Stereospecific Features of the Conformative Response of L-Asparaginase†

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ABSTRACT: The interaction of L-asparaginase with substrates and products was investigated by three independent procedures of differential inactivation. Rates of inactivation of the enzyme by iodination, proteolysis, and heat were determined in the presence of varying concentrations of the specific ligands. The following parameters were derived for L- and D-asparagine and for L-aspartic acid: n, R, and K_{cr}. The first parameter (n) is an index of site interaction; a value of $n = 1.0$ was obtained for all ligands, indicating no interaction between the four binding sites of the enzyme. R is defined as the ratio of the rate of inactivation at saturating ligand concentrations to the rate in absence of ligands. The R values were distinct for each ligand, ranging from $R = 1.0$ (no effect) to $R = 0$ (complete protection). The concentrations at which the half-maximal effect of each ligand is observed (K_{cr}) were determined by the same procedures. The K_{cr} values were compared to the corresponding catalytic constants. The results support the view that the conformative response of L-asparaginase is stereospecific and indicate a further change in conformation in the course of the catalytic reaction.

The stereospecificity of L-asparaginase of *Escherichia coli* has been recently investigated (Campbell and Mashburn, 1969; Wriston, 1971; Nakamura *et al.*, 1971; Citri and Zyk, 1972) and it is now well established that a single site catalyzes the reaction with L- and D-asparagine. We found, however, that a transient change in the stereospecificity is obtained when the enzyme is allowed to recover from thermal disruption. On the basis of this and related observations (Citri and Zyk, 1972) we suggested that the active site responds to the presence of each of its ligands by a characteristic change in conformation (conformative response). The stereospecificity of the conformative response might, furthermore, determine the relative rates of the catalytic reaction with each of the antipode substrates.

An analysis of the conformational aspects of stereospecificity has not been reported before, and L-asparaginase may provide a suitable model. In this report we present a quantitative approach which permits the derivation of parameters describing the conformative response of the enzyme to its ligands. Such parameters can be meaningfully related to the corresponding parameters based on catalytic activity.

Materials and Methods

Chemicals. L-Asparaginase from *E. coli* B (EC 3.5.1.1, L-asparagine amidohydrolase) and trypsin (twice crystallized) were purchased from Worthington Biochemical Corporation.

All other chemicals were C.P. grade commercial preparations.

Assay of L-Asparaginase. The standard assay and the determination of residual activity were as previously described (Citri and Zyk, 1972).

Methods of Treatment. All procedures involving treatment with heat, trypsin, and iodination were as previously described (Citri and Zyk, 1972) unless stated otherwise in the Results.

Results

Inactivation of L-Asparaginase by Iodine. Exposure to iodine at pH 8.0 causes progressive loss of L-asparaginase activity. The rate of inactivation was measured at 20-sec intervals and found to follow first-order kinetics with respect to the enzyme. The rate constant of inactivation (k_0) was derived by applying the integrated equation for first-order kinetics, $k_0 = 1/t \ln E_0/E_t$, where E_0 is the initial enzyme activity and E_t the residual activity at time t. The dependence of k_0 values on the concentration of iodine is shown in Figure 1. In the range of 0–0.05 mM I_2 the k_0 values increase in direct proportion to the iodine used in the treatment. A further increase in the concentration of iodine had, however, little effect, and the concentration of 0.1 mM I_2 was used in subsequent studies.

The effect of pH on the susceptibility of the enzyme to inactivation by iodine is shown in Figure 2. There is no loss of activity at pH values below 5.2, and in the range of pH 5.5–7.3 the extent of inactivation increases reaching a maximum in the alkaline range. Such pH dependence is consistent with the absence of free sulfhydryl groups in the enzyme molecule (Wriston, 1971) (Glossmann and Bode, 1971). On the other hand, the pH profile shown in Figure 2 is characteristic of an inactivation pattern resulting from iodination of tyrosine residues essential for the catalytic activity.

Effect of Substrates on the Rate of Iodination of L-Asparaginase. The effect of L-asparagine and D-asparagine on the susceptibility of the enzyme to inactivation by iodine was determined as shown in Figure 3. As pointed out above, the rate of inactivation in the absence of substrates follows first-order kinetics over the period of observation, giving a linear slope in the semilogarithmic plot. A linear slope is also obtained in the presence of the substrates. (We found no detectable hydrolysis of the substrates under the conditions of the treatment, *i.e.*, within 120 sec at 0°.) In the presence of D-asparagine (up to 2.0 mM) the slope is identical with that of the control (without substrate). In contrast, L-asparagine has a clear protective effect and even at much lower concentrations (20 μM) causes a striking decrease in the rate of inactivation. In the presence of both L- and D-asparagine (20

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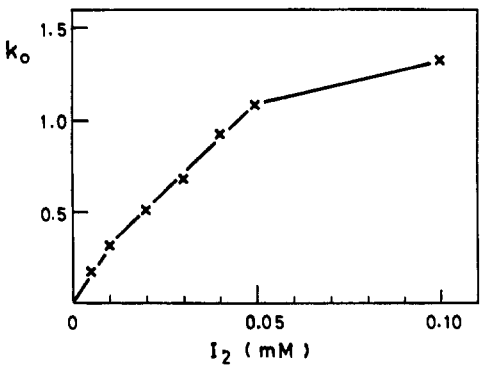

FIGURE 1: Inactivation of L-asparaginase as a function of iodine concentration. Samples of L-asparaginase (0.075 unit) were exposed at 0° to the indicated concentrations of iodine (1:5 in KI). The precooled treatment solutions contained Tris-HCl buffer (80 mM, pH 8.0) and the final volume of each sample was 0.5 ml. The samples were assayed at 20-sec intervals (see Methods) and the k_0 values determined as described in the text.

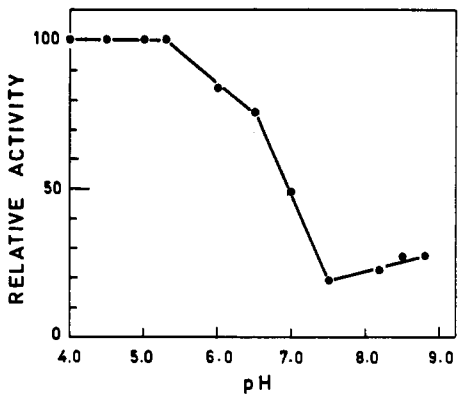

FIGURE 2: Effect of pH on iodination of L-asparaginase. Samples of L-asparaginase (0.075 unit) were exposed to iodinating solutions for 3 min at 0°. The iodinating solutions (total volume 0.5 ml) contained 0.01 μmole of iodine, 0.05 μmole of potassium iodide, and 40 μmoles of the appropriate buffer. The buffers used were: phthalate–NaOH (pH 4.0–5.3), potassium phosphate (pH 6.0–7.5), and Tris-HCl (pH 7.5–8.8). The residual activity of each sample was assayed as described in Methods and plotted as a per cent of the control sample, which was exposed to the buffer alone.

and 30 μM, respectively) the rate of inactivation is intermediate, indicating that at these concentrations D-asparagine effectively competes with L-asparagine for the active site of the enzyme. L-Aspartic acid had a protective effect (see below) whereas D-aspartic acid (up to 2.0 mM) had no effect in either protecting or preventing protection by L-asparagine.

Determination of F_{cr} Values. Substrate-induced modification of the susceptibility of an enzyme to iodination was previously shown to provide a sensitive tool for a quantitative study of the effect of the substrate on the conformation of the enzyme (Zyk and Citri, 1967). In a series of observations on the interaction of penicillinase with various substrates, the change in iodine sensitivity was clearly correlated with the *conformative response, i.e.*, the substrate-induced change in the conformation of the active site (Citri and Zyk, 1967).

The evidence for a conformative response in the L-asparaginase system has been presented elsewhere (Citri and Zyk, 1972), and it is reasonable to assume that resistance to iodination is a valid parameter of the conformative response to L-asparagine (see Discussion). As expected, the rate of inactivation was found to decrease with increasing substrate throughout the range of subsaturating substrate concentrations tested.

In relating the conformative response to the concentration of the ligand we shall use the following parameters. The fraction of the enzyme which has responded in a change in conformation at a given ligand concentration is termed F_{cr}. The molar concentration of the ligand which causes half-maximal conformative response is designated as K_{cr}. Thus, by definition, $F_{cr} = 0.5$ is observed when the ligand concentration (M) is equal to K_{cr}.

We have shown previously (Zyk *et al.*, 1969) that if the conformative response increases the stability of the enzyme, F_{cr} can be derived from

$$F_{cr} = \frac{1 - (V/V_0)}{1 - R} \qquad (1)$$

where V and V_0 are the rates of inactivation in the presence and absence of the ligand, respectively; R is the ratio V_s/V_0, where V_s is the rate of inactivation in the presence of saturating ligand.

The relative rates of inactivation (V/V_0) for a series of concentrations of L-asparagine were determined from slopes obtained as shown in Figure 3, and are listed in Table I. The relative rates of inactivation in the presence of saturating L-asparagine gave $R = 0.23$. Table I lists the F_{cr} values obtained by substituting the respective data for V/V_0 and R in eq 1.

Determination of K_{cr} for L-Asparagine. The concentration of L-asparagine which gave a half-maximal protection against inactivation by iodine was determined as shown in Figure 4a. In terms of conformative response, at that concentration half of the enzyme is found in the ligand-induced conformation ($F_{cr} = 0.5$). Thus the molar concentration of the ligand which is required for half-maximal response and has

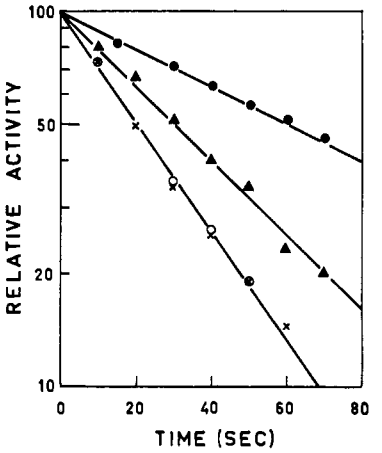

FIGURE 3: Effect of D- and L-asparagine on iodination of L-asparaginase. Samples of L-asparaginase (0.075 unit) were exposed at 0° to an iodinating solution consisting of 0.1 μmole of I_2, 0.5 μmole of KI, 40 μmoles of Tris-HCl buffer (pH 8.0), and substrates as indicated, in a total volume of 0.5 ml. The reaction was terminated at the indicated time intervals by the addition of 1.0 μmole of sodium thiosulfate. The residual activity (see Methods) is plotted as per cent of the activity of the corresponding uniodinated samples. Substrates present are (○) D-asparagine (1 μmole), (●) L-asparagine (0.01 μmole), (▲) D- and L-asparagine (0.015 and 0.010 μmole, respectively), and (×) none.

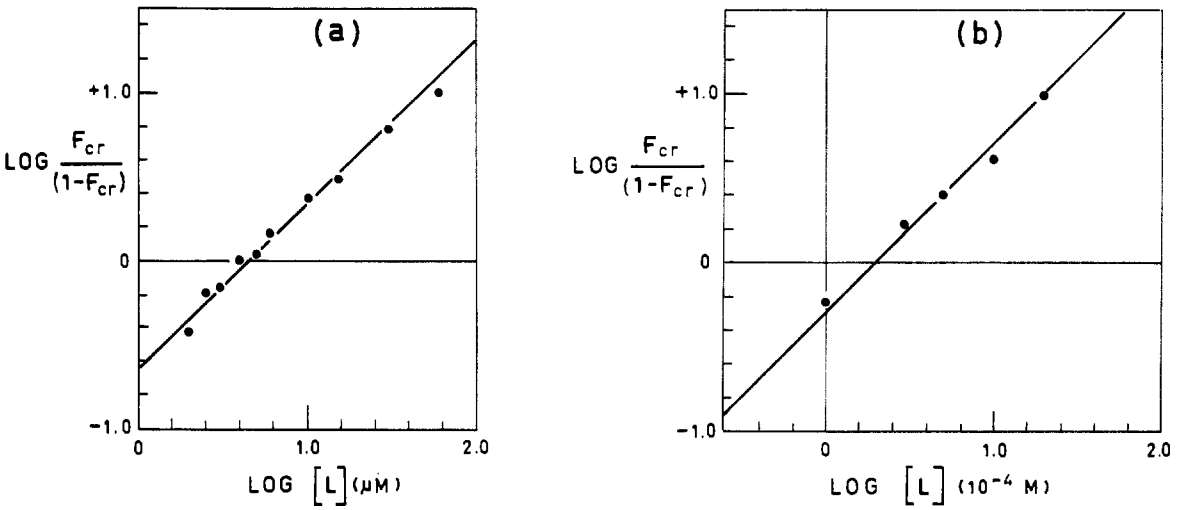

FIGURE 4: Determination of the K_{cr} value and the number of interacting sites in the conformative response. The values listed in Table I are plotted as log $[F_{cr}/(1 - F_{cr})]$ *vs.* log [L], where [L] represents the concentration of (a) L-asparagine and (b) L-aspartic acid. The slopes, $n = 1.0$, show no interaction between the binding sites. The horizontal intercepts are equal to log K_{cr}. The K_{cr} values are 4.2 and 180 μM for L-asparagine and L-aspartic acid, respectively. For other details, see text.

been termed K_{cr} can be obtained by plotting the reciprocals of F_{cr} against the reciprocals of ligand concentrations (Zyk *et al.*, 1969). For the present determinations we employed an alternative graphic method which also provides information on the number of sites interacting in the conformative response. The method is analogous to the Hill plot, and it is based on the following considerations. By definition, the fraction of enzyme showing conformative response to ligand L is

$$F_{cr} = \frac{[L]}{K_{cr} + [L]} \tag{2}$$

TABLE I: Relative Rates of Inactivation and F_{cr} Values in the Presence of L-Asparagine and L-Aspartic Acid.[a]

L-Asparagine (μM)	V/V_0	F_{cr}	L-Aspartic Acid (mM)	V/V_0	F_{cr}
2.0	0.79	0.27	0.1	0.69	0.37
2.5	0.70	0.39	0.3	0.47	0.62
3.0	0.69	0.41	0.5	0.40	0.71
4.0	0.62	0.50	1.0	0.32	0.80
5.0	0.60	0.52	2.0	0.23	0.91
6.0	0.54	0.60	10.0	0.14	1.00
10.0	0.44	0.74	50.0	0.15	1.00
15.0	0.42	0.75			
30.0	0.34	0.86			
60.0	0.30	0.91			
2000.0	0.23	1.00			

[a] Samples of L-asparaginase (0.075 unit) were iodinated as described in Figure 3, in the presence of increasing concentrations of L-asparagine or L-aspartic acid. The relative rates of inactivation (V/V_0) were calculated from the linear slopes obtained by plotting log residual activity as a function of time of treatment (*cf.* Figure 3). The F_{cr} values were derived as described in the text.

and a plot of F_{cr} as a function of ligand concentration should follow a rectangular hyperbola.

If, however, more than one site in the molecule binds L, and if there is interaction between the sites which modifies the conformative response, the shape of the curve will change. The following expression which is analogous to the Hill equation for hemoglobin (Monod *et al.*, 1963) includes this possibility since the exponent n reflects the number of interacting sites. Similarly

$$F_{cr} = \frac{[L]^n}{K_{cr} + [L]^n} \tag{3}$$

$$1 - F_{cr} = 1 - \frac{[L]^n}{K_{cr} + [L]^n} = \frac{K_{cr}}{K_{cr} + [L]^n} \tag{4}$$

By dividing eq 3 by eq 4 and converting to the logarithmic form, we obtain

$$\log\left(\frac{F_{cr}}{1 - F_{cr}}\right) = n \log [L] - \log K_{cr} \tag{5}$$

Hence, a plot of log $[F_{cr}/(1 - F_{cr})]$ *vs.* log [L] will give a straight line with a slope of n and a horizontal intercept equal to log K_{cr}. This is illustrated in Figure 4a for F_{cr} values taken from Table I. The K_{cr} value thus obtained is 4.2 μM, and the slope which is equal to 1.0 shows that no interaction between the binding sites is involved in the conformative response to L-asparagine.

The same procedure was used for the determination of K_{cr} for L-aspartic acid, which was also found to protect the enzyme against inactivation by iodine (Table I). A straight line with a slope of $n = 1.0$ was obtained, and the K_{cr} value was 180 μM (Figure 4b).

Determination of K_{cr} for D-*Asparagine.* In contrast to L-asparagine, the D isomer has no effect on the rate of inactivation of the enzyme by iodine. Since both isomers compete for the same site (Citri and Zyk, 1972), D-asparagine should prevent the protective effect of L-asparagine. This is

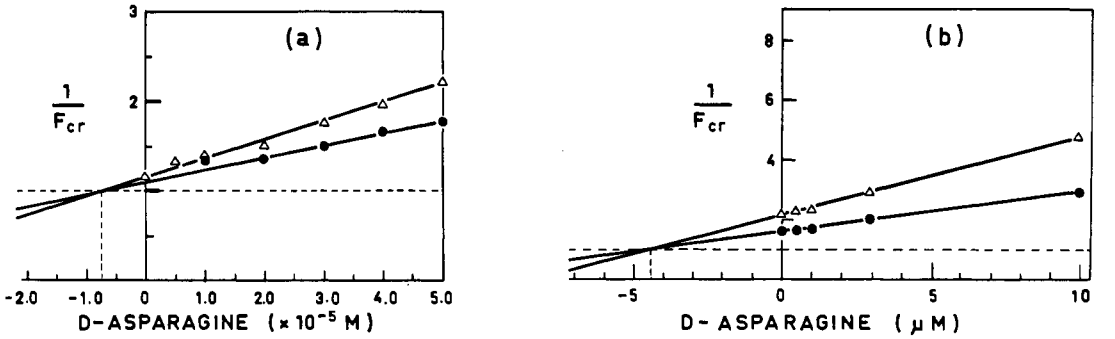

FIGURE 5: Determination of K_{cr} for D-asparagine. (a) A graphic presentation of the data listed in Table II. The reciprocals of the F_{cr} values are plotted against the respective concentrations of D-asparagine, at two concentrations of L-asparagine (△, 10 μM; ●, 20 μM). The K_{cr} for D-asparagine derived from the graph is 7.5 μM. (b) The F_{cr} values are from Table III and were obtained with two concentrations of L-aspartic acid (△, 100 μM; ●, 200 μM) and varying concentrations of D-asparagine. The K_{cr} for D-asparagine in the presence of L-aspartic acid is 4.4 μM.

indeed shown in Figure 3. The competitive relationship between the two isomers is further illustrated by the data in Table II, which lists the results obtained with two concentrations of L-asparagine and varying concentrations of D-asparagine. The rates of inactivation by iodination were determined as before (see Table I) and found to depend on the ratio of the two isomers present in the treatment. These results indicated that a K_{cr} value for D-asparagine could be derived in strict analogy to the derivation of a competitive inhibitor constant (K_i) in a catalytic reaction.

Indeed, a plot of the reciprocal F_{cr} values against the concentration of D-asparagine gives a straight line, which is intersected by a second straight line obtained with a different concentration of the L isomer (Figure 5a). The plot, which is an adaptation of the graphical method of Dixon (1953), gives directly the $-K_{cr}$ value for the inhibitor at the point of intersection of the two lines. This point is at the height of $1/F_{cr} = 1.0$, confirming the purely competitive character of the interaction as well as the value of $K_{cr} = 7.5$ μM.

The same procedure was used for the determination of K_{cr} for D-asparagine when L-aspartic acid was the protective ligand. The data are listed in Table III and the graphic derivation is given in Figure 5b. The K_{cr} value obtained from the graph is 4.4 μM.

Determinations of K_{cr} based on Thermostability. Alternative methods for the determination of K_{cr} for D-asparagine and for L-aspartic acid have been suggested by the observation that these ligands protect L-asparaginase against thermal and proteolytic inactivation.

The rates of thermal inactivation at 62° were determined at 1-min intervals in the presence of varying concentrations of D-asparagine. The rates were found to follow first-order kinetics as shown elsewhere (Citri and Zyk, 1972). The relative rates of inactivation (V/V_0) were derived from the linear slopes obtained in a semilogarithmic plot, and the F_{cr} values calculated as before. The results are listed in Table IV. The graphic derivation of the n and K_{cr} values from these data is given in Figure 6. The corresponding data for L-aspartic acid (Table IV) have been obtained in the same manner and the graphic derivation of n and K_{cr} for this ligand is also included in Figure 6. The two sets of data give an excellent

TABLE II: Relative Rates of Inactivation and F_{cr} Values in the Presence of D- and L-Asparagine.[a]

L-Asparagine (μM)	D-Asparagine (μM)	V/V_0	F_{cr}
10	0	0.44	0.76
	5	0.42	0.75
	10	0.45	0.72
	20	0.49	0.66
	30	0.56	0.57
	40	0.61	0.51
	50	0.65	0.45
20	0	0.39	0.79
	10	0.42	0.75
	20	0.44	0.73
	30	0.49	0.66
	40	0.54	0.60
	50	0.57	0.56

[a] L-Asparaginase (0.075 unit) was iodinated at 0° in the presence of 40 μmoles of Tris-HCl (pH 8.0), 0.1 μmole of iodine, 0.1–0.2 μmole of L-asparagine, and 0.1–0.5 μmole of D-asparagine (see Methods). For other details, see Table I and text.

TABLE III: Relative Rates of Inactivation and F_{cr} Values in the Presence of D-Asparagine and L-Aspartic Acid.[a]

L-Aspartic Acid (μM)	D-Asparagine (μM)	V/V_0	F_{cr}
200	0	0.61	0.46
	0.5	0.63	0.44
	1.0	0.63	0.43
	3.0	0.71	0.34
	10.0	0.82	0.21
400	0	0.47	0.62
	0.5	0.49	0.61
	1.0	0.50	0.59
	3.0	0.58	0.50
	10.0	0.69	0.35

[a] L-Asparaginase (0.075 unit) was iodinated at 0° in the presence of 40 μmoles of Tris-HCl (pH 8.0), 0.1 μmole of iodine, 2.0–4.0 μmoles of L-aspartic acid, and 0.005–0.10 μmole of D-asparagine (see Methods). For other details, see Table I and text.

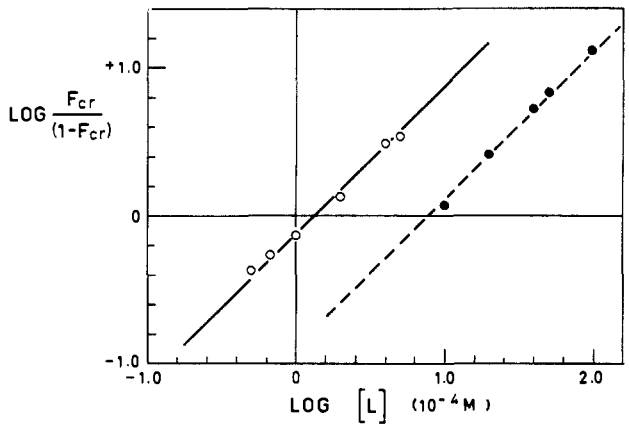

FIGURE 6: Determination of K_{cr} and n values based on differential heat inactivation. Data from Table IV are plotted as $\log[F_{cr}/(1 - F_{cr})]$ *vs.* log [L], where [L] represents the concentration of (○) D-asparagine and (●) L-aspartic acid. The slopes are $n = 1.0$, and the horizontal intercepts give the K_{cr} values of 130 and 790 μM for D-asparagine and L-aspartic acid, respectively.

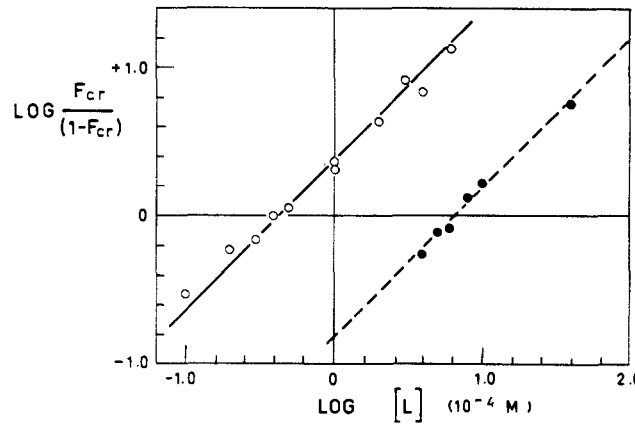

FIGURE 7: Determination of K_{cr} and n values based on differential tryptic inactivation. Data from Table V are plotted as in Figure 6. The slopes and intercepts give the following values: $n = 1.0$ for both ligands; $K_{cr} = 43$ μM for D-asparagine (○); $K_{cr} = 650$ μM for L-aspartic acid (●).

fit with slopes of $n = 1.0$. The K_{cr} values obtained from the respective horizontal intercepts are 130 μM for D-asparagine and 790 μM for L-aspartic acid.

Determinations of K_{cr} Based on Proteolytic Inactivation. As shown elsewhere (Citri and Zyk, 1972) L-asparaginase is readily inactivated by trypsin, and the initial rate of inactivation follows first-order kinetics. A decrease in that rate was observed in the presence of D-asparagine or L-aspartic acid. The initial kinetics of inactivation were followed at 1-min intervals in the presence of varying concentrations of the ligands. The relative rates of inactivation and the F_{cr} values were calculated as shown above and are presented in Table V. The graphic derivation given in Figure 7 confirms that $n = 1.0$ for both ligands. The respective K_{cr} values are 43 μM for D-asparagine and 650 μM for L-aspartic acid.

Comparison of Catalytic and Conformative Response Parameters. The parameters of conformative response obtained as described in the previous sections are listed in Table VI for comparison. The K_{cr} values for D-asparagine and L-aspartic acid which have been obtained by three independent procedures point to a significant difference between iodination and other methods. The values obtained with thermal and proteolytic procedures of inactivation are in fairly close agreement, whereas the K_{cr} values derived by iodination are considerably lower.

On the other hand, the K_i values are anomalously high as pointed out in a previous communication (Citri and Zyk, 1972). By contrast, the "K_i" values derived from the competitive inhibition of the conformative response listed as "indirect" K_{cr} values show no such anomaly. Finally, the individual differences in the R values will be noted. The possible significance of these divergent data will be discussed below.

Discussion

In a previous communication (Citri and Zyk, 1972) we have presented evidence that the same site in L-asparaginase

TABLE IV: Rates of Thermal Inactivation and F_{cr} Values in the Presence of D-Asparagine and L-Aspartic Acid.[a]

D-Asparagine (mM)	V/V_0	F_{cr}	L-Aspartic Acid (mM)	V/V_0	F_{cr}
0.05	0.79	0.21	1	0.55	0.54
0.067	0.75	0.25	2	0.40	0.72
0.1	0.58	0.42	4	0.30	0.84
0.2	0.37	0.63	5	0.28	0.87
0.4	0.25	0.75	10	0.24	0.93
0.5	0.23	0.77			

[a] Samples of L-asparaginase (0.1 unit) in 0.2 ml of Tris-HCl buffer (0.04 M, pH 8.0) were exposed to 62° in the presence of varying concentrations of the ligands, as indicated. The rates of inactivation were determined as previously described (Citri and Zyk, 1972). The relative rates of inactivation (V/V_0) and the F_{cr} values were derived as in Table I.

TABLE V: Rates of Proteolytic Inactivation and F_{cr} Values in the Presence of D-Asparagine and L-Aspartic Acid.[a]

D-Asparagine (mM)	V/V_0	F_{cr}	L-Aspartic Acid (mM)	V/V_0	F_{cr}
0.01	0.84	0.23	0.4	0.72	0.35
0.02	0.74	0.37	0.5	0.65	0.44
0.03	0.71	0.41	0.6	0.59	0.45
0.04	0.65	0.50	0.8	0.48	0.57
0.05	0.63	0.53	1.0	0.44	0.62
0.10	0.53	0.67	4.0	0.12	0.85
0.20	0.43	0.81			
0.30	0.38	0.89			
0.40	0.39	0.87			
0.60	0.35	0.93			

[a] Samples of L-asparaginase (0.1 unit) in 0.5 ml of Tris-HCl buffer (0.04 M, pH 8.0) were treated with trypsin (7.5 μg/ml) in the presence of varying concentrations of the ligands, as indicated. The rates of inactivation were determined as previously (Citri and Zyk, 1972). For other details, see Table I and text.

catalyzes the hydrolysis of D- and L-asparagine. We have shown that the site is flexible and demonstrated that the stereospecificity of that site can be reversibly altered. On the basis of that and additional evidence we suggested that each of the two isomers has a distinct effect on the conformation of the active site. In other words, the conformative response (*i.e.*, the ligand-induced change of conformation) is determined by the configuration of the substrate. We further suggested that the rate of the catalytic reaction with each isomer is determined by the specific response to that isomer. In those terms L-asparagine is the better substrate because the conformative response to that isomer is more favorable for the catalytic reaction. More generally, the stereospecificity of a catalytic reaction may be determined by the stereospecificity of the conformative response of the enzyme.

In order to explore this possibility in L-asparaginase, it was necessary to describe in quantitative terms the interactions of this enzyme with its ligands. In this report we analyzed data derived from studies of "differential inactivation," *i.e.*, inactivation rates measured in the absence and presence of ligands. The approach of differential inactivation is based on the assumption that a conformative response is very likely to alter the stability of the enzyme molecule. Conversely, the change in the rate of inactivation of an enzyme which is observed in the presence of a specific ligand may serve as a sensitive and accurate criterion for the conformative response to that ligand. The more trivial alternative, whereby the protective effect of a ligand is simply due to shielding of a susceptible site, can be reasonably ruled out by applying several independent procedures of differential inactivation (Zyk *et al.*, 1969, 1970). The procedures applied here, namely iodination, proteolysis, and thermal inactivation, are indeed unrelated; thus a parallel change in the rate of inactivation would not be expected unless a change in the conformation of the enzyme was involved.

The graphic method of derivation of the parameters of conformative response proposed here is particularly suited for the detection of cooperative effects between sites. L-Asparaginase is known to be composed of subunits (Frank *et al.*, 1970; Kirschbaum *et al.*, 1969; Greenquist and Wriston, 1970), and there is good evidence that there are four active sites per molecule (Jackson and Handschumacher, 1970). The question of interaction between the sites has been naturally raised, and results recently obtained with a substrate analog, 5-diazo-4-oxa-L-norvaline, suggested negative cooperativity in binding of this analog (Jackson and Handschumacher, 1970). Our results show conclusively that there is no cooperativity in the binding of L-asparagine, D-asparagine, or L-aspartic acid. Thus, values of $n = 1.0$ were obtained for these ligands by all three procedures of differential inactivation. The K_{cr} values obtained by the above procedures are compared in Table VI. The values based on iodination are lower than others, and very likely the most accurate (Citri and Zyk, 1965).

The higher values obtained by the thermal inactivation method are believed to reflect the fact that binding is less efficient at the high temperature of 62°. A related observation has been so interpreted by Theorell (Theorell and Tatemoto, 1971). Similarly, proteolytic enzymes may perceptibly impair the efficiency of binding, by perhaps splitting bonds not protected by the conformative response.

A further insight into that question may be provided by comparing the R values. As shown above, the parameter R is defined as the ratio of the rate of inactivation at saturating ligand concentrations to the rate of inactivation in the absence of ligands. When $1.0 > R > 0$ protection by the ligand is observed, but the protection is incomplete. A difference in R observed with two ligands, under otherwise identical conditions, means that the two ligands differ in their ability to stabilize the enzyme and suggests a difference in conformative response. The most striking illustration in the present case is provided by the contrasting iodination results obtained with D- and L-asparagine. The K_{cr} values are low and quite close confirming the earlier conclusion (Citri and Zyk, 1972) that the active site can accommodate both isomers with nearly equal ease. However, the optimal substrate (L-asparagine) shows a good protective R value (0.23) whereas the poor substrate (D-asparagine) has no protective effect ($R = 1.0$).

TABLE VI: Summary of Catalytic and Conformative Parameters.

Parameter (μM)	L-Asparagine	D-Asparagine	L-Aspartic Acid
K_i		630	2500[a] 2200[b]
K_m (indirect)	72[c] 15[d]	70[d]	
K_m (direct)	12	42	
K_{cr} (heat)		130 ($R = 0$)	790 ($R = 0.17$)
K_{cr} (proteolysis)		43 ($R = 0.30$)	650 ($R = 0.08$)
K_{cr} (indirect) (iodination)	2.7[e]	7.5[e] 4.4[d]	230[e]
K_{cr} (direct) (iodination)	4.2 ($R = 0.23$)	($R = 1.0$)	180 ($R = 0.15$)

[a] Substrate, L-asparagine (Citri and Zyk, 1972). [b] Substrate, D-asparagine (Citri and Zyk, 1972). [c] Derived from Dixon plot, as suggested by Dixon and Webb (1966); inhibitor, D-asparagine. [d] As in footnote *c*; inhibitor, L-aspartic acid. [e] From Figure 5a.

The evidence reviewed so far is thus fully consistent with the stereospecific conformative response suggested by the catalytic behavior of the enzyme. However, the most interesting conclusion from the data compared in Table VI is likely to emerge from the striking discrepancy between the K_i and the corresponding K_{cr} values.

We have shown that in the presence of competing ligands the apparent dissociation constants based on the catalytic activity (K_m and K_i) are anomalously high (Citri and Zyk, 1972). The observation was entirely consistent with the assumption that the conformational transitions are slower than the turnover of the ligands (Zyk and Citri, 1968a,b; Frieden, 1970). Thus, the conformative response induced by one ligand may lower the affinity of the active site for the competing ligand. A further illustration of this point is provided by the example of L-aspartic acid and presented here. The K_{cr} based on protection against iodination is 180 μM, whereas the K_i with either D- or L-asparagine as the substrate is over ten times that value.

In the present report we derived, in addition, parameters for the competitive inhibition of the conformative response. Unlike the corresponding K_i values which are anomalously high, the K_{cr} values based on competition show no such deviation (Table VI). Since the K_{cr} values are based on non-

catalytic interactions, the anomalous values appear to be restricted to the conditions of the catalytic reaction. It appears indeed (N. Citri and N. Zyk, in preparation) that the change is synchronous with the catalytic activity ("syncatalytic," Christen and Riordan, 1970) and that a distinction may now be possible between the stereospecific conformative response associated with the stage of binding, and the further, syncatalytic response which accompanies the catalytic activity of L-asparaginase.

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Determination of the h-Protein in Transformable and Transformed Cells in Culture†

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ABSTRACT: The h-protein, to which carcinogenic hydrocarbons are specifically bound, has been demonstrated in transformable cells in culture. The h-protein is present in cells of all the various species of rodents so far examined, but not in human fibroblasts. The isolation procedures for the h-protein consisted of three steps: Sephadex G-25 and DEAE-cellulose column chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis, by which the h-protein subunit was isolated as a sharp radioactive peak with a molecular weight of 22,000. The binding of hydrocarbons to the h-protein was firm, presumably covalent. When the binding to the h-protein of K-region derivatives of dibenz[*a*,*h*]anthracene was compared in mouse embryonic cells, the epoxide was bound about eight times more than the parent hydrocarbon, while with the *cis*-dihydrodiol and phenol negligible radioactivity was found in the h-protein region. This suggests that metabolism of hydrocarbon is involved in the binding to the h-protein. In transformed cells, a distinct band was present at a molecular weight of 22,000 (the h-protein region) in the same quantity as that of the normal or control cells, but no significant radioactivity was found in this band.

Studies of the interaction between carcinogenic hydrocarbons and macromolecules have been carried out extensively in mouse skin (Wiest and Heidelberger, 1953; Heidelberger and Moldenhauer, 1956; Abell and Heidelberger, 1962; Brookes and Lawley, 1964; Goshman and Heidelberger, 1967) and in systems of chemical carcinogenesis *in vitro* (Kuroki and Heidelberger, 1971). It now appears that the carcinogens are bound covalently to DNA, RNA, and proteins both *in vivo* and *in vitro*. However, the question remains open as to which of these interactions, if any, is most significant in carcinogenesis. A change produced in the genetic material could be causal in the heritable processes of transformation and mutation. However, as postulated by Pitot and Heidelberger (1963), it is also conceivable that the binding of a carcinogen to a specific protein (possibly a repressor) could give rise to a perpetuated change. This theory has been revived and amplified by Huebner and Todaro (1969). Early

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