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A Convenient Method for Determination of Dissociation Constants of Enzyme-Ligand Complexes Based on Difference Absorption Spectra.

A Model Case of Ribonuclease F1-Guanosine 2'-Monophosphate

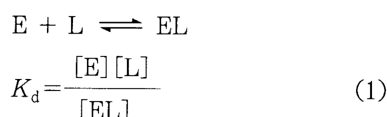
(enzyme-ligand interaction ／ difference absorption spectrum ／ ribonuclease F1)

Hiroshi YOSHIDA* and Mari IIZUKA*

A convenient method has been developed for investigating interaction of an enzyme with a specific ligand on the basis of the difference absorption spectrum. The experimental procedure has been described in detail. Briefly, to a solution of the enzyme, a concentrated solution of the ligand was added successively in small volume aliquots and the increase in absorbance after each addition was measured at two wavelengths which corresponded to a maximum and a cross-over point of the difference absorption spectrum. Processing the obtained data gave the difference at the maximum wavelength as a function of the total ligand concentration. The data set thus generated was fitted to the equilibrium equation and the best fit values for the dissociation constant, K_d and the molar difference absorption coefficient at the maximum wavelength, $\Delta\varepsilon_{max}$, were determined. The method was successfully applied to ribonuclease F1-guanosine 2'-phosphate system and gave the following parameters: $K_d = 2.5\ \mu M$ and $\Delta\varepsilon_{290} = 3890\ M^{-1}cm^{-1}$.

Introduction

Interaction of an enzyme with specific ligands is an imoportant subject in biochemistry. The affinity of an enzyme (E) for a specific ligand (L) is customarily expressed in terms of the dissociation constant of the following equilibrium reaction:



$$E + L \rightleftharpoons EL$$

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

where [EL], [E], and [L] are the concentrations of the enzyme-ligand complex, the free enzyme, and the free ligand, respectively. Determination of K_d is, therefore, a matter of basic importance.

When E and L are chromophoric, the so called difference absorption spectrum is often observed: the spectrum of the mixture of E and L is not equal to the sum of the spectra of E and L measured separately. This is caused by perturbation of chromophore(s) in E and/or L upon binding and the difference absorbance, ΔA, at a given wavelength is proportional to the concentration of EL:

$$\Delta A = \Delta\varepsilon\,[EL] \qquad (2)$$

where $\Delta\varepsilon$ is the molar difference absorption coefficient. From equations (1) and (2), the following relationship is obtained:

$$K_d = \frac{([E]_T - \Delta A/\Delta\varepsilon)([L]_T - \Delta A/\Delta\varepsilon)}{\Delta A/\Delta\varepsilon} \qquad (3)$$

where the suffix T shows the total concentration of the indicated component.

Experimentally, enzyme solutions of a constant concentration $[E]_T$ are mixed with ligand solutions of variable concentrations $[L]_T$. At each mixing, the difference absorbance at the maximum wavelength, ΔA_{max}, is determined, yielding a data set (ΔA_{max}, $[L]_T$). This data set is fitted to equation (3) and the best value for K_d and $\Delta\varepsilon_{max}$ are determined by an appropriate method. A typical example of this type of work is that of Walz and Hooverman (1), who examined interaction of guanine ligands with ribonuclease T1.

*Department of Chemistry

We have been working for some time on ribonuclease F1, a homolog of ribonuclease T1 and tried to carry out a similar type of study to that of Walz and Hooverman. Then we realized that the above method, though theoritically sound, had a practical drawback: a considerable amount of the enzyme was needed. With a limited amount of the enzyme available, we devised a new method which consisted of continuous spectrophotometric titration on a single enzyme solution, hence utilizing less amount of the enzyme. The principle of the method was beriefly described in a previous report (2). Using this method, we could determine K_d of ribonuclease T1-guanosine 2'-phosphate (2'-GMP) complex as 4.6 μM at pH 5.5 and 25 ℃, which is in good agreement with the value 6.9 μM determined by Walz and Hooverman under similar, but not identical, conditions. Thereafter, considerable improvement has been made in the process of determination of K_d and $\Delta\varepsilon_{max}$, due to development of computer hard- and softwares. Here, we describe the step-by-step procedure of our method. Although the method is described for a special case of ribonuclease F1-2'-GMP system, it can be applied to a wide range of EL complex.

Experimental and Results

Materials and Instruments —— Ribonuclease F1 was prepared as described previously (3). The ligand, 2'-GMP, was prepared from a commercially available mixture of 2'- and 3'-GMP (Kohjin) through chromatography on a Dowex 1 × 4 column. A buffer, 10 mM sodium acetate (pH 5.5) containing 100 mM NaCl, was used as a solvent throughout this study. All the solutions were filtered through chromatodisc 4N filters with a pore size of 0.45 μm (Kurabo) before measurements. Spectrophotometric measurements were made in capped quartz cuvettes with a Hitachi double beam spectrophotometer U-2000 equipped with a tandem cell holder and thermostatted at 25 ℃. Before recording a spectrum, a sample solution was kept in the apparatus for a few minute to ensure an equilibrium. The scan speed was 200 nm /min.

Measurement of the Difference Spectrum —— This was carried out in the following steps.

1) Solutions (each 1.5 ml) of ribonuclease F1 (E) and 2'-GMP (L), both at a concentration of about 50 μM, were put into separate cuvettes, then the cuvettes were placed in tandem at the sample side of the spectrophotometer. The spectrum, which is the addition of the spectra of E and L, was memorized as a user-defined base line.
2) The two solutions were mixed thoroughly, then repartitioned into the two cuvettes placed in tandem at about equal volumes. The spectrum was then recorded against the user-defined base line. Although both components were diluted twice by the mixing, the dilution was compensated by the doubling of the optical path. Therefore, the recorded spectrum is the difference spectrum itself (Fig. 1).

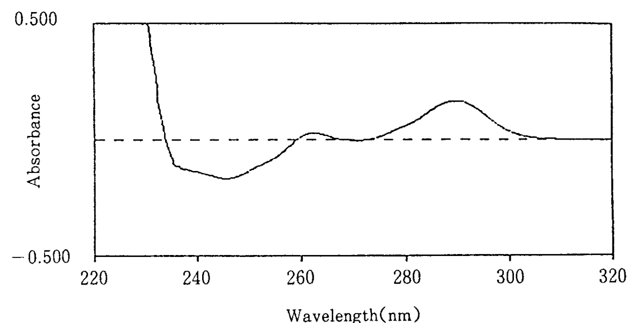


Fig. 1. The difference spectrum for the ribonuclease F1-2'-GMP complex. Experimental details are described in the text. The initial concentrations of ribonuclease F1 and 2'-GMP were 52.0 μM and 50.8 μM, respectively.

The wavelengths, λ_{max} and λ_O at which the difference spectrum reached a maximum and crossed over the bqse line, respectively, were read as follows: λ_{max}=290 nm, λ_O=271 nm.

Titration —— This was done in the following steps.

1) A user-defined base line was drawn with cuvettes filled with the buffer placed at the reference and sample sides.
2) The content of the cuvette at the sample side was replaced with 1.5 ml of E solution and the spectrum was recorded. From A_{280} corrected for the background by subtracting A_{320}, the total

concentration of E, $[E]_T$, was determined: $[E]_T = 50.9\,\mu M$. At 320 nm, the enzyme should not exhibit any absorbance. Thus slight absorbance (usually less than 0.005) at this wavelength was probably due to light scattering and was regarded as a background.

3) The spectrum was then memorized as a new user-defined base line. This enabled further measurements at high net absorbances possible.

4) A concentrated (10.2 mM) solution of L was added successively in 1-μl aliquots until a total of 15 μl was reached. After each addition followed by thorough mixing, A_{320}, A_{290}, and A_{271} were read, yielding a data set, (A_{320}, A_{290}, A_{271}).

5) The true absorbances corrected for light scattering were calculated:

$$A_{290}(\text{corr.}) = A_{290} - A_{320}$$

$$A_{271}(\text{corr.}) = A_{271} - A_{320}$$

During the titration, the A_{320} gradually increased and reached a value of 0.046 because of slight turbidity which developed in the mixture from an unknown reason. Although light scattering is wavelength-dependent, the degree of increase should be small in a wavelength range of 270 − 320 nm. So the A_{320} is taken as a universal background in this region. Fig. 2 shows the plot of the corrected values of A_{290} and A_{271} as a function of the volume of L solution added.

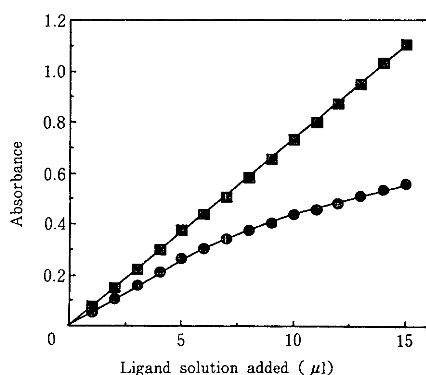


Fig. 2. The absorbance increases at 290 and 271 nm as a function of ligand (2'-GMP) solution added. Experimental details are described in the text. (●), A_{290}; (■), A_{271}.

As expected, A_{271} increased linearly with the ligand solution added, whereas A_{290} gave an upward curvature due to the difference absorption spectrum.

6) The total concentration of L, $[L]_T$, and the difference absorbance caused by the formation of EL complex, ΔA_{290}, were calculated according to the following equation:

$$[L]_T = A_{271} / \varepsilon_{271} \quad (4)$$

$$\Delta A_{290} = A_{290} - A_{271} \times (\varepsilon_{290} / \varepsilon_{271}) \quad (5)$$

where the absorbances were the corrected ones. The values, ε_{290} and ε_{271}, had been determined in advance by taking the spectrum of the ligand dissolved in the buffer at a known concentration. Thus, a new data set ($[L]_T$, ΔA_{290}) was generated as shown in Fig 3.

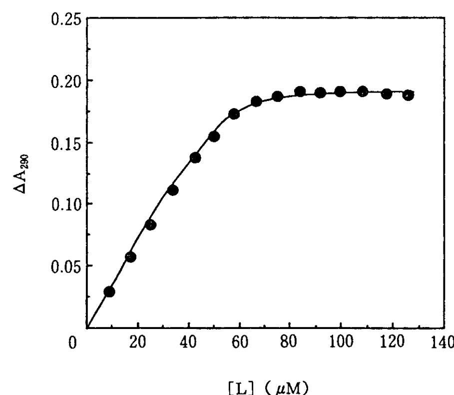


Fig. 3. The difference absorbance at 290 nm (ΔA_{290}) as a function of the total ligand concentration ($[L]_T$). Experimental details are described in the text.

The data set gave a familiar saturation curve. Curves of this type have usually been obtained by the previous method as shown in Fig. 2 of reference (1).

7) The data set was fitted to the following equation using KaleidaGraph software with a Macintosh Classic II computer.

$$\Delta A_{290} = (\Delta\varepsilon_{290}/2)\,\{[E]_T + [L]_T + K_d - \sqrt{([E]_T+[L]_T+K_d)^2 - 4\,[E]_T[L]_T}\,\} \quad (6)$$

This equation was obtained by solving equation (3) with respect to ΔA_{290}. A general curve fit was carried out after substituting $[E]_T$ with the determined value and rewriting equation (6) to conform to the software as follows:

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y = m1 / 2 * (50.9 + M0 + m2
        - SQRT ((50.9 + M0 + m2) *
        (50.9 + M0 + m2) - 203.6 * M0))
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The following values were then obtained:

$K_d = 2.5 \pm 0.7\ \mu M$

$\Delta\varepsilon_{290} = 0.00389 \pm 0.00007\ \mu M^{-1}cm^{-1}$

$= 3890 \pm 70\ M^{-1}cm^{-1}$

Discussion

The present method enables speedy, accurate determination of the dissociation constant K_d of an EL complex based on the difference absorption spectrum. Although the method has its ground on a known principle, it has some advantages over the previous method (1).

First, the experiment is done on a single enzyme solution. Thus only a minimum amount of the enzyme is required. Further, the total concentration of the enzyme, $[E]_T$, is kept constant. When a series of experiments are carried out on many enzyme solutions as in the previous method (1), $[E]_T$ is subject to experimental variation, though it should be kept constant. Also in the present method, $[E]_T$ is not absolutely constant because of the volume increase caused by addition of the ligand solution. But the increase is only 1% of the total volume even after the final addition, so the change in $[E]_T$ due to the dilution can be safely neglected.

Second, our method is free from pipetting errors, because $[L]_T$ is calculated from the actual increase in absorbance at the cross-over wavelength according to equation (4) and not from the nominal volume of ligand solution added. In fact, accurate addition of a volume as small as 1 μl is difficult and prone to an experimental error. Therefore, calculation of $[L]_T$ according to equation (4) is essential for accurate determination of K_d.

Using the present method, we were able to study the interaction of various guanine ligands with ribonuclease F1. The results will be published elsewhere in near future.

References

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