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MODIFICATION OF L-ASPARAGINASE EC-2 BY HOMOLOGOUS ANTIBODIES

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SUMMARY

1. Antibodies to L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) decrease the activity, and increase the stability, of the enzyme to thermal and proteolytic inactivation. A similar effect is observed with the corresponding Porter fragments.

2. Several catalytic and conformative response parameters have been determined for the enzyme–antibody complex and compared with those of the native enzyme. The activation energy (12.9 kcal/mole) and the pH and temperature optima are identical for both preparations. The K_m values for L- and D-asparagine and the complex (68 and 120 μM, respectively) are slightly higher than those of the free enzyme. The K_{CR} values, however, are identical (4.4 and 180 μM for L-asparagine and L-aspartic acid, respectively).

3. The relative rate of hydrolysis of D-asparagine by the free enzyme (0.07) is slightly increased (0.11), and there is little change in the competitive inhibition constants for D-asparagine and for L-aspartic acid.

4. The relation between the effect of antibodies on the stability and on the catalytic properties of the enzyme is discussed in terms of the conformational constraint presumably imposed by the antibody.

INTRODUCTION

In recent years a considerable number of studies has been reported on the properties of L-asparaginases in general[1], and in particular on L-asparaginase EC of *Escherichia coli*[1–3]. While the main interest in this enzyme lies in its anti-neoplastic activity[4–8], much has also been learned about the physical[9–14], catalytic[15–18] and conformative response[18,19] characteristics of the EC-2 L-asparaginase[9].

We have previously shown that specific antibodies constrain the conformation of an enzyme (penicillinase) with resulting changes in stability, activity, and affinity for its substrates[20,21]. In this report we provide further evidence showing the constraining effect of homologous antibodies on L-asparaginase EC-2 derived from *E. coli*.

The properties of the constrained enzyme molecule are described and the significance of the observed changes is discussed. The preservation of the catalytic activity in the highly stabilized complex is of particular interest in relation to the anti-neoplastic activity of the EC-2 L-asparaginase.

MATERIALS AND METHODS

Chemicals

L-Asparaginase from *E. coli* B (EC 3.5.1.1, L-asparagine amidohydrolase, 33 I.U./mg) was purchased from Worthington Biochemical Corp. The preparation has the properties of L-asparaginase EC-2, as described by Campbell *et al.*[9].

Trypsin was purchased from the same source. Pronase (protease from *Streptomyces griseus*, Type V, purified) was purchased from Sigma Chemical Co.

L-Asparagine (monohydrate crystalline) was purchased from Nutritional Biochemical Corp. D-Asparagine (monohydrate crystalline) and L-aspartic acid were purchased from Sigma Chemical Co.

Iodine, KI and $Na_2S_2O_3$ (all C.P. grade) were purchased from Agan Chemical Co.

Preparation of antisera

Antiserum to L-asparaginase was prepared by injecting 20 I.U. of the commercial enzyme preparation, homogenized with Freund's adjuvant, into the subscapular region of a rabbit[22]. Injections were repeated four times at 10-day intervals, and the rabbits were bled from the ear 10 days after the last injection. The blood was allowed to clot overnight and the serum was separated and stored at −20 °C. One month after the last injection, the rabbits were given a booster injection of 20 I.U. of the enzyme preparation and the serum was collected and stored as above.

Preparation of anti-asparaginase γ-globulin fraction

The immune sera, prepared as described above, provided the γ-globulin fraction which was isolated by the Rivanol procedure[23]. 35 vol. of 0.4% Rivanol were added to 10 volumes of antiserum and stirred at pH 8.0, at room temperature, for 30 min. Excess Rivanol was removed by charcoal. The γ-globulin fraction was concentrated by aquacid and stored at −20 °C.

Preparation of anti-asparaginase Porter fragments

F_{AB} fragments were prepared by papain digestion of the γ-globulin fraction of the immune sera according to the method described by Porter[24].

Assay of asparaginase

The standard assay and the determination of residual activity were as previously described[18].

Methods of treatment

All procedures involving treatment with heat, trypsin, pronase and iodination were as previously described[18] unless stated otherwise in Results and Discussion.

RESULTS AND DISCUSSION

Precipitation, neutralization and stabilization

The asparaginase–anti-asparaginase complex is characterized by the quantitative precipitin reaction. The results, presented in Table I, indicate that at the

TABLE I

PRECIPITIN REACTION FOR L-ASPARAGINASE AND ITS HOMOLOGOUS ANTIBODY

The indicated amounts of L-asparaginase were incubated with 0.1 ml of the homologous antiserum (prepared as described in Materials and Methods) in 1.0 ml of borate–saline[25] at 37 °C for 1 h, and overnight at 4 °C. The precipitate was washed with borate–saline and resuspended in 0.1 M NaOH. The protein content was estimated by measuring absorption at 280 nm[25].

Antigen added (μg)	*Total protein precipitated (μg)*	*Antibody protein (μg)*	*Antibody : Antigen ratio*	
			Weight	*Molar**
10	78	68	6.8	5.6
20	141	120	6.0	4.9
40	208	168	4.2	3.4
80	398	318	3.9	3.2
100	429	329	3.3	2.7
140	447	307	2.2	1.8
160	480	320	2.0	1.6
175	519	344	1.9	1.6
200	492	292	1.5	1.2
225	539	314	1.4	1.1
250	495	245	1.0	0.8
360	409	49	—	—
600	234	—	—	—

* Mole ratio was estimated by assuming molecular weights of 130 000 for the antigen[13] and 160 000 for the antibody.

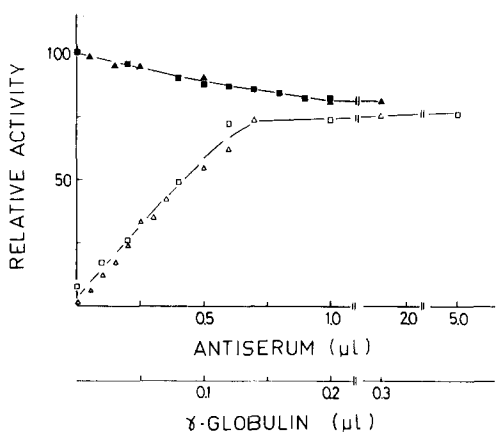

Fig. 1. Effect of antibodies on the thermostability and catalytic activity of L-asparaginase EC-2. Samples of the enzyme (0.063 unit) were incubated for 15 min at 37 °C with the indicated amounts of antiserum or γ-globulin fraction and 40 μmoles Tris–HCl (pH 8.0) in a total volume of 0.5 ml, made up with normal rabbit serum or normal γ-globulin. The residual activity was assayed (a) before and (b) after, exposure to heat, (see below), as described in Materials and Methods. It is expressed as percent initial activity. (a) Neutralization: ■, whole antiserum; ▲, γ-globulin fraction. (b) Stabilization: △, γ-globulin fraction, 62 °C for 5 min; □, whole antiserum, 62 °C for 2 min.

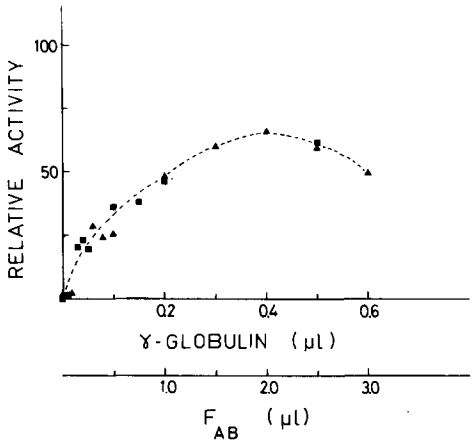

Fig. 2. Stabilization against proteolytic inactivation as a function of antibody concentration. Samples of L-asparaginase (0.06 unit) were incubated for 15 min at 37 °C with the indicated amounts of γ-globulin (4.7 mg/ml) (▲) or F_{AB} fraction (1.3 mg/ml) (■) and 40 μmoles Tris–HCl buffer (pH 8.0) in a total volume of 0.5 ml. The incubation was terminated by the addition of 2.5 μg of Pronase. The residual activity was assayed 3 min later as described previously[18].

equivalence zone (200–225 μg of antigen), the molar ratio of antibody to antigen is 1:1. At this ratio the catalytic activity is about 80% of that of the free enzyme. As shown in Fig. 1, this is close to the value obtained for the maximally neutralized enzyme.

More pronounced than the effect on catalytic activity is the increased thermostability observed with increasing antibody concentrations (Fig. 1). Similar specific stabilization to proteolysis is illustrated in Fig. 2, where the F_{AB} fraction prepared according to Porter[24] is shown to effectively replace the intact γ-globulin, derived from the immune serum. Thus the effect of the antibody does not depend on lattice formation, and can best be explained on the basis of constraint of the tertiary structure of the enzyme molecule[20,21].

The stabilizing effect of antibody is further demonstrated in Fig. 3 where the rates of inactivation of the native enzyme and the enzyme–antibody complex (see below) are compared. The complex is stable to proteolytic and especially to thermal inactivation. The antibody, however, had no protective effect against inactivation of the enzyme by iodine.

In the experiments described below, the catalytic and conformative response properties of the native and antibody-bound enzyme are compared. For this purpose, a "standard" enzyme–antibody complex was prepared by incubating aliquots of L-asparaginase (10–20 units) and the homologous antibody in 0.05 M Tris–HCl buffer (pH 8.0) for 15 min at 37 °C. The amount of antibody was adjusted so as to give a 2:1 antibody to antigen complex. The preparations were tested and found to be maximally neutralized and stabilized and showed no change in these properties for at least six months of storage at −20 °C.

Catalytic properties: comparison of free and bound L-*asparaginase*

Effect of temperature. Arrhenius plots for the free and bound enzyme are shown

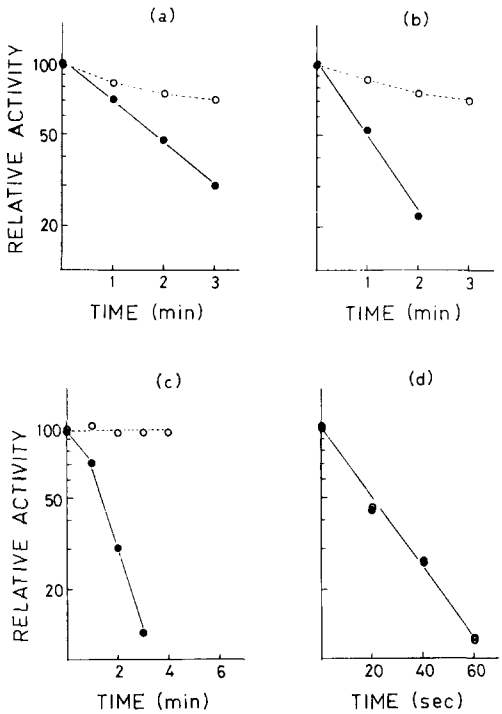

Fig. 3. Comparison of rates of inactivation of free and antibody-bound L-asparaginase. Aliquots (0.065 unit) of free (●) and antibody-bound (○) L-asparaginase in 0.5 ml Tris–HCl buffer (0.08 M, pH 8.0) were exposed to (a) trypsin (10 μg/ml; 37 °C), (b) Pronase (7.5 μg/ml; 37 °C), (c) heat (62 °C), (d) iodination (0.25 mM I_2 and 1.25 mM KI at 0 °C). The free enzyme was treated in the presence of normal rabbit serum. The rates of inactivation were determined by assay of residual activity (see Material and Methods) at the indicated time intervals.

in Fig. 4. The plots coincide in the temperature range of 23–65 °C, and the temperature (45 °C), above which the initial rates no longer obey the Arrhenius equation, is also common to both preparations. Above 65 °C, however, a difference is observed, as the catalytic activity of the free enzyme begins to decline rapidly. The energy of activation was calculated from the linear portions of the slopes and a value of 12.9 kcal/mole was obtained for both the free enzyme and the enzyme–antibody complex.

Effect of pH. The pH-activity profiles of the free and bound enzyme are essentially similar, showing the characteristic broad pH optimum of *E. coli* L-asparaginase[17,26]. A slight difference was observed between pH 4.0–5.0 where the enzyme–antibody complex appeared to be slightly more stable. Below pH 4.0 there is a rapid decline in the activity of both preparations.

Kinetic parameters. The relative rate of hydrolysis of D-asparagine was significantly higher (0.10–0.11) than that observed with the free enzyme[17,18]. This slight but reproducible change in stereospecificity is also observed when the enzyme is constrained by other means *e.g.* glutaraldehyde (in preparation). It is also fully consistent with earlier conclusions concerning the role of conformative response in stereospecificity.

The conformative response (*i.e.* the substrate-induced change in the confor-

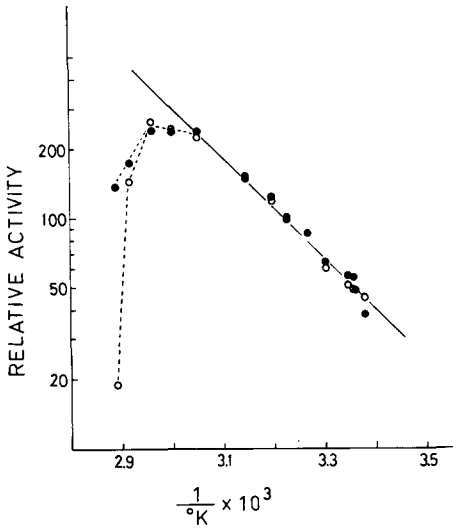

Fig. 4. Arrhenius plot for the hydrolysis of L-asparaginase by the free and antibody-bound enzyme. Samples (0.034 unit) of free (○) and bound (●) asparaginase were assayed (see Material and Methods) at the temperatures indicated. All reagents were preincubated at the respective temperatures for 5 min before use. The activity at each temperature was determined from the initial kinetics, calculated as percent of the activity at 37 °C, and plotted on a logarithmic scale against the reciprocal values of the absolute temperature, 1/°K.

mation of the active site) caused by the L-isomer differs from that observed with the D-isomer, and is more favourable for the catalytic reaction[18,19]. Thus the difference in the relative rates of hydrolysis reflect a difference in the conformative response to the two isomers. By imposing a constraint on the flexibility of the enzyme, we expect to reduce the difference in the relative V of the two isomers[18–21].

The K_m values for L- and D-asparagine and the enzyme–antibody complex have been determined from their initial rates of hydrolysis. The results, presented in Table II, give values of 68 and 120 μM with L- and D-asparagine, respectively. The values are slightly higher than those obtained with the free enzyme (*cf.* Table II and refs 18, 27 and 28).

Substrate inhibition with L-asparagine which we have noted with the free enzyme is observed in the enzyme–antibody complex as well. Thus, in the presence of large excess (0.01 M) of L-asparagine, the activity of both preparations is about 25% lower than at optimal substrate concentrations. With D-asparagine as the substrate there was no evidence of inhibition by excess of D-asparagine.

The competitive inhibition constants, K_I, for D-asparagine and L-aspartic acid were determined by the method of Dixon[29]. Values of 0.4 and 3.5 mM for D-asparagine and L-aspartic acid, respectively, were obtained. These are essentially similar to the K_I values obtained for the free enzyme (*cf.* Table II).

Conformative response properties

A quantitative analysis of the conformative response, has been shown to provide a series of parameters which permit a detailed comparison of the conformational properties of closely related enzyme molecules[30,31]. The methods used for the native enzyme have been previously described[19,30,32].

TABLE II

COMPARISON OF CATALYTIC AND CONFORMATIVE PARAMETERS OF FREE ASPARAGINASE EC-2 AND THE ENZYME-ANTIBODY COMPLEX

Parameter	*Ligand*	*Free enzyme*	*Enzyme–antibody complex*
K_m (μM)	L-asparagine	12	68
	D-asparagine	42	120
K_I (μM)	D-asparagine	630	400
	L-aspartic acid	2500	3500
R (iodination)	L-asparagine	0.23	0.16
	D-asparagine	1.00	1.00
	L-aspartic acid	0.15	0.05
K_{CR} (μM)	L-asparagine	4.4	4.4
	D-asparagine	7.5*	10.0*
		4.4**	3.0**
	L-aspartic acid	180	180

* Determined in the presence of L-asparagine (see text).
** Determined in the presence of L-aspartic acid (see text).

In the case of the enzyme–antibody complex presented here, the criterion of susceptibility to inactivation by iodine was used for the determination of K_{CR} values (the concentration at which half-maximal effect is observed) for the various ligands. The complex was protected against iodination at 0 °C and pH 8.0 by L-asparagine or L-aspartic acid, but not by D-asparagine (Fig. 5). The pattern of protection was thus similar to that observed with the free enzyme[19]. The rates of inactivation in the absence (V_0) and in the presence of varying ligand concentrations (V) were deter-

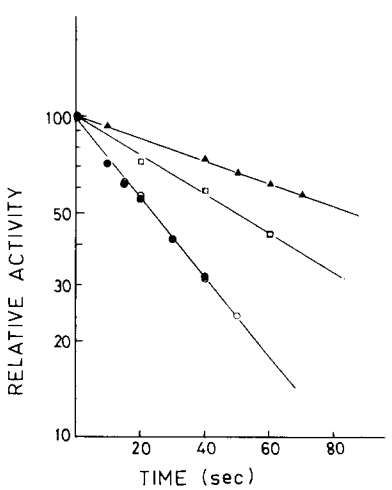

Fig. 5. Effect of ligands on iodination of the enzyme–antibody complex. Samples (0.08 unit) of the enzyme–antibody complex were exposed at 0 °C to an iodinating solution consisting of 0.13 μmole I_2, 0.63 μmole KI, 40 μmoles Tris–HCl buffer (pH 8.0) and ligands as indicated, in a total volume of 0.5 ml. The reaction was terminated at the indicated time intervals by the addition of 1.0 μmole of $Na_2S_2O_3$. The residual activity (see Material and Methods) is plotted as percent of the uniodinated samples. Ligand present: ●, none; ○, D-asparagine (0.2 mM); ▲, L-asparagine (0.1 mM); □, L-aspartic acid (0.2 mM).

mined for the enzyme–antibody complex as previously described[18,19]. Results with L-asparagine and L-aspartic acid as protective ligands are given in Table II. As can be seen, the K_{CR} values obtained with the bound enzyme are identical with those obtained with the free enzyme (180 and 4.4 μM for L-aspartic acid and L-asparagine, respectively).

As described above, D-asparagine has no effect on the rate of iodination of the enzyme (Fig. 5). Since both the D- and L-isomers compete for the same site, D-asparagine will prevent the protective effect of L-asparagine[19]. This competitive relationship was used to derive the K_{CR} value for D-asparagine as previously described[19]. The K_{CR} value obtained for D-asparagine was 10 μM (Table II).

The same procedure was used for the determination of K_{CR} for D-asparagine where L-aspartic acid was the protective ligand. The K_{CR} value obtained was 3 μM (Table II).

Other methods of determining conformative response parameters are based on ligand induced changes in the susceptibility to thermal or proteolytic inactivation[19,33]. As shown above, the antibody-bound enzyme is very stable (Fig. 3); however at higher temperatures, where partial inactivation of the complex is obtained, stabilization by L- and D-asparagine and L-aspartic acid can be observed.

In spite of several significant differences the overall picture which emerges from this study is one of a remarkable similarity between the free and bound enzyme preparations (*cf.* Table II). This is particularly evident in the conformative response parameters which are very sensitive for the detection of subtle differences between related enzyme preparations[18,34,35]. The observations opened the interesting possibility of using the enzyme–antibody complex *in vivo* as a stabilized derivative of L-asparaginase which retains the antineoplastic activity of the native enzyme.

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