Measuring Positive Cooperativity using the Direct ESI-MS Assay. Cholera Toxin B Subunit Homopentamer Binding to GM1 Pentasaccharide

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Abstract

The direct electrospray ionization mass spectrometry (ESI-MS) assay was used to investigate the stepwise binding of the GM1 pentasaccharide β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→4)[α-D-Neu5Ac-(2→3)]-β-D-Gal*p*-(1→4)-β-D-Glc*p* (GM1os) to the cholera toxin B subunit homopentamer (CTB_5) and to establish conclusively whether GM1os binding is cooperative. Apparent association constants were measured for the stepwise addition of one to five GM1os to CTB_5 at pH 6.9 and 22°C. The intrinsic association constant, which was established from the apparent association constant for the addition of a single GM1os to CTB_5, was found to be $(3.2 \pm 0.2) \times 10^6$ M^{-1}. This is in reasonable agreement with the reported value of $(6.4 \pm 0.3) \times 10^6$ M^{-1}, which was measured at pH 7.4 and 25 °C using isothermal titration calorimetry (ITC). Analysis of the apparent association constants provides direct and unambiguous evidence that GM1os binding exhibits small positive cooperativity. Binding was found to be sensitive to the number of ligand-bound nearest neighbour subunits, with the affinities enhanced by a factor of 1.7 and 2.9 when binding occurs next to one or two ligand-bound subunits, respectively. These findings, which provide quantitative support for the binding model proposed by Homans and coworkers, highlight the unique strengths of the direct ESI-MS assay for measuring cooperative ligand binding.

Introduction

To carry out their functions, proteins must bind to ligands (e.g. other proteins, carbohydrates, lipids, DNA, metal ions or small molecules). Many proteins, in particular enzymes and multisubunit protein complexes, can bind simultaneously to multiple ligand molecules; this may involve multiple copies of the same ligand (homotropic binding) or different ligands (heterotropic binding). Often, binding of one ligand influences the affinities of other ligands. This phenomenon, which represents an important regulatory mechanism in biological systems, is referred to as cooperative binding [1-5]. Cooperativity can be described quantitatively on the basis of Gibbs energy (ΔG) couplings of binding events at different sites [6-7]. Positive cooperativity occurs when ligand binding at one site increases the affinity (association constant, K_a) and decreases ΔG at another site; negative cooperativity arises when ligand binding reduces the affinity (and increases ΔG) at another site [3]. If the binding sites are independent of one another, binding is referred to as non-cooperative [5,8-9].

Despite the recognized importance of cooperative binding in biological processes, quantifying theses effects is challenging [10-11]. The most direct approach to quantifying cooperativity is based on the microscopic association constants (K_a for binding at a specific site) and the changes in K_a (and ΔG) for ligand binding at a given site when another site is occupied [12-14]. However, there are no generally-applicable methods available for monitoring the occupancies of individual ligand binding sites and, consequently, microscopic K_a values are often not experimentally accessible [15-16]. Therefore, a common approach used to evaluate cooperative binding is to compare the trend in macroscopic K_a values (K_a for stepwise ligand binding) with the trend expected for statistical binding. However, most commonly used binding assays, such as isothermal titration calorimetry (ITC) and

spectrophotometric techniques, do not directly provide a quantitative measure of the K_a values (or corresponding thermodynamic parameters) for stepwise ligand binding. Consequently, it is often only possible to establish the K_a values by fitting an assumed binding model to the experimental data. In some cases, simplifying assumptions must be made in order to reduce the number of unknown terms [15,17].

In recent years, electrospray mass spectrometry (ESI-MS) has proven to be a useful tool to detect and quantify protein-ligand interactions, as well as other non-covalent biological complexes, *in vitro* [18-21]. The direct ESI-MS assay [22], which is based on the detection and quantification of the gas-phase ions of free and ligand-bound protein, has been used to measure affinities for a variety of protein-ligand complexes, and in many instances the K_a values agree well with affinities measured by other analytical methods [23-26]. An important feature of the ESI-MS assay is the ability to study multiple binding equilibria, including stepwise ligand binding, simultaneously. Given that the relative concentrations of the different ligand-bound protein forms can (in principle) be measured directly and the macroscopic K_a values quantified [22], the assay is well suited for studying cooperative binding. Despite this obvious potential, there are surprisingly few examples where the direct ESI-MS assay was used to probe cooperative interactions. In one of the earliest reported examples, Rogniaux *et al.* deduced cooperative ligand binding to a series of enzymes [27]. More recently, Klassen and coworkers used ESI-MS to demonstrate enhanced carbohydrate substrate binding to a glycosyltransferase in the presence of bound donor [21], Zenobi and coworkers identified from ESI-MS measurements a new ligand binding site resulting from ligand-induced protein conformational changes [28], and Sharon and coworkers used ESI-MS

to elucidate the allosteric mechanism for stepwise binding of ATP to the multisubunit protein complex GroEL [29].

Here, we exploit the direct ESI-MS assay to quantify the stepwise binding of the GM1 pentasaccharide β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→4)[α-D-Neu5Ac-(2→3)]-β-D-Gal*p*-(1→4)-β-D-Glc*p* (referred to here as GM1os) to the B subunit homopentamer of cholera toxin (CT) and to establish whether ligand binding is cooperative. Cholera toxin, which is a member of the AB_5 class of cytotoxins [30], is composed of a catalytically active A subunit and doughnut-shaped homopentamer of B subunits (B_5), which is responsible for the recognition of host cell receptors and binds selectively to the GM1 ganglioside (β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→4)[α-D-Neu5Ac-(2→3)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-Cer) [31-32]. The structure, kinetics and thermodynamics of the interactions of CT holotoxin and CTB_5 with GM1 ganglioside or the soluble GM1os pentasaccharide have been extensively investigated [14,31-37]. According to the crystal structure of the (CTB_5 + 5GM1os) complex (PDB id 3CHB), each of the five GM1os binding sites (one per subunit) is made up primarily from a B single subunit, with 18 direct or solvent mediated H-bonds between GM1os and amino acid residues located within the subunit and one H-bond with residue Gly33 from an adjacent subunit [31].

Affinity measurements have been performed for CTB_5 binding to soluble GM1os and to GM1 ganglioside in supported bilayers [38] and in vesicles [39] using a variety of techniques, including ITC [14] and surface plasmon resonance (SPR) spectroscopy [37,40-41]. The binding data are found to be highly dependent on solution conditions (buffer, ionic strength and pH) and on membrane chemistries. Intrinsic association constants ($K_{a,int}$) of between 10^6 and 10^7 M^{-1} have been reported for GM1os [14,42] and 10^4 to 10^8 for GM1

ganglioside [37,42-43]. Wiegandt and coworkers, based on the observation of nonlinear Scatchard plots constructed from equilibrium dialysis data, were the first to report that GM1os binding to CTB_5 exhibits positive cooperativity (cooperativity coefficient of 1.25) [34]. Re-analysis of these same data by Schafer and Thakur, using a model comprising seven independent stepwise association reactions, led to the suggestion of a 2-fold increase in the affinity for the addition of a second (or subsequent) GM1os to CTB_5 [44]. Schon and Freire, from ITC data and a binding model that assumed that cooperativity would manifest itself only through nearest neighbor interactions, reported a 4-fold increase in affinity when the ligand binds adjacent to a subunit that is already occupied [36]. According to this model binding is enhanced further (by a factor 8) when both nearest neighbours are ligand-bound. More recently, Homans and coworkers analyzed ITC data using a simplified form of the nearest neighbour model (Figure 1) and reported affinity enhancement factors of 1.9 and 3.4 for GM1os binding to subunits with one or two ligand-bound nearest neighbours, respectively [14]. The ESI-MS binding data reported here serve as direct and conclusive evidence that GM1os binding to CTB_5 exhibits small, positive cooperativity and support the binding model proposed by Homans and coworkers [14].

Experimental

Materials and Methods

Cholera toxin B subunit pentamer (CTB_5, MW 58 020 Da) was purchased from Sigma-Aldrich Canada (Oakville, ON). The protein was concentrated and dialyzed against aqueous 50 mM ammonium acetate and stored at 4°C if not used immediately. The protein concentration was determined using the bicinchoninic acid (BCA) assay calibrated with bovine serum albumin (Pierce, Rockford, USA). The GM1 pentasaccharide β-D-Gal*p*-(1→3)-

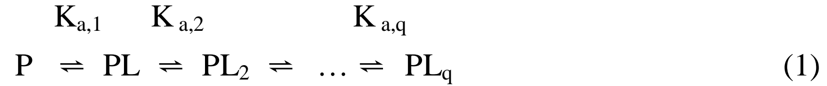
β-D-Gal*p*NAc-(1→4)[α-D-Neu5Ac-(2→3)]-β-D-Gal*p*-(1→4)-β-D-Glc*p* (GM1os, MW 998.4 Da) was purchased from Elicityl SA (Crolles, France). A stock solution of GM1os was prepared by dissolving a known amount of the solid sample in ultrafiltered water (Milli-Q, Millipore) to yield a concentration of 1 mM. The solution was stored at -20°C until needed.

Mass spectrometry

The binding measurements were carried out with a Bruker 9.4T ApexQe FTICR mass spectrometer (Billerica, MA). Nanoflow ESI (nanoESI) was performed in positive ion mode using borosilicate tubes (1.0 mm o.d., 0.68 mm i.d.), pulled to ~5 µm o.d. at one end using a Sutter Instruments P-2000 micropipette puller (Novato, CA). The electric field required to spray the solution was established by applying a voltage of ~1.0 kV. The droplets and gaseous ions produced by ESI were introduced into the mass spectrometer through a metal sampling capillary (0.5 mm i.d.). Nitrogen gas at a flow rate of 1.0 L min^{-1} and 90 ^{o}C was used as a drying gas. Both the capillary entrance and exit voltages were held at 280 V. A deflector voltage of 250 V was used. Gaseous ions were transmitted through the first funnel and skimmer held at 150 V and 20 V, respectively, and then through the second funnel and skimmer held at 7.6 V and 5.3 V, respectively. The ions were stored electrodynamically in an rf hexapole for 0.9 s and then accumulated in a hexapole collision cell for 1.2 s. Following accumulation, the ions were transferred into the ion cell for detection. The front and back trapping plates of the cell were maintained at 0.9 and 1.0 V, respectively. The typical base pressure for the instrument was ~1 x 10^{10} mbar. Data acquisition and analysis were performed using ApexControl, version 4.0 (Bruker Daltonics). A minimum of 50 transients with 32k data points per transient were used for each acquisition.

Determination of ligand affinities from ESI-MS data

The general expression for the apparent association constants, $K_{a,q}$, for the stepwise binding of L to P (eq 1) is given by eq 2:



$$\mathrm{P} \underset{}{\overset{K_{a,1}}{\rightleftharpoons}} \mathrm{PL} \overset{K_{a,2}}{\rightleftharpoons} \mathrm{PL_2} \rightleftharpoons \ldots \overset{K_{a,q}}{\rightleftharpoons} \mathrm{PL_q} \quad (1)$$

$$K_{a,q} = \frac{R_q}{R_{q-1}\left([\mathrm{L}]_0 - \dfrac{(R_1 + 2R_2 + \ldots qR_q)[\mathrm{P}]_0}{1 + R_1 + R_2 + \ldots R_q}\right)} \quad (2)$$

where $[\mathrm{L}]_0$ and $[\mathrm{P}]_0$ are the initial concentration of L and P, respectively, and the R_q terms represent the concentration ratios of ligand-bound to free protein, i.e., $[\mathrm{PL_q}]/[\mathrm{P}]$. Provided that the ionization and detection efficiencies (i.e., response factors) of the ions corresponding to the ligand-bound and free protein species are equivalent, the R_q terms can be determined from the abundances (*Ab*) of the gaseous ligand-bound and free P ions, summed over all charge states (n), eq 3:

$$R_q = \frac{Ab\,(\mathrm{PL_q})}{Ab\,(\mathrm{P})} = \frac{\sum_n Ab\,(\mathrm{PL_q})^{n+}}{\sum_n Ab\,(\mathrm{P})^{n+}} = \frac{[\mathrm{PL_q}]}{[\mathrm{P}]} \quad (3)$$

For a protein with Q equivalent and independent binding sites, the apparent $K_{a,q}$ values are related by statistical factors that reflect the number of occupied and unoccupied binding sites, eq 4:

$$K_{a,q} / K_{a,q-1} = (q - 1)(Q - q + 1)/(q(Q - q + 2)) \quad (4)$$

$K_{a,int}$ can be found from any of the $K_{a,q}$ values using the general expression:

$$K_{a,int} = qK_{a,q} / (Q - q + 1) \quad (5)$$

If the ligand binding sites are not equivalent or in cases of cooperative binding (positive or negative), the relationship given by eq 5 will not be valid, *vide infra*.

Results and discussion

Measurements were carried out on aqueous ammonium acetate (30 mM, pH 6.9) solutions of CTB_5 (8.5 μM) and GM1os at concentrations ranging from 0 to 55 μM. Shown in Figure 2 are representative mass spectra acquired in the absence of GM1os and presence of GM1os (at concentrations of 6 μM, 17.5 μM and 50 μM). In the absence of GM1os, the only protein ions detected correspond to protonated homopentamer, $(CTB_5 + nH)^{n+} \equiv CTB_5^{n+}$ at charge states n = 14 – 17. At low GM1os concentrations (6 μM), ions corresponding to CTB_5 bound to 1, 2 or 3 GM1os are detected, i.e., $(CTB_5 + qGM1os)^{n+}$ ions where q = 1 - 3, while at the higher concentrations investigated $(CTB_5 + 4GM1os)^{n+}$ and $(CTB_5 + 5GM1os)^{n+}$ ions were also detected. It should be noted that a reference protein (P_{ref}) was also added to the solutions in order to monitor the occurrence of nonspecific ligand-protein binding during the ESI process [45]. However, there was no evidence of nonspecific binding in any of the measurements.

Apparent $K_{a,q}$ values for the stepwise addition of GM1os to CTB_5 were determined from the ESI mass spectra as described in the Experimental section. Listed in Table 1 are the $K_{a,q}$ values measured at twelve different GM1os concentrations and the corresponding average values. Inspection of the values in Table 1 reveals that the apparent $K_{a,q}$ values decrease with increasing q. This effect is expected due to the reduction in the number of available binding sites. However, the actual reduction in $K_{a,q}$ is less than expected in the case where the binding sites are equivalent and independent (Table 1). Assuming that the five binding sites are equivalent, $K_{a,int}$, which corresponds to $(1/5)K_{a,1}$, is found to be $(3.2 \pm 0.2) \times 10^6$ M^{-1}. This value is slightly smaller (by a factor of 1/2) than reported by Homans and coworkers ($(6.4 \pm 0.3) \times 10^6$ M^{-1}), although the solution conditions used in their study, most notably pH, were slightly different [14]. From $K_{a,int}$, the $K_{a,q}$ values expected in the absence of

cooperative binding can be calculated: $K_{a,2}$ = (4/2)$K_{a,int}$ = (6.4 ± 0.5) × 10^6 M^{-1}, $K_{a,3}$ = (3/3)$K_{a,int}$ = (3.2 ± 0.2) × 10^6 M^{-1}. $K_{a,4}$ = (2/4)$K_{a,int}$ = (1.6 ± 0.1) × 10^6 M^{-1}, $K_{a,5}$ = (1/5)$K_{a,int}$ = (6.4 ± 0.5) × 10^5 M^{-1}. It can be seen that the measured and calculated values do not agree, the measured $K_{a,q}$ values being larger. This comparison establishes conclusively that GM1os binding to CTB_5 exhibits positive cooperativity, in agreement with previous proposals [14,44]. The ESI-MS derived $K_{a,q}$ values also provide a means of directly quantifying the magnitude of this effect.

In order to quantify the magnitude of the cooperative binding effects, the $K_{a,q}$ values were analyzed using the model proposed by Homans and coworkers (Figure 1) [14]. According to this model, stepwise ligand binding can be described using three intrinsic association constants, K_1, K_2 and K_3, which represent the case of ligand binding to a subunit with zero, one or two ligand-bound nearest neighbours, respectively. It follows that the equilibrium concentrations of the eight distinct species present in solution (referred to here as P, PL, $PL_2^{'}$, $PL_2^{''}$, $PL_3^{'}$, $PL_3^{''}$, PL_4 and PL_5) are related by eqs 6a – 6j:

$$5K_1 = \frac{[PL]}{[P][L]} \tag{6a}$$

$$K_2 = \frac{[PL_2^{'}]}{[PL][L]} \tag{6b}$$

$$K_2 = \frac{[PL_3^{'}]}{[PL_2^{'}][L]} \tag{6c}$$

$$K_2 = \frac{[PL_4]}{[PL_3^{'}][L]} \tag{6d}$$

$$K_3 = \frac{[PL_4]}{[PL_3^{''}][L]} \tag{6e}$$

$$\frac{K_3}{5} = \frac{[PL_5]}{[PL_4][L]} \tag{6f}$$

$$K_1 = \frac{[PL_2^{''}]}{[PL][L]} \tag{6g}$$

$$K_2 = \frac{[PL_3^{''}]}{[PL_2^{''}][L]} \tag{6h}$$

$$K_1 = \frac{[PL_3^{''}]}{[PL_2^{'}][L]} \tag{6i}$$

$$K_3 = \frac{[PL_3^{'}]}{[PL_2^{''}][L]} \tag{6j}$$

It should be noted that the $PL_2^{'}$ and $PL_2^{''}$ species, as well as the $PL_3^{'}$ and $PL_3^{''}$ species can't be distinguished by ESI-MS because they have identical molecular weights. It follows that K_1 and K_3 can be calculated directly from $K_{a,1}$ and $K_{a,5}$, eqs 7 and 8:

$$K_{a,1} = 5K_1 = \frac{[PL]}{[P][L]} \tag{7}$$

$$K_{a,5} = \frac{K_3}{5} = \frac{[PL_5]}{[PL_4][L]} \tag{8}$$

while K_2 is related to K_1 and K_3 by eq 9:

$$K_1K_2K_3=K_2^3 \tag{9}$$

Using this approach, the values of K_1, K_2 and K_3 were determined to be $(3.2 \pm 0.2) \times 10^6$ M^{-1}, (5.5 ± 1.2) x 10^6 M^{-1} and (9.5 ± 3.5) x 10^6 M^{-1}, respectively. According to this analysis, binding is enhanced by a factor of 1.7 when one of the neighbouring subunits is already bound to GM1os and a factor of 2.9 when both neighbouring subunits are ligand-bound. These values are in reasonable agreement with enhancement factors of 1.9 and 3.4, inferred from ITC data, reported by Homans and coworkers [14].

Although the similarities in the enhancement factors obtained from the ESI-MS and ITC data offer support for the proposed binding model (Figure 1), they do not, on their own, conclusively establish that the model correctly describes the experimental data. To further test the appropriateness of this model, the K_1, K_2 and K_3 values extracted from the ESI-MS data were used to predict the corresponding $K_{a,2}$, $K_{a,3}$ and $K_{a,4}$ values. It can be seen that the predicted $K_{a,q}$ values ($K_{a,2} = K_1 + K_2 = (8.7 \pm 1.2) \times 10^6$ M^{-1}; $K_{a,3} = (K_2 + K_3)/(K_2/K_1 + 1) = (5.5 \pm 1.6) \times 10^6$ M^{-1}; $K_{a,4} = K_3/(K_3/K_2 + 1) = (3.4 \pm 1.6) \times 10^6$ M^{-1}) are in good agreement with the values obtained from ESI-MS measurements. These findings confirm that the proposed binding model properly describes the stepwise binding of GM1os to CTB_5.

Conclusions

In summary, apparent $K_{a,q}$ values were measured for the stepwise binding of GM1os to CTB_5 at pH 6.9 and 22°C using the direct ESI-MS assay. Analysis of the binding data provides direct evidence that GM1os binding exhibits small, positive cooperativity. The ESI-MS data are consistent with a binding model, proposed by Homans and coworkers, in which ligand binding to CTB_5 can be described by three intrinsic K_a values that represent the case of ligand binding to a subunit with zero, one or two ligand-bound nearest neighbours (Figure 1). According to the ESI-MS results, binding to a subunit located next to a single ligand-bound subunit results in an affinity enhanced by a factor of 1.7, while binding is enhanced by a factor of 2.9 when both nearest neighbors are bound to ligand. These results highlight the strength of the direct ESI-MS assay for studying stepwise ligand binding to proteins and quantifying cooperativity effects.

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Table 1. Apparent association constants ($K_{a,q}$) for the stepwise binding of GM1os to CTB_5 measured at 22 °C and pH 6.9 by ESI-MS.[a,b]

[GM1os] (µM)	$K_{a,1}$ (10^6 M^{-1})	$K_{a,2}$ (10^6 M^{-1})	$K_{a,3}$ (10^6 M^{-1})	$K_{a,4}$ (10^6 M^{-1})	$K_{a,5}$ (10^6 M^{-1})
6.0	15.6 ± 0.7	6.6 ± 0.4	3.0 ± 0.1	-	-
7.5	13.8 ± 0.6	5.8 ± 0.2	2.9 ± 0.1	-	-
10.0	14.1 ± 1.1	6.5 ± 0.8	3.6 ± 0.8	-	-
12.5	14.4 ± 0.6	5.9 ± 0.5	3.5 ± 0.1	3.4 ± 0.1	-
15.0	16.2 ± 0.7	7.1 ± 0.6	4.1 ± 0.3	2.6 ± 0.2	-
17.5	16.7 ± 0.9	7.5 ± 0.9	3.8 ± 0.8	2.3 ± 0.4	3.2 ± 0.2
22.5	15.6 ± 0.4	8.7 ± 0.6	5.2 ± 0.2	2.8 ± 0.7	1.1 ± 0.5
27.5	16.0 ± 0.6	8.7 ± 0.5	4.8 ± 0.2	2.8 ± 1.1	1.3 ± 0.2
35.0	-	9.1 ± 0.7	3.2 ± 0.2	3.8 ± 0.9	1.7 ± 0.6
45.0	-	-	-	3.7 ± 0.6	1.6 ± 0.1
50.0	-	-	-	3.0 ± 0.2	1.9 ± 0.2
55.0	-	-	-	-	2.0 ± 0.1
Average	16.0 ± 1.2	7.3 ± 1.3	3.8 ± 0.8	3.1 ± 0.5	1.9 ± 0.6
Calculated[c]	16.0 ± 1.2	6.4 ± 0.5	3.2 ± 0.2	1.6 ± 0.1	0.6 ± 0.05

a. CTB_5 (8.5 µM) in 30 mM ammonium acetate.
b. Errors correspond to one standard deviation.
c. Calculated, assuming that the five binding sites are equivalent and independent, apparent association constants based on the $K_{a,int}$ value determined by ESI-MS.

Figure captions

Figure 1. Proposed model for the stepwise binding of GM1os (L) to the CTB_5 homopentamer (P) (adapted from [14]). Binding is described by three intrinsic association constants, K_1, K_2 and K_3, which represent the case of L binding to a subunit with zero, one or two ligand-bound nearest neighbours, respectively. The equilibrium concentrations of the eight distinct species (P, PL, $PL_2^{'}$, $PL_2^{''}$, $PL_3^{'}$, $PL_3^{''}$, PL_4 and PL_5) are related through eqs 6a to 6j.

Figure 2. ESI mass spectra acquired for aqueous ammonium acetate (30 mM) solutions (22 °C at pH 6.9) of CTB_5 (8.5 μM) and varying concentrations of GM1os (a) 0 μM (b) 6 μM (c) 17.5 μM and (d) 50 μM. A reference protein (P_{ref}, 4.5 μM) was added into the solution to identify the occurrence of nonspecific ligand binding. The number of molecules of GM1os bound to CTB_5 is indicated by q.

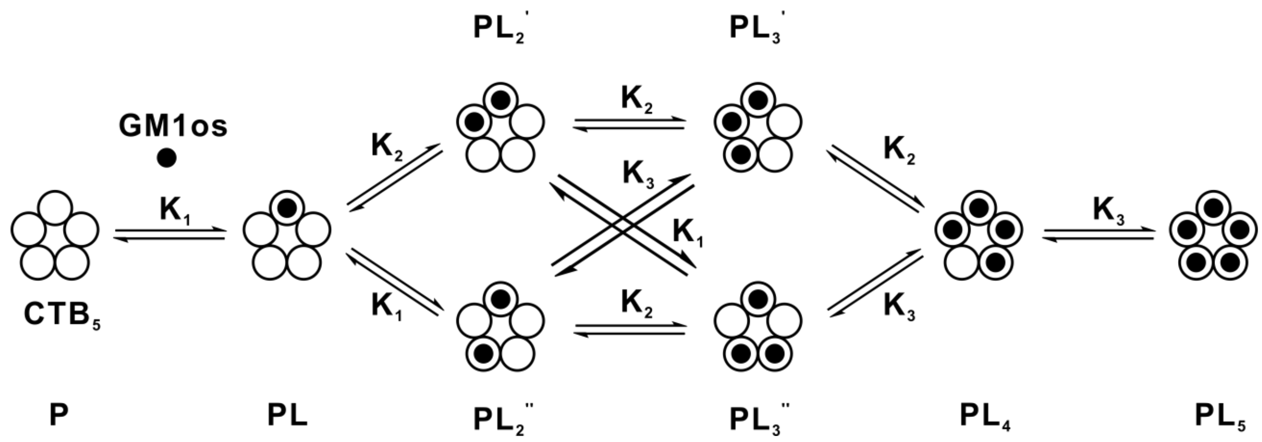

Figure 1

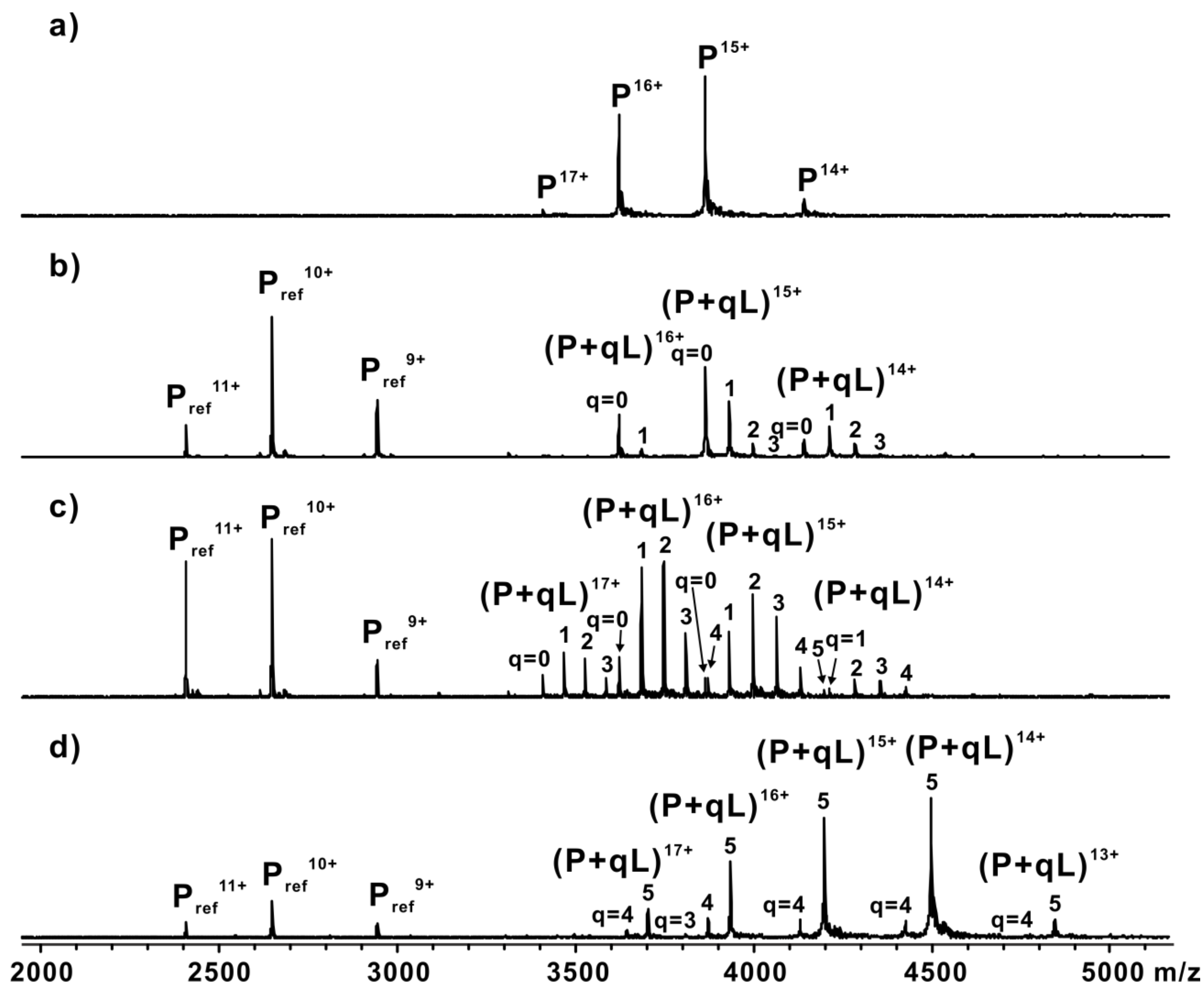

Figure 2