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Analysis of biochemical reactions in equilibrium: Hydrolysis of penicillin and ampicillin

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Abstract

A general equilibrium model for a class of biochemical reactions was formulated, using the formalism of binding polynomials for the evaluations of the various species. Thermodynamic equilibrium constant was obtained from the observed equilibrium total concentrations of reactants at specified pH and certain ligand concentrations. Results for the hydrolysis of penicillin and ampicillin were analyzed with this equilibrium model. Predicted fractions of hydrolysis products at various pH values were calculated.

Keywords: Binding polynomials; Reference reaction; Equilibrium constant; Penicillin; Ampicillin; Hydrolysis

1. Introduction

Biochemical reactions generally involve many specific chemical reactions. Analysis of biochemical reactions and the calculations of its equilibrium composition entail calculations of reactions of various species. This was illustrated clearly in the classical cases involving adenosine triphosphate [1-3]. Multiple equilibrium calculations on these complicated systems has been formulated and implemented either by minimizing the free energies or by solving the simultaneous equilibrium expressions with conservation equations [4,5]. In certain kind of experiment of biochemical reactions, where pH and concentrations of free ligands are kept constant, the calculation of the equilibrium compositions can conveniently be done in terms of the formalism of the binding studies of biological macromolecules [6,7]. The purpose of this brief paper is to develop this formalism in general and to apply it to the analysis of our recent results on the enzymatic hydrolyses of penicillin and ampicillin [8].

2. The equilibrium model

Consider a reactant, R_i, with J independent binding sites for a proton, in a solution at equilibrium at a given pH. Then there are 2^J species of R_i in the solution, ranging from the completely dissociated species, $R_i^{z_i^0}$, to $R_i^{z_i^0+J}$ which has J bound protons (z_i^0 is a signed quantity). The concentration of the various species of R_i are given by C_{i0}, the concentration of $R_i^{z_i^0}$, multiplied by the corresponding terms, with appropriate k_{ijH}

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and powers of a_{H}, of the following binding polynomial (partition function):

$$Z_i = \prod_{j=1}^{J} (1 + k_{ij\mathrm{H}} a_{\mathrm{H}}), \tag{1}$$

where a_{H} denotes the activity of a proton. In the above equation we have used the microscopic binding constants $k_{ij\mathrm{H}}$ [10]. These are related to the macroscopic step-wise dissociation constants, $K_{i\mathrm{H}n}$ by

$$K_{i\mathrm{H}n} = \sum_{j_1=1}^{J-n+1} \sum_{j_2>j_1}^{J-n+2} \cdots \sum_{j_{n-1}>j_{n-2}}^{J} \prod_{\nu=1}^{n-1} k_{ij_\nu\mathrm{H}} \times \left(\sum_{j_1=1}^{J-n} \sum_{j_2>j_2}^{J-n+1} \cdots \sum_{j_n>j_{n-1}}^{J} \prod_{\nu=1}^{n} k_{ij_\nu\mathrm{H}} \right)^{-1},$$

and Z_i can be written as $Z_i = 1 + \sum_{j=1}^{J} a_{\mathrm{H}}^{j} \prod_{n=1}^{j} K_{i\mathrm{H}n}^{-1}$. If there are L types of ligands, including protons, competing for the J binding sites, then the binding polynomials must be replaced by

$$Z_i = \prod_{j=1}^{J} \left(1 + \sum_{l=1}^{L} k_{ijl} a_l \right), \tag{2}$$

where a_l is the activity of the lth ligand.

If the bindings at the sites are not independent, i.e. there are interactions between the sites, then the binding polynomial is no longer simply a product of linear factors as written above [7]. In this case Z_i can be formulated in terms of the intrinsic constants plus various interaction parameters [10–12]. For small J, Z_i can easily be written alternatively as a polynomial in terms of the macroscopic dissociation constants, e.g. the case of ATP [1–3].

The concentration of all the species of R_i with m_{H} bound proton is given by $c_{i,m_{\mathrm{H}}} = (c_{i0} a_{\mathrm{H}}^{m_{\mathrm{H}}} / m_{\mathrm{H}}!)(\partial^{m_{\mathrm{H}}} Z_i / \partial a_{\mathrm{H}}^{m_{\mathrm{H}}})_{a_{\mathrm{H}}=0}$, and in general the concentration of species of R_i with m_l ($l = 1$ to L) of the lth ligand bound is given by

$$c_{i,[m_l]} = c_{i0} \left(\partial^{\Sigma m_1} Z_i / \partial a_1^{m_1} \ldots \partial a_L^{m_L} \right)_{a_1=0,\ldots,a_L=0} \times \prod_{l=1}^{L} (a_l^{m_l} / m_l!). \tag{3}$$

The average number of lth ligand bound per molecule of R_i is $\bar{\nu}_{il} = \partial \ln Z_i / \partial \ln a_l$, and the average charge on R_i is $z_{i0} + \sum_{l=1}^{L} \bar{\nu}_{il} Z_l$, where z_l is the charge of the lth ligand.

Next we consider a reference chemical reaction involving the unligated species of the reactants:

$$\sum \nu_i R_i^{z_i^0} = 0,$$

where the stoichiometric coefficient, ν_i, is positive for reactants and negative for products. The equilibrium constant for the reference chemical reaction is

$$K_{\mathrm{ref}} = \prod_i (c_{i0} \gamma_{i0})^{\nu_i}, \tag{4}$$

where γ_{i0} denotes activity coefficient of $R_i^{z_i^0}$. However the commonly measured apparent equilibrium constant of the corresponding biochemical reaction includes the equilibrium concentrations of all the species of the reactants, and is given by

$$K_{\mathrm{obs}} = \prod{}' C_i^{\nu_i} = \prod{}' (C_{i0} Z_i)^{\nu_i}, \tag{5}$$

where $C_i = c_{i0} Z_i$ is the total concentration of R_i, and the prime on the product sign indicates that the product includes only the biochemical species in the biochemical reaction. The total concentrations, C_i, are related by mass conservations, and are given by the following set of relations:

$$C_i = C_i^0 + (\nu_1/\nu_i)(C_1 - C_1^0) \quad \text{for} \quad i = 2, \ldots, I, \tag{6}$$

where C_i^0 is the initial value of the concentration C_i. Therefore all the equilibrium concentrations, C_i, are determined from K_{obs} and C_i^0, using Eqs. (5) and (6). The concentrations of the various species can then be calculated from Z_i. The thermodynamic equilibrium constant for the reference reaction, Eq. (4), is then determined from the c_{i0}, with the activity coefficients calculated from the Debye–Hukel equation for the given ionic strength I_m.

3. Hydrolysis of penicillin and ampicillin

Penicillin. Structures of penicillin G and of penicillinoic acid are shown in Fig. 1 and their titration curves are shown in Fig. 2, where pH of

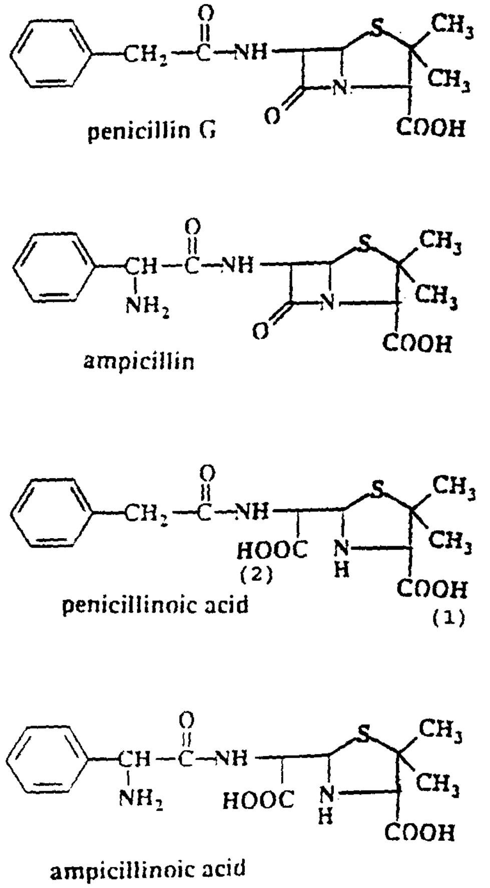

Fig. 1. Structural formula of penicillin, ampicillin, penicillinoic acid, and ampicillinoic acid.

the solution is plotted versus Δ, the change in proton bound per mole of penicillin. For penicillin the only ionizable group for pH < 10 is the carboxyl group with log $k < 3$. Its binding polynomial is simply $Z_{PEN} = 1 + k_{pen}a_H$ with the microscopic binding constant, $k_{pen} = 10^{2.93}$. For penicillinoic acid, there is an ionizable group with log $k = 5.64$; this is assumed to be the cyclic amine group (see Fig. 1) and that it binds independently of the two carboxyl groups which have log $k < 3$. Therefore $Z_{PA} = (1 + k_{PA1}a_H)[1 + (k_{PA2} + k_{PA3})a_H + \alpha k_{PA2}k_{PA3}a_H^2]$ with $k_{PA1} = 10^{5.64}$. The values we determined for k_{PA2}, k_{PA3} and the interaction parameter α are less accurate due to the larger uncertainties in both the experiments and analyses of the data at pH < 3. However, for the practical pH range of 4 < pH < 10, these carboxyl groups are almost fully dissociated; and thus the determinations of the concentrations of the species and of the equilibrium constant of the reference reaction are relatively insensitive to the precise values for α, k_{PA2}, and k_{PA3}. For calculations herein we shall assume $\alpha = 1$, and take $k_{PA2} = 10^{2.81}$, $k_{PA3} = 10^{1.80}$, values derived from the literature [8]; the values used for the microscopic constants are summarized in Table 1.

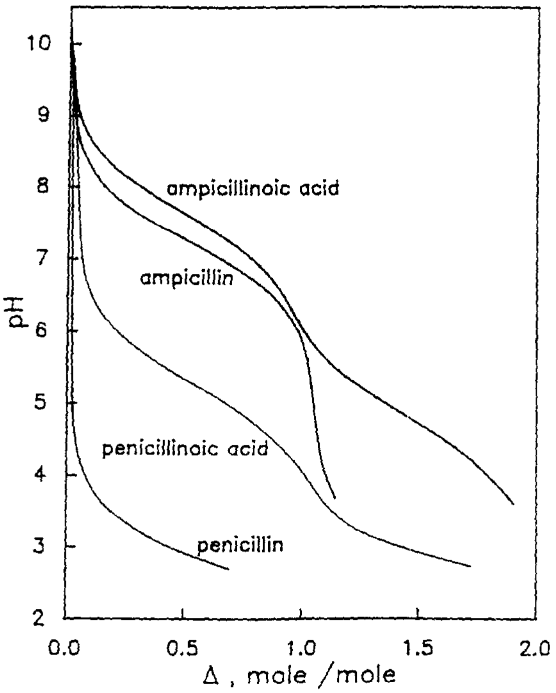

Fig. 2. Titration curves of penicillin, penicillinoic acid, ampicillin, and ampicillinoic acid. Δ is the increment in mole of bound proton per mole of reactant between pH 10 and the given pH.

Consider the biochemical reaction of the hy-

Table 1
Values of log k used in the calculations

	penicillin	penicillinoic acid	ampicillin	ampicillinoic acid
carboxyl (1)	2.93	2.81	2.54	2.81
carboxyl (2)		1.81		1.81
cyclic amine		5.64		5.03
primary amine			7.71	8.48

drolysis of penicillin to penicillinoic acid PEN + H_2O = PA. The reference chemical reaction is

$$\mathrm{PEN}^{-1} + H_2O = \mathrm{PA}^{-2} + H^+$$

The apparent equilibrium constant $K_{\mathrm{obs}} = C_{\mathrm{PA}}/C_{\mathrm{PEN}}$ was determined to be 2.00 at pH = 6.01 [8]. Therefore in a solution initially 0.05 molar in penicillin and contains no penicillinoic acid, at equilibrium it will have $C_{\mathrm{PEN}} = 0.0167$, and $C_{\mathrm{PA}} = 0.0333$, using Eqs. (5,6). The concentrations of various species are calculated from Eq. (1), and are listed in Table 2. From these we calculated an equilibrium constant for the reference chemical reaction,

$$K_{\mathrm{Pen}} = C_{\mathrm{PA}^{-2}}\gamma_{\mathrm{PA}^{-2}}a_{\mathrm{H}}/C_{\mathrm{PEN}^{-1}}\gamma_{\mathrm{PEN}^{-1}}a_{\mathrm{H_2O}}$$
$$= 5.14\times10^{-7}$$

where we have assumed that $a_{\mathrm{H}} = 10^{-\mathrm{pH}}$, and that the activity of water is 1 for these calculations. Whereas the thermodynamic equilibrium constant K_{Pen} is pH independent, the apparent equilibrium constant $K_{\mathrm{obs}} = K_{\mathrm{Pen}}a_{\mathrm{H_2O}}Z_{\mathrm{PA}}\gamma_{\mathrm{Pen}}/a_{\mathrm{H}}Z_{\mathrm{Pen}}\gamma_{\mathrm{PA}}$ is obviously a function of pH, and this is shown in Fig. 3. *Ampicillin.* Consider next the hydrolysis of ampicillin to ampicillinoic acid, AMP + H_2O = AA. The titration curve of ampicillin in Fig. 2 shows an ionizable group at pH ≈ 7.5 besides the carboxyl group with log $k < 3$. We assume this to be the primary amine, see Fig. 1. The binding polynomial is then

$$Z_{\mathrm{AMP}} = (1 + k_{\mathrm{ampl}}a_{\mathrm{H}})(1 + k_{\mathrm{amp2}}a_{\mathrm{H}})$$

with $k_{\mathrm{ampl}} = 10^{7.71}$ and $k_{\mathrm{amp2}} = 10^{2.54}$ [8]. In the case of ampicillinoic acid Fig. 2 shows an additional proton binding site at pH ≈ 5, which is ≈ −3 pH units from that of the primary amine and ≈ +2 pH units from the carboxyl groups, this is taken again to be the secondary amine group of the thiazolidine ring. Therefore,

$$Z_{\mathrm{AA}} = (1 + k_{\mathrm{AA1}}a_{\mathrm{H}})(1 + k_{\mathrm{AA2}}a_{\mathrm{H}})$$
$$\left[1 + (k_{\mathrm{AA3}} + k_{\mathrm{AA4}})a_{\mathrm{H}} + \beta k_{\mathrm{AA3}}k_{\mathrm{AA4}}a_{\mathrm{H}}^2\right]$$

with $k_{\mathrm{AA1}} = 10^{8.48}$, and $k_{\mathrm{AA2}} = 10^{5.03}$. As in the

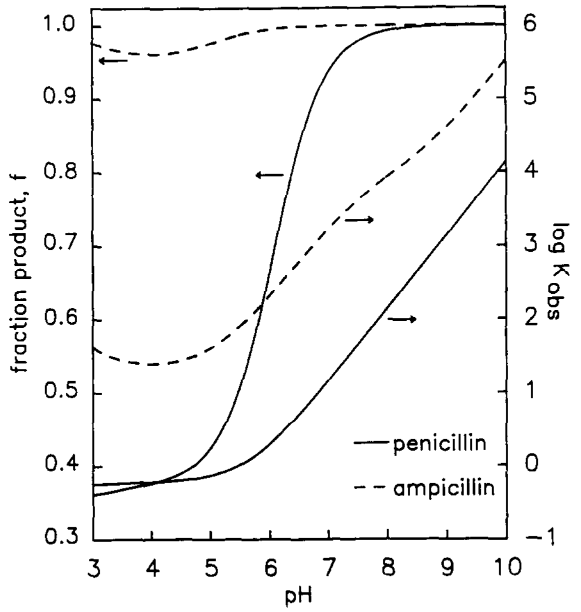

Fig. 3. log K_{obs} and fraction of hydrolysis products, $K_{\mathrm{obs}}/(1+K_{\mathrm{obs}})$, as functions of pH.

Table 2
Concentrations, in mol kg^{-1}, of various species at equilibrium in the hydrolysis of penicillin and ampicillin

	(A) Penicillin at 298.2 K pH 6.01, $I_m = 0.25$		(B) Ampicillin at 282.4 K pH 5.58, $I_m = 0.25$	
	penicillin	penicillinoic acid	ampicillin	ampicillinoic acid
total:				
initial	0.05	0	0	0.05
equil.	0.0167	0.0333	0.000538	0.0495
species:				
valence −2	–	0.02335	–	0.000048
−1	0.01665	0.00997	0.00000395	0.03848
0	0.00001	$< 10^{-5}$	0.0005332	0.01091
+1	–	$< 10^{-9}$	0.0000005	0.00002
+2	–	–	–	$< 10^{-8}$

case of the penicillinoic acid above we assume again that $\beta = 1$, and take $k_{AA3} = 10^{2.81}$, $k_{AA4} = 10^{1.80}$. A reference chemical reaction for this biochemical reaction of ampicillin is

$$\mathrm{Amp}^{-1} + H_2O = \mathrm{AA}^{-2} + H^+.$$

The measured apparent equilibrium constant of the biochemical reaction at pH = 5.58 is $K_{obs,Amp} = C_{AA}/C_{Amp} = 92$. Therefore in an equilibrium solution initially 0.05 molar in ampicillinoic acid, we have $C_{Amp} = 0.000538$ and $C_{AA} = 0.0495$. The concentrations of various species are given in Table 2 (B). From these the thermodynamic equilibrium constant for the reference chemical reaction is determined to be $K_{Amp} = C_{AA}\gamma_{AA}a_H/C_{Amp}\gamma_{Amp}a_{H_2O} = 9.73 \times 10^{-6}$.

The apparent equilibrium constant $K_{obs,Amp}$ at various pH are calculated and illustrated in Fig. 3.

The slopes of the log K_{obs} versus pH curves in Fig. 3 are given by

$$\frac{d\log K_{obs,Pen}}{d\,pH} = 1 + \frac{k_{Pen}a_H}{1 + k_{Pen}a_H} - \frac{k_{PA1}a_H}{1 + k_{PA1}a_H} - \frac{(k_{PA2} + k_{PA3})a_H + 2k_{PA2}k_{PA3}a_H^2}{1 + (k_{PA2} + k_{PA3})a_H + k_{PA2}k_{PA3}a_H^2}$$

for penicillin, and

$$\frac{d\log K_{obs,Amp}}{dpH} = 1 + \frac{k_{Amp1}a_H}{1 + k_{Amp1}a_H} + \frac{k_{Amp2}a_H}{1 + k_{Amp2}a_H} - \frac{k_{AA1}a_H}{1 + k_{AA1}a_H} - \frac{k_{AA2}a_H}{1 + k_{AA2}a_H} - \frac{(k_{AA3} + k_{AA4})a_H + 2k_{AA3}k_{AA4}a_H^2}{1 + (k_{AA3} + k_{AA4})a_H + k_{AA3}k_{AA4}a_H^2}$$

for ampicillin. The slopes of both curves approach 1 at high pH and −1 at low pH. Thus log K_{obs}, for both, passed through a minimum at an intermediate pH where the slope is 0, this occurs at pH 2.92 for penicillin and at pH 4.01 for ampicillin. Fig. 3 also shows the fraction of hydrolysis products, f, as functions of pH. These are given by $f_{Pen} = C_{PA}/(C_{PEN} + C_{PA}) = (1 + a_H Z_{Pen}\gamma_{PA}/K_{Pen}Z_{PA}\gamma_{Pen})^{-1}$ for penicillin and $f_{Amp} = C_{AA}/(C_{Amp} + C_{AA}) = (1 + a_H Z_{Amp}\gamma_{AA}/K_{Amp} Z_{AA}\gamma_{Amp})^{-1}$ for ampicillin. Thus both f_{Pen} and f_{Amp} approach 1 at high pH, i.e. a_H approaches 0. And they also approach 1 at low enough pH where $a_H Z_{Pen}/Z_{PA}$ and $a_H Z_{Amp}/Z_{AA}$ both approaches 0. The derivatives of the f versus pH curves, given by $df/d\,pH = f(1-f)\,d\log K_{obs}/d\,pH$, is 0 also at the same pH where $d\log K_{obs}/d\,pH = 0$. Thus the fraction of hydrolyses products is a minimum at this pH (2.92 for penicillin and 4.01 for ampicillin) and increases as the pH of the solution is increased or decreased.

4. Discussion

The reference reaction defined above as the chemical reaction between unligated species is of course not unique, it could have been defined as chemical reaction between dominant species, for example, with proper balance of charges and protons [8]. However, since we employ the formalism of binding polynomial in this treatment, the definition of reference chemical reaction as that between the unligated species is less cumbersome and perhaps more general and logical. Obviously, the variously defined reference reactions are simply related to one another and the thermodynamic equilibrium constant of one is easily obtained from that of the other. Due to the fact that our measurement of the total concentrations of the reactants at equilibrium were done at pH > 5.5, the uncertainties in the bindings to the carboxyl groups cause a relatively small error of < 1% in the values of the K_{ref}'s determined, and an estimated uncertainty in the predicted values of K_{obs} of ≈ 1% at pH4 which decreases as the pH increases.

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