Brazilian Journal of Medical and Biological Research (1998) 31: 1105-1111
ISSN 0100-879X

Flavianate, an amino acid precipitant, is a competitive inhibitor of trypsin at pH 3.0

J.M. Schneedorf, M.M. Santoro and M. Mares-Guia

Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

Abstract

Textile dyes bind to proteins leading to selective co-precipitation of a complex involving one protein molecule and more than one dye molecule of opposite charge in acid solutions, in a process of reversible denaturation that can be utilized for protein fractionation. In order to understand what occurs before the co-precipitation, a kinetic study using bovine ß-trypsin and sodium flavianate was carried out based on reaction progress curve techniques. The experiments were carried out using α-CBZ-L-Lys-*p*-nitrophenyl ester as substrate which was added to 50 mM sodium citrate buffer, pH 3.0, containing varying concentrations of ß-trypsin and dye. The reaction was recorded spectrophotometrically at 340 nm for 30 min, and the families of curves obtained were analyzed simultaneously by fitting integrated Michaelis-Menten equations. The dye used behaved as a competitive inhibitor of trypsin at pH 3.0, with Ki = 99 µM; kinetic parameters for the substrate hydrolysis were: Km = 32 µM, and kcat = 0.38/min. The competitive character of the inhibition suggests a specific binding of the first dye molecule to His-57, the only positively charged residue at the active site of the enzyme.

Correspondence
M. Mares-Guia
BioMM, Inc.
14775 SW 132 Place
Miami, FL 33186-7642
USA
E-mail: maresguia@aol.com

Research supported by CNPq and BioBRAS S/A.

Received September 1, 1997
Accepted May 21, 1998

Key words
• Flavianic acid
• Tripsin inhibition
• Textile dyes

Introduction

Interaction studies on systems of protein co-precipitation with dyes have regained attention in the last decade for the understanding of crystallization processes and bioseparations (1). The analysis and interpretation of kinetic data can be useful to investigate physical phenomena in such associating systems, such as conformational changes or macromolecular polymerization that precede the co-precipitation events (2). Kinetic parameters of enzyme-catalyzed reactions can be evaluated by measuring initial velocities or monitoring progress curves (3). The progress curve records the extent of product formation or substrate depletion at increasing times after initiation of the reaction, and employs non-linear regression techniques in integrated Michaelis-Menten equations to estimate kinetic parameters. The advantages of this technique compared to reaction rate determinations are that progress curves eliminate pipetting errors, require less substrate consumption, are faster than initial rate methods, and permit the evaluation of kinetic

parameters from a single curve with a large number of experimental points, only limited by the system of data acquisition used. The technique has also attracted attention because it appears to offer the opportunity to detect time-dependent features produced during association of ligands with proteins, such as slow transitions or conformational changes arising in response to changes in ligand concentration (4).

In this paper, we report kinetic properties of a dye-protein system in a concentration range below the values observed for co-precipitation, using as a model the inhibition of the well-known serine protease ß-trypsin by flavianate, a yellow textile dye used in the past to fractionate basic amino acids from crude protein extracts (5).

Material and Methods

General

All chemicals used in this study were analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO). The concentration of purified flavianic acid, twice-crystallized as the monosodium salt, was determined spectrophotometrically. Extinction coefficients were determined for the solvent system described below. The experiments were carried out using α-CBZ-L-Lys-*p*-nitrophenyl ester without further purification as a stock solution in dimethylsulfoxide. ß-Trypsin was purified from commercial trypsin according to Schroeder and Shaw (6) and Dias and Rogana (7). Concentrations of ß-trypsin were determined according to Chase and Shaw (8). Enzyme samples dissolved in HCl, pH 3.0, were prepared daily, filtered through a nitrocellulose membrane and centrifuged in a Sorvall instrument with an SS-34 rotor at 45,000 *g* for 45 min to remove a small insoluble residue. The enzyme was then added at a final concentration of 0.2 mg/ml in dilutions of sodium flavianate solubilized in 50 mM sodium citrate buffer, pH 3.0, and placed in the reference and sample cuvettes after a pre-incubation period of 15 min. The samples were carefully mixed and the activity was recorded with a Shimadzu UV-160 spectrophotometer during a steady-state of 30 min at 340 nm at a constant temperature of 37.0°C. Spontaneous substrate hydrolysis was recorded separately. From every progress curve nine equally spaced points were then measured in triplicate. The full assay included a range of initial substrate concentrations of 47, 94, 140 and 185 µM, and a range of initial dye concentrations of 47, 94, 141, 264 and 367 µM.

$$\text{(a)}\quad P = Vm \times t + Km\left(1+\frac{I}{Ki}\right)\left(\ln\frac{S_t}{S_0}\right)$$

$$\text{(b)}\quad P = Vm \times t\left(1+\frac{I}{Ki}\right) + Km\left(\ln\frac{S_t}{S_0}\right)$$

$$\text{(c)}\quad P = \left[Vm \times t + Km\left(\ln\frac{S_t}{S_0}\right)\right]\left(\frac{1}{1+\frac{I}{Ki}}\right)$$

Figure 1 - Integrated Michaelis-Menten equations for general inhibition systems. *a*, Competitive; *b*, non-competitive; *c*, uncompetitive.

Selwyn's test

A test for undesired inactivation of ß-trypsin during the period of kinetic experiment was done according to Selwyn (9). The kinetic assays were conducted as previously described, varying concentrations of ß-trypsin up to 0.6 mg/ml, in the absence of dye. Results were analyzed by plotting the enzyme concentration multiplied by time against the amount of released product.

Non-linear regression

Michaelis-Menten parameters were estimated by fitting values of S_0, S_t, I, P and t to the integrated equations for pure competitive, non-competitive and uncompetitive inhibition models (Figure 1). In these equa-

tions, S_0, S_t, I and P were concentrations of initial substrate, substrate at time t, inhibitor and product, respectively. The data obtained were analyzed with the help of non-linear packages (Sigma Plot release 5.01, Jandel Corporation, San Rafael, CA, and Statistica release 4.5, StatSoft, Inc., Tulsa, OK) able to apply non-linear regression to equations introduced by the user. The values for the initial Km and kcat parameters were calculated according to Lee and Wilson (10). Non-linear fitting was initially done simultaneously with twenty curves to discriminate between competing patterns of inhibition. Adjustments were done with the reciprocal of square product concentration as the statistical weight for loss function. A refined procedure was further applied according to Duggleby and Nash (11) to improve the fitting, placing the previous unadjusted residual range in the loss function of the algorithm. All accepted adjustments converged, with tolerance satisfied, and no ill-matrix conditions.

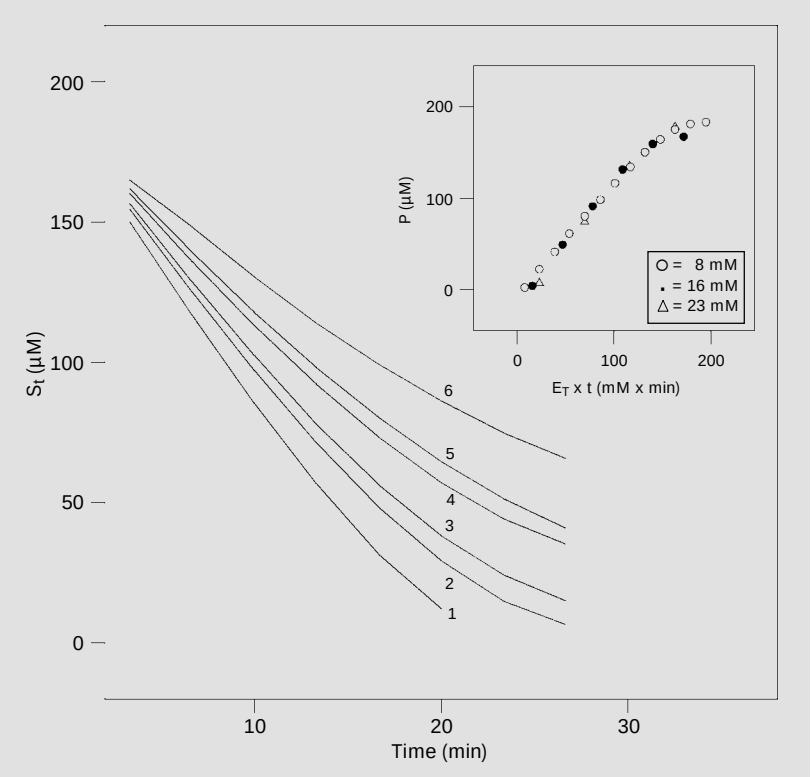

Figure 2 - ß-Trypsin-catalyzed hydrolysis of α-CBZ-L-Lys-*p*-nitrophenyl ester obtained with increasing flavianate concentrations of 47 (1), 94 (2), 141 (3), 264 (4) and 367 (6) µM, at an S_0 of 185 mM. *Ordinate*, Remaining substrate concentration at each time (t). *Inset*, Progress curves of released product plotted against time multiplied by amount of enzyme (Selwyn's test). Symbols represent increasing concentrations of trypsin.

Time-dependence analysis

The flavianate-trypsin system was also investigated for time-dependence indicators like slowness of association-dissociation reactions and hysteretic effect due to molecular transconformations. A decay parameter of substrate depletion was thus assigned to each progression curve. The relations below describe the substrate consumption as a function of time (t):

$$v_i = -\frac{dS}{dt} = kS \qquad \text{Equation 1}$$

Integration of Equation 1 between limits of S_0 and S_t leads to Equation 2:

$$S_t = S_0 e^{-kt} \qquad \text{Equation 2}$$

First-order non-linear adjustments of Equation 2 were applied to substrate consumption data measured in kinetic assays carried out at different enzyme concentra-

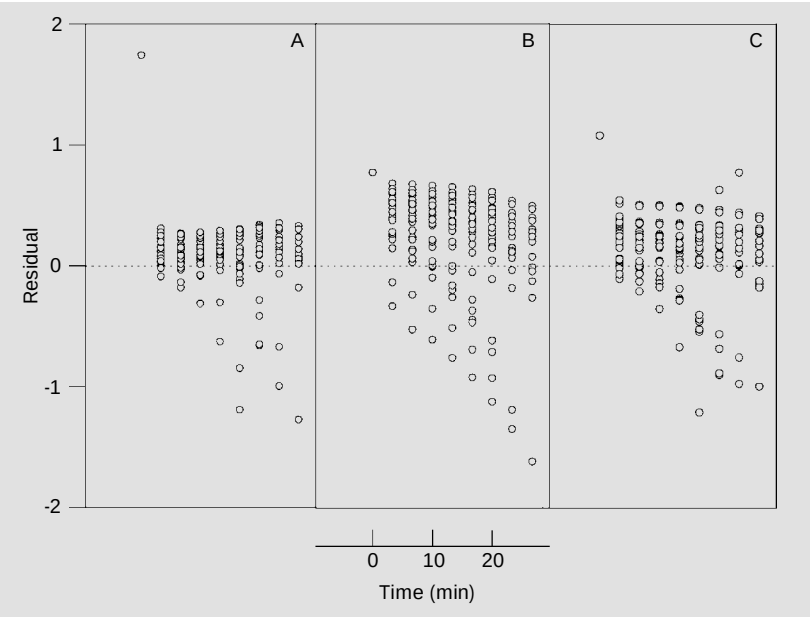

Figure 3 - Residual plots from fitting to competitive (A), non-competitive (B) and uncompetitive (C) kinetic models of integrated Michaelis-Menten equations.

tions and different preincubation periods of flavianate with trypsin, at fixed S_0. Under these experimental conditions, the S_0/Km ratio was in the range of 2-6 x 10^{-3}. The kinetic parameter k obtained was then plotted against these variables and analyzed (4).

Results and Discussion

The interaction of flavianate with trypsin is maximized at pH 3.0. α-CBZ-L-Lys-*p*-nitrophenyl ester was chosen as an adequate substrate for ß-trypsin-catalyzed hydrolysis at this low pH. A sample of primary hydrolysis data shown in Figure 2 reveals that ß-trypsin activity can be easily monitored. In fact, trypsin is inactive only at pH ranges lower than 2 (12). The inset in Figure 2 shows the results of the application of Selwyn's (9) test, which requires that the experimental points should fall on a single curve, indicating that no denaturation of the enzyme occurred.

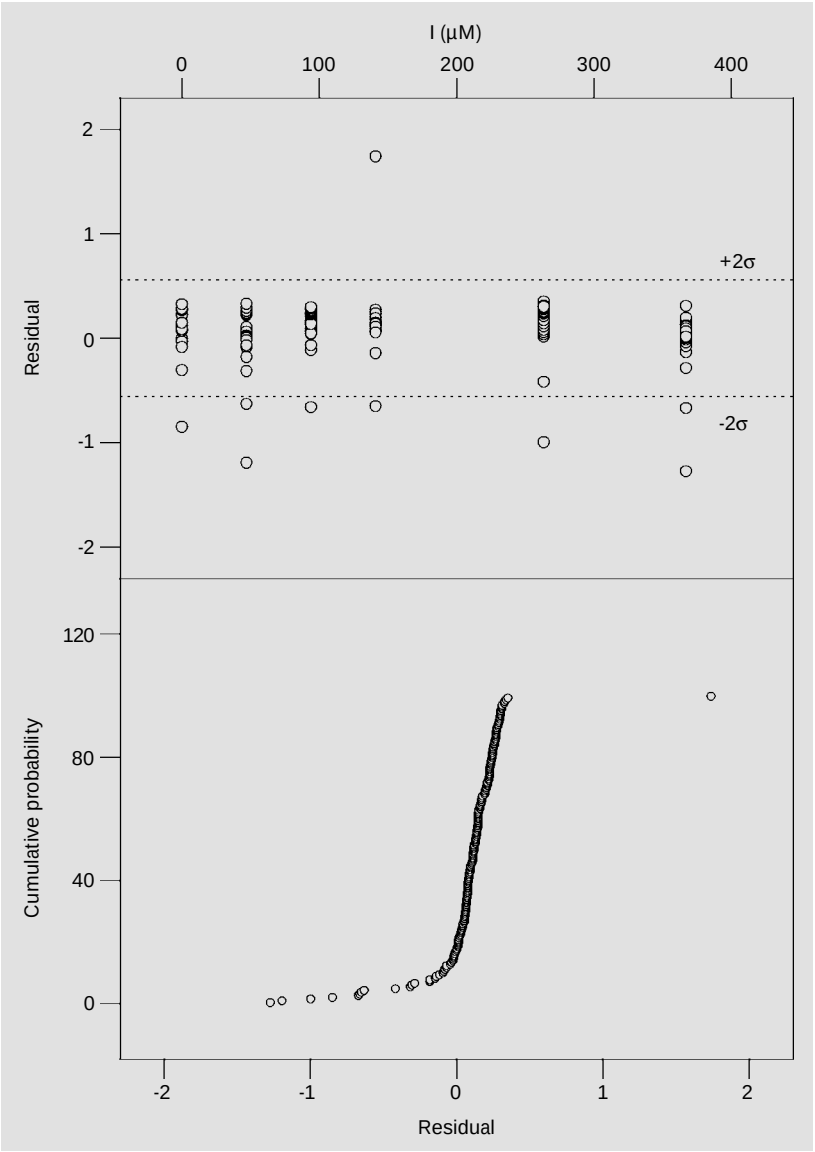

Figure 4 - Residual analysis for the competitive model adjustment. *Upper panel*, Residue plot obtained from adjustments of the integrated competitive model to the data (180 points). *Lower panel*, Cumulative normal probability plot of residues.

Alternative models

The kinetic parameters and standard errors obtained from non-linear fitting of the three inhibition models tested are listed in Table 1. The choice between alternative models was based on the chi-square (χ^2) test and non-linear correlation coefficents (R), since analysis of residual plots alone could not lead to a best model (Figure 3). An F-test based on the chi-square ratio (13) was then applied as the criterion of statistical acceptability for kinetic models. The chi-square ratios for non-competitive/competitive and uncompetitive/competitive models were 2.50 and 1.39, respectively. The results led to the selection of the pure competitive model for inhibition of trypsin by flavianate since the value of the chi-square ratio for this model was higher than the 1.28 value predicted from F-distribution (N = 180, P<0.05).

Having defined the model, further refining of kinetic parameters was done according to Duggleby and Nash (11), with data depleted of outliers. These very extreme values were identified by examination of residues and probability plots (14), the latter a test for normality of residuals (Figure 4, lower panel). This plot represents a set of expected points from a normal distribution with a mean of zero versus cumulative probability. Plotting the residuals against the normal scores gives the usual cumulative nor-

mal probability plot. If the errors are normally distributed, these points should lie on a straight line; otherwise, in the case of non-normality or in the presence of oversized residuals, the data will not follow a general linear trend. Nine extreme residuals in the residue plot (Figure 4, upper panel) were uncovered, and eleven residuals (Figure 4, lower panel), clearly not on a straight line, were detected and deleted. Detection was carried out iteratively as follows: first, we constructed half-normality plots with r and r_{+1} sets of deleted residues that resulted from non-linear adjustments for competitive inhibition. We then applied a linear regression to both data sets and computed their weighted chi-square values. Finally, we tested for the best population of deleted residues by comparing these values using an F-test. Re-fitting was then performed, and the new parameters were determined (Table 1). Applying Duggleby and Nash procedures (11) to this set of data leads to the significant decrease in chi-square value shown in Table 1. Re-fitting by only excluding outliers leads to a chi-square of 3.75. The agreement between observed and calculated data from this new fitting is shown in the lower panel of Figure 5. Michaelian parameters finally evaluated at pH 3.0 (Table 1, last line) were at variance with data reported in the literature at a similar pH range (12,15).

Thus, Bender et al. (15) reported kcat = 0.84/min, Km = 27.8 µM, with kcat/Km = 3.0×10^{-2} min^{-1} μM^{-1}, at pH 2.66, and Ascenzi et al. (12) reported kcat = 3.0/min, Km = 500 µM, with kcat/Km = 6.0×10^{-2} min^{-1} μM^{-1}, at pH 2.55. It is interesting to compare these parameters to those calculated at pH 7.8 (12): kcat = 140/s, Km = 89 µM, and kcat/Km = 1.6 s^{-1} μm^{-1} On the other hand, this is the only study in which purified ß-trypsin was used. Early authors used crystalline trypsin known to contain inactive material (8). The pH dependence of kinetic parameters shows that at acid pH values (2.6-4.8) the deacylation step is rate-limiting in catalysis, with the binding constant Ks defined by the ionization state of the Asp-177 carboxyl group (16). The Duggleby and Nash (11) treatment and outlier depletion led to better results: a linear regression applied to the data in Figure 5 (lower panel) gave a slope of 0.84, suggesting that the pure competitive model chosen to explain the inhibition of ß-trypsin by flavianate is the best approximation of the real situation. This slope, how-

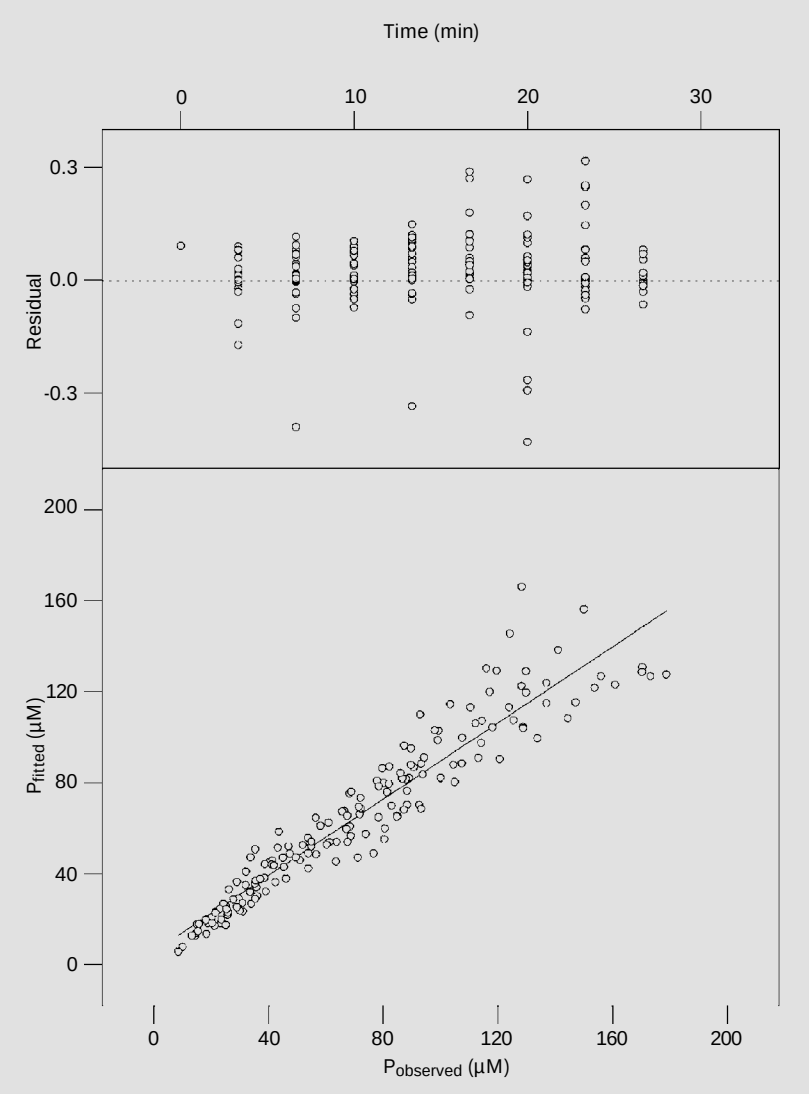

Figure 5 - *Upper panel*, Residual plot relative to the refitting of the integrated competitive model with refined procedures after deletion of outliers. *Lower panel*, Comparison between experimental data for the released product and those calculated from the new adjustments. The continuous line represents a linear regression applied to the data, with a slope of 0.84.

Table 1 - Statistical and kinetic parameters for non-linear adjustments of integrated equations of inhibition models: the ß-trypsin-catalyzed reaction of α-CBZ-L-Lys-*p*-nitrophenyl ester inhibited by flavianate.

χ^2: Chi-squares for residues after fitting. R: Non-linear correlation coefficient for fitting.

Model	Km (µM)	kcat (min)	Ki (µM)	10^2 kcat/Km (min^{-1} μM^{-1})	χ^2	R
Competitive	25 ± 3	0.33 ± 0.02	93 ± 9	1.32	14.89	0.90
Noncompetitive	$5x10^{-8}$ ± $4x10^{-8}$	0.12 ± 0.01	$1x10^7$ ± $2x10^7$	$2.40x10^8$	37.23	0.51
Uncompetitive	45 ± 7	0.42 ± 0.04	136 ± 19	0.93	20.64	0.82
Refitting:						
Competitive	32 ± 2	0.38 ± 0.01	99 ± 4	1.19	1.72	0.94

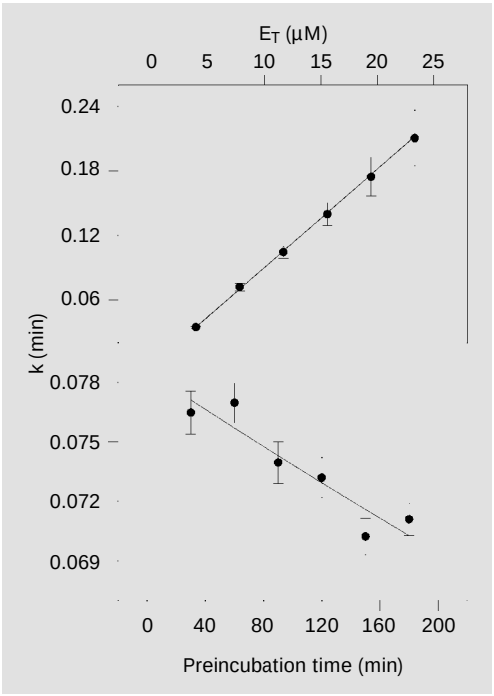

Figure 6 - Time dependence of the activity decay parameter k in the flavianate-trypsin system. *Upper panel*, Linear relationship of the activity decay parameter k with increasing enzyme concentrations at 100 mM flavianate without preincubation. The line represents a weighted linear regression applied to the data. *Lower panel*, Profile of the k parameter at increasing preincubation periods with 150 mM flavianate and trypsin at 0.6 mg/ml. The line is a single exponential decay fitted to the data.

ever, indicates that only 16% of the data were not explained by pure competitive inhibition, with an increased dispersion of points occurring at higher product concentrations (Figure 5, lower panel). Product inhibition can be ruled out as a cause of the bias found at high product formation due to the low substrate concentration employed and the magnitude of systematic errors found in this range, where substrate depletion reached more than 95% of the initial one. Even so, enzyme activity was measured in the presence of high concentrations of *p*-nitrophenol, with no inhibition.

Another explanation for the deviations from the experimental data would be the correctness of the models, since we did not test other patterns of integrated Michaelis-Menten equations because these integrated rate equations are generally available only for simple systems. For even moderately complex systems they become difficult or impossible to derive analytically (17,18). The competitive model found indicated that a dye molecule binds to ß-trypsin at its active center, competing with α-CBZ-L-Lys-*p*-nitrophenyl ester. With the assumptions that flavianate binds to the protein through coulombic attraction between independent charged centers on the polymer and the anion (19), it follows that flavianate may be bound to His-57 (20). This catalytic residue is the only positively charged one located at the active center of ß-trypsin at pH 3.0, with a pKa of 6.8 (21). The His-57 residue can probably bind to either of the negative ionic charges on the dye, the sulfonate and the hydroxide, the sulfonic group and the hydroxyl one, the latter having a pK value of 1.7.

Time-dependence analysis

Figure 6 shows the results obtained by modifying the enzyme concentrations (up-

per panel) or preincubation times (lower panel) of the flavianate-trypsin system before the kinetic assays. The upper panel in Figure 6 clearly presents the expected linear relationship between trypsin concentration and the k parameter, indicating that no force is operative in the reaction other than the simple mass action law. These results rule out possible protein association-dissociation reactions occurring during complex formation, or any hysteretic effect due to slow conformational changes caused by increasing protein concentration (22).

The lower panel of Figure 6 appears to represent a curvilinear profile rather than a linear one. In fact, non-linear adjustments for polynomial, hyperbolic, logistic and exponential models applied to the data resulted in statistical superiority of a single exponential decay for the fitting ($P<0.05$), suggesting a continuous transition for the macromolecule or aggregate. The magnitude of changes in k reflects the responsiveness of the system to an apparent reduction of 7% of active centers or of the catalytic efficiency in ß-trypsin that occurred during the period of preincubation. This reduction could be due to a very small aggregation of flavianate-trypsin complexes occurring during preincubation.

References

1. Lovrien RE, Conroy MJ & Richardson TI (1995). Molecular basis for protein separation. In: Gregory RB (Editor), *Protein-Solvent Interactions*. Marcel Dekker, New York.
2. Conroy MJ & Lovrien RE (1992). Matrix coprecipitating and cocrystallizing ligands (MCC ligands) for bioseparations. *Journal of Crystal Growth*, 122: 213-222.
3. Dagys R, Pauliukonis A, Kazlauskas D, Mankevicius M & Simutis R (1986). Determination of initial velocities of enzymic reactions from progress curves. *Biochemical Journal*, 237: 821-825.
4. Kenneth EN & Ainslie GR (1980). Hysteretic enzymes. *Methods in Enzymology*, 64: 192-226.
5. Bunney HE & Rose WC (1928). Growth upon diets practically devoid of arginine, with some observations upon the relation of glutamic and aspartic acids to nutrition. *Journal of Biological Chemistry*, 76: 521-535.
6. Schroeder D & Shaw E (1968). Chromatography of trypsin and its derivatives. Characterization of a new active form of bovine trypsin. *Journal of Biological Chemistry*, 243: 2943-2949.
7. Dias CLF & Rogana E (1986). Autolysis of ß-trypsin at pH 3.0. *Brazilian Journal of Medical and Biological Research*, 19: 11-18.
8. Chase T & Shaw E (1967). *p*-Nitrophenyl-*p'*-guanidinobenzoate-HCl: A new active site titrant for trypsin. *Biochemical and Biophysical Research Communications*, 29: 508-514.
9. Selwyn MJ (1965). A simple test for inactivation of an enzyme during assay. *Biochimica et Biophysica Acta*, 105: 193-195.
10. Lee H & Wilson IB (1971). Enzymic parameters: measurement of V and Km. *Biochimica et Biophysica Acta*, 242: 519-522.
11. Duggleby RG & Nash JC (1989). A single-parameter family of adjustments for fitting enzyme kinetic models to progress-curve data. *Biochemical Journal*, 257: 57-64.
12. Ascenzi P, Menegatti E, Bortolotti F, Guarneri M & Antonini E (1981). Steady-state and pre-steady-state kinetics of the trypsin-catalysed hydrolysis of α-CBZ-L-lysine-*p*-nitrophenyl ester. *Biochimica et Biophysica Acta*, 658: 158-164.
13. Sprague ED, Larrabee CE & Halsall HB (1980). Statistical evaluation of alternative models: application to ligand-protein binding. *Analytical Biochemistry*, 101: 175-181.
14. Fry JC (1993). One-way analysis of variance. In: Fry JC (Editor), *Biological Data Analysis. A Pratical Approach*. IRL Press, Oxford.
15. Bender ML, Kézdy J & Feder J (1965). The kinetics of the trypsin-catalyzed hydrolysis of *p*-nitrophenyl α-N-benzyloxycarbonyl-L-lysinate hydrochloride. *Journal of the American Chemical Society*, 87: 4953-4954.
16. Antonini E & Ascenzi P (1981). The mechanism of trypsin catalysis at low pH. Proposal for a structural model. *Journal of Biological Chemistry*, 256: 12449-12455.
17. Waley SG (1988). Integrated 2:2 and 3:3 rate equations of enzyme kinetics. *Biochimica et Biophysica Acta*, 955: 272-273.
18. Gutheil WG, Kettner CA & Bachovchin WW (1994). Kinlsq: a program for fitting kinetics data with numerically integrated rate equations and its application to the analysis of slow, tight-binding inhibition data. *Analytical Biochemistry*, 223: 13-20.
19. Colvin JR (1955). A note on the stoichiometry of adsorption of anions by lysozyme. *Canadian Journal of Biochemistry and Physiology,* 33: 651-653.
20. Mares-Guia M, Shaw E & Cohen W (1967). Studies on the active center of trypsin. Further characterization of the hydrophobic binding site. *Journal of Biological Chemistry*, 242: 5777-5781.
21. Bartunik HD, Summers LJ & Bartsch HH (1989). Crystal structure of bovine ß-trypsin at 1.5 Å resolution in a crystal form with low molecular packing density. Active site geometry, ion pairs and solvent structure. *Journal of Molecular Biology*, 210: 813-828.
22. Avilán L & Garcia P (1994). Hysteresis of cytosolic NADP-malic enzyme II from *Trypanosoma cruzi*. *Molecular and Biochemical Parasitology*, 65: 225-232.