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Gel chromatographic evaluation of the binding constant for the interaction of thiamin diphosphate with magnesium ion

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ABSTRACT

A simple gel chromatographic procedure is devised for characterizing the interactions of nucleotides and other coenzymes with metal ions. Its application is illustrated by determining the binding constant for the interaction of thiamin diphosphate with Mg^{2+} ion by frontal gel chromatography on Sephadex G-10. An association constant of 3200 ($\pm$400) M^{-1} is obtained for the interaction in 0.1 *M* Tris–HCl buffer (pH 7.6) supplemented with poly(ethylene glycol) (50 mg/ml) and mercaptoethanol (25 m*M*).

INTRODUCTION

Because all enzymes that are dependent upon thiamin diphosphate also require a divalent cation for activity, it is generally considered that the active cofactor is the complex of coenzyme with metal ion. In experimental studies Mg^{2+} is most frequently included with thiamin diphosphate to generate active holoenzyme; and yet, surprisingly, the binding constant for this interaction does not seem to have been reported. Some information is available on the interaction of thiamin diphosphate with manganous ion, a metal ion substitution that has allowed resort to electron paramagnetic resonance for establishing the 1:1 stoichiometry and association constant of 5000 M^{-1} for the Mn^{2+}–coenzyme interaction [1]. Associated proton magnetic resonance studies revealed the existence of that complex as a folded structure with the pyrophosphate looped back over the thiazole ring [1].

From the physiological viewpoint it is clearly important to characterize the interaction of thiamin diphosphate with Mg^{2+} ion rather than with some other metal ion that happens to be convenient for study by a particular method. In that regard a subsequent ^{31}P nuclear magnetic resonance study [2] has confirmed the 1:1 stoichiometry of the Mg^{2+}–coenzyme interaction, and has also signified the likelihood that this complex is folded in similar fashion to its manganous counterpart. However, no attempt was made to evaluate the equilibrium constant. Gel chromatography is a general procedure that has the capacity to provide this quantification; and has, indeed, been recommended for the study of metal–nucleotide interactions [3]. As noted previously [4], that recommendation was premature in the sense that no allowance was made for the consequences of the above-mentioned change in shape/size of the coenzyme that accompanies complex formation in many metal–nucleotide interactions —a phenomenon that should give rise to a difference in gel chromatographic migration rates for thiamin diphosphate in free and complexed states.

Determination of association constants for acceptor–ligand interactions by gel chromatography is relatively simple in instances where the acceptor is

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macromolecular and hence where acceptor and acceptor–ligand complex comigrate [5–7]. However, the situation in which both reactants are small molecules introduces complexity into the analysis because of the different velocities, or elution volumes, exhibited by the acceptor and acceptor–ligand species [4,8]. Although procedures have been devised to cover these circumstances [4,6,8–10], the sophistication of those analyses may well deter the biochemist whose prime interest is the magnitude of the equilibrium constant rather than the physicochemical elegance of the procedure by which it is obtained. In response to a situation requiring the assignment of a magnitude to the binding constant for the interaction of magnesium ion with thiamin diphosphate, a simple gel chromatographic procedure has been devised to obtain the first direct estimate of the equilibrium constant for this physiologically important metal–nucleotide interaction.

EXPERIMENTAL[a]

Reactant solutions

Separate stock solutions of thiamin diphosphate (Sigma) and reagent-grade $MgCl_2$ in buffer medium [0.1 *M* Tris–HCl (pH 7.6) supplemented with poly (ethylene glycol) 600 (50 mg/ml) and β-mercaptoethanol (25 m*M*)] were prepared on a weight/volume basis. Mixtures comprising a fixed total concentration (100 μM) of thiamin diphosphate ($[\bar{A}]_0$) and a range (0–0.1 *M*) of magnesium ion concentrations ($[\bar{S}]_0$) were then prepared from these stock solutions. Because only small volumes of stock magnesium ion solution were required for the preparation of these mixtures, atomic absorption spectroscopy was used to establish the precise magnitudes of $[\bar{S}]_0$. The incorporation of the poly(ethylene glycol) and mercaptoethanol into the buffer medium was a legacy of conditions required for apotransketolase stability in the enzyme kinetic studies that precipitated the need for evaluation of the Mg^{2+}–coenzyme binding constant.

[a] Symbols: $[\bar{A}]_0$ = total concentration of acceptor (thiamin diphosphate) in applied mixture; $[\bar{S}]_0$ = corresponding total concentration of ligand (Mg^{2+} ion); $[A]_0$ = concentration of uncomplexed acceptor in applied mixture; $\bar{V}_A$, $\bar{V}_S$ = constituent elution volumes of acceptor and ligand components, respectively; V_A, V_S, V_C = elution volumes of acceptor, ligand and acceptor–ligand complex.

Gel chromatographic procedure

Mixtures (100 ml) of thiamin diphosphate and Mg^{2+} ion were subjected to frontal gel chromatography [11–15] at room temperature (20–22°C) on a column (20 × 2.5 cm) of Sephadex G-10 that had been preequilibrated with the appropriate Mg^{2+}-supplemented buffer medium. The column effluent, maintained at a nominal flow-rate of 2 ml/min by means of a peristaltic pump, was monitored continuously at 280 nm, a wavelength that allowed measurement of the total concentration ($[\bar{A}]$) of thiamin diphosphate constituent (irrespective of its presence as free coenzyme or as Mg^{2+}–coenzyme complex). During application of each mixture, the precise flow-rate was determined by collecting the column effluent in a previously tared vessel over an accurately defined time interval.

The constituent elution volume ($\bar{V}_A$) of the thiamin diphosphate component of each mixture was obtained as the median bisector [6,12] of the advancing elution profile via the expression

$$\bar{V}_A = \{\Sigma([\bar{A}]_0 - [\bar{A}])\Delta V\}/[\bar{A}]_0 \quad (1)$$

where $[\bar{A}]$ denotes the constituent (total) concentration of acceptor (thiamin diphosphate) in a volume increment ΔV at effluent volume V in an experiment with applied concentration $[\bar{A}]_0$ of coenzyme component. Since the advancing elution profile sufficed for determination of $\bar{V}_A$, the trailing profile generated by application of buffer medium was not subjected to analysis.

Method of analysis

The above frontal gel chromatographic procedure establishes the magnitude of the constituent elution volume, $\bar{V}_A$, of thiamin diphosphate in each applied mixture with respective total concentrations $[\bar{A}]_0$ and $[\bar{S}]_0$ of coenzyme and ligand (magnesium ion). In the absence of ligand ($[\bar{S}]_0 = 0$) the measured parameter defines V_A, the elution volume of thiamin diphosphate, whereas a similar experiment conducted with a very high concentration of Mg^{2+} (sufficient to saturate the single binding site [1,2] on the coenzyme) defines V_C, the elution volume of the Mg^{2+}–coenzyme complex. For intermediate concentrations of magnesium ion the elution volume defined by eqn. 1 is related to V_A and V_C by

$$\bar{V}_A = \{V_A[A]_0 + V_C([\bar{A}]_0 - [A]_0)\}/[\bar{A}]_0 \quad (2)$$

which allows the concentration of free thiamin diphosphate, $[A]_0$, in the applied mixture to be evaluated from the expression

$$[A]_0 = [\bar{A}]_0(\bar{V}_A - V_C)/(V_A - V_C) \quad (3)$$

The association equilibrium constant, K, for the acceptor–ligand interaction is then obtained from its definition as

$$K = [AS]_0/[A]_0[S]_0 \quad (4a)$$

$$= ([\bar{A}]_0 - [A]_0)/\{[A]_0([\bar{S}]_0 - [\bar{A}]_0 + [A_0)\} \quad (4b)$$

A value of K is therefore obtained by evaluating $\bar{V}_A$ for each mixture with defined composition ($[\bar{A}]_0$, $[\bar{S}]_0$).

RESULTS

Fig. 1 presents advancing elution profiles for thiamin diphosphate (100 μM) in the absence of metal ion, and also for the coenzyme component in mixtures containing 0.245 mM and 0.1 M magnesium ion. The first point to note is the skewed nature of the elution profile in each case. Asymmetry of the profile for thiamin diphosphate in the absence of Mg^{2+} was certainly an unexpected finding, because symmetrical boundaries are normally observed for single solutes. Secondly, although this skewness led to location of the median bisector of the boundary at $0.66[\bar{A}]_0$ rather than the $0.50[\bar{A}]_0$ for a symmetrical boundary, the elution profiles obtained in experiments with mixtures of coenzyme and Mg^{2+} ion exhibited similar asymmetry (Fig. 1). Any contribution to the skewness resulting from heterogeneity of the thiamin diphosphate is therefore exhibiting a similar bias on $\bar{V}_A$ in all experiments. Thirdly, the elution volume corresponding to the median bisector ($\bar{V}_A$) increases with increasing concentration of metal ion, a finding consistent with the existence of the Mg^{2+}–coenzyme complex as a more folded, compact structure than the free coenzyme [1,2].

Fig. 2 summarizes the inverse dependence of the constituent elution volume of thiamin diphosphate, $\bar{V}_A$, upon total Mg^{2+} concentration, $[\bar{S}]_0$. It is apparent that the elution volume of 58.3 ml obtained from the experiment with 0.1 M Mg^{2+} ion on the present Sephadex G-10 column (Fig. 1) suffices to define the elution volume of Mg^{2+}–coenzyme complex (V_C), which is the limiting value of $\bar{V}_A$ as $[\bar{S}]_0 \rightarrow \infty$. Experiments conducted in the absence of metal ion established the elution volume of thiamin diphosphate (V_A) as 52.4 ml. These values of V_A and V_C were therefore used in conjunction with eqn. 3 and the measured elution volumes ($\bar{V}_A$) for other reaction mixtures to obtain the concentration of free thiamin diphosphate, $[A]_0$, in each applied mixture. Results of those calculations, and also of the consequent evaluation of K via eqn. 4b, are summarized in Table 1. The interaction between thiamin diphosphate and Mg^{2+} under the present conditions is thus described by an association constant of 3200 ($\pm$ 400) M^{-1}. This value is of the same order of magnitude as, but smaller than, the binding con-

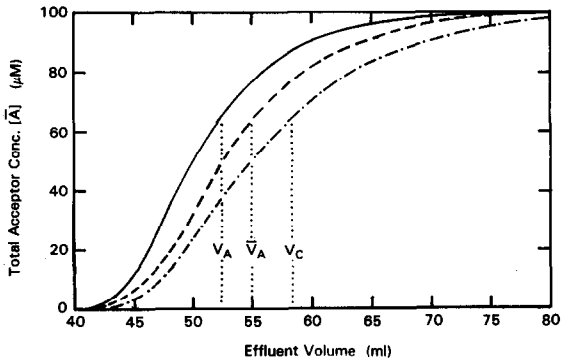

Fig. 1. Advancing elution profiles obtained in frontal gel chromatography of thiamin diphosphate (100 μM) on a column (20 × 2.5 cm) of Sephadex G-10 in the absence of metal ion (——) and in the presence of 0.245 mM (– – –) and 0.1 M $MgCl_2$ (– · –).

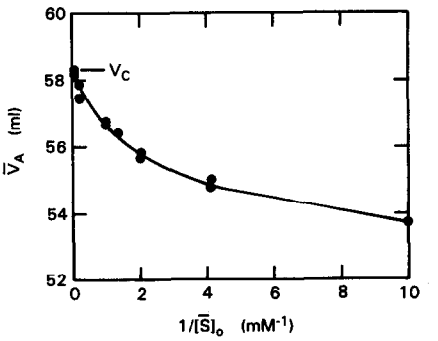

Fig. 2. Evaluation of the elution volume for Mg^{2+}–coenzyme complex (V_C) as the limiting value of the constituent elution volume ($\bar{V}_A$) of thiamin diphosphate in the limit of infinite magnesium ion concentration.

TABLE I

GEL CHROMATOGRAPHIC EVALUATION OF THE BINDING CONSTANT FOR THE INTERACTION OF MAGNESIUM ION WITH THIAMIN DIPHOSPHATE

Conditions: 0.1 *M* Tris–HCl (pH 7.6) supplemented with poly (ethylene glycol) (50 mg/ml) and mercaptoethanol (25 m*M*).

$[\bar{S}]_0$ (m*M*)	$\bar{V}_A$ (ml)[a]	$[A]_0$ (μM)[b]	K (M^{-1})[c]
0	52.4	–	–
100	58.3	–	–
0.098	53.7	78.0	3700
0.245	55.0	55.9	3900
0.245	54.8	59.3	3400
0.490	55.6	45.8	2700
0.490	55.8	42.4	3100
0.735	56.4	32.2	3200
0.980	56.6	28.8	2700
0.980	56.7	27.1	3000

[a] Elution volume in frontal chromatography of mixtures of thiamin diphosphate (100 μM) and the indicated Mg^{2+} concentrations on a column (20 × 2.5 cm) of Sephadex G-10.

[b] Equilibrium concentration of thiamin diphosphate calculated from eqn. 3.

[c] Equilibrium constant calculated from eqn. 4b.

stant of 5000 (± 200) reported [1] for the interaction of manganous ion with thiamin diphosphate.

DISCUSSION

Resort to frontal chromatography to define the dependence of the constituent elution volume ($\bar{V}_A$) of the acceptor (thiamin diphosphate) as a function of mixture composition has yielded a relatively direct and simple gel chromatographic procedure for evaluating the binding constant describing the interaction of magnesium ion with thiamin diphosphate. In that regard the zonal gel chromatographic procedure [5] that has been recommended by Colman [3] could also have been used. However, as noted previously [4], the non-identity of V_A and V_C introduces the requirement that the apparent steady-state values, K_{ss}^{app}, so obtained be extrapolated to zero acceptor concentration (infinite dilution) to obtain the true steady-state parameter, K_{ss}^{∞}, which must then be converted to the thermodynamic association constant, K, via the expression

$$K = K_{ss}^{\infty}(V_S - V_A)/(V_S - V_C) \quad (5)$$

Rigorous evaluation of K by the Hummel–Dreyer technique [3,5] thus requires not only extrapolation of data to infinite dilution but also a magnitude for the elution volume of ligand (V_S) in addition to those of V_A and V_C.

Having established that the present frontal gel chromatographic procedure for evaluating K is much more readily applied than its zonal counterpart [3–5], we conclude by showing that it also surpasses existing frontal chromatographic procedures for simplicity of application. One such procedure for evaluation of the association constant is based on the expression [8–10]

$$[A]_0 = \{[\bar{A}]_0(\bar{V}_A - V_S) - [\bar{S}]_0(\bar{V}_S - V_S)\}/(V_A - V_S) \quad (6)$$

which involves $\bar{V}_S$, the constituent elution volume of ligand, and thus requires delineation of separate elution profiles for ligand and acceptor components. It does, however, have the advantage of being applicable in instances where direct measurement of V_C is impractical. Another frontal gel chromatographic technique employs the relationship [6,16]

$$[A]_0 = \{[\bar{A}]_0(\bar{V}_A - V_S) - ([\bar{S}]_0 - [S]^{\beta})(\bar{V}_S - V_S)\}/(V_A - V_S) \quad (7)$$

and hence relies on an ability to resolve accurately the trailing elution profile into a reaction boundary (across which all acceptor species are eluted) and a second boundary corresponding to elution of a pure ligand (Mg^{2+} ion) phase β with concentration $[S]^{\beta}$.

From the foregoing discussion of existing methods, the present procedure based solely on dependence of the constituent elution volume of acceptor ($\bar{V}_A$) upon mixture composition is clearly the simplest gel chromatographic method available for the determination of binding constants for acceptor–ligand interactions when $V_C \neq V_A$. It is, however, only applicable to interactions for which the concentration of ligand can be varied over a sufficiently wide range for V_C to be determined either directly (Fig. 1) or by extrapolation of $\bar{V}_A$ to infinite ligand concentration (Fig. 2). Whereas the frontal methods discussed in the preceding paragraph were developed in the context of protein-protein interactions, for which evaluation of V_C by such means is precluded, the requirement that V_C be determined

experimentally could be met for the Mg^{2+}–thiamin diphosphate system. Furthermore, this requirement is unlikely to be a limiting factor in the quantitative study of other metal–nucleotide interactions.

In summary, the difficulties inherent in the evaluation of ligand-binding constants by gel chromatography when both reactants are small have been resolved satisfactorily in the sense that measurement of the constituent elution volume of acceptor as a function of mixture composition is likely to provide a straightforward and rigorous method of evaluating K. On the basis of its application to frontal gel chromatographic results for mixtures of thiamin diphosphate and magnesium ion on Sephadex G-10, the dissociation constant for the interaction of metal ion with coenzyme is 0.31 mM under the conditions examined [0.1 M Tris–HCl (pH 7.6) supplemented with poly(ethylene glycol) and mercaptoethanol]. In view of the physiological importance of the interaction between thiamin diphosphate and Mg^{2+}, it seems extraordinary that this is the first occasion on which the binding has been quantified. Finally, this very simple gel chromatographic method of evaluating equilibrium constants for metal—nucleotide interactions only requires delineation of the elution profile for the nucleotide component, and therefore has potential for quantifying the binding of any nucleotide to the metal ion of physiological relevance rather than one for which there happens to be a more convenient assay.

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