Simple and universal method to determine dissociation constants for enzyme/ligand complexes

Agnieszka Bzowska* and Lucyna Magnowska

Department of Biophysics, Institute of Experimental Physics, University of Warsaw, Żwirki i Wigury 93, 02-089 Warsaw, Poland

ABSTRACT

A simple and in principle universal method is proposed for measuring enzyme/ligand dissociation constants. The method is based on measuring enzyme activity remaining after heat treatment in the absence and in the presence of ligands. The method is especially suitable for enzymes interacting with nucleosides, nucleosides and oligonucleotides since for such enzymes convenient spectrophotometric assays are available.

INTRODUCTION

When new compounds are synthesized often one of the task is to quantify their binding properties with some target enzyme. One of the methods of measuring inhibition constants, but those values are not necessarily equal to dissociation constants. Purine nucleoside phosphorylase (PNP, EC 2.4.1.1) - the key enzyme of the purine salvage pathway [1] - displays a non-Michaelis kinetics, hence discrepancies between inhibition and dissociation constants (Kd) are expected [2]. For such systems an easy and universal method to determine dissociation constants would be very helpful. Here we propose such an approach and test it by measuring Kd for various ligands of mammalian PNP, a well-known drug target due to its role in some immunological diseases and in the intracellular degradation of some antitumour and antiviral drugs [1].

MATERIALS AND METHODS

Calf spleen PNP (~30 U/mg, Sigma) was desalted and previously described [2]. (S)-PMP-DAP was synthesized according to [3]. Guanine, 7-methylguanosine and hepes were from Sigma or Roth. PNP (3 μM subunits) in a total volume of 20 μl was incubated in 10 mM Hepes buffer pH 7.0 for 3 min at 50°C, 10 min at 46°C, 25 min at 43°C or 60 min at 40°C, in the absence (control) and presence of ligands (0.1-100 mM for P1, 1-200 μM for Gua, 0.5-350 μM for (S)-PMP-DAP). Activity of PNP was determined before (ν0) and after (ν) heat treatment, spectrophotometrically with 7-methylguanosine (0.3 mM) as a substrate, in 50 mM phosphate buffer pH 7 at 25°C [2]. A hyperbolic dose-response curve was fitted to the data (% activity remaining after heat treatment vs ligand concentration, [c]):

\[ \frac{\nu}{\nu_0} = A_0 + \frac{A_{\text{max}} [c]}{C + [c]} \]  

where A0 is the % activity observed after heat treatment in the absence of ligand, \( A_{\text{max}} \) is the maximal protection provided by a ligand, C is the concentration of ligand providing half-maximal protection. C may be regarded as the reciprocal of the apparent association constant at a given temperature: C = 1/Kd.

RESULTS AND DISCUSSION

PNP catalyzes the reversible phosphorolysis of purine nucleosides: β-purine nucleoside + orthophosphate = purine base + α-D-ribose-1-phosphate [1]. PNP ligands embraced in this study are shown below: guanine (Gua, left), orthophosphate (P1, middle) and 2,6-diaminopurine ((S)-PMP-DAP, right). For all of them Kd at 298 K determined by various methods and/or various laboratories were reported in literature.

Protective effect of ligands against the heat treatment of PNP was measured as described above. Data for P1 are shown in Figure 1. Similar data were obtained for Gua and (S)-PMP-DAP. Concentrations of ligand providing half-maximal protection, C, obtained from fitting equation (1), are listed in Table 1, and shown in Fig. 2 in the form of the van’t Hoff plot ln[C] vs 1/T (T is temperature in kelvins).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Effects of P1 on the thermal stability of calf spleen PNP. Experimental data, % activity remaining after 60 min incubation at 40°C (O), 25 min at 43°C (●), 10 min at 46°C (○) and 3 min at 50°C (■), and least-squares fits (solid lines) to equation (1).
Figure 2. van’t Hoff plots for the specific interactions of P₆ (left), Gua (middle) and (S)-PMP-DAP (right) with calf PNP. Non-linear regression analysis (solid line) was conducted by fitting the non-linear van’t Hoff equation (2) to the data points shown in Table 1.

Table 1. Concentrations, C, of ligands providing half-maximal protection against heat treatment of calf spleen PNP. C may be regarded as the reciprocal of the apparent association constant, Kₐ⁰, at a given temperature: C = 1/Kₐ⁰.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>T [K]</th>
<th>(C ± S.E.) [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₆</td>
<td>313</td>
<td>943 ± 77</td>
</tr>
<tr>
<td>P₆</td>
<td>316</td>
<td>1989 ± 244</td>
</tr>
<tr>
<td>P₆</td>
<td>319</td>
<td>4463 ± 723</td>
</tr>
<tr>
<td>P₆</td>
<td>323</td>
<td>15040 ± 4970</td>
</tr>
<tr>
<td>Gua</td>
<td>313</td>
<td>3.90 ± 0.90</td>
</tr>
<tr>
<td>Gua</td>
<td>316</td>
<td>12.30 ± 1.60</td>
</tr>
<tr>
<td>Gua</td>
<td>319</td>
<td>25.90 ± 4.40</td>
</tr>
<tr>
<td>Gua</td>
<td>323</td>
<td>116.60 ± 14.30</td>
</tr>
<tr>
<td>(S)-PMP-DAP</td>
<td>313</td>
<td>1.58 ± 0.30</td>
</tr>
<tr>
<td>(S)-PMP-DAP</td>
<td>316</td>
<td>5.18 ± 0.55</td>
</tr>
<tr>
<td>(S)-PMP-DAP</td>
<td>319</td>
<td>10.44 ± 0.42</td>
</tr>
<tr>
<td>(S)-PMP-DAP</td>
<td>319</td>
<td>11.29 ± 0.26</td>
</tr>
<tr>
<td>(S)-PMP-DAP</td>
<td>323</td>
<td>67.33 ± 9.47</td>
</tr>
</tbody>
</table>

S.E. are the standard errors of the fitted parameters.

The van’t Hoff dependence of ln[C⁻¹] vs 1/T exhibits non-linear behaviour, especially for P₆ and (S)-PMP-DAP (Figure 2), therefore the non-linear van’t Hoff equation (4) was fitted to the data (Tₛ and Tₑ are critical temperatures in which, respectively, ∆Hₛ = 0 and ∆Sₛ = 0; index "s" refers to the pseudo-standard state at concentrations of 1 mol/L): 

ln[C⁻¹] = ln(Kₐ⁰) = (∆Cₛ/R) [Tₑ/T + ln(Tₑ/T) - 1]  (2)

Formation of complexes between PNP and P₆, Gua and (S)-PMP-DAP is characterized by the negative value of the heat capacity change, ∆Cₛ, the phenomenon reported for many enzyme-ligand complexes [5,6]. Using the parameters obtained form the fits (ΔCₛ, Tₛ, Tₑ, Kₐ) at a desired temperature may be calculated. For 298 K (25 °C) this leads to Kₐ = 91μM for P₆, Kₐ = 0.06μM for Gua, and Kₐ = 0.35μM for (S)-PMP-DAP. These results, despite the fact that a very simple model (eq.1) was used to analyze the heat-treatment data, are in fairly good agreement with the Kₐ values reported previously: 170-290μM [7] and 220±120μM [2] for P₆; 0.08±0.02μM [7] and 0.12±0.02μM [8] for Gua; 0.23 - 3.5μM (depending on enzyme concentration) for (S)-PMP-DAP [9].

The analysis performed here yields, in addition to dissociation constants, also some important thermodynamic parameters of the complexes examined, ΔCₛ, Tₛ, Tₑ, not discussed here due to limited space for the presentation.

CONCLUSION

The method to determine dissociation constants for enzyme/ligand complexes - proposed and tested here for mammalian PNP and some of its ligands - is easy and does not require any sophisticated equipment. It is in principle universal and may be easily adopted to determine dissociation constants for other enzyme/ligand complexes. This is especially true if the assay for the enzyme activity measurement is easy and well established and this in turn is often the case with the nucleoside, nucleotide and oligonucleotide binding enzymes for which convenient spectrophotometric assays are mostly available.

ACKNOWLEDGEMENTS

We are indebted to Prof. Antonín Holý for the sample of (S)-PMP-DAP and to the Polish Ministry of Science and Higher Education (grants N301 003 31-0042 and BW-1724/BF) for the support.

REFERENCES


*Corresponding author: Agnieszka Bzowska, E-mail: abzowska@biogeo.uw.edu.pl