Stereospecificity of the Catalytic Reaction of L-Asparaginase†

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ABSTRACT: The stereospecificity of L-asparaginase of *Escherichia coli* was investigated. D-Asparagine is a competitive inhibitor of the hydrolysis of L-asparagine (K_i = 0.63 mM) and is itself hydrolyzed at a slow rate (relative V_{max} = 0.06–0.08) by the native enzyme. However, partial thermal disruption caused a transient increase in the relative activity on D-asparagine. The involvement of two distinct sites in the hydrolysis of the two isomers was eliminated by the application of site-specific protection against inactivation by heat, iodination, and proteolytic enzymes. The reversible change in the specificity of the catalytic site pointed to the possible role of the substrates in guiding the conformation of the enzyme. Indeed both substrates promote the refolding of reversibly denatured enzyme. The two isomers differ, however, in their effect on the conformation of the active site. The difference is reflected in the temperature dependence, and in the parameters, of the catalytic reaction.

It has been reported (Campbell and Mashburn, 1969) that purified preparations of L-asparaginase from *Escherichia coli* B catalyze the hydrolysis of the amide group of D-asparagine. Under optimal conditions, the rate of hydrolysis of D-asparagine was found to be about 6% of that of L-asparagine, and evidence has been presented indicating that both activities are carried out by a single catalytic site. Similar evidence has been recently obtained for the activity of a single L-asparaginase derived from *Escherichia coli* strain A-1-3 on both isomers of asparagine (Nakamura *et al.*, 1971).

In this communication we provide further evidence concerning the stereospecificity of L-asparaginase derived from *E. coli* B. The evidence confirms and extends the previous observations that a single enzyme catalyzes the hydrolysis of D- and L-asparagine. It will be shown, however, that a transient change in the stereospecificity of L-asparaginase accompanies the recovery of the enzyme after thermal disruption. The change in stereospecificity can be explained on the assumption of a single, flexible site. Evidence consistent with that assumption will be presented below. Stereospecific effects of the substrates on the conformation of that site will be described in a subsequent report (Citri *et al.*, 1972).

Materials and Methods

Chemicals. L-ASPARAGINE (monohydrate crystalline) was purchased from Nutritional Biochemical Corp. D-Asparagine (monohydrate crystalline) was purchased from Sigma Chemical Co.

L-ASPARAGINASE from *E. coli* (EC 3.5.1.1 L-asparagine amidohydrolase, 33 IU/mg) was purchased from Worthington Biochemical Corp.

IODINE, POTASSIUM IODIDE, AND SODIUM THIOSULFATE (all C.P. grade) were purchased from Agan Chemical Co.

PRONASE (protease from *Streptomyces griseus*, Type V, purified) was purchased from Sigma Chemical Co.

TRYPSIN, twice crystallized, was purchased from Worthington Biochemical Corp.

Assay of Asparaginase. The standard assays were carried out at 37° in 2.0 ml of Tris-HCl buffer (0.05 M, pH 8.0) containing 2 μmoles of substrate, as described by Meister (1955). To determine initial rates of hydrolysis of L- and D-asparagine, samples were taken every 30 sec and every 2.5–5.0 min, respectively. The liberated ammonia was determined by direct nesslerization and the optical density measured at 420 mμ in a Klett-Summerson colorimeter.

One unit of enzyme releases 1 μmole of ammonium N/min at 37° and pH 8.0.

Iodination and Assay of Residual Activity. Iodination was carried out at 0° in 0.5 ml of 0.04 M Tris-HCl buffer (pH 8.0), containing 0.1 mM I_2, 0.5 mM KI, and the indicated amount of the ligands. The reaction was terminated by injecting 0.05 ml of 0.01 M sodium thiosulfate. In control samples the sodium thiosulfate was added before the iodine.

The residual activity was assayed by direct nesslerization with reagent solutions prewarmed to 37°.

Heat Treatment and Assay of Residual Activity. The treatment was carried out in test tubes immersed in a water bath with the temperature regulated by a Thermomix II immersion thermostat within 0.1°. L-Asparaginase (0.1 unit) was incubated in 0.5 ml of Tris-HCl buffer (0.04 M, pH 8.0), containing the substrate as indicated. The temperature and time of incubation are stated in the description of each experiment. The treatment was terminated by immersing the test tubes in an ice bath for 1 min.

The assay of residual activity was started by the addition of the substrate solution (in 1.5 ml of Tris-HCl, pH 8.0) prewarmed to 37°. In experiments involving protection by a ligand, the ligand left out in the treatment was included in the substrate solution.

Pronase Treatment. Pronase solutions (1 mg/ml) were freshly prepared in 0.01 M $CaCl_2$ prior to each experiment. Proteolysis was carried out at 37° in 0.5 ml of Tris-HCl (0.04 M, pH 8.0) containing 0.1 unit of L-asparaginase and ligands as indicated. The concentration of Pronase used is given in each experiment. The treatment was terminated by the initiation of the assay as described above.

Trypsin Treatment. The procedure for proteolysis by trypsin was identical with that described for Pronase. Trypsin (7.5 μg/ml) was used.

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TABLE I: Partial Inactivation of L-Asparaginase: Protection by Substrates and Specificity of Remaining Enzyme.

Treatment	Protective Ligand	Act. on D-Asparagine[a,b]	Act. on L-Asparagine[a,c]	Ratio D:L
1. None	None	a. 62	107	0.058
		b. 125	182	0.069
2. Pronase (4.0 μg/ml), 3 min, 37°	None	a. 28	48	0.058
		b. 56	94	0.060
	D-AspN (2 mM)	a. 17	30	0.057
3. Trypsin (7.5 μg/ml), 3 min, 37°	None	a. 15	23	0.065
		b. 27	42	0.064
	D-AspN (2 mM)	a. 38	68	0.056
		b. 76	112	0.068
	L-Aspartic acid (10 mM)	a. 35	52	0.067
		b. 73	109	0.067
	D-Aspartic acid (10 mM)	a. 21	33	0.064
		b. 41	65	0.063
4. Iodination (0.1 mM I_2), 30 sec, 0°	None	a. 21	32	0.066
		b. 45	69	0.065
	L-AspN (50 μM)	a. 25	38	0.066
		b. 52	87	0.060

[a] Identical aliquots of the enzyme preparations were assayed with D- and L-asparagine. [b] Δ Klett units determined at (a) 5 min and (b) 10 min. [c] Δ Klett units determined at (a) 30 sec and (b) 60 sec.

Results

The Stereospecificity of Partially Inactivated L-Asparaginase. It is well known that various preparations of L- asparaginase from *E. coli* catalyze the hydrolysis of D-asparagine. There is also excellent evidence that the hydrolysis of L-asparagine and D-asparagine is carried out by the same enzyme (Campbell and Mashburn, 1969; Nakamura *et al.*, 1971). The evidence rests in part on the observation that the two activities are inseparable in the course of purification or in the passage through the circulation of experimental animals. As far as we know no report has been made of attempts to separate the two activities by selective inactivation.

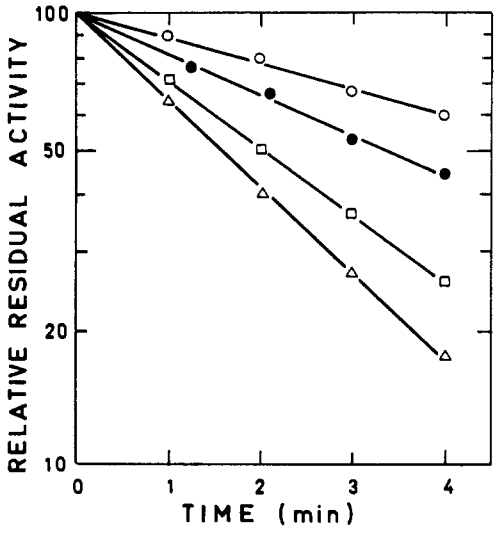

FIGURE 1: Rates of tryptic inactivation of unprotected and protected L-asparaginase. Samples of L-asparaginase (0.1 unit) in 0.5 ml of Tris-HCl (0.04 M, pH 8.0) were treated with 7.5 μg/ml of trypsin in the absence and presence of ligands, as indicated. The residual activity was assayed at the indicated time intervals. For details see Methods. (△) No ligand; (○) D-asparagine (0.15 μmole); (●) L-aspartic acid (0.20 μmole); (□) D-aspartic acid (5.0 μmoles).

The effect of partial inactivation on the stereospecificity of the residual activity is summarized in Table I. Treatments leading to partial loss of activity included iodination (Citri *et al.*, 1972) and proteolysis by trypsin and by Pronase. The conditions of iodination and proteolysis were as described in Methods, and the pertinent details are given in Table I. In all such treated preparations the stereospecificity of the remaining enzyme was not significantly different from that of the untreated preparation.

Protection of L-Asparaginase by Substrates and Products of the Reaction. The possibility of selective inactivation was more specifically tested by applying the following observations. As shown elsewhere (Citri *et al.*, 1972) inactivation of L-asparaginase by iodine can be prevented by L-asparagine, while D-asparagine has no effect.

We presently studied the effect of D-asparagine and the products of the reaction on the rate of inactivation by proteolysis and by heat. The effect of L-asparagine was not tested since this substrate is rapidly broken down by the enzyme at the temperatures used here. Under the conditions described in Methods, the initial rates of inactivation by the proteolytic enzymes or by heat follow first-order kinetics. The effect of the specific ligands on tryptic inactivation is shown in Figure 1. In the presence of D-asparagine the rate of inactivation is slowed down considerably. A somewhat less pronounced protective effect is shown by L-aspartic acid.

TABLE II: Effect of Partial Heat Inactivation on the Stereospecificity of L-Asparaginase.[a]

Protective Ligand	Duration of Exposure to 81° (sec)	Act. on D-Asparagine	Act. on L-Asparagine	Ratio of Act. (D:L)
None	0	30.5	470	0.06
	60	0	0	
	60*	0	0	
D-Asparagine	0	31.5	455	0.07
	30	12.8	64	0.20
	60	11.7	68	0.18
	60*	33.0	440	0.075
L-Asparagine	0	31.5	445	0.07
	30	12.6	66	0.19
	60	9.8	49	0.20
	60*	31.0	475	0.065

[a] Samples of the enzyme in 0.2 ml of Tris-HCl (0.04 M, pH 8.0) were exposed to 81° for 30 or 60 sec in the absence and presence of protective ligands (2.0 μmoles). Treatment was terminated by dilution with 1.8 ml of the buffer prewarmed to 37°. Assay was started immediately by the addition of either substrates (20 μmoles in 0.1 ml of buffer) except for samples marked (*) which were assayed after overnight storage at 4°.

With 0.20 μmole of D-aspartic acid no effect was observed, but at a higher concentration (5.0 μmoles) partial protection against tryptic inactivation is obtained. The results are similar when trypsin is replaced with Pronase, although the specificity of that proteolytic preparation is different. Typical rates of inactivation are shown in Figure 2.

The effect of D-asparagine and the reaction products on the rate of thermal inactivation is shown in Figure 3. At 62°, complete protection is observed with 10 mM D-asparagine, and partial protection is found with the same concentration of the product, D-aspartic acid. The other product, L-aspartic acid, gives excellent protection.

As pointed out before, the effect of L-asparagine could not be tested at 62°. Thus a higher temperature (81°) was used to compare the protective effects of L- and D-asparagine. At that temperature there is no perceptible catalytic reaction with either substrate (see Figure 5, below). As shown in Table II, both ligands are equally effective in protecting the enzyme in these conditions.

The Stereospecificity of Selectively Protected Preparations. In view of the above observations we tested the stereospecificity of preparations partially inactivated in the presence of protecting ligands.

Iodination was carried out at 0° and pH 8.0 as described in Methods. The rate of inactivation in the absence and presence of the substrates was monitored at 20-sec intervals. Under these conditions there was no detectable breakdown of either substrate.

Proteolytic inactivation was tested with two proteases of different specificity, trypsin and Pronase. The rate of inactivation at 37° was followed at intervals of 60 sec for 4 min.

The residual activity of samples partially inactivated by iodination or by proteolytic enzymes was assayed with L-

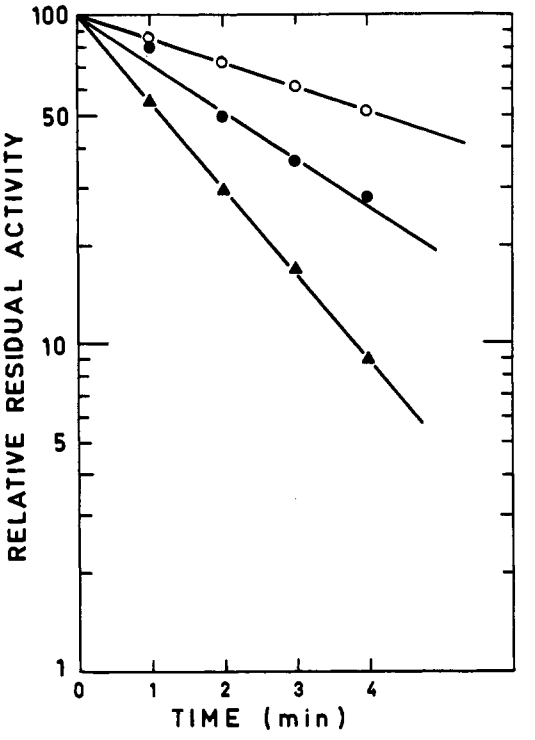

FIGURE 2: Rates of pronatic inactivation of unprotected and protected L-asparaginase. Samples of L-asparaginase (0.1 unit) in 0.5 ml of Tris-HCl (0.04 M, pH 8.0) were treated with 10 μg/ml of Pronase in the absence and presence of ligands as indicated. The residual activity was assayed at the indicated times. See also Methods. (▲) No ligand; (○) D-asparagine (0.04 μmole); (●) L-aspartic acid (1.0 μmole).

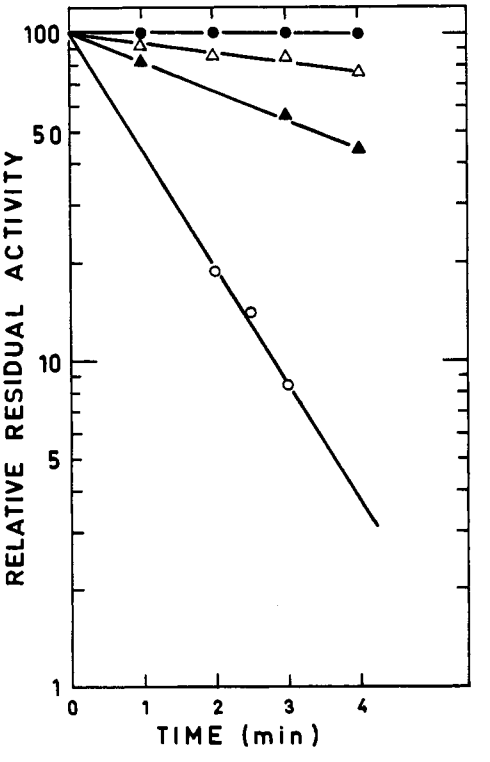

FIGURE 3: Rates of thermal inactivation of L-asparaginase in the absence and presence of ligands. Samples of L-asparaginase (0.1 unit) in 0.5 ml of Tris-HCl (0.04 M, pH 8.0) were exposed to 62° in the absence and presence of the ligands (5.0 μmoles). The residual activity was assayed at the indicated intervals as described in Methods. (○) No ligand; (●) D-asparagine; (△) L-aspartic acid; (▲) D-aspartic acid.

TABLE III: Partial Loss and Recovery of Stereospecificity of L-Asparaginase.

Recov Time[a] (min)	Act.[b] on D-Asparagine	Act.[b] on L-Asparagine	Ratio of Act. (D:L)
1	3.5	9	0.390
2	8	33	0.242
3	15	66	0.227
4	21	108	0.194
5	27	150	0.180
6	36	204	0.176
7	45	258	0.174
8	57	340	0.168
9	72	445	0.162
10	92	643	0.143

[a] The heat treatment (6 min at 66°) was terminated and the residual activity assayed as described in Methods. The assay was stopped at the indicated times by the addition of 0.1 ml of trichloroacetic acid (1.5 M). [b] Expressed as Δ Klett units per minute.

asparagine and D-asparagine as substrates. Typical results are included in Table I, where the relative activity on the two substrates (ratio D:L, last column) is seen to be unchanged by the treatments. Even more significant is the observation that either substrate protects both activities to the same extent.

Thermal inactivation was carried out at 81° and pH 8.0 in the absence and presence of L-asparagine and D-asparagine. The results which are summarized in Table II bring out the following points. In the absence of substrates the treatment leads to irreversible inactivation of the enzyme. Treatment in the presence of either substrate causes incomplete inactivation, which is also fully reversible. Thus after overnight storage in the cold, total recovery of activity was observed. The stereospecificity of the recovered enzyme is not altered by the presence of either D- or L-asparagine in the treatment and is the same as that of the untreated enzyme.

These results are fully consistent with all previous observations and with the results summarized in Table I. Thus even under conditions of specific protection the activities on D- and L-asparagine are inseparable, confirming the conclusion that both activities are carried out by the same enzyme molecule.

However, when the specificity of the residual enzyme was tested immediately after the termination of the treatment, there was a clear indication of a relative increase in the activity against D-asparagine. The increase was the same (threefold) when either substrate was used to protect the enzyme against complete inactivation. This unexpected observation was further investigated as described below.

A Transient Change in the Stereospecificity of L-*Asparaginase.* As shown before, exposure to 81° in the absence of substrates caused complete and irreversible inactivation of the enzyme. However, a milder heat treatment permits recovery of the enzymic activity, even if the enzyme is not protected by substrate. The effect of such treatment on the specificity of the enzyme was investigated as follows. Samples of the enzyme in 0.5 ml of Tris-HCl (0.04 M, pH 8.0) were exposed to 66° for 6 min, and the treatment terminated as described in Methods. The residual activity against L-asparagine and D-asparagine was compared at 1-min intervals for 10 min. The results, which are presented in Table III, show progressive reactivation of the enzyme and a parallel change in stereospecificity. The initial relative activity against D-asparagine is very high as compared to that of the native enzyme and it decreases gradually as the enzyme recovers from thermal disruption.

Role of Substrates in Reactivation of Heat-Denatured Enzyme. The spontaneous recovery of the original activity and specificity after thermal inactivation appears to be very slow as indicated by the data in Table II. We presently examined the effect of the substrates on the rate of recovery. The kinetics of the catalytic reaction with either substrate was followed for 10 min *immediately* after the heat treatment and compared to the kinetics observed after 10 min of incubation in the *absence* of substrates. The results presented in Figure 4a,b, show that the course of the catalytic activity of the heat-treated enzyme, unlike that of the native enzyme, is not linear with time. The rate of the reaction of the heat-treated enzyme increases with time, when either L-asparagine (Figure 4a) or D-asparagine (Figure 4b) is the substrate. Preincubation without substrate for the same length of time (10 min at 37°) has no perceptible effect on the subsequent initial velocity of the reaction or on the rate of recovery. It is thus clear that the substrates promote the reactivation of the reversibly inactivated enzyme.

Temperature Dependence of the Catalytic Reaction with L- *and* D-*Asparagine.* The initial velocities of the reaction at various temperatures were determined as described in Methods.

The Arrhenius plot with L-asparagine and D-asparagine as substrates (Figure 5) shows that the activation energy for both substrates is identical (12.9 kcal/mole) throughout the range of 23–45°. There is, however, a clear difference at temperatures above 45°, where the catalytic activity on D-asparagine begins to decline, while the activity on L-asparagine continues to rise with no change in the activation energy up to 55°. This is followed by a slight decline in the activation energy in the range of 55–65°, and a subsequent sharp onset of inactivation.

In the experiment presented in Figure 5 the assay of activity was carried out with equal amounts of enzyme and longer incubation times for D-asparagine as substrate (see Methods). A similar experiment was done in which the incubation times with both substrates were equal whereas the amounts of enzyme were not. In that experiment ten times as much enzyme was used with D-asparagine as substrate. Samples were taken at the same intervals (every 30 sec) to determine the activity with either substrate. The results were in close agreement with those presented in Figure 5.

The agreement between the results obtained by the two assay procedures confirms that the divergent portion of the Arrhenius plot does not reflect a difference in the conditions of the assay. Rather, it indicates that at temperatures above 50° the catalytic efficiency of the enzyme with D-asparagine as the substrate declines more rapidly. On the assumption of a single site the further implication is that the conformation of the enzyme in complex with D-asparagine differs from that in the presence of L-asparagine.

Competitive Inhibition and Comparison of Constants. The involvement of a single site with a stereospecific change in conformation was also suggested by the following observations. We found that the product of one of the reactions, namely L-aspartic acid, acts as a competitive inhibitor of the enzyme. The inhibition constant, K_i, was determined by

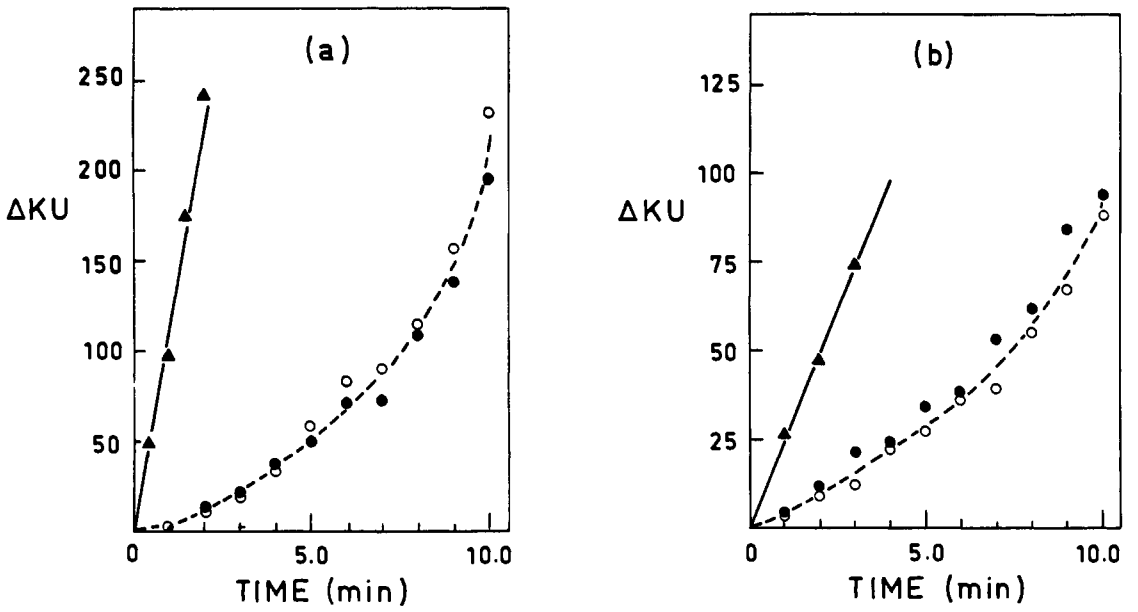

FIGURE 4: Effect of substrates on the reactivation of heated L-asparaginase. Samples of L-asparaginase (0.5 or 1.5 units, see below) in 0.5 ml of Tris-HCl buffer (0.04 M, pH 8.0) were exposed to 66° for 6 min, as described in Methods. The treatment was terminated by dilution with 1.5 ml of the Tris buffer, prewarmed to 37°. The assay was started immediately (○) or after 10 min at 37° (●) by the addition of 20 μmoles of either substrate (see below) in 0.1 ml of the buffer. The assay was terminated at the indicated time intervals by addition of 0.1 ml of trichloroacetic acid (1.5 M). (a) Enzyme, 0.5 unit; substrate, L-asparagine. (b) Enzyme, 1.5 units; substrate, D-asparagine. Broken line, heat-treated enzyme; solid line, untreated control.

the method of Dixon (1953) with L-asparagine and D-asparagine as substrates. The K_i value for L-aspartic acid and L-asparagine was 2.5 mM, whereas the corresponding value with D-asparagine as the substrate was 2.2 mM. The close agreement between the two K_i values is consistent with the involvement of the same site in both reactions.

The competition of the two substrates for the active site was tested by the method of Whittaker and Adams (1949). The relative rates of hydrolysis of D-asparagine and L-asparagine and of the equimolar mixture of both substrates are given in Table IV. The rate of hydrolysis of L-asparagine is reduced in the presence of D-asparagine as expected in a situation when both substrates compete for single site. Furthermore, in such a situation the ratio of the K_m values for the competing substrates can be obtained from these data (see Table IV). In the present case the ratio of K_m (D-asparagine): K_m (L-asparagine) is 10.7. Since the K_m value for L-asparagine is 12 μM, as previously reported (Ho *et al.*, 1970; Broome,

TABLE IV: Relative Rates of Hydrolysis of L-Asparagine and D-Asparagine and of an Equimolar Mixture of Both Substrates.[a]

Substrate (1.0 mM)	Rate of Hydrolysis (μmoles/min)	Ratio of Catalytic Constants
L-Asparagine	$V_L = 0.62$	
D-Asparagine	$V_D = 0.037$	$\frac{V_D}{V_L} = 0.06$
L-Asparagine + D-asparagine	$V_M = 0.57$	$\frac{K_m^D}{K_m^L} = \frac{V_M - V_D}{V_L - V_M} = 10.7$

[a] Initial rates of reaction were determined with 2.0 μmoles of the respective substrates in 2.0 ml of Tris-HCl buffer (0.05 M, pH 8.0). V_L, V_D, and V_M represent the initial rates of hydrolysis of L-asparagine, D-asparagine, and the equimolar mixture of both, respectively. K_m^D and K_m^L represent the respective K_m values for D- and L-asparagine. The ratio K_m^D:K_m^L is derived as suggested by Whittaker and Adams (1949).

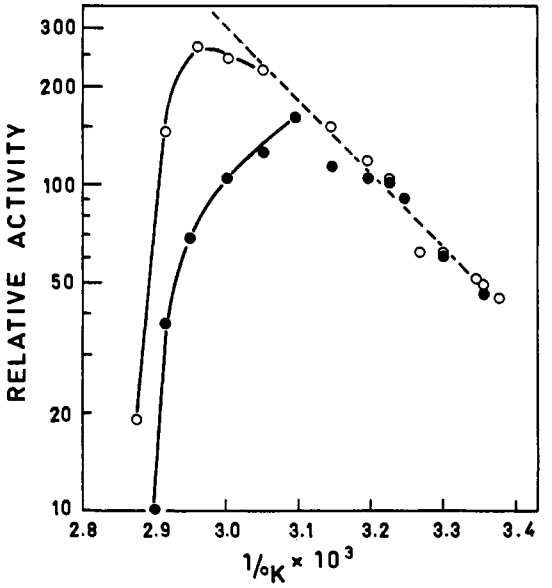

FIGURE 5: Arrhenius plot for the hydrolysis of L- and D-asparagine. All reagents were preincubated at the respective temperatures for 5 min before assay. Relative activities determined from the initial kinetics were plotted on a logarithmic scale against the reciprocal values of the absolute temperature 1/°K. Other details are described in Materials and Methods. Substrates (○) L-asparagine and (●) D-asparagine.

1968), the K_m value for D-asparagine derived from the above data is 128 μM. The K_m value for D-asparagine was also determined directly by the conventional method and found to be considerably lower (42 μM).

The discrepancy between the K_m values obtained independently, and the results based on equimolar substrate mixtures, raised the possibility that the K_m value for one substrate is modified by the presence of the competing substrate (see Discussion). In that case the K_i value for the inhibition of the hydrolysis of L-asparagine by D-asparagine was expected to be significantly higher than the K_m value for D-asparagine. The K_i was determined by the method of Dixon (1953), and corrected for the effect of the hydrolysis of the inhibitor on the observed inhibition according to Webb (1963). The value of 630 μM thus obtained and the anomalous ratio of K_m values derived from the mixed-substrate system are consistent with the notion (Zyk and Citri, 1968a) that the two substrates exert a powerful and antagonistic effect on the conformation of the active site (see Discussion).

Discussion

The study of the interaction of an enzyme with two substrates competing for a single site offers interesting possibilities (Zyk and Citri, 1968a–c). This is particularly true of substrates which are steric isomers and which are consequently metabolized at different rates, as in the case of the hydrolysis of D- and L- asparagine by L-asparaginase of *E. coli*. It is generally accepted that both activities are carried out by the same active site. That was also the point of departure of the present study. We found, however, that a change in stereospecificity is observed when the enzyme preparation is allowed to recover after thermal disruption. In order to interpret that observation it was necessary to reexamine the question whether a single site is involved in the hydrolysis of both isomers of asparagine.

Evidence indicating that one enzyme is involved has been previously reported (Campbell and Mashburn, 1969). The evidence consisted of a series of attempts to separate the two activities by electrofocusing, various other purification procedures or by partial thermal inactivation. No dissociation of the activities was observed in such experiments. Similarly, there was a parallel increase of both activities in the circulation of mice injected with L-asparaginase, which was followed by a parallel decrease as the enzyme was eliminated.

A similar study has been recently reported, in which the specificity of L-asparaginase from a related strain of *E. coli* was investigated (Nakamura *et al.*, 1971). The authors observed that the pH–activity curve for D-asparagine as the substrate differed significantly from that obtained with L-asparagine. They concluded, nevertheless, that a single site is involved in the hydrolysis of both substrates. The evidence for that included the very pertinent observation that both reactions are competitively inhibited by a nonmetabolizable substrate analog, α-*N*-ethylasparagine, and that the same K_i value is obtained in the presence of either substrate. The evidence presented here corroborates and extends the previous observations that the two activities are inseparable under conditions of partial inactivation by heat, iodination, or proteolysis. The possibility remained, however, that distinct sites on a single molecule or on separate, but otherwise nearly identical, molecules, carry out the two reactions.

Although rendered unlikely, that possibility was not entirely eliminated by the observation that the product of one of the reactions, L-aspartic acid, inhibited both reactions to the same extent, as witnessed by the respective K_i values.

A test more stringent than the generally accepted criteria (Dixon and Webb, 1966) was therefore devised. The test depends on the well-known observation that specific ligands (substrates and structurally related compounds) frequently modify the stability or reactivity of the active site. This can be applied to the selective protection or elimination of active sites which differ in their binding characteristics (Citri *et al.*, 1972). In the present case use was made of the protective effect of the substrates and products of the reaction of L-asparaginase. The effect depends on the experimental procedure; thus conditions have been defined where either L-asparagine or D-asparagine or both can protect the enzyme against inactivation. This provides selective means of destruction of unprotected active sites. However, the stereospecificity of the remaining enzyme was totally unaffected by the presence or the structure of the protective ligand. In view of these results, the assumption of two distinct sites would require that such sites be identical not only in stability, but also in their binding specificity for all four ligands tested. Since the two activities proved inseparable under a variety of conditions and the sites are indistinguishable in the most specific tests, the existence of a separate enzyme or catalytic site for the hydrolysis of D-asparagine can be reasonably eliminated.

A further conclusion which must be reached is that the single site involved in both activities is sufficiently flexible to accommodate either isomer, and to undergo reversible changes in stereospecificity.

We shall presently examine the hypothesis that the stereospecificity of the catalytic reaction is determined by the conformative response of the enzyme to the isomer substrates. The term "conformative response" designates the reversible change in the conformation of the active site, which is associated with the binding of the substrate (Citri and Zyk, 1967). In the present case, the conformative response is clearly manifested in the ability of both substrates to promote reactivation of the thermally disrupted enzyme.

The similar efficiency of the two isomers in promoting reactivation (Figure 4) is consistent with the finding that K_m values for the two ligands are quite low and fairly close (12 and 42 μM for L- and D-asparagine, respectively). This indicates that the active site can provide a close fit for either isomer. The details of the conformative response depend, however, on the structure of the ligand, as shown elsewhere (Citri, 1972). The difference in the conformative response to the two isomers is reflected in the divergent portion of the Arrhenius plot (Figure 5) and is consistent with the observed difference in the protective effect of the isomers. It also explains the reported incongruity of the pH–activity curve for the two substrates (Nakamura *et al.*, 1971). Moreover, a stereospecific response in conformation provides the simplest explanation for the remaining observations reported here.

In the course of following the kinetics of reactivation we tested the specificity of the enzyme and found an apparent, transient increase of activity on D-asparagine. This seemingly unexpected observation is in fact entirely consistent with the view that the L isomer is the better substrate because it can induce an optimal conformative response. It is reasonable to assume that an optimal response will require a more precise alignment than a suboptimal response. Hence the efficiency of the hydrolysis of the optimal substrate will be more affected by the partial disruption of the conformation of the enzyme. Indeed, recovery of the catalytic efficiency after heat inactiva-

tion is accompanied by increase in the relative rate of hydrolysis of the L isomer, indicating that more molecules are capable of approaching an optimal conformative response.

The last set of results brings out an apparent anomaly in the dissociation constants for the two isomers. Since the isomers are hydrolyzed at sufficiently different rates, it was possible to view the D isomer as both a competing substrate and as a competitive inhibitor of the hydrolysis of the L isomer. In the first case the K_m ratio was derived from a study of the rate of hydrolysis of an equimolar mixture of the substrates. In the second case the K_i value for D-asparagine was derived from its effect on the rate of hydrolysis of L-asparagine. Since the relative rate of hydrolysis of the inhibitor was taken into account, there was no *a priori* reason why the K_i value should be significantly different from the K_m value for D-asparagine. Yet the ratio $K_i:K_m$ (D-asparagine) gave the anomalously high value of 15. Similarly, the ratio of the K_m values obtained from the mixed substrate system was considerably higher (10.7) than expected on the basis of the independent K_m determinations.

A similar anomaly was observed in the interaction of penicillinase with two competing substrates (Zyk and Citri, 1968a). In that system the anomaly could be clearly traced to the difference in the conformative response to each substrate, which had been previously demonstrated (Citri and Garber, 1961, 1962; Citri and Zyk, 1965).

The interpretation here would be analogous on the assumption that the stereospecific response to one isomer decreases the affinity of the enzyme for the competing isomer. Such a situation is likely to be observed if the conformational transitions are slower than the substrate turnover (Zyk and Citri, 1968a).

In the absence of a more manifest "hysteretic behavior" (Frieden, 1970) a kinetic anomaly such as observed here may be the simplest diagnostic indication of a relatively slow conformational transition (Citri, 1972).

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