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Determination of binding affinity of metal cofactor to the active site of methionine aminopeptidase based on quantitation of functional enzyme

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Abstract

Determination of metal affinity to the active site of metalloenzymes constitutes an integral part in the understanding of enzyme catalysis and regulation. Nonlinear curve fitting of metal titration curves using the multiple independent binding sites (MIBS) model was adapted to determine K_D values based on functional enzyme concentrations. This approach provides a more accurate evaluation of K_D compared to existing methods that are based on total protein concentrations. We applied this concept to methionine aminopeptidase from *Mycobacterium tuberculosis* and showed that it is a monometalated enzyme with K_D of 0.13 μM for Co^{2+}.

Keywords

cofactor affinity; K_D determination; enzyme activity; binding curve; metal titration

Metal ions carry out diverse and essential functions in biology, including the vital role of serving as enzyme cofactors for catalysis [1;2;3]. Many metallohydrolases can be activated by several different cations, and it has become evident that determination of metal affinity to the active site is a prerequisite for a complete understanding of the catalytic and regulatory roles of these metals. For accurate determination of the equilibrium binding constants, assay conditions are adjusted such that the enzyme concentration is relatively much smaller than the K_D for the interaction [4], where it is assumed that the titrated metal concentration corresponds to the amount of free metal ions in solution. This would be described as a "K_D controlled" scenario, because the binding curve becomes more susceptible to changes in K_D [5]. However, optimal conditions that meet this requirement often cannot be met due to a number of factors, including lack of sensitivity of the enzymatic assay, instability of substrate or product, degradation and aggregation of enzyme. As a result, the enzyme concentration is generally increased to a point that total metal added is no longer equivalent to free metal concentration, and therefore a standard Langmuir-type binding isotherm model becomes less reliable as it deviates from a true "K_D controlled" situation. This setback can be overcome by incorporating total protein concentrations into the binding model, which is utilized to obtain free metal

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concentrations from total metal added [6]. However, estimation of total protein can be unreliable depending on the technique and protein standard used, and the presence of interfering agents [7]. In addition, the assumption does not always hold true that the determined protein concentration is the same as the concentration of functional enzyme that can bind and be activated by the metal ion. We propose an iterative approach that calculates equilibrium binding constants based on the amount of functional enzyme instead of total protein concentration. This method makes use of nonlinear regression curve fitting with the multiple independent binding sites (MIBS) model [5], and it is applied to a methionine aminopeptidase (MetAP) derived from *Mycobacterium tuberculosis* that is activated by Co^{2+} ions. MetAP exploits divalent cations to catalyze the maturation of nascent proteins by removing the N-terminal methionine, an essential process demonstrated to be vital for cell survival [8].

A well-established approach for the determination of binding affinity is to monitor spectral changes during ligand-receptor interactions [4]. Fluorescence signal resulting from hydrolysis of the fluorogenic substrate methionyl 7-amino-4-methylcoumarin, MetAMC, [9] (20 μM in 50 mM MOPS, pH 7.5) at increasing metal c0.oncentrations produces a rectangular hyperbola (Fig. 1), which can be analyzed using the adapted model for MIBS [5], taking into consideration total protein $[P]_T$ and total metal concentrations $[M]_T$:

$$F=F_0+(F_{MAX}-F_0)\times\frac{(n[P]_T+[M]_T+K_D)-\sqrt{(n[P]_T+[M]_T+K_D)^2-4n[P]_T[M]_T}}{2n[P]_T} \quad (1)$$

where F is the fluorescence signal resulting from MetAMC hydrolysis, F_0 is the signal from a blank, F_{MAX} corresponds to the maximal fluorescence intensity resulting from maximal enzyme activation by the metal cofactor, K_D is the dissociation constant and n is the number of independent binding sites.

Our proposed approach entails the use of two metal titrations, at high and low enzyme concentrations, respectively. In order to accurately measure functional MetAP concentration, a first titration curve was generated under tight-binding situation where the protein concentration is more than 10 fold of the estimated K_D. The reported K_D for Co^{2+} is 0.3 μM for a homologous MetAP of *Escherichia coli* [10], so that 20 μM of *M. tuberculosis* MetAP protein was used. The protein concentration was determined by the Bradford assay with bovine serum albumin as the standard [11]. Under this tight-binding condition, cations initially bind stoichiometrically to the enzyme as the metal concentration increases, resulting in the observed linear onset of the curve (Fig. 1A). Functional protein concentration and n can be accurately determined under this circumstance, based on the metal concentration. The titration curve clearly showed that only one equivalent of Co^{2+} is required to fully activate *M. tuberculosis* MetAP, as we have observed for *E. coli* MetAP [12]. After estimating an initial value for K_D and holding n = 1, Eq. 1 was fitted to the first titration curve by adjusting F_0, F_{MAX} and $[P]_T$. Accurate K_D values cannot be determined under the tight-binding situation [5]. Instead, enzyme concentration should be lowered with the intention to create varying proportions of free and bound metals [4]. Therefore, in order to determine K_D, a second titration curve was obtained at a lower MetAP concentration where it is close to the estimated K_D. In this case, 0.5 μM of *M. tuberculosis* MetAP protein was used, generating a more rounded rectangular hyperbolic curve (Fig. 1B). The estimated $[P]_T$ obtained from the first titration was used in the analysis of the second titration curve (after adjusting for dilution), and Eq. 1 was fitted to the second titration curve by refining F_0, F_{MAX} and K_D. The newly generated K_D is then fed to the first titration data, and the cycle is repeated until convergence is achieved by noting that $[P]_T$ and K_D values remain constant after further iterations. The results revealed functional MetAP protein concentrations $[P]_T$ of 21.76 μM and 0.54 μM for the first and second titration

curves, respectively, and a K_D value of 0.13 μM for the binding of the metal Co^{2+} to the active site of *M. tuberculosis* MetAP.

Although K_D estimation can be accurately obtained based on total protein concentration, it requires that all of the protein is fully functional. However, in most cases that information is unknown, and the K_D calculation described here based on functional enzyme concentrations is appropriate. This simple method requires only generating two titration curves, and the concentration of functional protein and the binding affinity of cofactor can be simultaneously determined. Potential application of this method holds great promises in the analysis of affinity of cofactors to functional proteins, particularly in the activation of metalloenzymes by cations. This approach can be expanded to include protein-protein and DNA-protein interactions, such as in the binding of cAMP receptor to a DNA fragment of the *lac* promoter [13].

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Abbreviations used

MetAP
: methionine aminopeptidase

MIBS
: multiple independent binding sites

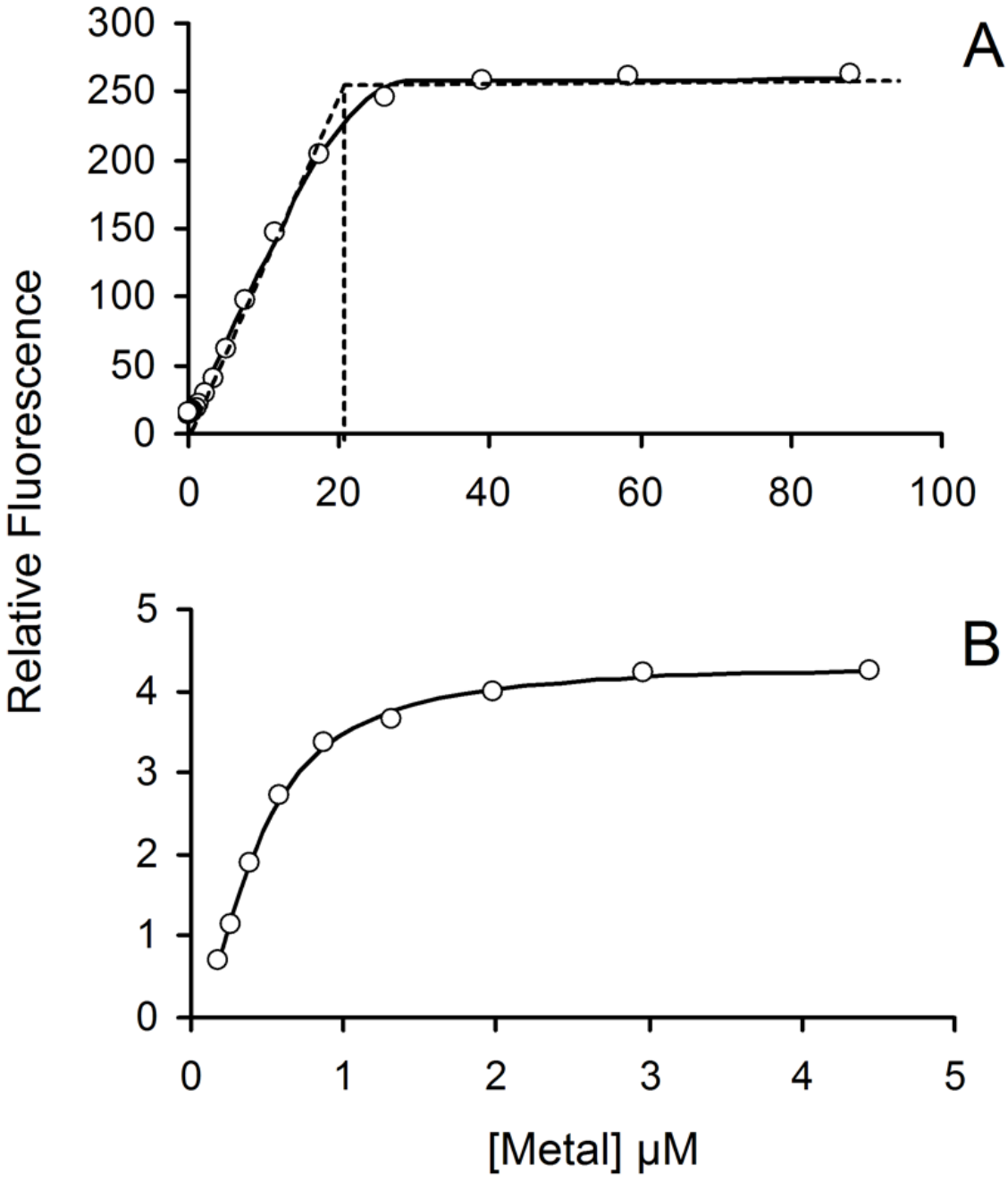

Fig 1.

Titration of MetAP enzyme with increasing concentrations of Co^{2+} cofactor, where enzymatic activity was monitored by fluorescence from the hydrolysis of a fluorogenic substrate. (A) Titration curve under tight-binding situation (20 μM MetAP was used). Raw data (open circle) was fitted with Eq. 1 (solid line). The linear segment (diagonal dash line) corresponding to data extrapolation intercepts the maximal activity (horizontal dash line) at the titration endpoint, indicating a 1:1 metal/protein molar ratio (vertical dash line). Therefore, $n = 1$ was kept constant when using Eq. 1. (B) Titration curve under "K_D controlled" situation (0.5 μM MetAP was used). Raw data (open circle) was fitted with Eq. 1 (solid line), giving a K_D value of 0.13 μM. Fitting parameters were calculated using the Solver feature in Microsoft Excel.