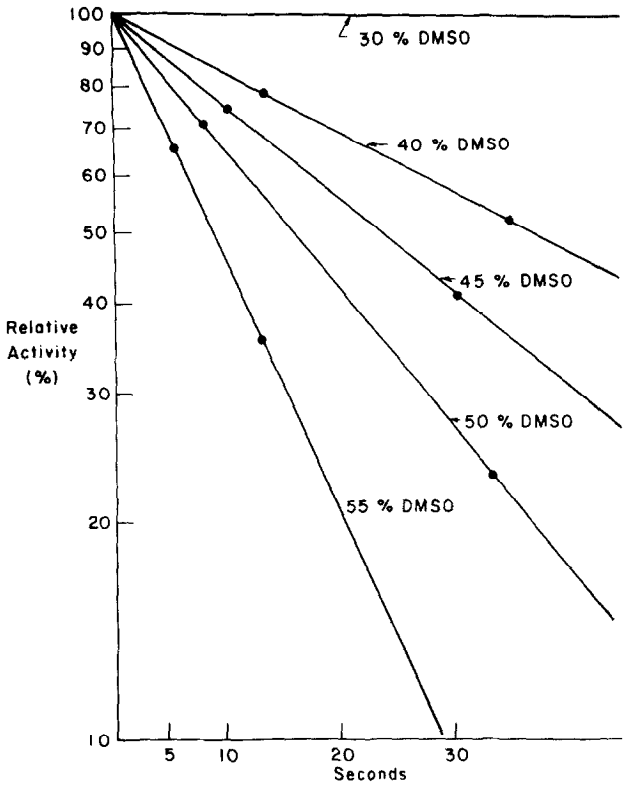

Figure II: Inactivation of asparaginase (0.16 mg/ml) by DONV (0.05 M) in DMSO:phosphate buffer (0.05 M, pH 7.5) solutions. The residual asparaginase activity was determined in a 0.026 ml sample by the GOT assay system.

ture of the active site in the presence of 50% DMSO derives from studies with the strong nucleophile hydroxylamine. This compound accelerates the rate of decomposition of DONV in aqueous buffer with a Km (NH_2OH) of 6 X 10^{-2} M; in 50% DMSO, decomposition of DONV is restored to a rate which is approximately 6% of that in aqueous NH_2OH solutions, and under these conditions the rapid inactivation of enzyme activity previously seen in 50% DMSO is essentially eliminated (Table I). The presence of the normal substrate asparagine (0.05 M) reduced by 50% the rate of decomposition of DONV in 50% DMSO and 0.1 M NH_2OH.

TABLE I

Kinetic Constants

	DONV Decomposition		Asparaginase Inactivation	
	K_m	V_{max} (umoles/min/mg)	$K_{inactivation}$	V_{max} inactivation (umoles enzyme/min)
Phosphate Buffer				
Aqueous Buffer	1×10^{-5} M	6.0	7×10^{-5} M	0.003
+ 0.1 M NH_2OH	1×10^{-4} M	18.0	N.D.	N.D.
50% DMSO - Buffer	---	< 0.3*	9×10^{-5} M	1.2
+ 0.1 M NH_2OH	1×10^{-3} M	1.2	5×10^{-3} M	0.0002

* The rapid inactivation of the enzyme precludes accurate rate measurements.

All reactions were conducted at 37° in 0.05 M sodium phosphate buffer pH 7.5.

Discussion

Increasing concentrations of DMSO cause a nearly parallel loss in the hydrolysis of asparagine and decomposition of DONV by asparaginase from E. coli. The slower rate of glutamine hydrolysis, 3% of that with asparagine, (1) probably reflects the poor fit into the active-site region associated with the extra methylene group which is made even less favorable by DMSO. Whether both DONV and asparagine interact with the same portions of the active site is uncertain since DONV inactivates asparaginase in a concentration of DMSO at which asparagine is no longer hydrolyzed. However, the catalytic site for both DONV and asparagine are not grossly distorted since asparagine can act as a competitive inhibitor of inactivation in 50% DMSO (5). The kinetics of inactivation by DONV (Fig. II) may reflect the sharp decline in the availability of water to act as a nucleophile in this reaction in solutions above 40% DMSO. Activation of the diazo function by the enzyme may generate a highly reactive species which alkylates an amino acid that serves as a nucleophile in the region of the active site, particularly in 50% DMSO when the effective concentration of water is reduced. It is important to note that water and DMSO form a 2:1 adduct (12) and therefore in 50% DMSO the concentration of free water is approximately 25%. Rammler (13) has shown that the catalytic activity of several enzymes increases in 10-40% DMSO but the current report documents a much greater increase in activity and at higher concentrations of DMSO.

Additional evidence that the catalytic site remains functional in 50% DMSO has been obtained using hydroxylamine (Table I). Hydroxylamine clearly accelerates the rate of DONV decomposition in aqueous buffer, and in 50% DMSO hydroxylamine restores the catalytic decomposition of DONV, which in effect protects the enzyme from inactivation. However, the Km value for DONV in buffer and 50% DMSO are significantly larger suggesting that hydroxylamine has an adverse effect on substrate binding. Ehrman et al., 1971 have shown that β-aspartohydroxamate is formed from the reaction of asparagine and hydroxyl-

amine with asparaginase. Similarly, DONV reacts under aqueous conditions to form 5-hydroxy-4-oxo-L-norvaline (HONV) (4), but in 0.1 M hydroxylamine-buffer several products differing from HONV have been detected by thin layer chromatography. These products undergo a rapid polymerization reaction that precludes chemical characterization.

Definitive description of the changes created by DMSO will require physical analysis of the spatial relationships in the active site.

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