ELSEVIER

Biochemical Education 27 (1999) 118–121

Biochemical Education

# Binding of proflavin to chymotrypsin: an experiment to determine protein–ligand interactions by direct nonlinear regression analysis of spectroscopic titration data

H. Stewart Hendrickson*

*Department of Chemistry, University of Washington, Seattle, WA 98195-1700, USA*

**Abstract**

The dissociation constant and stoichiometry of a proflavin–chymotrypsin complex are determined by spectroscopic titration and direct nonlinear regression data analysis in a simple experiment during one laboratory period. 

## 1. Introduction

The determination of dissociation constants and stoichiometries of protein–ligand complexes is of general interest in biochemical research. Many physical methods rely on changes in absorbance or fluorescence properties of a ligand as it binds to a protein. Traditional methods of data analysis have involved approximations, linear transforms of binding equations, and end-point titrations involving large amounts of protein. The current sophistication of desk-top computing now allows exact fitting of spectroscopic data to mathematical models by nonlinear regression analysis.

This laboratory exercise was developed at St. Olaf College, Northfield, MN, USA, for an undergraduate biochemistry laboratory and later updated as computational methods became more sophisticated. It was based on a published study by Bernhard et al. [1] on the binding of proflavin (Fig. 1) to the active site of α-chymotrypsin. A nonlinear regression program was originally written in FORTRAN to analyze the data by an iterative process; this eliminated the need for linear transforms that tend to skew the errors. More recently, data analysis was modeled after an elegent method by Wang et al. [2] that allows for dilution effects by fitting data to an exact quadratic equation, and a commercial program (SigmaPlot) was used for the analysis. The experimental simplicity and ease of data analysis makes this method attractive for an undergraduate biochemistry teaching laboratory as well as a powerful technique in the research laboratory.

Proflavin binds strongly to α-chymotrypsin (24,300 kDa) and is a competitive inhibitor of the enzyme. Binding occurs at the active site with one molecule of dye bound per active site [1]. The dye has an absorption spectrum with a maximum at 444 nm which, upon binding to the enzyme active site, is shifted to a longer wavelength with a slightly larger extinction. This shift is characteristic of the dye moving to a more hydrophobic environment (on the enzyme). The most accurate way to measure this shift is to record the difference spectrum — that is, with dye and enzyme combined in the sample beam and separate in the reference beam. Since the enzyme has negligible absorption in the visible region ($> 350$ nm) it does not need to be present in the reference beam. Data from a single titration of chymotrypsin into proflavin ($\Delta A$ vs the volume of enzyme added) are analyzed directly by nonlinear regression to give both the dissociation constant for the complex and the stoichiometry of binding.

## 2. Materials and methods

### 2.1. Instrumentation

*Spectroscopic measurements were made with a Perkin*-Elmer Lambda 3B UV/VIS spectrometer. The cell holder was thermostated at 23°C. Data were collected through

* E-mail: hend@u.washington.edu

0307-4412/99/$20.00 
PII: S0307-4412(98)00267-2

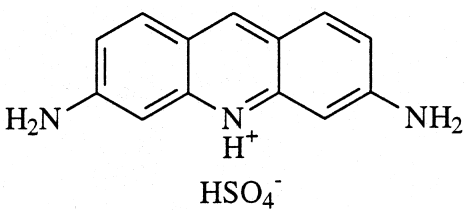


Fig. 1. Structure of proflavin.

a computer interface using Perkin-Elmer's PECSS software. Data were analyzed by nonlinear regression using SigmaPlot software (SPSS, Chicago, IL, USA) on a personal computer operating under Windows 95.

### 2.2. Reagents and solutions

*Proflavin hemisulfate,* α-chymotrypsin (type II), and Tris base were purchased from Sigma Chemical Co. (St. Louis, MO, USA). A stock solution of approximately 0.01 M proflavin hemisulfate (65 mg/20 ml of 0.1 M Tris buffer, pH 8) was prepared and stored in the dark. This was diluted to about 80 μM in the same Tris buffer; the exact concentration was determined from the absorption of a 1/3 dilution at 444 nm ($\varepsilon = 3.34 \times 10^4\ M^{-1}\ cm^{-1}$) A stock solution of chymotrypsin was prepared by dissolving about 40 mg in 1 ml of Tris buffer; the exact concentration was determined from the absorbance of a 1/100 dilution at 280 nm ($\varepsilon = 2.04\ ml\ mg^{-1}$ or $5.01 \times 10^4\ M^{-1}\ cm^{-1}$).

### 2.3. Procedure

Exactly 2.0 ml portions of 80 μM proflavin (in 0.1 M Tris buffer, pH 8.0) were placed in both the sample and reference cuvettes. The baseline was corrected and then recorded from 550 to 350 nm. A 10 μl portion of chymotrypsin solution was added to the sample cuvette, mixed, and the difference spectrum was again recorded. This procedure was repeated with two more 10-μl additions, three 20-μl additions, three 50-μl additions, and finally 100-μl additions until the peak absorption decreased due to dilution of proflavin.

## 3. Calculations

Binding of proflavin to the enzyme can be represented by the following equilibrium and dissociation constant:

$$E + L \leftrightharpoons EL$$

where E represents free enzyme and L represents the ligand, and

$$K_d = \frac{[E][L]}{[EL]} \quad (1)$$

If more than one molecule of proflavin is bound to the enzyme, then

$$[L] = [TL]/N \quad (2)$$

where TL is total ligand (proflavin) and $N$ is the number of proflavin molecules bound per enzyme molecule. The experimental data, change in absorbance and volume of enzyme solution added ($\Delta A$ and $V_c$), were analyzed according to the method of Wang et al. [2] as follows:

$$E_t = [E] + [EL] \text{ and } L_t = [L] + [EL] \quad (3)$$

where $E_t$ and $L_t$ represent the total concentrations of enzyme and ligand respectively. The dissociation constant is described by

$$K_d = \frac{(E_t - [EL])(L_t - [EL])}{[EL]} \quad (4)$$

Eq. (4) can be rearranged using the mathematical solution to a quadratic equation to give

$$[EL] = \tfrac{1}{2}\{E_t + L_t + K_d - [(E_t + L_t + K_d)^2 - 4E_tL_t]^{1/2}\} \quad (5)$$

Using the difference molar extinction coefficient, $\Delta\varepsilon = \Delta A/[EL]$, Eq. (5) can be rearranged to

$$\Delta A = \Delta\varepsilon\,\tfrac{1}{2}\{(E_t + L_t + K_d - [(E_t + L_t + K_d)^2 - 4E_tL_t]^{1/2}\} \quad (6)$$

If the initial volume and concentration of ligand are $V_0$ and $[L]_0 = [TL]_0/N$ respectively, and the concentration of stock enzyme solution is $[E]_0$, then the total concentration of ligand and enzyme can be obtained by accounting for the total volume of enzyme, $V_c$, added during the titration:

$$E_t = \frac{[E]_0 V_c}{V_0 + V_c} \quad (7)$$

and

$$L_t = \frac{([TL]_0/N)V_0}{V_0 + V_c} \quad (8)$$

The experimental data, $\Delta A$ and $V_c$, along with values for $[E]_0$, $[TL]_0$, and $V_0$, are fitted to Eqs. (6)–(8) by nonlinear regression analysis using estimates of $\Delta A_{max} = \Delta\varepsilon[TL]_0/N$, $K_d$, and $N$. Best results are obtained when $[TL]_0$ is several times the value of $NK_d$. In the initial phase of the titration of enzyme into ligand, where [total ligand] ≫ [E], all or most of the enzyme is bound to the interface. $N$ can be estimated from extrapolation of the initial linear slope of the plot of $\Delta A$ vs $V_c$ to the estimated value of $\Delta A_{max}$. The total ligand concentration at this intersect divided by the concentration of enzyme is an estimate of $N$.

Data are analyzed by the nonlinear regression program SigmaPlot. The following is the script (.FIT file) used for the regression analysis.

```
jsv4R
[Parameters]
;Prolig1—Ligand–protein binding: titration of protein
   into ligand (probe),
;monitored by fluorescence or absorbance. Use to fit
   exp data.
;cell(4,1) conc of stock protein
;cell(4,2) initial conc of ligand
;cell(4,3) initial vol of ligand
;col(1) values of vol protein added, Vc
;col(2) values of change in absorbance or fluorescence
i = cell(4,4) ;est. max change in absorbance, delta Amax
k = cell(4,5) ;est. Kd (dissoc. const.)
n = cell(4,6) ;est. N, max ligands bound per protein
[Variables]
pt = col(1)*cell(4,1)/(cell(4,3) + col(1))
lt = cell(4,2)*cell(4,3)/(cell(4,3) + col(1))
y = col(2)
[Equations]
f = 0.5*(i*n/cell(4,2))*(pt + (lt/n) + k
    − sqrt((pt + (lt/n) + k)^2 − 4*pt*(lt/n)))
fit f to y
[Constraints]
[Options]
iterations = 50
```

Initial estimates are needed to begin the analysis. $\Delta A_{max}$ is estimated by a value somewhat larger than the largest observed $\Delta A$ value; for chymotrypsin–proflavin binding, $N$ is estimated as 1, and $K_d$ is estimated as the concentration of chymotrypsin that gives half the maximal observed $\Delta A$ value; otherwise these parameters are estimated by judicious trial and error, rejecting any fits with negative or otherwise unreasonable constants, and comparing theoretical curves with experimental points.

## 4. Results

The titration of 1.28 mM chymotrypsin into 2 ml of 79.6 μM proflavin was monitored by difference absorption spectroscopy as shown in Fig. 2. The difference spectra are indicative of a shift in proflavin absorbance to longer wavelengths and somewhat higher extinction. A single isosbestic point was observed, indicating a simple change between two states of the probe (bound and unbound). A plot of $\Delta A$ vs volume of chymotrypsin added is shown in Fig. 3. The data fitted well to a nonlinear regression with $\Delta A_{max} = 0.948 \pm 0.038$ ($\Delta\varepsilon = 1.29 \pm 0.05 \times 10^4\ M^{-1}\ cm^{-1}$), $K_d = 21.5 \pm 6.8$ μM, and $N = 1.08 \pm 0.13$. Nonlinear regression analysis of the data by SigmaPlot gives standard errors that are defined as 'the square root of the mean of the squares of the differences from their mean of all the data samples for each curve'. These values compare favorably to those published by Bernhard et al. [1]: $\Delta\varepsilon = 1.85 \pm 0.02 \times 10^4\ M^{-1}\ cm^{-1}$, $K_d = 39 \pm 3$ μM and $N = 0.96 \pm 0.1$. Also shown in this plot (dotted line) is the limiting curve for stoichiometric binding ($K_d = 0$).

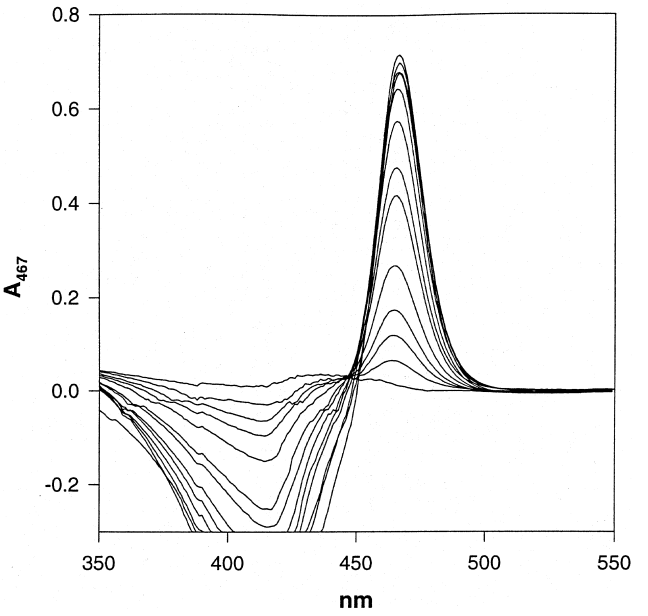


Fig. 2. Difference spectra: titration of 1.28 mM chymotrypsin into 2 ml of 79.6 μM proflavin. Additions of 0, 10, 20, 30, 50, 70, 90, 140, 240, 340, 440, and 540 μl.

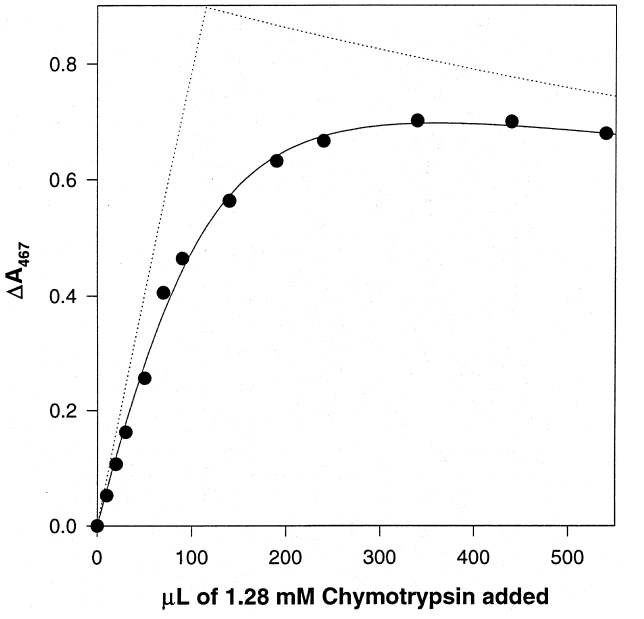


Fig. 3. Binding curve: titration of 1.28 mM chymotrypsin into 2 ml of 79.6 μM proflavin. Solid line, fit to $\Delta A_{max} = 0.948$, $K_d = 21.5$ μM, and $N = 1.08$; dotted line, limiting curve for stoichiometric binding ($K_d = 0$). $T = 23°C$.

## 5. Discussion

This experiment is easily performed in one laboratory period and gives results comparable to those originally published in the literature [1], which involved multiple titrations and complex data analysis using linear transforms. Since chymotrypsin is readily available at a low cost, it is a good choice of protein for this experiment. This experiment uses the program SigmaPlot to analyze the data; however, other nonlinear regression analysis programs are available and should work just as well. A good 'practical and nonmathematical review' of nonlinear regression analysis was published by Motulsky and Ransnas 3.

Kantrowitz and Eisele 4 described other spectroscopic experiments with chymotrypsin that utilize proflavin as a probe; these are also based on the work of Bernhard et al. [1]. Chymotrypsin catalyzes the hydrolysis of esters or amides by a two-step mechanism. In the first step an acyl–enzyme complex is formed with the release of the first product, $P_1$ (the alcohol or amine). In the second step the acyl–enzyme is hydrolyzed with release of the second product, $P_2$ (the acid):

$$E + S \leftrightharpoons ES \rightharpoonup P_1 + EP_2 \rightharpoonup E + P_2.$$

Using a poor substrate such as *N-trans*-cinnamoyl imidazole, the deacylation step can be looked at separately since it is sufficiently slower than the first acylation step. The deacylation is monitored by the use of the enzyme–proflavin difference spectrum. This second phase is a first-order reaction and a rate constant can be obtained from analysis of the data, $\Delta A$ vs time. Another experiment uses proflavin to monitor the activation of chymotrypsinogen by trypsin.

## References

[1] Bernhard, S.A., Lee, B.F. & Tashjian, Z.H. (1966) On the interaction of the active site of α-chymotrypsin with chromophores: proflavin binding and enzyme conformation during catalysis. J. Mol. Bio., 18, 405–420.

[2] Wang, Z.-X., Kumar, N.R. & Srivastava, D.K. (1992) A novel spectroscopic titration method for determining the dissociation constant and stoichiometry of protein–ligand complex. Anal. Biochem., 206, 376–381.

[3] Motulsky, H.J. & Ransnas, L.A. (1987) Fitting curves to data using nonlinear regression: a practical and nonmathematical review. FASEB J., 1, 365–374.

[4] Kantrowitz, E.R. & Eisele, G. (1975) Two spectrophotometric experiments with alpha-chymotrypsin. J. Chem. Educ., 52, 410–413.