Kinetic studies of chicken and turkey liver mitochondrial aspartate aminotransferase

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The kinetic behaviour of chicken liver and turkey liver aspartate aminotransferases (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) was studied. Steady-state data were obtained from a wide range of concentrations of substrates and product L-glutamate. The data were fitted by rational functions of degree 1:1, 1:2 and 2:2 with respect to substrates and 0:1, 1:1, 0:2 and 1:2 with regard to product (L-glutamate), by using a non-linear regression program that guarantees the fit. The goodness of fit was improved by the use of a computer program that combines model discrimination parameter refinement and sequential experimental design. It was concluded that aspartate aminotransferase requires a minimum velocity equation of degree 2:2 for L-aspartate, 2:2 for 2-oxoglutarate and 1:2 for L-glutamate. Finally, a plausible kinetic mechanism that justifies these experimental results is proposed.

INTRODUCTION

Aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) from eukaryotic cells exists in two isoenzymic forms, one within the mitochondria and the second in the cytosol (Braunstein, 1973).

Spectrophotometric, chemical and steady-state kinetic studies support the view that both isoenzymes follow a Ping-Pong Bi Bi kinetic mechanism (Braunstein, 1973; Cheng *et al.*, 1971; Michuda & Martinez-Carrion, 1969), and are inhibited by several inorganic anions (Harruff & Jenkins, 1978), dicarboxylic acids (Jenkins, 1982) and high concentrations of substrates and products (Wada *et al.*, 1968).

The enzyme binding sites for anions and dicarboxylic acids and their relationship to those sites responsible for substrate or product binding have been largely studied and discussed (Cheng & Martinez-Carrion, 1972). However, the studies carried out by Martinez-Carrion *et al.* (1973) with the use of ^1H and ^{19}F n.m.r. provided strong evidence of two binding sites: a histidine residue in the active centre and the protonated coenzyme-protein internal aldimine linkage. In accordance with this, ^{19}F -n.m.r. studies carried out with trifluoroacetate and the pseudo-substrate 2-methylaspartate showed the existence of aspartate aminotransferase-pseudo-substrate-anion complexes (Martinez-Carrion *et al.*, 1973).

On the other hand, formation of ternary enzyme-substrate-dicarboxylic acid complexes has been suggested to justify the inhibition of pig heart mitochondrial aspartate aminotransferase by C_4 dicarboxylic acids (Michuda & Martinez-Carrion, 1970).

The results obtained by Jenkins & d'Ari (1966) on the inhibition of aspartate aminotransferase by ions and dicarboxylic acids strongly suggest that enzyme-product abortive complexes, frequently characterized in transaminases, are also able to react directly with the substrate and form an intermediary ternary complex containing both substrate and product bound to the enzyme. In spite of that, the apparent inhibition by excess of substrates or products reported for this enzyme has never been associated with the possible existence of ternary

complexes. That is because lineal plots are obtained when concentrations close to K_m values of both substrates, or highly inhibitory concentrations of one substrate, are used. These results agree with the Ping-Pong mechanism with excess substrate inhibition due to the abortive binary complexes described for aspartate aminotransferase (Henson & Cleland, 1964; Boyde, 1968; Braunstein, 1973).

Although it has been recently demonstrated that many oligomeric enzymes, which follow a Michaelis-like behaviour when an insufficient range of substrate concentrations is employed, can present a more complex mechanism when kinetic studies with a complete substrate concentration range are done (Hill *et al.*, 1977; Bardsley *et al.*, 1980), no attempts of this kind have hitherto been reported for aspartate aminotransferase.

In the present investigation the kinetic behaviour of chicken liver and turkey liver mitochondrial aspartate aminotransferase was studied. Steady-state data obtained from a wide range of concentrations of substrates and product L-glutamate were analysed by using a program that combines model discrimination, parameter refinement and sequential experimental design (Franco *et al.*, 1986). The experimental strategy employed permits positive results in only a single experimental session with the aid of a computer.

EXPERIMENTAL

Reagents

L-Aspartate, 2-oxoglutarate and L-glutamate were purchased from Merck. NADH and mitochondrial malate dehydrogenase from pig heart were obtained from Boehringer Mannheim. All other chemicals used were the purest commercially available.

Preparation of the enzymes

Mitochondrial aspartate aminotransferases were prepared from turkey (*Meleagris gallopavo*) and chicken (*Gallus domesticus*) livers as previously described (Cascante *et al.*, 1987). The enzymes, buffered with 50 mM-triethanolamine/acetate buffer, pH 7.4, con-

taining 0.05% NaN_3 , were stable at 4 °C for at least 6 months.

Enzyme assay

All spectrophotometrical measurements were carried out with a Hewlett-Packard 8450A spectrophotometer, in 1 cm-light-path cells.

Aspartate aminotransferase activity was measured in a 3.0 ml reaction mixture containing 50 mM-triethanolamine/acetate buffer, pH 7.4, 0.07 mM-NADH and 10 μg of dialysed mitochondrial malate dehydrogenase/ml at 30 ± 0.1 °C; the oxaloacetate produced was detected by the method of Karmen (1955). Various concentrations of substrates L-aspartate (50 μM –100 mM), 2-oxoglutarate (50 μM –50 mM) and product L-glutamate (1 mM–100 mM) were used. In all cases the reaction was started by addition of 0.1 ml of enzyme (3 $\mu\text{g}/\text{ml}$).

To check that inhibition of aspartate aminotransferase activity observed at high concentrations of substrates or product L-glutamate is not due to the increase of ionic strength, control experiments (with non-inhibitory concentrations of substrates or L-glutamate) with sodium acetate (0–250 mM) were carried out. In all cases no differences in aspartate aminotransferase activity were observed.

One enzyme unit is defined as the amount of enzyme producing the conversion of 1 μmol of substrate/min, under the conditions of the assay.

For each experimental point a minimum of five replicates were made. The obtained replicates were analysed by means of a program (Lopez-Cabrera, 1987) based on Akaike's information criteria (AIC) to detect outliers, which were discarded. Standard deviation (s.d.) mean value were calculated for each point. In all cases s.d. values less than 0.005 unit were obtained; when necessary more replicates were made until standard deviation was improved.

Analysis of the steady-state data

Data were fitted by rational functions of degree 1:1, 1:2 and 2:2 with regard to substrates and 0:1, 1:1, 0:2 and 1:2 with regard to product (L-glutamate), by using a non-linear-regression program that guaranteed the fit (Canela, 1984).

Experiments planned to discriminate among rival models were carried out with a sequential design using the method described by Franco *et al.* (1986) and Bruguera *et al.* (1988), based on the D-optimality criterion (Fedorov, 1972), which combines determination of the true model with refinement of the estimates of the parameter values. In this design, experimental points, performed initially, were geometrically distributed among a wide range of concentrations of each substrate or product, and data were fitted to the corresponding models. From the information obtained, a new point was chosen and a new measure was carried out. The set of data including this new measure was then fitted to the corresponding equation. The process was repeated until no more improvement was achieved. The goodness of the chosen equation, according to this program, was confirmed by following the criteria proposed by other authors, as the AIC minimum value (Jones *et al.*, 1984), the *F* test (Pettersson & Pettersson, 1970; Burguillo *et al.*, 1983) and the lower sum of squares (Mannervik, 1982).

In experiments designed to get the most accurate kinetic parameters, the set of optimal experimental points was chosen by using a program based on the maximization of the determinant of the system information matrix (Lopez-Cabrera, 1987).

All the programs, written in FORTRAN77, used were implemented on a VAX 11/750 computer.

RESULTS

Study of the error structure

As a previous step to model discrimination, error structure of the experimental system was studied.

The set of data were 115 different initial velocities ranged over a complete experimental interval.

The s.d. in each experimental point was estimated by five replicate measurements.

The curve was fitted by non-linear regression analysis by using the equation:

$$\text{s.d.} = O_1 + O_2 \cdot v^2 \quad (1)$$

where O_1 and O_2 are parameters and v is the mean velocity.

The parameter values of the experimental-error structure for kinetic data obtained with mitochondrial aspartate aminotransferase from chicken liver were $O_1 = 1.75 \times 10^2$ and $O_2 = 1.40 \times 10^{-2}$.

These values were assumed as valid also for mitochondrial aspartate aminotransferase from turkey liver.

The experimental-error function found was included in previous reported programs (Franco *et al.*, 1986; Lopez-Cabrera, 1987) in order to take into account the error associated with the points before they were chosen.

Variation of the initial velocities with respect to L-aspartate concentration

The preliminary experiment included 14 points, each one with a different L-aspartate concentration between 50 μM and 100 mM. The concentration of 2-oxoglutarate was held constant (0.2 mM). The experimental velocities obtained were respectively fitted to the following equations:

$$v = \frac{O_1 \cdot [\text{S}]^2 + O_2 \cdot [\text{S}]}{[\text{S}]^2 + O_3 \cdot [\text{S}] + O_4} \quad (2)$$

$$v = \frac{O'_1 \cdot [\text{S}]}{[\text{S}]^2 + O'_2 \cdot [\text{S}] + O'_3} \quad (3)$$

where $O_1, \dots, O_4$ and $O'_1, \dots, O'_3$ are parameters and $[\text{S}]$ is [substrate].

After the 20th determination the probability for the degree 2:2 rational function (eqn. 2) was 1. For mitochondrial aspartate aminotransferases from chicken and turkey, the goodness of eqn. (2) with regard to that of degree 1:2 (eqn. 3) was supported by three different discrimination tests: the sum of squares (Mannervik, 1982), the *F* test (Pettersson & Pettersson, 1970) and AIC (Jones *et al.*, 1984). Table 1(a) shows the results of such a test for mitochondrial aspartate aminotransferase from chicken liver. Also, the logarithmic plot of experimental and theoretical [according to eqns. (2) and (3)] initial velocities versus L-aspartate concentration is shown in Fig. 1. However, when the constant concentration of

Table 1. Discrimination among $n:n$ functions of degree 1:2 and 2:2 corresponding to L-aspartate:2-oxoglutarate aminotransferase reaction with respect to the varied-concentration substrate

(a) L-Aspartate is the varied-concentration substrate (total points: 20); (b) 2-oxoglutarate is the varied-concentration substrate (total points: 17). The probability *a posteriori* was 1 for the model 2:2, and this model fitted better at the 99% confidence level with the *F* test as suggested by Pettersson & Pettersson (1970) in both cases.

Model	Weighted sum of squares	AIC
(a) [L-Aspartate] = 50 μ M–100 mM; [2-oxoglutarate] = 0.2 mM		
1:2	22.7	83.5
2:2	13.5	55.0
(b) [2-Oxoglutarate] = 50 μ M–50 mM; [L-aspartate] = 0.2 mM		
1:2	8.7	13.3
2:2	5.2	6.2

2-oxoglutarate was high (50 mM) the experimental data only fitted to an equation of degree 1:1 as the following:

$$v = \frac{O_1 \cdot [S]}{[S] + O_2} \quad (4)$$

Variation of the initial velocities with respect to 2-oxoglutarate concentration

As described in the previous case, the preliminary experiment included 14 points, each one with a different 2-oxoglutarate concentration between 50 μ M and 50 mM. The L-aspartate concentration was held constant at 0.2 mM. For both enzymes, the goodness of fit of the equation of degree 2:2 (eqn. 2) was demonstrated by means of the above-mentioned tests.

Table 1(b) shows the results of these tests for mitochondrial aspartate aminotransferase from chicken liver, performed after two equations (eqns. 2 and 3) had been fitted to a set of data. For equation of degree 2:2 all tests indicated a good fit. It may be pointed out that the probability 1 for the equation of degree 2:2 was obtained after the 17th determination. The logarithmic plot of the initial velocities versus the 2-oxoglutarate concentration appears in Fig. 2.

Also, for this varied-concentration substrate, when the concentration of the L-aspartate held constant was high (100 mM), the experimental initial velocities obtained only fitted to an equation of degree 1:1 with respect to the oxoglutarate concentration.

Variation of the initial velocities simultaneously with both substrate concentrations in the absence of reaction products

The preliminary experiment included 49 points: seven series of L-aspartate concentrations ([L-aspartate] 57.6, 192, 700, 2400, 8000, 28 600 and 100 000 μ M) with that of 2-oxoglutarate held constant ([2-oxoglutarate] 72.2, 190, 640, 1900, 5600, 16 700 and 500 000 μ M). The experimental velocities obtained were fitted to the equation:

$$v = \frac{O_1 \cdot [A]^2 \cdot [B] + O_2 \cdot [A] \cdot [B]^2 + O_3 \cdot [A] \cdot [B]}{O_4 \cdot [A]^2 \cdot [B] + O_5 \cdot [B]^2 \cdot [A] + [A] \cdot [B] + O_6 \cdot [A]^2 + O_7 \cdot [A] + O_8 \cdot [B]^2 + O_9 \cdot [B]} \quad (5)$$

where $O_1, \dots, O_9$ are parameters, [A] is [L-aspartate] and [B] is [2-oxoglutarate].

So, an estimate of the parameters $O_1, \dots, O_9$ was obtained, which made it possible to select, by using the program mentioned above (Lopez-Cabrera, 1987), the set of points optimal for estimating parameter values.

Fig. 1. Curve fitting to experimental data for the L-aspartate:2-oxoglutarate aminotransferase reaction obtained with different L-aspartate concentrations and a constant 2-oxoglutarate concentration (0.2 mM)

The points were fitted by using a 2:2 rational polynomial function (curve a) and a 1:2 rational polynomial function (curve b). [S] is [L-aspartate] (M); *v* is initial velocity (units).

Fig. 2. Curve fitting to experimental data for the L-aspartate:2-oxoglutarate aminotransferase reaction obtained with different 2-oxoglutarate concentrations and a constant L-aspartate concentration (0.2 mM)

The points were fitted by using a 2:2 rational polynomial function (curve a) and a 1:2 rational polynomial function (curve b). [S] is [2-oxoglutarate] (M); *v* is initial velocity (units).

The experiments performed with the optimal 18 points and the kinetic parameters obtained for eqn. (5) for both mitochondrial aspartate aminotransferases appear in Table 2.

Product inhibition studies

Inhibition by L-glutamate, one of the reaction products, was studied. The effect of oxaloacetate, the other reaction product, on the process rate was not determined because in the activity measurements the spectrophotometric method of Karmen (1955) was used. This method is based on coupling with exogenous malate dehydrogenase, which catalyses the reduction of the oxaloacetate formed by NADH.

Finally, when the 2-oxoglutarate concentration held constant was high (50 mM) the experimental initial velocities only fitted to an equation of degree 0:2 in accordance with eqn. (7).

Variation of the initial velocities with 2-oxoglutarate and L-glutamate concentrations simultaneously

Preliminary experiments were performed with varied concentrations for 2-oxoglutarate (100 μ M–50 mM) and L-glutamate (1 mM–100 mM). The concentration of L-aspartate was held constant at 0.2 mM.

The experimental velocities obtained were fitted respectively to the following equations:

$$v = \frac{O_1 \cdot [S]^2 + O_2 \cdot [S] + O_3 \cdot [S] \cdot [Q]}{[S]^2 + O_4 \cdot [S] + O_5 \cdot [S] \cdot [Q] + O_6 \cdot [Q]^2 + O_7 \cdot [Q] + O_8 + O_9 \cdot [S]^2 \cdot [Q] + O_{10} \cdot [Q]^2 \cdot [S]} \quad (9)$$

$$v = \frac{O'_1 \cdot [S]^2 + O'_2 \cdot [S]}{[S]^2 + O'_3 \cdot [S] + O'_4 \cdot [S] \cdot [Q] + O'_5 \cdot [Q]^2 + O'_6 \cdot [Q] + O'_7 + O'_8 \cdot [Q] \cdot [S]^2} \quad (10)$$

$$v = \frac{O''_1 \cdot [S]^2 + O''_2 \cdot [S]}{[S]^2 + O''_3 \cdot [S] + O''_4 \cdot [S] \cdot [Q] + O''_5 \cdot [Q]^2 + O''_6 \cdot [Q] + O''_7} \quad (11)$$

Variation of the initial velocities in the presence of the product L-glutamate

The preliminary experiment included 11 points, each with a different L-glutamate concentration between 1 mM and 100 mM. The corresponding concentrations of L-aspartate and 2-oxoglutarate were held constant at 0.2 mM. The experimental velocities were fitted respectively to the following equations:

$$v = \frac{O_1 \cdot [Q] + O_2}{O_3 \cdot [Q]^2 + [Q] + O_4} \quad (6)$$

$$v = \frac{O'_1}{O'_2 \cdot [Q]^2 + [Q] + O'_3} \quad (7)$$

where $O_1, \dots, O_4$ and $O'_1, \dots, O'_3$ are parameters and $[Q]$ is [substrate].

After the 16th determination the probability for the rational function of degree 1:2 (eqn. 6) was 1 for both enzymes. The goodness of eqn. (6) with respect to that of degree 0:2 (eqn. 7) was supported by the usual tests. So, for chicken mitochondrial aspartate aminotransferase, the weighted sum of squares and AIC values for the degree 1:2 model are 1.1 and 4.8 respectively in front of values of 1.9 and 11.9 for the degree 0:2 model. Also, the degree 1:2 model fitted better at 99% confidence level with the *F* test as suggested by Pettersson & Pettersson (1970).

On the other hand, when the L-aspartate concentration held constant was inhibitory (100 mM) the experimental data only fit to an equation of degree 0:1 in accordance with the following equation:

$$v = \frac{O_1}{O_2 + [Q]} \quad (8)$$

where $[S]$ is [varied-concentration], $[Q]$ is [L-glutamate] and $O_1, \dots, O_{10}, O'_1, \dots, O'_8$ and $O''_1, \dots, O''_7$ are parameters that depend on the substrate concentration held constant.

By following the normal procedure, the usual discriminatory tests used proved that the experimental initial velocities fitted best to eqn. (9). The probability *a posteriori* was 0.94 for eqn. (9) and 0.06 for eqn. (10). Also, eqn. (9) fitted better at the 95% confidence level with the *F* test as suggested by Pettersson & Pettersson (1970).

Variation of the initial velocities with L-aspartate and L-glutamate concentrations

Experiments were performed with varied concentrations of L-aspartate (100 μ M–100 mM) and L-glutamate (1 mM–100 mM). The concentration of 2-oxoglutarate was held constant at 0.2 mM.

The experimental initial velocities obtained only fitted to an equation of degree 0:2 with respect to the L-glutamate concentration in accordance with eqn. (11). This equation degree apparently contradicted previous results obtained when the concentrations of both substrates were held low and constant (not inhibitory). However, very probably this surprising result can be attributed to insufficiency of information available in the working experimental conditions to detect the degree 1 with respect to L-glutamate in the numerator of the equation of velocity.

Variation of the initial velocities with both substrate concentrations simultaneously in the presence of the product L-glutamate

The results described so far have made it evident that, theoretically and as a maximum, the global equation to which initial reaction velocities can fit will be of 18 parameters, as follows:

$$v = \frac{O_1 \cdot [A]^2 \cdot [B] + O_2 \cdot [B]^2 \cdot [A] + O_3 \cdot [A] \cdot [B] + O_4 \cdot [A] \cdot [B] \cdot [Q]}{O_5 \cdot [A]^2 \cdot [B] + O_6 \cdot [B]^2 \cdot [A] + [A] \cdot [B] + O_7 \cdot [A] \cdot [B] \cdot [Q] + O_8 \cdot [B]^2 + O_9 \cdot [B] + O_{10} \cdot [B]^2 \cdot [Q] + O_{11} \cdot [B] \cdot [Q]^2 + O_{12} \cdot [B] \cdot [Q] + O_{13} \cdot [A]^2 + O_{14} \cdot [A] + O_{15} \cdot [A] \cdot [Q] + O_{16} \cdot [Q]^2 + O_{17} \cdot [Q] + O_{18} \cdot [A] \cdot [B]^2 \cdot [Q]} \quad (12)$$

Table 2. Parameters of the global model in the absence of reaction products

Parameter	Chicken enzyme	Turkey enzyme	Units
O_1	12.5 ± 3.9	13.7 ± 4.1	units $\cdot$ M ⁻¹
O_2	16.1 ± 4.1	31.1 ± 2.2	units $\cdot$ M ⁻¹
O_3	$8.56 \times 10^{-1} \pm 4.0 \times 10^{-2}$	$8.960 \times 10^{-1} \pm 3.6 \times 10^{-3}$	units
O_4	24.3 ± 6.5	15.7 ± 4.8	M ⁻¹
O_5	23.8 ± 5.9	33.8 ± 2.5	M ⁻¹
O_6	$3.33 \times 10^{-2} \pm 4.7 \times 10^{-3}$	$1.37 \times 10^{-2} \pm 3.4 \times 10^{-3}$	
O_7	$3.68 \times 10^{-4} \pm 1.3 \times 10^{-5}$	$4.09 \times 10^{-4} \pm 1.1 \times 10^{-5}$	M
O_8	$1.21 \times 10^{-2} \pm 3.1 \times 10^{-3}$	$5.4 \times 10^{-2} \pm 2.6 \times 10^{-3}$	
O_9	$3.54 \times 10^{-4} \pm 1.3 \times 10^{-5}$	$3.70 \times 10^{-4} \pm 1.0 \times 10^{-5}$	M

Table 3. Parameters of the global model in the presence of the reaction product L-glutamate

Parameter	Chicken enzyme	Turkey enzyme	Units
O_1	8.3 ± 5.8	8.4 ± 6.1	units $\cdot$ M ⁻¹
O_2	12.2 ± 8.3	30.5 ± 4.1	units $\cdot$ M ⁻¹
O_3	$8.20 \times 10^{-1} \pm 5.0 \times 10^{-2}$	$8.90 \times 10^{-1} \pm 1.2 \times 10^{-2}$	units
O_4	10.6 ± 8.1	10.1 ± 7.8	M ⁻¹
O_5	15.5 ± 17.5	32.9 ± 9.1	M ⁻¹
O_6	$1.35 \times 10^{-1} \pm 1.5$	1.4 ± 1.0	M ⁻¹
O_7	$3.01 \times 10^{-2} \pm 2.5 \times 10^{-2}$	$5.32 \times 10^{-2} \pm 6.4 \times 10^{-3}$	
O_8	$3.51 \times 10^{-4} \pm 6.0 \times 10^{-5}$	$3.65 \times 10^{-4} \pm 2.4 \times 10^{-5}$	M
O_9	$1.48 \times 10^{-1} \pm 3.1 \times 10^{-1}$	$2.39 \times 10^{-1} \pm 1.5 \times 10^{-1}$	M ⁻¹
O_{10}	$1.51 \times 10^{-2} \pm 8.1 \times 10^{-2}$	$6.55 \times 10^{-2} \pm 7.4 \times 10^{-2}$	M ⁻¹
O_{11}	$1.38 \times 10^{-2} \pm 1.0 \times 10^{-2}$	$5.75 \times 10^{-2} \pm 4.7 \times 10^{-3}$	
O_{12}	$7.22 \times 10^{-3} \pm 5.0 \times 10^{-3}$	$6.22 \times 10^{-3} \pm 4.1 \times 10^{-3}$	
O_{13}	$3.10 \times 10^{-4} \pm 9.2 \times 10^{-5}$	$4.12 \times 10^{-4} \pm 1.9 \times 10^{-5}$	M
O_{14}	$8.60 \times 10^{-3} \pm 4.1 \times 10^{-3}$	$2.10 \times 10^{-3} \pm 1.1 \times 10^{-3}$	M
O_{15}	$4.13 \times 10^{-5} \pm 2.5 \times 10^{-5}$	$6.48 \times 10^{-5} \pm 3.1 \times 10^{-5}$	
O_{16}	$1.24 \times 10^{-5} \pm 2.0 \times 10^{-5}$	$1.30 \times 10^{-5} \pm 7.7 \times 10^{-6}$	M

where [A] is [L-aspartate], [B] is [2-oxoglutarate], [Q] is [L-glutamate] and $O_1, \dots, O_{18}$ are parameters.

They also permitted calculation of the theoretical estimates of the corresponding parameters, from which it was possible to design the appropriate experiment to obtain better estimates of these parameters by using the adequate program.

The initial velocities obtained did not fit to eqn. (12), but did so to an equation of 16 parameters as follows:

$$v = \frac{O_1 \cdot [A]^2 \cdot [B] + O_2 \cdot [A] \cdot [B]^2 + O_3 \cdot [A] \cdot [B]}{O_4 \cdot [A]^2 \cdot [B] + O_5 \cdot [B]^2 \cdot [A] + [A] \cdot [B] + O_6 \cdot [A] \cdot [B] \cdot [Q] + O_7 \cdot [B]^2 + O_8 \cdot [B] + O_9 \cdot [B]^2 \cdot [Q] + O_{10} \cdot [B] \cdot [Q]^2 + O_{11} \cdot [B] \cdot [Q] + O_{12} \cdot [A]^2 + O_{13} \cdot [A] + O_{14} \cdot [A] \cdot [Q] + O_{15} \cdot [Q]^2 + O_{16} \cdot [Q]} \quad (13)$$

where [A] is [L-aspartate], [B] is [2-oxoglutarate], [Q] is [L-glutamate] and $O_1, \dots, O_{16}$ are parameters.

The estimates of the parameters obtained permitted calculation again of the best points in order to estimate the values of the parameters as precisely as possible.

The experiments were performed with the optimal 20 points.

The kinetic parameters obtained for eqn. (13) for both mitochondrial aspartate aminotransferases appear in Table 3.

DISCUSSION

The Ping-Pong Bi Bi kinetic mechanism with inhibition by excess of substrates and products caused by the formation of abortive enzyme-substrate binary complexes has been generally accepted for mitochondrial aspartate aminotransferases from various organisms (Braunstein, 1973; Cheng *et al.*, 1971; Michuda & Martinez-Carrion, 1969).

This mechanism has been confirmed by several authors, not only by the parallel lines obtained at low substrate concentrations in double-reciprocal plots, but also by substrate inhibition studies (Henson & Cleland, 1964; Boyde, 1968). However, these authors neither studied a wide range of substrate concentrations simultaneously nor tested other models of a higher degree.

The experimental results obtained in the present work can explain the linearity of the classical double-reciprocal plots, since for eqn. (4) it is clear that obtaining steady-

Scheme 1. Kinetic mechanism of chicken and turkey liver mitochondrial L-aspartate:2-oxoglutarate aminotransferases

E and F represents aldimine form and amino form respectively of the enzyme; A is L-aspartate, B is 2-oxoglutarate, P is oxaloacetate and Q is L-glutamate. Dashed boundaries around areas represents the two rapid-equilibrium regions.

state data at only low or high substrate will always lead to a failure to differentiate 2:2 from 1:1 rate equations. At the same time, to justify these results, the demonstrated inhibition of activity by high substrate and product concentrations cannot be explained only by the abortive enzyme-substrate binary complexes classically described for the aspartate aminotransferase. Thus the mechanism necessary to justify the steady-state findings must lead to a rate equation of degree 2:2 with respect to each substrate concentration, and of degree 1:2 with respect to L-glutamate concentration.

The kinetic experimental results obtained in the present work lead us to propose that inhibition by excess of

substrate and product can be due to formation of abortive binary complexes that can originate productive ternary complexes by addition of a second molecule of substrate (Scheme 1). Hence the product formation is possible by the transformation not only of the binary complexes of the Ping-Pong mechanism characteristic but also of the above-described productive ternary complexes.

The rate equations were derived by application of the combined rapid equilibrium steady-state treatment described by Cha (1968), considering the two equilibrium regions shown in Scheme 1. The combined rapid equilibrium steady-state method (Cha, 1968) rather than

steady-state kinetics was used because the steady-state mechanism provides equations of higher degree that cannot be easily reduced and too many denominator and numerator terms appear in the rate equation. Further, higher-degree equations should be not detected experimentally (Burguillo *et al.*, 1983). The expression obtained is the following:

$$\frac{v}{[E_0]} = \frac{\left(\sum_{i=19}^{i=28} f_i \cdot k_{+i}\right) \left(\sum_{i=9}^{i=13} f_i \cdot k_{+i}\right) - \left(\sum_{i=9}^{i=18} f_i \cdot k_{+i}\right) \left(\sum_{i=19}^{i=23} f_i \cdot k_{+i}\right)}{\left(\sum_{i=19}^{i=28} f_i \cdot k_{+i}\right) + \left(\sum_{i=9}^{i=18} f_i \cdot k_{+i}\right)}$$

where v is the velocity of the product oxaloacetate formation and $[E_0]$ = concentration of total enzyme, being:

$$\begin{aligned} f_9 &= \frac{[A] \cdot [B] \cdot k_{+1} \cdot k_{+3}}{k_{-1} \cdot k_{-3} \cdot d_1}, & f_{10} &= \frac{[A]^2 \cdot k_{+1} \cdot k_{+2}}{k_{-1} \cdot k_{-2} \cdot d_1}, \\ f_{11} &= \frac{[A] \cdot k_{+1}}{k_{-1} \cdot d_1}, & f_{12} &= \frac{[A] \cdot [P] \cdot k_{+1} \cdot k_{+6}}{k_{-1} \cdot k_{-6} \cdot d_1}, \\ f_{13} &= \frac{[A] \cdot [Q] \cdot k_{+1} \cdot k_{+5}}{k_{-1} \cdot k_{-5} \cdot d_1}, & f_{14} &= \frac{[A] \cdot [Q] \cdot k_{+2} \cdot k_{+4}}{k_{-2} \cdot k_{-4} \cdot d_1}, \\ f_{15} &= \frac{[B] \cdot [Q] \cdot k_{+3} \cdot k_{+4}}{k_{-3} \cdot k_{-4} \cdot d_1}, & f_{16} &= \frac{[Q] \cdot k_{+4}}{k_{-4} \cdot d_1}, \\ f_{17} &= \frac{[Q]^2 \cdot k_{+4} \cdot k_{+5}}{k_{-4} \cdot k_{-5} \cdot d_1}, & f_{18} &= \frac{[P] \cdot [Q] \cdot k_{+4} \cdot k_{+6}}{k_{-4} \cdot k_{-6} \cdot d_1}, \end{aligned}$$

$$\begin{aligned} d_1 &= 1 + \frac{[A] \cdot [B] \cdot k_{+1} \cdot k_{+3}}{k_{-1} \cdot k_{-3}} + \frac{[A]^2 \cdot k_{+1} \cdot k_{+2}}{k_{-1} \cdot k_{-2}} \\ &+ \frac{[A] \cdot k_{+1}}{k_{-1}} + \frac{[A] \cdot [P] \cdot k_{+1} \cdot k_{+6}}{k_{-1} \cdot k_{-6}} + \frac{[A] \cdot [Q] \cdot k_{+1} \cdot k_{+5}}{k_{-1} \cdot k_{-5}} \\ &+ \frac{[A] \cdot [Q] \cdot k_{+2} \cdot k_{+4}}{k_{-2} \cdot k_{-4}} + \frac{[B] \cdot [Q] \cdot k_{+3} \cdot k_{+4}}{k_{-3} \cdot k_{-4}} + \frac{[Q] \cdot k_{+4}}{k_{-4}} \\ &+ \frac{[Q]^2 \cdot k_{+4} \cdot k_{+5}}{k_{-4} \cdot k_{-5}} + \frac{[P] \cdot [Q] \cdot k_{+4} \cdot k_{+6}}{k_{-4} \cdot k_{-6}} + \frac{[A] \cdot k_{+2}}{k_{-2}} \\ &+ \frac{[B] \cdot k_{+3}}{k_{-3}} + \frac{[Q] \cdot k_{+5}}{k_{-5}} + \frac{[P] \cdot k_{+6}}{k_{-6}} \end{aligned}$$

$$\begin{aligned} d_{11} &= 1 + \frac{[P] \cdot [B] \cdot k_{+3} \cdot k_{+8}}{k_{-3} \cdot k_{-8}} + \frac{[A] \cdot [P] \cdot k_{+2} \cdot k_{+8}}{k_{-2} \cdot k_{-8}} \\ &+ \frac{[P] \cdot k_{+8}}{k_{-8}} + \frac{[P]^2 \cdot k_{+6} \cdot k_{+8}}{k_{-6} \cdot k_{-8}} + \frac{[P] \cdot [Q] \cdot k_{+5} \cdot k_{+8}}{k_{-5} \cdot k_{-8}} \\ &+ \frac{[A] \cdot [B] \cdot k_{+2} \cdot k_{+7}}{k_{-2} \cdot k_{-7}} + \frac{[B]^2 \cdot k_{+3} \cdot k_{+7}}{k_{-3} \cdot k_{-7}} + \frac{[B] \cdot k_{+7}}{k_{-7}} \\ &+ \frac{[B] \cdot [Q] \cdot k_{+5} \cdot k_{+7}}{k_{-5} \cdot k_{-7}} + \frac{[B] \cdot [P] \cdot k_{+6} \cdot k_{+7}}{k_{-6} \cdot k_{-7}} + \frac{k_{+5} \cdot [Q]}{k_{-5}} \\ &+ \frac{k_{+6} \cdot [P]}{k_{-6}} + \frac{k_{+2} \cdot [A]}{k_{-2}} + \frac{k_{+3} \cdot [B]}{k_{-3}} \end{aligned}$$

Substitution of f_i values and of K_i for k_{+i}/k_{-i} ratio leads to an equation of degree 3:3 with respect to the concentrations of each substrate and product, which can be expressed as follows:

$$\begin{aligned} v &= \frac{[E_0] \cdot \{(k_{+26} + k_{+24} \cdot K_2 \cdot [A] + k_{+25} \cdot K_3 \cdot [B] + k_{+28} \cdot K_6 \cdot [P] + k_{+27} \cdot K_5 \cdot [Q]) \cdot K_7 \cdot [B] \cdot (k_{+11} + k_{+10} \cdot K_2 \cdot [A] + k_{+9} \cdot K_3 \cdot [B] \\ &+ k_{+12} \cdot K_6 \cdot [P] + k_{+13} \cdot K_5 \cdot [Q]) \cdot K_1 \cdot [A] - (k_{+16} + k_{+14} \cdot K_2 \cdot [A] + k_{+15} \cdot K_3 \cdot [B] + k_{+18} \cdot K_6 \cdot [P] \\ &+ k_{+17} \cdot K_5 \cdot [Q]) \cdot K_4 \cdot [Q] \cdot (k_{+21} + k_{+20} \cdot K_2 \cdot [A] + k_{+19} \cdot K_3 \cdot [B] + k_{+22} \cdot K_6 \cdot [P] + k_{+23} \cdot K_5 \cdot [Q]) \cdot K_8 \cdot [P]\}} \\ &\{(k_{+21} + k_{+20} \cdot K_2 \cdot [A] + k_{+19} \cdot K_3 \cdot [B] + k_{+22} \cdot K_6 \cdot [P] + k_{+23} \cdot K_5 \cdot [Q]) \cdot K_8 \cdot [P] + (k_{+26} + k_{+24} \cdot K_2 \cdot [A] + k_{+25} \cdot K_3 \cdot [B] \\ &+ k_{+28} \cdot K_6 \cdot [P] + k_{+27} \cdot K_5 \cdot [Q]) \cdot K_7 \cdot [B]\} \cdot (1 + K_1 \cdot [A] + K_2 \cdot [A] + K_3 \cdot [B] + K_6 \cdot [P] + K_4 \cdot [Q] + K_5 \cdot [Q] \\ &+ K_1 \cdot K_2 \cdot [A] \cdot [A] + K_1 \cdot K_3 \cdot [A] \cdot [B] + K_1 \cdot K_6 \cdot [A] \cdot [P] + K_2 \cdot K_4 \cdot [A] \cdot [Q] + K_5 \cdot K_1 \cdot [A] \cdot [Q] + K_3 \cdot K_4 \cdot [B] \cdot [Q] \\ &+ K_4 \cdot K_6 \cdot [P] \cdot [Q] + K_4 \cdot K_5 \cdot [Q] \cdot [Q]) + \{(k_{+11} + k_{+10} \cdot K_2 \cdot [A] + k_{+9} \cdot K_3 \cdot [B] + k_{+12} \cdot K_6 \cdot [P] + k_{+13} \cdot K_5 \cdot [Q]) \cdot K_1 \cdot [A] \\ &+ (k_{+16} + k_{+14} \cdot K_2 \cdot [A] + k_{+15} \cdot K_3 \cdot [B] + k_{+18} \cdot K_6 \cdot [P] + k_{+17} \cdot K_5 \cdot [Q]) \cdot K_4 \cdot [Q]\} \cdot (1 + K_2 \cdot [A] + K_3 \cdot [B] \\ &+ K_7 \cdot [B] + K_6 \cdot [P] + K_8 \cdot [P] + K_5 \cdot [Q] + K_2 \cdot K_7 \cdot [A] \cdot [B] + K_2 \cdot K_8 \cdot [A] \cdot [P] + K_3 \cdot K_7 \cdot [B] \cdot [B] + K_6 \cdot K_7 \cdot [B] \cdot [P] \\ &+ K_3 \cdot K_8 \cdot [B] \cdot [P] + K_5 \cdot K_7 \cdot [B] \cdot [Q] + K_6 \cdot K_8 \cdot [P] \cdot [P] + K_5 \cdot K_8 \cdot [P] \cdot [Q]) \end{aligned} \quad (14)$$

$$\begin{aligned} f_{19} &= \frac{[P] \cdot [B] \cdot k_{+3} \cdot k_{+8}}{k_{-3} \cdot k_{-8} \cdot d_{11}}, & f_{20} &= \frac{[A] \cdot [P] \cdot k_{+2} \cdot k_{+8}}{k_{-2} \cdot k_{-8} \cdot d_{11}}, \\ f_{21} &= \frac{[P] \cdot k_{+8}}{k_{-8} \cdot d_{11}}, & f_{22} &= \frac{[P]^2 \cdot k_{+6} \cdot k_{+8}}{k_{-6} \cdot k_{-8} \cdot d_{11}}, \\ f_{23} &= \frac{[P] \cdot [Q] \cdot k_{+5} \cdot k_{+8}}{k_{-5} \cdot k_{-8} \cdot d_{11}}, & f_{24} &= \frac{[A] \cdot [B] \cdot k_{+2} \cdot k_{+7}}{k_{-2} \cdot k_{-7} \cdot d_{11}}, \\ f_{25} &= \frac{[B]^2 \cdot k_{+3} \cdot k_{+7}}{k_{-3} \cdot k_{-7} \cdot d_{11}}, & f_{26} &= \frac{[B] \cdot k_{+7}}{k_{-7} \cdot d_{11}}, \\ f_{27} &= \frac{[B] \cdot [Q] \cdot k_{+5} \cdot k_{+7}}{k_{-5} \cdot k_{-7} \cdot d_{11}}, & f_{28} &= \frac{[B] \cdot [P] \cdot k_{+6} \cdot k_{+7}}{k_{-6} \cdot k_{-7} \cdot d_{11}} \end{aligned}$$

This rate equation deduced from the mechanism of Scheme 1 has a high degree (3:3) that has not been detected experimentally. To solve the discrepancy between the theoretical eqn. (14) and the experimental eqn. (13) a reduction of the degree of eqn. (14) is necessary.

Although other hypotheses might exist, the only possibility found for simplifying eqn. (14) is that the following constants are numerically indistinguishable:

$$\begin{aligned} k_{+19} &= k_{+25} = k_{+9} = k_{+15} \\ k_{+20} &= k_{+24} = k_{+10} = k_{+14} \\ k_{+21} &= k_{+26} = k_{+11} = k_{+16} \\ k_{+22} &= k_{+28} = k_{+12} = k_{+18} \\ k_{+23} &= k_{+27} = k_{+13} = k_{+17} \end{aligned}$$

Substituting, we readily discovered that there is a common factor of the numerator and denominator of

eqn. (14), which is $(k_{+9} \cdot K_3 \cdot [B] + k_{+10} \cdot K_2 \cdot [A] + k_{+11} + k_{+12} \cdot K_6 \cdot [P] + k_{+13} \cdot K_5 \cdot [Q])$. Some of the equalities assumed among rate constants should be intuitively accepted because of the symmetry of the mechanism, but other ones are not obvious. However, if the constants although not identical have similar values, no increment in the degree would be experimentally detectable according to the low probabilities (0.43 for the 3:3 mechanism) of detection of high rate-equation degrees of complex kinetic mechanisms as described by Burguillo *et al.* (1983).

If we also consider that [oxaloacetate] is zero the rate equation becomes:

$$v = \frac{[E_0] \cdot (k_{+10} \cdot K_2 \cdot K_7 \cdot K_1 \cdot [A]^2 \cdot [B] + k_{+11} \cdot K_7 \cdot K_1 \cdot [A] \cdot [B] + k_{+9} \cdot K_1 \cdot K_3 \cdot K_7 \cdot [A] \cdot [B]^2 + k_{+13} \cdot K_1 \cdot K_5 \cdot K_7 \cdot [A] \cdot [B] \cdot [Q])}{K_1 \cdot [A] + K_7 \cdot [B] + (K_7 \cdot K_2 + 2 \cdot K_7 \cdot K_1 + K_3 \cdot K_1) \cdot [A] \cdot [B] + K_2 \cdot K_1 \cdot [A] \cdot [A] + K_3 \cdot K_7 \cdot [B]^2 + 2 \cdot K_1 \cdot K_2 \cdot K_7 \cdot [A]^2 \cdot [B] + 2 \cdot K_3 \cdot K_7 \cdot K_1 \cdot [A] \cdot [B]^2 + (K_5 \cdot K_1 + K_2 \cdot K_4) \cdot [A] \cdot [Q] + (K_5 \cdot K_7 + 2 \cdot K_4 \cdot K_7 + K_3 \cdot K_4) \cdot [B] \cdot [Q] + K_4 \cdot [Q] + K_5 \cdot K_4 \cdot [Q]^2 + 2 \cdot K_5 \cdot K_7 \cdot K_4 \cdot [B] \cdot [Q]^2 + (2 \cdot K_2 \cdot K_4 \cdot K_7 + 2 \cdot K_5 \cdot K_1 \cdot K_7) \cdot [A] \cdot [B] \cdot [Q] + 2 \cdot K_4 \cdot K_3 \cdot K_7 \cdot [B]^2 \cdot [Q]}$$

(15)

This expression (eqn. 15) and the global equation experimentally obtained (eqn. 13) only differ in the additional presence of the term $[A] \cdot [B] \cdot [Q]$ at the numerator. However, this difference can be perfectly explained by considering that the existence of a $[A] \cdot [B] \cdot [Q]$ term in the numerator of the experimental equation is detected when low concentrations of the substrate L-aspartate are used (see Results section).

So, the mechanism described at Scheme 1 is proposed by us as the minimum that justifies the experimental results for the studied enzymes. However, it is possible that the degree of the actual equation was higher and the statistical significance of the terms that are theoretically plausible was not sufficient to detect it.

Also, the working methodology used in these studies (Franco *et al.*, 1986; Bruguera *et al.*, 1988) shows that, although the assignment of a complex correct mechanism is not possible from the kinetic studies, these can be a powerful tool in order to confirm or discard particular hypotheses about a plausible mechanism.

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