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PROGRESS CURVE ANALYSIS IN ENZYME KINETICS

MODEL DISCRIMINATION AND PARAMETER ESTIMATION

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Summary

The method of progress curve analysis for enzyme-catalyzed reactions (Duggleby, R.G. and Morrison, J.F. (1977) Biochim. Biophys. Acta 481, 297—312) has been extended to a two substrate, reversible reaction through the use of enzyme-catalyzed recycling of one of the products. The reaction investigated was that catalyzed by aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) and the product, α-ketoglutarate was recycled to glutamate using NADH and NH_4Cl in the presence of glutamate dehydrogenase. The values determined for the kinetic parameters of the aminotransferase were found to agree well with those obtained from steady-state velocity measurements. The standard errors of the parameters, as calculated by the procedure originally described, were found to underestimate the observed variation between different experiments. Therefore, a procedure of data compression was devised which leads to more realistic values for standard errors. The compressed data obtained with aspartate aminotransferase have been fitted to the integrated rate equations that describe a variety of kinetic mechanisms. The best fit was obtained with the Ping-Pong model which is applicable to the aspartate aminotransferase reaction. Thus, progress curve analysis may be used to determine the kinetic mechanism of, and values of the kinetic parameters associated with, an enzyme-catalyzed reaction.

Introduction

In a recent publication [1], a procedure was reported for the analysis by non-linear regression of progress curves for irreversible enzyme-catalyzed reactions. The prime objective of that study was the determination of the values and associated standard errors of the kinetic parameters for a reaction conform-

ing to an assumed kinetic mechanism. This objective was achieved, but subsequently it was realized that the data obtained from any given progress curve are not statistically independent and the procedure adopted for calculating standard errors was probably underestimating these values. A method has now been developed for the calculation of what appear to be realistic standard errors, as judged by comparison of these statistics with the variation of the kinetic parameters obtained from different progress curve experiments.

In kinetic investigations on enzyme-catalyzed reactions, evaluation of the kinetic parameters is frequently of secondary interest only while the main aim is to elucidate the kinetic mechanism of the reaction. This objective can be achieved by the application of steady-state kinetic techniques, but it was thought that it may also be possible to reach conclusions about the kinetic mechanism of a reaction by analysis of progress curve data. Thus studies have been undertaken on the reaction catalyzed by aspartate aminotransferase (EC 2.6.1.1). This enzyme catalyses a reversible reaction and is not well suited to progress curve analysis using the procedure described earlier [1]. A technique of product recycling has been developed which facilitates the analysis of such systems. From the results obtained by fitting progress curve data to integrated rate equations for a variety of sequential and non-sequential mechanisms, it is concluded that the reaction conforms to a Ping-Pong mechanism.

Materials and Methods

Pig heart aspartate aminotransferase was purchased from Calbiochem, as were oxaloacetic acid and L-aspartic acid. Bovine liver glutamate dehydrogenase (L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3) with a specific activity of 50 U/mg was obtained from Sigma as a suspension in 2 M ammonium sulphate from which it was removed by centrifugation before use. L-Glutamic acid was obtained from Fluka and PL Biochemicals supplied NADH. Pig heart malate dehydrogenase (L-malate:NAD$^+$ oxidoreductase, (EC 1.1.1.37) was purchased from Boehringer Mannheim and other chemicals were high purity preparations from the usual commercial sources.

Assay of aspartate aminotransferase. Aspartate aminotransferase was assayed using oxaloacetate and glutamate as substrates, with the product α-ketoglutarate being reconverted to glutamate using NADH and NH_4Cl in the presence of glutamate dehydrogenase. The reaction was monitored at 30° C by following the disappearance of NADH at 340 nm with a Cary 118 spectrophotometer. The absorbance data were digitized automatically and collected on magnetic tape as described previously [1]. Reaction mixtures contained 0.1 M sodium arsenate buffer (pH 7.4), 0.2 mM NADH, 20 mM NH_4Cl, 0.4 mg/ml glutamate dehydrogenase in addition to oxaloacetate, glutamate and aspartate at the concentrations specified in the text.

Data analysis. Progress curve data were analysed using the PROCURA programs [1] which are based on the Gauss-Newton method for non-linear regression. These programs usually utilise analytical expressions to calculate values for the partial derivatives, but is was convenient in some cases to approximate these values using numerical methods of differentiation. A more detailed description of the programs may be obtained from the authors.

Results

Establishment of experimental conditions

Studies on any enzyme reaction for which oxaloacetate is a substrate can be complicated because of the existence of this compound in solution as an equilibrium mixture of enol and keto forms. At neutral pH, the latter form predominates and it is very likely that this form always functions as the substrate. However, significant amounts of the enol form of oxaloacetate would be in solution and thus it becomes important to establish that the enol to keto tautomerism does not limit the rate of the aspartate aminotransferase reaction. Since arsenate catalyses the interconversion of the enol and keto forms of oxaloacetate [2], sodium arsenate (pH 7.4) was chosen as the buffer for studies on aspartate aminotransferase and determinations were made of the half-life of the enol form under these conditions. The determinations were conducted by monitoring the oxidation of NADH by oxaloacetate in the presence of a large excess of malate dehydrogenase. There was an initial rapid oxidation of NADH which corresponded to the reduction of the predominant keto form of oxaloacetate, followed by a slower oxidation of NADH which represented the conversion of the enol to the keto form of oxaloacetate. The weighted mean value of the half-life from three experiments was calculated to be 8.88 ± 0.03 s. This rate is very much faster than those of the transamination reactions, which typically took 5—15 min to utilize half of the starting concentration of oxaloacetate, so the concentration of the active substrate could be considered as being a constant fraction of the total oxaloacetate concentration. As the fraction of the inactive enol form was not determined with precision, all oxaloacetate concentrations reported are the total of the two tautomers.

The recycling assay for the aspartate aminotransferase reaction was based on the conversion of enzymatically-formed α-ketoglutarate back to glutamate by NADH and NH_4Cl in the presence of glutamate dehydrogenase. With such a coupled assay it is important that the α-ketoglutarate arising from glutamate be maintained at negligible concentrations for the extent of NADH oxidation to reflect accurately the extent of the aminotransferase reaction. In the present work it was even more important that this condition be met since it was assumed that recycling would keep glutamate concentrations constant at their initial values. Tests indicated that the aforementioned requirements were satisfied by using glutamate dehydrogenase at a concentration of 0.1 mg/ml because there was no lag in the oxidation of NADH and the reaction rate was not increased by further additions of glutamate dehydrogenase. However, to ensure that the recycling system was always fully operative, the glutamate dehydrogenase was used at a concentration of 0.4 mg/ml for all progress curve studies.

Kinetic parameters for aspartate aminotransferase

To facilitate understanding of the experimental procedures that were adopted to determine values for kinetic parameters from the analysis of progress curve data, the following terms are defined: Progress curve: a single curve which is obtained as a result of monitoring product formation or substrate disappearance as a function of time. Progress curve experiment: consists of several progress curves which are obtained at different substrate concentrations either

in the absence or presence of product. Data point: a single measurement of the amount of product formed by reaction (z) at time t. Data set: refers to a number of data points which are taken from one progress curve.

Under the conditions chosen to study the aspartate aminotransferase reaction, five kinetic parameters can be evaluated. These are the maximum velocity, the Michaelis constants for oxaloacetate and glutamate and the inhibition constants for oxaloacetate and aspartate. Values for these parameters may be determined by fitting progress curve data to Eqn. 1:

$$V \cdot t = \left\{1 + \frac{K_{glu}}{glu_0}\left[1 + \frac{(asp_0 - K_{ioaa})}{K_{iasp}}\right]\right\} z + \left\{\frac{K_{glu}}{glu_0 K_{iasp}}\right\} \frac{z^2}{2} - \left\{K_{oaa} + \frac{K_{ioaa}K_{glu}(oaa_o + asp_o)}{glu_o K_{iasp}}\right\} \ln\left[1 - \frac{z}{oaa_o}\right] \quad (1)$$

where oaa_0, glu_0 and asp_0 are the initial concentrations of these reactants, and the remaining symbols represent the kinetic parameters. This equation is the integrated form of the steady state rate equation (Eqn. 2) that

$$v = \frac{V}{1 + \frac{K_{oaa}}{oaa} + \frac{K_{glu}}{glu} + \frac{K_{ioaa}K_{glu}asp}{K_{iasp}oaa\ glu} + \frac{K_{glu}asp}{K_{iasp}glu}} \quad (2)$$

applies to a Bi-Bi-Ping-Pong mechanism in the absence of one of the products. For the purposes of integration, it was assumed that the glutamate concentration remains constant while oxaloacetate is depleted and aspartate accumulates as the reaction progresses. It can be shown that the five parameters of Eqn. 1 may be determined from a minimum of two progress curves obtained at different concentrations of glutamate. However, as it was planned to fit progress curve data to the equations for other mechanisms, progress curves were run at various initial concentrations of the three reactants. Three progress curve experiments were performed and each experiment consisted of 15—20 progress curves, with oxaloacetate in the range of 45—105 μM, glutamate in the range of 2—10 mM and aspartate in the range of 0—4 mM. Under all conditions there was no inactivation of the enzyme during the course of the reaction as judged by the fact that plots of product formed against enzyme concentration multiplied by time gave superimposable curves with different enzyme concentrations [3]. For any one experiment, 40—60 data points were taken from each progress curve and then all data points were fitted to Eqn. 1. The values obtained for the five kinetic parameters are listed in Table I together with their associated errors. Although there is good agreement between the three experiments, the standard errors appear to be too small. If the standard error of a parameter is a realistic estimate of the variation to be expected in the parameter value between experiments, then the absolute values of the normalized errors (as defined in Table I) should be mostly below a value of 1. However, the data (Table I) indicate that these values are very much higher than 1 with one-third of them being greater than 6. Clearly, the procedure used to determined standard errors underestimates the parameter variation between experiments.

TABLE I

KINETIC PARAMETERS FOR ASPARTATE AMINOTRANSFERASE

Three separate progress curve experiments were performed as described in the text, and analysed by fitting to Eqn. 1 using a weighting factor of $1/A_0$. Values (Θ) and standard errors (S.E. (Θ)) for each of the five kinetic parameters which result from this analysis were used to calculate weighted means, ($\overline{\Theta}$) from the relationship $\overline{\Theta} = \Sigma\omega\Theta/\Sigma\omega$, where $\omega = [1/\text{S.E.}(\Theta)]^2$. Normalised errors for each parameter were then calculated from the expression $(\Theta - \overline{\Theta})/\text{S.E.}(\Theta)$.

Kinetic constant	Value ± standard error (normalised error)			Weighted mean
	Experiment 1	Experiment 2	Experiment 3	
V (U/mg)	803 ± 14 (3.9)	690 ± 9 (−6.6)	788 ± 9 (4.3)	749
K_{oaa} (μM)	46.7 ± 1.6 (2.3)	36.4 ± 0.9 (−7.3)	49.2 ± 1.0 (6.2)	43.0
K_{ioaa} (μM)	27.9 ± 1.3 (2.0)	30.0 ± 1.5 (3.1)	21.7 ± 1.0 (−3.6)	25.3
K_{glu} (mM)	8.05 ± 0.16 (−6.7)	8.42 ± 0.13 (−5.4)	10.98 ± 0.15 (12.4)	9.12
K_{iasp} (mM)	3.88 ± 0.07 (−2.7)	4.26 ± 0.09 (2.1)	4.13 ± 0.07 (0.9)	4.07

Analysis of compressed data

In an endeavour to obtain more realistic estimates of the standard errors of kinetic parameters, a process of data compression was introduced. The data from a single progress curve were fitted to Eqn. 3 which is the general form of an integrated rate equation that describes the progress curve of virtually any reaction that can be considered to involve only a single substrate (cf. ref. 1). In Eqn. 3,

$$R_1 t = z + \frac{R_2 z^2}{2} - R_3 \ln\left(1 - \frac{z}{A_0}\right) \qquad (3)$$

A_0 is the initial concentration of the substrate which becomes depleted while R_1, R_2 and R_3 denote the parameters to be estimated. The resulting values for the three parameters were used to calculate three idealized data points which corresponded to the first and last time intervals for which real data points were collected as well as the mean of the two time intervals. In effect the whole progress curve of 40–60 data points is time-averaged to give three new data

TABLE II

KINETIC PARAMETERS FOR ASPARTATE AMINOTRANSFERASE, VALUES DERIVED USING COMPRESSED DATA SETS

Three separate progress curve experiments were performed, and each data set was compressed into three idealised data points as described in the text. The compressed data sets for each experiment were combined and fitted to Eqn. 1 using weighting factors which were determined during compression (see Discussion). Weighted means and normalised errors were calculated as described in Table I.

Kinetic constant	Value ± standard error (normalised error)			Weighted mean
	Experiment 1	Experiment 2	Experiment 3	
V (U/mg)	780 ± 50 (0.72)	715 ± 31 (−0.94)	761 ± 33 (0.52)	744
K_{oaa} (μM)	41.3 ± 5.4 (−0.31)	40.5 ± 3.6 (−0.69)	46.4 ± 3.6 (0.94)	43.0
K_{ioaa} (μM)	25.6 ± 4.0 (0.08)	26.3 ± 4.9 (0.20)	24.3 ± 4.0 (−0.25)	25.3
K_{glu} (mM)	7.94 ± 0.56 (−1.86)	8.61 ± 0.44 (−0.84)	10.52 ± 0.54 (2.85)	8.98
K_{iasp} (mM)	3.79 ± 0.23 (−0.74)	3.95 ± 0.31 (−0.03)	4.22 ± 0.27 (0.96)	3.96

points which contain all the relevant information on the shape of the curve but which are largely free of the noise contained in any real data point. The compressed data sets from each progress curve experiment were then fitted to the integrated form of the appropriate rate equation.

When data for the three experiments on the aspartate aminotransferase reaction were treated as described above and then fitted to Eqn. 1, the results reported in Table II were obtained. From a comparison of these results with those of Table I, it is apparent that while there is little change in the values for the kinetic parameters, the absolute values of the normalized errors are now mostly less than unity. Thus the process of data compression leads to standard errors which are good estimates of the variation to be expected from one experiment to another.

Comparison of data obtained from progress curve and steady-state velocity studies

To confirm that comparable values for kinetic parameters are obtained from progress curve and steady-state velocity studies, a limited number of steady-state velocity experiments, in the absence and presence of aspartate, were undertaken. The Michaelis constants for oxaloacetate (K_{oaa}) and glutamate (K_{glu}) were determined to be 43.5 ± 11.6 μM and 7.06 ± 1.32 mM, respectively, while the apparent inhibition constants for aspartate (app K_{iasp}), as determined from a plot of v^{-1} against aspartate concentration, was found to be 6.10 ± 0.63 mM with glutamate and oxaloacetate at fixed concentrations of 5 mM and 102 μM, respectively. By substituting the above values of the Michaelis constants for glutamate and oxaloacetate, as well as a K_{iasp} value of 4.0 mM (cf. Table V), into the expression for app K_{iasp} as derived from Eqn. 2, K_{ioaa} was calculated to be 32 μM. Thus the values obtained for the kinetic parameters from analysis of steady-state velocity data are in agreement with those listed in Tables I and II.

Model discrimination using progress curve data

For the foregoing analysis it was accepted that the aspartate aminotransferase reaction conforms to a one-site Ping-Pong mechanism and the progress curve data were fitted to the integrated form of the equation for a Ping-Pong model which allows the recycled substrate-product pair (glutamate and α-ketoglutarate) to combine with different enzyme forms. However, in principle, the data could be fitted also to a number of other models. These include a two-site Ping-Pong model in which the recycled reactants compete for the same enzyme form, four ordered models which differ with respect to the order of substrate addition and product release, four Theorell-Chance models in which there is variation in the order of substrate combination and product dissociation and two rapid equilibrium, random models which differ by the formation of a dead-end complex involving enzyme, glutamate and aspartate. The compressed data were fitted to each of the models described and the residual sums of squares calculated (Table III). On the assumption that this quantity provides a useful criterion for choosing between different models, then the results of Table III indicate that the models which best describe the data are models 2 and 12. Closer examination of model 12, which is described by six parameters, revealed

TABLE III

MODEL DISCRIMINATION USING PROGRESS CURVE DATA. COMPARISON OF VALUES FOR RESIDUAL SUM OF SQUARES WHEN DIFFERENT BI-BI MODELS ARE ASSUMED

Compressed data sets from each of three progress curve experiments were fitted to each of the twelve models listed, and the residual sums of squares were calculated. A and P refer to the first substrate bound and the first product released, where such a distinction may be made.

Model				10^4 × sum of squares		
	Mechanism (number of parameters)	A	P	Experiment 1	Experiment 2	Experiment 3
1.	Two-site Ping-Pong (5)	oaa	α-kG	2.814	0.748	1.218
2.	One-site Ping-Pong (5)	oaa	asp	0.401	0.246	0.238
3.	Ordered (5)	glu	α-kG	1.017	0.398	0.327
4.	Ordered (5)	oaa	α-kG	3.644	0.914	1.688
5.	Ordered (6)	glu	asp	3.644 [c]	0.757	1.444
6.	Ordered (6)	oaa	asp	1.017 [d]	0.398 [d]	0.327 [d]
7. [a]	Theorell-Chance (5)	glu	α-kG	1.017	0.398	0.327
8. [b]	Theorell-Chance (5)	oaa	α-kG	3.644	0.914	1.688
9. [b]	Theorell-Chance (5)	glu	asp	3.644	0.914	1.688
10. [a]	Theorell-Chance (5)	oaa	asp	1.017	0.398	0.327
11.	Rapid equilibrium random (5)	oaa	asp	2.840	1.069	1.233
12.	Rapid equilibrium random with asp · glu complex (6)	oaa	asp	0.401 [e]	0.246 [e]	0.234

[a] Models 7 and 10 are equivalent to model 3, except for the definitions of kinetic constants.
[b] Models 8 and 9 are equivalent to model 4, except for the definitions of kinetic constants.
[c] The values of the kinetic constants cause this model to degenerate into model 4.
[d] The values of the kinetic constants cause this model to degenerate into model 3.
[e] The values of the kinetic constants cause this model to degenerate into model 2.

that the parameter values were such that the equation degenerates into one which is indistinguishable from the five parameter equation for the Ping-Pong model. Thus progress curve data may be used to determine kinetic mechanisms for enzyme-catalyzed reactions.

Discussion

In principle, progress curve studies can give the same sort of information as steady-state velocity studies, and moreover, this information may be obtained with less experimentation. Further, progress curve studies can facilitate the study of the inhibition by products which are difficult to obtain in pure form. Finally, progress curve measurements involve no subjective estimation of tangents which may, on occasion, introduce considerable uncertainty. On the other hand, progress curve analysis requires that the enzyme, substrates and products be stable under assay conditions, and is not very amenable to graphical representation of the data.

It has been demonstrated [1] that analysis of progress curve data for enzyme-catalyzed reactions is a useful method for determining values of the kinetic parameters. However, algebraic considerations tend to restrict the application of the method to single substrate, irreversible reactions. Two substrate

reversible reactions may be studied by raising the concentration of one substrate to a sufficiently high level that its concentration remains approximately constant and the reaction becomes essentially irreversible. The latter approach may be impractical, or undesirable because of substrate inhibition. An alternative approach to the problem of reversible Bi-Bi reactions involves reconverting one of the products back to substrate by either chemical or enzymic means. Provided that the recycling system is essentially irreversible then the enzyme reaction under study becomes effectively irreversible. Further, such recycling allows the concentration of the substrate involved in the recycling to be held constant at any desired concentration.

The recycling system used for the present work is one which can be utilized with any aminotransferase that will accept glutamate as an amino group donor as it involves the reconversion of α-ketoglutarate to glutamate by NADH and NH_4Cl in the presence of glutamate dehydrogenase. It offers a convenient method for continuous monitoring of an aminotransferase reaction by following the oxidation of NADH, and it has been used to obtain progress curve data for the reaction catalyzed by aspartate aminotransferase. These data have been analyzed to yield information about the kinetic mechanism of the reaction and values for a number of kinetic parameters. Naturally, values for the kinetic constants associated with α-ketoglutarate could not be determined. For both steady-state velocity and progress curve studies which employ a coupling or recycling assay system, information regarding the kinetic effects of one of the products can never be obtained as these assays rely on maintaining the concentration of that product close to zero.

Data compression and calculation of standard errors of kinetic parameters

In the calculation of standard errors of parameters, it is normally assumed that the variance (σ^2) is approximately equal to the residual variance (s^2) which is calculated from the relationship: $s^2 = RSS/(n-p)$ where RSS is the minimum residual sum of squares, n is the number of experimental points and p is the number of parameters being estimated. The standard errors of the parameters are then approximately equal to the limits of a hyperellipsoid in parameter space which encloses all sets of parameter values for which the residual sum of squares is less than or equal to $s^2\,(n-p+1)$. When a continuous assay method is available to monitor an enzyme-catalyzed reaction, it is possible to collect a very large number of experimental points which would have the effect of reducing the size of this hyperellipsoid. However, it must be recognized that data from a single progress curve will show correlations which will not exist between data from different curves. Thus variation in enzyme concentration will affect all data gathered from one progress curve in a systematic manner but these systematic effects will be different with data from different curves. If such correlations are ignored, there will be no major effect on the values for the kinetic parameters, but the estimated values of the standard errors will be too small because the number of degrees of freedom $(n-p)$ will have been overestimated. Ideally, the standard errors of parameters, as calculated from data for a single progress curve experiment, should be indicative of the variation in parameter values which are to be expected from experiment to experiment. This criterion does not hold for the results recorded in Table I and it becomes

apparent that fewer points must be taken from each curve if realistic standard errors are to be calculated. The questions that now arise relate to how many points should be taken from the progress curve and from where along the curve should they be chosen.

Most progress curves can be described by a three parameter general equation (Eqn. 3) and consequently are defined completely by three data points, provided that they are without error. A method of approximating this condition is to fit a large number of data points from a progress curve to Eqn. 3 and use the resulting estimates of R_1, R_2 and R_3 to calculate the expected value of the dependent variable for each of three time intervals. The effect of this procedure is to compress the data from one curve and eliminate, as far as possible, the intra-experimental variation. The three idealized data points for any one progress curve then embody all the essential information about the shape of the complete curve and may be combined with compressed data from other curves for complete analysis of the reaction under investigation. The procedure cannot eliminate completely the variation within a single progress curve and error will be associated with the values for each of the R parameters. These errors will, in turn, affect the precision of the idealized data points which should be assessed so as to determine the weighting factors for each point in subsequent analysis. The variance of each point may be calculated from a generalized form of the formula given by Wilkinson [4]. Specifically, the variance of z, the amount of product formed at any selected time, is given by $x^T \cdot [s^2 (X^T \cdot X)^{-1}]$. x where $s^2 (X^T \cdot X)^{-1}$ is the variance-covariance matrix, x is the vector of partial derivatives with respect to the parameters (dz/dR) evaluated at point z, and x^T is the transform of x. Each of these quantities is normally calculated during the fitting procedure and so it is a relatively simple matter to determine the variance of z and hence its reciprocal as the weighting factor (w) for each idealized data point. These weighting factors do not take into account variation which is related to substrate concentration and which will, therefore, affect the curves to differing extents. For this reason, the results of Tables II, III and IV were obtained using weighting factors which were calculated such that the relative weights of each data point within a compressed data set were proportional to w, while the sum of the weights within a compressed data set was equal to $1/A_0$, where A_0 represents the initial concentration of oxaloacetate.

For the analyses performed in the present study, the three idealized data points were chosen to correspond to the beginning, middle and end of the time period over which the data were collected. To some extent these choices were arbitrary, but they had some basis in the results of the re-analysis of data obtained for the reaction catalyzed by prephenate dehydratase [1]. For all practical purposes this is an Uni-Uni reaction. Thus any progress curve is described by a simplified form of Eqn. 3 which does not contain a z^2 term because R_2 must be equal to zero. Consequently, only two idealized points are required to define the curve and these may be taken from within any region of the progress curve including those beyond the range of the data. Idealized points were calculated over the range from 5 to 95% substrate utilization at intervals of 5%. Each possible data pair from one curve was taken and combined with equivalent data pairs from other curves. The combined data were fitted to the integrated equation for an Uni-Uni model and from each fit the

product of the relative standard errors of the three parameters V, K_a and K_p was calculated. Table IV shows part of this analysis and indicates that this quantity (α) was smallest when data points were chosen from each end of the progress curves. It was concluded that, for this model, the two points should be chosen from each end of the progress curve, and that extrapolation into regions beyond the extremes of the experimental curves offered no advantage. Thus, the two points should be chosen to correspond to the first and last time interval at which data were actually obtained. For progress curves described by a three-parameter general equation it seemed reasonable to choose one data point from the beginning of the curve, one from the end of the curve and a third from a point somewhere between the other two. The data point at the mean, on the time axis, of the other two points was chosen for computational simplicity.

The use of the aforementioned analytical procedure is vindicated by the results obtained from progress curve studies with aspartate aminotransferase (Table II). The standard errors of the kinetic parameters give a good indication of the variation to be expected in the values of the parameters as determined from different experiments. Comparison of the results of Tables I and II indicates that data compression makes little difference to the estimated values for the kinetic parameters in any individual experiment and virtually no difference to the weighted mean values as determined from three experiments (Table V). These values for the kinetic parameters associated with the aminotransferase reaction have been compared with those obtained by Henson and Cleland [2] from an analysis of steady-state velocity data (Table V). The values

TABLE IV

VALUES OF ALPHA (α) DETERMINED BY ANALYSIS OF PAIRS OF IDEALISED DATA POINTS OBTAINED FROM PROGRESS CURVES FOR THE PREPHENATE DEHYDRATASE REACTION

Each of five progress curves for prephenate dehydratase [1] were compressed into two idealised data points as described in the text. These were combined and fitted to the integrated rate equation for a Uni-Uni reaction. Alpha (α) was calculated from the expression

$$\alpha = \frac{[\text{S.E. } (V)][\text{S.E. } (K_a)][\text{S.E. } (K_p)]}{[V][K_a][K_p]}$$

and is equal to the product of the relative standard errors of the three parameters V, K_a and K_p. The experimental data covered the ranges of 18—87%, 17—85%, 21—94%, 18—93% and 12—87% substrate utilisation.

$10^3 \times \alpha$

Point 2	Point 1				
	Percent substrate utilisation at idealised points				
	5	25	45	65	85
15	1.89	2.96	0.59	0.32	0.25
35	0.53	4.17	5.37	0.67	0.26
55	0.32	0.64	6.42	7.10	0.59
75	0.25	0.32	0.68	7.06	n.d. [a]
95	0.24	0.24	0.28	0.46	n.d.

[a] n.d., not determined.

TABLE V

COMPARISON OF THE KINETIC PARAMETERS ASSOCIATED WITH THE ASPARTATE AMINOTRANSFERASE REACTION

Progress curves were analysed as described in Tables I and II, and the weighted means of three experiments were calculated. The steady-state velocity data of Henson and Cleland [2] are the values reported for the anionic and cationic forms of the enzyme. Other steady-state velocity results were determined as described in the text.

Kinetic constant	Method of analysis			
	Progress curves		Steady state velocity	
	Raw data	Compressed data	Present work	Henson and Cleland (ref. 2)
K_{oaa} (μM)	43	43	44	88—95
K_{ioaa} (μM)	25	25	32	48—50
K_{glu} (mM)	9.1	9.0	7.1	8.9—9.6
K_{iasp} (mM)	4.1	4.0	n.d. [a]	3.5—3.9

[a] n.d., not determined.

obtained for the amino acid reactants are in good agreement while the kinetic constants for oxaloacetate are lower as determined from progress curve data. These discrepancies may be due to slight differences in assay conditions, as it has been noted elsewhere [5] that the kinetic parameters for the substrates of the aspartate aminotransferase reaction, and particularly those for oxaloacetate, are very sensitive to relatively minor changes in solvent composition. This explanation is supported by the observation that analysis of steady-state velocity or progress curve data obtained under identical experimental conditions yield similar values of the kinetic constants for oxalacetate, glutamate and aspartate (Table V).

Progress curve analysis and model discrimination

The data derived from progress curves contain the same type of information as those which are acquired from steady-state velocity studies and in principle, either experimental approach can be used for the elucidation of the kinetic mechanism of an enzyme-catalyzed reaction. Therefore, an attempt was made to use progress curve data to deduce kinetic mechanisms. The compressed data for the aspartate aminotransferase reaction were fitted to the integrated rate equations that describe a variety of Bi-Bi models and conclusions about the goodness of fit were made on the basis of a single criterion, the residual sum of squares (Table III). The values for this quantity indicated that the reaction conforms to a one-site Ping-Pong mechanism, the established mechanism for the aspartate aminotransferase reaction [2], or to a rapid equilibrium, random mechanism which involves the formation of a dead-end enzyme · aspartate · glutamate complex (model 12). It should be noted that the fitting of data to the equations describing these two models yielded consistently lower values for the residual sums of squares than when the data were fitted to equations for the other models. This was true in spite of the fact that several of the latter

equations contained an additional parameter. Closer examination of the values of the kinetic parameters obtained by fitting the data to the integrated equation describing model 12 revealed that their magnitudes were such as to cause degeneration of this equation to one of the same form as that which describes the Ping-Pong mechanism (model 2). While the rapid equilibrium, random mechanism cannot be definitively excluded, the values of the kinetic parameters make it appear unlikely. In this connection it should be mentioned that the use of a recycling (or coupling) system will preclude differentiation between mechanisms wherever such differentiation relies on the kinetic effects of the recycled product. The problem is not unique to studies with progress curves and applies equally to those employing steady-state velocity measurements.

References

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