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Application of the continuous variation method to cooperative interactions: mechanism of Fe(II)–ferrozine chelation and conditions leading to anomalous binding ratios[☆]

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Abstract

The method of continuous variation, often known as the Job plot, has long been used for determining the stoichiometry of two interacting components. The correct binding ratio, n, is generally obtained when the total concentration of the reactants, C_o, is much greater than the dissociation constants involved. For non-cooperative binding systems, the stoichiometry varies between one and n as C_o increases; whereas for positive cooperative systems, values larger than n may be observed at low C_o. In this report, we present examples to illustrate how the changing apparent stoichiometries as a function of C_o can provide clues for differentiating various binding mechanisms. To test these concepts, we examined the chelation of Fe(II) with ferrozine in the range of $C_o = 7$ to 210 μM with Fe(II) expressed in molar concentration or in terms of its binding equivalents (three in this case). The results were analyzed according to several models and found to be most consistent with the mechanism of one-step complex formation or infinite cooperativity with a K_d of 8 μM.

Keywords: Continuous variation; Job plot; Anomalous stoichiometry; Differentiation of cooperative systems; Iron-ferrozine interaction

1. Introduction

Characterization of molecular interactions has long been a subject of interest to chemists and biologists. For instance, the theoretical and experimental aspects of such interactions have been treated in-depth by Edsall and Wyman [1]. The method of continuous variation, often known as the Job plot [2], for determining the stoichiometry of two interacting components has been in use for quite some time. In fact, publications dealing with the basic principles of the method predate Job's 1928 article by nearly two decades. With this method, the total molar concentration of the two reactants is held constant while their molar ratios are continuously varied. A measurable parameter

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[☆] We wish to dedicate this manuscript in memory of professor John T. Edsall, a pioneer in biophysical chemistry much-admired by us.

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that is linearly proportional to the complex(es) formed is plotted against the mole fractions of the reactants to generate a curve. The binding stoichiometry is then determined from the ratio of the mole fractions of the two components found at the maximum of the curve. For determining binding stoichiometry alone, it is evident that the method is valid when the total concentration is much greater than the dissociation constant(s) involved in the interaction. At total concentrations comparable to the dissociation constants, the maximum of the Job curves may not permit accurate calculation of the binding ratio, but may allow dissociation constants and mechanisms of interaction to be determined. A random survey of recent literature reveals, however, that the method is generally carried out in a rather casual manner in the simplest way.

Application of the continuous variation method to the study of different binding mechanisms has been reviewed by Huang [3]. From the equations derived by Huang for cooperative binding systems, it became apparent that anomalous combining ratios, usually higher than the true one, may be observed under certain conditions, although this feature was not highlighted at the time [3]. Li et al. have pointed this out and interpreted their data on the reconstitution of 5-oxoprolinase by a cooperative binding mechanism [4]. In the present study, we re-examine the binding of Fe(II) with ferrozine using the continuous variation method performed at varying total reactant concentrations to determine the dissociation constants and mechanisms involved, including the anomalous ratios observed in one of the experiments. Several conditions not dealt with previously [3] that can lead to anomalous ratios and how the changing ratios as a function of C_o may provide clues to the mechanisms involved are discussed.

2. Experimental procedures

2.1. Materials

$FeSO_4 \cdot 7H_2O$ and ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine] were purchased from Allied Chemicals and Hach Chemical Co., respectively. HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethinesulfonic acid)] and other chemicals were from Sigma.

2.2. Methods

Ferrozine (Fz) was dissolved in water and $FeSO_4$ in 1.2 mM HCl. Various portions of Fz stock solutions were added to 50 mM HEPES buffer, pH 7.0, containing 0.1 M KCl and mixed with freshly prepared $FeSO_4$ solution to a final volume of 30 ml. In view of the rather slow reaction between Fe(II) and Fz, sufficient incubation time (>10 min) of the two components was allowed before the complex was measured at 562 nm [5] at 25 °C by a Cary 219 spectrophotometer with a thermostated cell compartment. Ten-cm light-path cells were used to increase the sensitivity of absorbency determination.

The method of continuous variation was carried out in two ways. In the first series of experiments, the molar ratios of the two reactants, Fe(II) and Fz, were varied while their total concentrations were kept at several constant levels. The concentrations used are given in the figure legends. In the second series of experiments, the same procedure was used except that the Fe(II) concentration was calculated in terms of its binding site by assigning three sites for Fz. Equations were derived to describe the reaction schemes of interest. All data were analyzed by curve-fitting methods using these equations and the root-finding operator of the MLAB software package (Civilized Software, Inc.) [6].

3. Theory

Fe(II) has been shown to combine with three molecules of ferrozine [7]. Here we apply the continuous variation method to study this binding mechanism according to three plausible models and to illustrate where the anomalous stoichiometry can occur.

1: The one-step or ‘infinite cooperative’ binding mechanism: Let us assign the two interacting components as A and B such that A binds n molecules of B in one step:

$$A + nB \overset{K_d^n}{\rightleftharpoons} AB_n$$

The continuous variation method requires that the total concentration of A and B be kept at a constant level, C_o:

$$A_o + B_o = C_o$$

$$\frac{A_o}{C_o} + \frac{B_o}{C_o} = 1$$

$$\text{or } X + Y = 1 \tag{1}$$

where X and Y are the mole fractions of A and B, respectively, and $Y = 1 - X$. In a Job plot, where the complex formed, AB_n, is plotted against X, the binding stoichiometry n is obtained from the ratio of mole fractions found at the maximum of the Job curve, R_m, according to the expression:

$$R_m = \frac{Y_m}{X_m} = \frac{1 - X_m}{X_m} = n \tag{2}$$

A hallmark of this one-step mechanism is that the correct binding stoichiometry can always be obtained regardless of the C_o employed. However, it is possible to obtain the same n value at different C_o if all of the C_o used are much greater than K_d (and the binding mechanism may not be infinite cooperative). It is useful, therefore, to perform a series of Job plots at different C_o by expressing A concentration in terms of its binding equivalent, i.e. $A_o = A + nAB_n$ (instead of $A_o = A + AB_n$). The situation results in the following equation:

$$K_d^n = \frac{(A_o - nAB_n)(B_o - nAB_n)^n}{nAB_n} \tag{3}$$

From Eq. (1) we have:

$$A_o = C_o X \text{ and } B_o = C_o Y = C_o(1–X) \tag{4}$$

Let $AB_n = \Sigma$ and substitute (4) into (3), we have:

$$n \sum K_d^n = (C_o X - n\Sigma)\left[C_o(1 - X) - n\Sigma\right]^n \tag{5}$$

Differentiation of Eq. (5) with respect to X yields:

$$\begin{aligned} nK_d^n(d\Sigma/dX) = {} & (C_o X - n\Sigma)n\left[C_o(1 - X) - n\Sigma\right]^{n-1} \\ & \times\left[-C_o - n(d\Sigma/dX)\right] \\ & + \left[C_o(1 - X) - n\Sigma\right]^n \\ & \times\left[C_o - n(d\Sigma/dX)\right] \end{aligned} \tag{6}$$

The maximum AB_n complex, Σ_m, is formed at a given C_o when $(d\Sigma/dX) = 0$. From Eq. (6) it can be shown that:

$$R_m = \frac{Y_m}{X_m} = \frac{nC_o - n(n-1)\Sigma_m}{C_o + n(n-1)\Sigma_m} \tag{7}$$

When $C_o \rightarrow 0$, $\Sigma_m \rightarrow 0$ and $R_m \rightarrow n$.

When $C_o \rightarrow \infty$, $\Sigma_m \rightarrow C_o/2n$ and $R_m \rightarrow 1$.

Thus, the stoichiometry calculated from R_m for the infinite cooperative binding case, when A concentration is expressed in terms of its binding equivalent, will shift from n to the true value of 1 as C_o increases. Clearly, anomalous stoichiometries between n and 1 may be obtained under this experimental condition and this kind of shift is indicative of this particular one-step binding mechanism.

2. Cooperative binding: Let us consider the stepwise binding mechanism according to the Adair model with $n = 3$:

$$A + B \overset{K_1}{\rightleftharpoons} AB + B \overset{K_2}{\rightleftharpoons} AB_2 + B \overset{K_3}{\rightleftharpoons} AB_3$$

Let the symbol Σ represent the summation of all AB complexes such that:

$$\sum = \sum_{i=1}^{n} = AB + 2AB_2 + 3AB_3$$

then we can write the saturation function as:

$$\frac{\Sigma}{A_o} = \frac{3(B/K_1)\left[(1 + (2B/K_2) + (B^2/K_2K_3))\right]}{1 + (3B/K_1) + (3B^2/K_1K_2) + (B^3/K_1K_2K_3)} \tag{8}$$

Eq. (8) can be expressed in terms of mole fractions by introducing the relationships defined in Eq. (4). It has been shown by Huang [3] that the binding stoichiometry, n, for this model can be calculated from the ratio of the mole fractions X_i and Y_i obtained at the intersection point of the two limiting slopes, $(d\Sigma/dX)_{X-0}$ and $(d\Sigma/dY)_{Y-0}$ when C_o is much larger than all of the dissociation constants involved (in this case, $C_o \gg K_1$, K_2, K_3).

Here the ratio, R_i, has the expression:

$$R_i = \frac{Y_i}{X_i} = \frac{1-X_i}{X_i} = \frac{K_1K_2K_3 + (2K_1+3K_2)K_3C_o + (K_1+6K_3)C_o^2 + 3C_o^3}{K_1K_2K_3 + 3K_2K_3C_o + 3K_3C_o^2 + C_o^3} \tag{9}$$

It can be seen that as C_o is large, $R_i \rightarrow 3$. When $C_o \rightarrow 0$, $R_i \rightarrow 1$. But, in between the two extremes, R_i can exceed the true n number of 3. Fig. 1 shows computer-generated Job plots at varying C_o concentrations with decreasing K_d's ($K_1 > K_2 > K_3$) conforming with positive cooperativity. Inset (a) of Fig. 1 shows how the n value calculated from R_i can increase from 1 to an anomalous 4.7 before falling back to the correct value of 3. The n value calculated from the maxima of the Job curves, R_m, on the other hand, increases from 1 to 3. If the concentration of A is expressed in terms of its binding equivalents, as can be seen from Inset (b) of Fig. 1, R_i increases from 1 to a peak value of 3.4 and then descends to the expected value of 1; whereas R_m increases from 1 to 1.7 and then reduces to 1.

From the two binding mechanisms presented above, it is clear that the n values obtained at different C_o can provide clues to permit distinction between these mechanisms.

3. Apparent cooperative binding arising from detecting only the final complex: So far we have tacitly assumed that the amount of complexes formed is linearly proportional to a measurable signal, e.g. absorbance at 526 nm for Fe(II)–Fz complexes where FeFz, $FeFz_2$ and $FeFz_3$ contribute in a 1:2:3 ratio. It is possible that only the $FeFz_3$ form contributes significantly to the absorbance at 526 nm. In this case, Eq. (8) becomes:

$$\frac{\Sigma}{A_o} = \frac{3(B^3/K_1K_2K_3)}{1+(3B/K_1)+(3B^2/K_1K_2)+B^3/(K_1K_2K_3)} \tag{10}$$

Eq. (10) predicts a skewed Job plot that is similar in shape to that of the infinite cooperative case, except that the n values calculated from R_m will vary with C_o at C_o concentrations comparable to the K_d's. This feature allows one to distinguish true infinite cooperativity from the apparent ones.

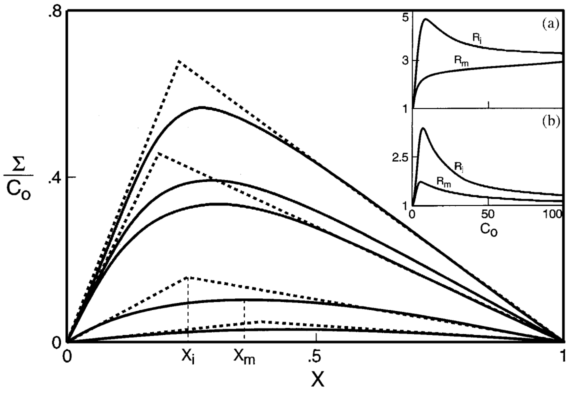

Fig. 1. Computer-simulated Job plot for positive-cooperative binding according to the Adair model. Relative magnitude of dissociation constants are: $K_1 = 30$, $K_2 = 5$, $K_3 = 1$. Total concentrations used are $C_o = 1$, 3, 11, 15 and 30. The mole fraction found at the maximum of the curve is designated X_m; whereas that found at the intersection point of the two limiting slopes is designated X_i. Inset (a): R_m and R_i, the binding stoichiometry calculated from X_m and X_i at different C_o according to the equations $R_m = (1-X_m)/X_m$ and $R_i = (1-X_i)/X_i$. Inset (b): R_m and R_i as a function of C_o with the reactant concentrations expressed in terms of binding equivalents.

4. Results

4.1. Job plots performed at different total concentrations of Fe(II) and Fz

Although Fe(II) has been shown to combine with three molecules of Fz [5,7], the mechanism of binding is not known. We, therefore, apply the continuous variation method at different C_o to re-examine the Fe(II)–Fz interaction. From the examples presented in Theory section, it is clear that a choice of mechanism can be made from the distinctive pattern of apparent binding stoichiometries as a function of C_o. Fig. 2 shows Job plots obtained at four total concentrations in the range of 7–105 μM. The data are indicative of the notion that the Fe(II)–Fz chelation conforms with the infinitely cooperative mechanism: (1) The maxima of all four curves appear at approximately 0.25 mole fraction of Fe(II), signifying a binding

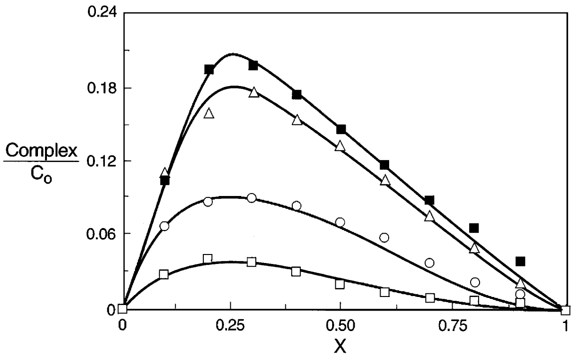

Fig. 2. Job plots of Fe(II)–Fz interaction performed at different C_o: □, 7 μM; ○, 14 μM; △, 52.5 μM; and ■, 105 μM. The lines are fitted by computer using the NIH MLAB program. Experimental conditions are described in Experimental procedures.

stoichiometry of 3. (2) The infinite cooperative mechanism is further supported by the skewed patterns seen near $X\rightarrow 1$ at $C_o=7$ and 14 μM. The solid lines are computer-generated using an equation derived for the one-step binding process.

4.2. Job plots performed at Fe(II) concentrations expressed in terms of binding equivalents

To further confirm that the mechanism of Fe(II)–Fz interaction is indeed that of infinite cooperativity, we carried out another series of experiments in which the concentration of Fe(II) was calculated in terms of its binding equivalent, i.e. one third of its molar concentration. From the Theory section, one expects the apparent binding stoichiometry, n, to shift from 3 to 1 as C_o increases. As can be seen in Fig. 3, the maximum occurs at $X=0.26(n=2.85)$ when $C_o=10.5$ μM and at $X=0.5(n=1)$ when $C_o=210$ μM. Thus, the shift in the n value also is consistent with the infinitely cooperative model. The solid lines are fitted by a computer program using an equation derived for these particular experimental conditions.

Treatment of data by the Hill plot. Both Figs. 2 and 3 provide support for an infinitely cooperative binding mechanism. This type of mechanism is a special case where the Hill plot should prove to be definitive since a linear line with a slope$=3$ should result. Fig. 4 shows a Hill plot using data from experiments included in Figs. 2 and 3. As expected, we obtained $n=3.0$ from the slop and a $K_d=7.9$ μM from the intercept, further demonstrating agreement with the one-step binding process.

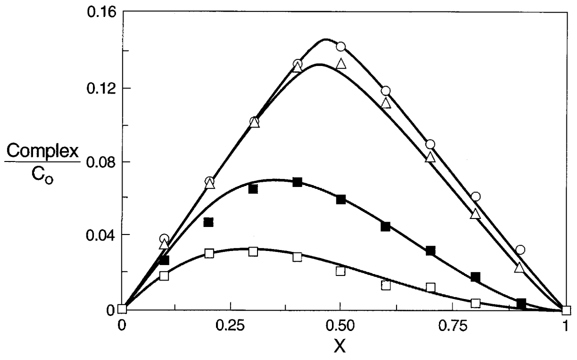

Fig. 3. Job plots of Fe(II)–Fz interaction carried out at different C_o in which the Fe(II) concentration is expressed in terms of binding equivalents: □, 10.5 μM; ■, 21 μM; △, 105 μM, and ○, 210 μM. The lines are fitted by computer using the NIH MLAB program. Experimental conditions are described in Experimental procedures.

5. Discussion

The method of continuous variation has been, in most cases, used to determine the stoichiometry of two reactants. For this reason, this method is best suited for tight-binding components because it requires a total concentration much greater than

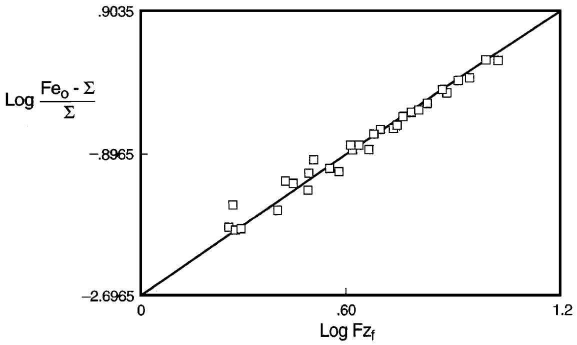

Fig. 4. Hill plot of Fe(II)–Fz interaction using data from Figs. 2 and 3.

Table 1
Computer analysis of Job plots of Fe(II)–Ferrozine interaction

Mechanism	Total concentration C_o (μM)	Dissociation constants (μM)			
Infinite Cooperative (one-step)					K_d
	7				7.99
	14				7.11
	52.5				8.28
	105				7.71
	10.5[a]				8.34
	21[a]				8.30
	105[a]				6.55
	210[a]				7.54
Adair (stepwise)		K_1	K_2	K_3	$K_d=(K_1K_2K_3)^{1/3}$
	7	1576	12.2	0.0275	7.97
	14	112	17.1	0.285	8.16
	10.5[a]	1560	11.5	0.0328	8.38
	21[a]	150	9.98	0.408	8.49
Adair (only $FeFz_3$ detected)	7	2980	9.22	0.0184	7.97
	14	129	7.77	0.370	7.17
	10.5[a]	1566	9.39	0.0387	8.29
	21[a]	180	5.35	0.449	7.63

[a] Concentrations of Fe(II) calculated in terms of binding equivalents (1/3 of molar concentration).

Experiments were carried out in 50 mM HEPES buffer, pH 7.0 at 25 °C. Equations pertaining to each model were used in the fitting by the NIH MLAB computer program [6]. Details have been described in Experimental procedures.

the K_d's involved. The advantages of the Job plot over titration of one component with another have been presented by Huang [3]. There are situations which make assigning a definitive n value difficult. For instance, if the maximum of a Job plot appears between $X_m=0.2$ and 0.25 (i.e. $n=0.8/0.2=4$ or $n=0.75/0.25=3$), the accuracy of the data may not allow one to make a choice between the two values. This is why the experiments may need to be repeated by expressing reactant concentrations in terms of the suspected number of binding sites to see if a Job plot with a maximum at the center of the curve (i.e. $n=1$) can be obtained [3]. But the continuous variation method need not be confined to stoichiometry determination. When carried out at different C_o, valuable information regarding K_d's and the mechanism of complex formation can be obtained. Theoretical treatment of applying continuous variation to various cooperative systems has been provided by Huang [3] in a rather general manner. In this report, we use computer simulations to illustrate how the mole fraction ratios, R_m and R_i, vary with C_o in different models and experimental designs. Two key points emerge from these theoretical considerations: First, using less than saturating levels of C_o to determine binding stoichiometry can lead to anomalous ratios substantially higher or lower than the true n value. Second, however, these shifting n values as a function of C_o are characteristic of their respective mechanisms and can be used as a basis for distinguishing various modes of reaction. For example, with the Adair model, R_m increases form 1 to n as C_o increases, while R_i increases from 1 to a peak value larger than n and finally settles on n (Fig. 1, Inset (a)). With C_o calculated in terms of binding equivalents, both R_m and R_i rise from 1 to higher values and fall back to 1 (Fig. 1, Inset (b)). With the infinitely cooperative model, $R_m=n$ regardless of C_o (Fig. 2, R_i cannot be obtained with this model because $d\Sigma/dY$ approaches zero as $Y\rightarrow 0$). When carried out in the 'binding-equivalent' procedure, R_m varies from $n\rightarrow 1$ as C_o increases (Fig. 3). In the case where only the final complex is detected, R_m does not remain constant even though the skewed shape of the curve often resembles that of the one-step mechanism.

In order to test the theory mentioned above, we set out to study the chelation of Fe(II) by Fz to elucidate the mechanism of complex formation and to determine the K_d's. The Job plots shown in Fig. 2 consistently yield an n value of 3, indicating a one-step binding process. With the 'binding-equivalent' approach, the n value shifts from 3 to 1 with increasing C_o as predicted by the one-step model. The mechanism is further confirmed by the Hill plot yielding a slope of 3 (Fig. 4).

To study the binding mechanism in greater detail, we fit the data shown in Figs. 2 and 3 to three plausible models: (1) the one-step, infinitely cooperative model; (2) the stepwise Adair model, where FeFz, $FeFz_2$, and $FeFz_3$ contribute signals linearly (i.e. 1:2:3); and (3) only $FeFz_3$ is detected spectrally. The results are summarized in Table 1. When fitted to the one-step mechanism, all eight curves at different C_o yield a K_d in the range of 7.80 ± 0.09 μM. With the stepwise binding in the Adair model, the values of K_1, K_2 and K_3 vary over a much wider range. However, it is clear that very strong cooperativity, $K_1\gg K_2\gg K_3$, is required in all cases in fitting the data. These results further support the notion that the cooperativity is so strong it actually approaches infinite cooperativity. By assuming $FeFz_3$ to be the only species contributing to the absorbance at 526 nm, we found that the dissociation constants needed to fit the data are essentially the same as those of the infinite cooperative case. This means that whatever FeFz and $FeFz_2$ are formed are likely present in insignificant amounts. And, despite the wide range of K_1, K_2 and K_3, a K_d calculated as $(K_1K_2K_3)^{1/3}$ almost always produces the value of 8.01 ± 0.43 μM. This K_d is comparable to that obtained with the infinite cooperativity model, 7.80 ± 0.09 μM, and that from the Hill plot, 7.90 μM. Thus, we conclude that Fe(II) interacts with Fz in a highly cooperative manner with a K_d of 8 μM.

References

[1] J.T. Edsall, J. Wyman, Biophysical Chemistry, Volume 1, Academic Press, New York, 1958.

[2] P. Job, Recherches sur la formation de complexes minéraux en solution, et sur leur stabilité, Ann. Chim. (Paris) 9 (1928) 113–134.

[3] C. Huang, Determination of binding stoichiometry by the continuous variation method: The Job plot, Methods Enzymol. 87 (1982) 509–525.

[4] L. Li, A.P. Seddon, A. Meister, Interaction of the protein components of 5-oxoprolinase. Substrate-dependent enzyme complex formation, J. Biol. Chem. 263 (1988) 6495–6501.

[5] L.L. Stookey, Ferrozine-A new spectrophotometric reagent for iron, Anal. Chem. 42 (1970) 779–781.

[6] G.D. Knott, MLAB—a mathematical modeling tool, Comput. Programs Biomed. 10 (1979) 271–280.

[7] C.R. Gibbs, Characterization and application of ferrozine iron reagents as a ferrous iron indicator, Anal. Chem. 48 (1976) 1197–1200.