Protein–Ligand Interactions

Stochastic Detection of Monovalent and Bivalent Protein–Ligand Interactions**

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The term "multivalency" refers to the phenomenon in which a molecule that contains multiple binding sites binds simultaneously to multiple ligands.[1] Multivalency can lead to significant enhancements in binding strength for weakly binding ligands.[2] Prominent examples of multivalent systems include binding by antibodies and lectins, and the receptor-ligand interactions involved in cell adhesion or viral attachment. Both for fundamental reasons and so that the phenomenon can be exploited in the design of inhibitors,[3–6] there is great interest in understanding multivalent binding in detail.

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Studying the physical chemistry of multivalency has been an enormous challenge, however. Although a wide variety of model systems and techniques have been employed to probe multivalent binding,[1,7–10] all of them have involved ensemble measurements in which specific characteristics of the multivalent systems are averaged and, thus, details of the interactions may be obscured. For example, monovalent and multivalent binding states cannot be observed independently within one experiment, and this complicates the kinetic analysis of multivalent binding. Herein, we describe a new approach to studying multivalent interactions. We show that the binding kinetics of a lectin to one or more ligands can be followed at the single-molecule level by monitoring the ionic current flow through a heptameric protein pore covalently modified with up to seven carbohydrates. This single-molecule approach permits the direct observation of individual monovalent and divalent binding events.

Chemically modified protein pores are powerful tools to study the binding kinetics of ligand receptor combinations at the single molecule level. An individual ligand is tethered to the protein pore and the reversible binding of the corresponding receptor produces transient current blockades in single-channel current recordings. In the past, this approach has been used to study the kinetics of monovalent binding of, for example, complementary DNA strands.[11–13] We wondered whether the single-molecule approach might also be applied to probe the multivalent interactions of carbohydrate binding proteins, with the aid of protein pores carrying

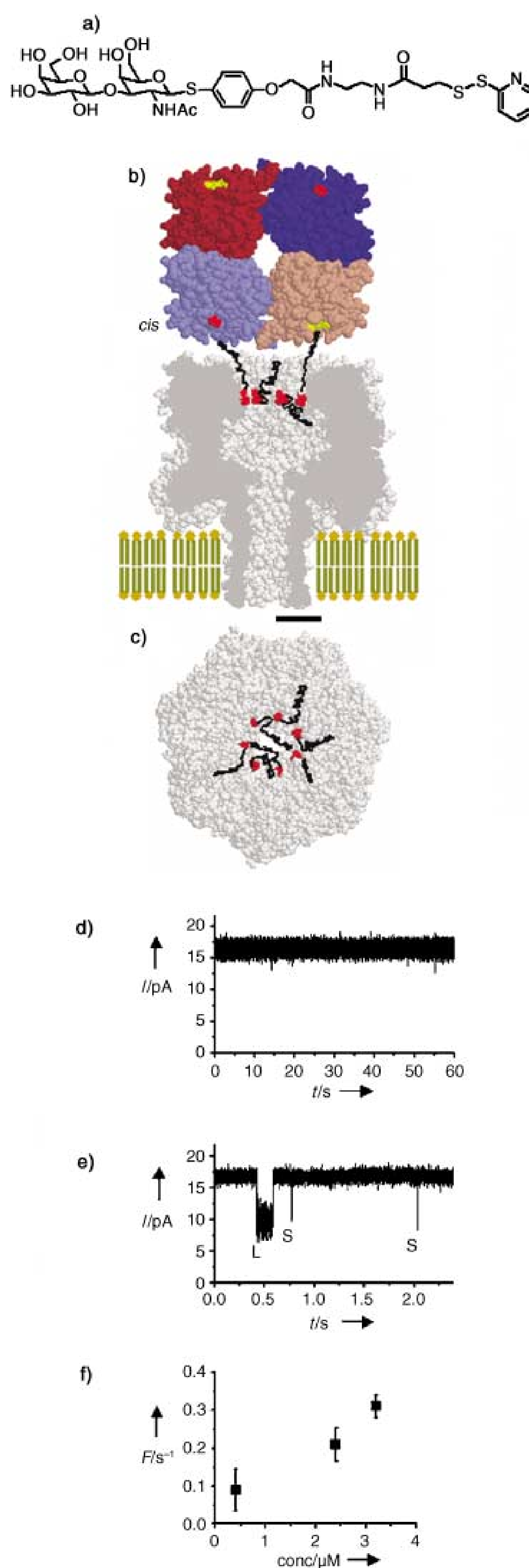

Figure 1. Protein pore 9C-NL$_7$, an engineered version of heptameric α-hemolysin (αHL) modified at seven sites with the disaccharide natural ligand, and its interaction with the tetrameric lectin from *Bauhinia purpurea*. a) Chemical structure of NL disaccharide D-gal-β-1,3-*N*-acetylgalactosamine with a 16-atom linker terminated with an *S*-thiopyridyl group used for the coupling to αHL. b) A cross section of a model of 9C-NL$_7$ carrying the NL disaccharide covalently attached through the linker to each of the seven engineered cysteines residues at position C9 (red). The model of BP is based on the crystal structure of *Griffonia simplicifolia* lectin (PDP ID: 1HQL), which has a high extent of sequence similarity to the BP lectin. In the diagonally arranged pale red and dark red subunits the binding sites (yellow) are on the front side of the model, while those in the pale blue and dark blue subunits are on the back. Their projected positions on the front side are indicated in bright red. The distance between the two lectin binding sites in the pale red and pale blue subunits, which are close to αHL, is 4.6 nm. This value is the distance between the C4 carbon atoms of the galactose units of the two ligands in the crystal structure. The distance for the diagonally positioned subunits of the lectin (e.g. pale red and dark red) is 7.6 nm. This compares to a maximum distance of 5.5 nm between the C4 carbon atoms in galactose of two NL disaccharides attached to distal (i.e., the first and fourth) subunits of 9C-NL$_7$.The corresponding distance for the first and third subunit is 5.1 nm. Bar, 2 nm. c) Top view of 9C-NL$_7$. d) Representative single-channel current trace of 9C-NL$_7$ at a transmembrane potential of +50 mV relative to the *cis* side of the bilayer. e) Representative trace of 9C-NL$_7$ after the addition of 3.2 µM BP to the *cis* side of the bilayer. Negative current deflection with short (S) and long (L) lifetimes represent individual monovalent and bivalent binding events, respectively. The individual events were not clustered into groups as indicated by the single exponential decay of the distribution in the inter-event interval histogram (data not shown). f) The frequency of occurrence (*F*) of all binding events depends linearly on the concentration of BP.

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multiple copies of the ligand. To address this question, we prepared the thiopyridyl derivative (Figure 1 a) of the disaccharide Gal-β-1,3-GalNAc (natural ligand, NL), which is a ligand for the carbohydrate binding protein *Bauhinia purpurea* (BP) lectin.[14,15] The NL disaccharide was then coupled to a cysteine residue engineered at position C9 of α-hemolysin (αHL), a bacterial polypeptide toxin, which forms heptameric pores of known structure .[16] Position C9 is located at the narrower part of the *cis* opening of the αHL pore (Figure 1 b, c). By using a procedure based on published work[13] we were able to generate three different αHL heptamers, $H_6$9C-NL_1, $H_5$9C-NL_2, and 9C-NL_7, containing one, two, and seven NL-modified subunits, respectively, and unmodified subunits, H.

Single-channel current recordings of the homoheptamer 9C-NL_7 were performed in 0.6 M KCl, 0.6 mM $CaCl_2$, 0.3 mM $MnCl_2$, and 6 mM Tris-HCl, pH 7.2, at a potential of +50 mV with the *cis* side of the chamber connected to ground. In the absence of BP lectin, the current trace for the channel containing seven chemically modified subunits showed no fluctuations (Figure 1 d) and the unitary conductance was 302 ± 50 pS ($n = 18$; n, number of independent recordings). This is lower than the conductance of the unmodified pore (Table 1) and suggests that the attached carbohydrates, which are tethered near a narrow part of the pore, can reduce the current flow through steric or electrostatic effects. Upon the addition of 3.2 μM BP lectin to the *cis* side of the homoheptamer, negative current deflections were observed with a mean amplitude of 110 ± 24 pS ($n = 5$) and a wide distribution of durations (Figure 1 e). The frequency of occurrence of the events increased with the concentration of BP lectin (Figure 1 f), but no events were observed in the presence of the lectin concanavalin A, which does not bind to Gal-β-1,3-GalNAc (data not shown). These results suggest that the current deflections are caused by specific binding of BP lectin to the carbohydrate ligands attached to the subunits. Consistent with this interpretation, the presence of the disulfide bond-breaking agent dithiothreitol led to a stepwise increase in mean conductance and the abolition of the binding events (data not shown). Furthermore, the presence of soluble *N*-acetyl-galactosamine (93 mM), which is known to compete with Gal-β-1,3-GalNAc for binding to BP lectin, led to a 2.4-fold decrease in the frequency of the events.

Two main types of binding events were discerned on further analysis of recordings of the carbohydrate-derivatized pore 9C-NL_7: short (Figure 1 e, symbol S) with a mean lifetime, τ_{off-1}, of 6.5 ± 1.2 ms (78 ± 5 % of all events) and long (Figure 1 e, symbol L) with a mean lifetime, τ_{off-2}, of 110 ± 30 ms (22 ± 5 % of all events; $n = 4$, Table 2). The two main types of events were separated based on the bi-exponential distribution of the lifetime histograms (see Supporting Information). The lifetime histogram also revealed a third component, and these rare events (less than 1 % of all events) were longer ($\tau_{off} \approx 200$ ms) than the main type of long events. The analysis of the current traces yielded values for the frequencies of occurrence for the two main types of binding events, $8.4 \pm 3.1 \times 10^{-2}$ s^{-1} for short and $2.4 \pm 0.9 \times 10^{-2}$ s^{-1} for long events (values normalized to 1 μM BP). We speculated that the short events reflect monovalent binding of BP lectin to the channel while the long events reflect bivalent binding. Molecular modeling confirmed that two disaccharides can bind simultaneously to the tetravalent BP lectin (Figure 1 b, see legend for the size of channel and approximate distances between disaccharide binding sites on the BP lectin).[17] The molecular models also indicate that trivalent binding is not possible.

To test the hypothesis that mono- and divalent binding events can be distinguished as short and long current blockades of derivatized channels, we evaluated the pattern of current flow through channels $H_6$9C-NL_1 and $H_5$9C-NL_2, which contain one and two chemically modified subunits, respectively. In the absence of BP lectin, no current fluctuations were observed and, as expected, the conductance values of $H_6$9C-NL_1 and $H_5$9C-NL_2 were higher than those of 9C-NL_7 (Table 1). In the presence of BP lectin, the pore containing a single carbohydrate gave rise to only short events ($\tau_{off-1} = 2.5 \pm 0.6$ ms, $n = 5$). The pore containing two

Table 2: The number of NL-disaccharides attached to αHL influences the type and the frequency of binding events.

Number of αHL subunits modified with NL	τ_{off-1} [ms][a]	τ_{off-2} [ms][a]	Frequency of occurrence for τ_{off-1} events [s^{-1}] [a,b]	Frequency of occurrence for τ_{off-2} events [s^{-1}][a,b]	τ_{on-app} [s][a,b]
1	2.5 ± 0.6	not observed	$3.5 \pm 1.4 \times 10^{-3}$	not observed	180 ± 70
2	2.5 ± 0.7	80 ± 40	$9.1 \pm 3.2 \times 10^{-3}$	$9 \pm 3 \times 10^{-4}$	79 ± 17
7	6.5 ± 1.2	110 ± 30	$8.4 \pm 3.1 \times 10^{-2}$	$2.4 \pm 0.9 \times 10^{-2}$	10 ± 3.7

[a] Measured at +50 mV in 0.6 M KCl, 0.6 mM $CaCl_2$, 0.3 mM $MnCl_2$, and 6 mM Tris-HCl, pH 7.2. The concentration of BP ranged from 0.4 to 3.2 μM in the *cis* chamber. The values for τ_{off-1} and τ_{off-2}, as well as τ_{on} were derived from the bi-exponential and mono-exponential fits, respectively, of lifetime histograms and represent the mean ± SD from five independent experiments for $H_6$9C-NL_1 and $H_5$9C-NL_2 and four experiments for 9C-NL_7. The frequencies of occurrence for τ_{off-1} and τ_{off-2} events were obtained by multiplying the frequency of occurrence for all events with fractions of the total events representing τ_{off-1} and τ_{off-2} events. The latter were obtained from the bi-exponential fits of the lifetime histograms. [b] Values are normalized to 1 μM BP.

Table 1: Conductance properties of αHL channels covalently modified with one, two and seven NL disaccharides at position 9C.

Number of αHL subunits modified with NL	Mean conductance of open channel [pS][a]	Mean amplitude of BP lectin binding event [pS][a,b]
1	550 ± 18 ($n = 9$)	194 ± 24 ($n = 5$)
2	536 ± 22 ($n = 7$)	164 ± 22 ($n = 5$)
7	302 ± 50 ($n = 18$)	110 ± 24 ($n = 5$)
0	578 ± 20 ($n = 4$)	not observed

[a] Measured at +50 mV in 0.6 M KCl, 0.6 mM $CaCl_2$, 0.3 mM $MnCl_2$, and 6 mM Tris-HCl, pH 7.2. [b] Comprises short and long binding events.

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carbohydrate derivatives gave rise to both short ($\tau_{off-1} = 2.5 \pm 0.7$ ms) and long events ($\tau_{off-2} = 80 \pm 40$ ms, $n = 5$), and, in line with expectations, the frequencies of occurrence of both were lower than for the pores containing seven modified subunits (Table 2). These results are consistent with the hypothesis that single-channel current recordings can report on mono- and bivalent binding of receptors to ligands attached to the channel.

To evaluate the validity of our single-molecule data on lectin binding, we derived kinetic and equilibrium constants from single-channel current recordings of 9C-NL_7 and compared these values with the literature value of the equilibrium constant from ensemble measurements. The rate constants for lectin binding and dissociation, k_{on} and k_{off}, were obtained from the intervals between binding events, τ_{on}, the concentration of BP lectin, and the mean lifetimes of binding events, τ_{off}.[18] The apparent association rate constant, k_{on-app}, was dominated by the monovalent events and gave the value $1.0 \pm 0.4 \times 10^5\, M^{-1}\, s^{-1}$. The dissociation rate constants for the monovalent and bivalent events were $k_{off-1} = 160 \pm 20\, s^{-1}$ and $k_{off-2} = 9.6 \pm 2.4\, s^{-1}$.[19] The kinetic data were used to derive the overall dissociation constant, $K_{d-1\&2}$, by assuming a kinetic model with a monovalent step that preceded a bivalent binding step (see Supporting Information). By using k_{off-1}, k_{off-2}, and k_{on-app}, a value for $K_{d-1\&2}$ of $3.1 \times 10^{-4}\, M$ was obtained. This compares well to the literature value[14] for macroscopic binding, K_{d-lit}, of $1.2 \times 10^{-5}\, M$, considering that K_{d-lit} was obtained by affinity chromatography and includes trivalent binding and rebinding, which are excluded from $K_{d-1\&2}$. The kinetic analysis of the single-channel recordings also yielded K_{d-1}, the dissociation constant for monovalent binding, with a value of $1.7 \times 10^{-3}\, M$. Further comparison of K_{d-1} and $K_{d-1\&2}$ with ensemble data affirmed the validity of the single-molecule data on lectin binding (see Supporting Information).

Our experiments show that ion channels derivatized with disaccharides are a valuable tool to study the binding kinetics of lectins. The single-molecule approach yields data in line with ensemble measurements but also reveals kinetic details, which would not have been discernible with conventional methods. For example, the dissociation rate constant for bivalent binding, k_{off-2}, with a value of $9.6 \pm 2.4\, s^{-1}$, is, by a factor of 17, smaller than the monovalent constant, k_{off-1}, with a value of $160 \pm 20\, s^{-1}$. The two dissociation rate constants k_{off-1} and k_{off-2} should not differ greatly given the similar molecular nature of the events. In further experiments, we will explore the reasons for the low value of k_{off-2} at the molecular level. By using our single-molecule approach we will also be able to test the binding model used to derive k_{off-2} from τ_{off-2}. In this model, a bivalent event is thought to reflect the following molecular changes: A lectin tetramer binds a first and then a second ligand before it dissociates from the two ligands in sequential steps with a bivalent binding state preceding a monovalent state. In an extended model, the dissociating lectin molecule goes through a succession of several dissociation–rebinding steps that cycle between the monovalent and bivalent states before the molecule eventually dissociates from the derivatized pore. Presently, the current recordings do not show conductance substeps indicative of these different binding states. In future experiments, we will improve the temporal and conductance resolution of the bivalent binding events to detect potential substates within one bivalent binding event. The strategy to increase the conductance resolution will employ NL-conjugates with longer linkers to achieve different pore blockades for the monovalent and bivalent states. Currently, the monovalently bound lectin is likely to reside very close to the pore opening and subsequent bivalent binding does not alter the extent of pore blockade.

In summary, our approach to the kinetics of binding of a tetravalent lectin to a ligand-modified pore resulted in the detection of monovalent and bivalent binding events at the single molecule level, and yielded kinetic details, which would not have been available through conventional ensemble methods.

Experimental Section

The synthesis of the disaccharide ligand for *Bauhinia purpurea* lectin is described in detail in the Supporting Information. Briefly, an S-glycoside was synthesized in which the disaccharide D-galactose-β-1,3-*N*-acetyl-galactosamine (natural ligand, NL) was coupled through the C1 carbon of galactosamine to 4-hydroxy-thiophenol .[20] The phenolic hydroxyl was further modified to yield a 16-atom linker, which terminated with a thiol. The thiol was modified with 2,2′-dithiopyridine to yield the *S*-thiopyridyl NL conjugate (Figure 1 a). The molecular weight of the conjugate is 678 Da, without the thiopyridyl group, and the effective length of the linker between the C1 atom of galactosamine and the thiol of the thiopyridyl group is 18 Å, assuming an extended conformation.

αHL heptamers modified with one, two, and seven NL-disaccharides were generated by using a published procedure[13] based on the formation of a disulfide linkage between an engineered cysteine residue of the protein and a compound containing a 2-pyridyl disulfide group. The heptamers with one and two NL-modified subunits were generated by combining modified and unmodified monomers and then isolating the heptameric assemblies containing the desired number of derivatized subunits. The heptamer containing seven modified subunits was prepared by using only modified monomers for the assembly. Details of the procedure are provided in the Supporting Information.

Single-channel current recordings of NL-modified heptamers were performed using a planar lipid bilayer apparatus[21,22] and details are available in the Supporting Information. The current recordings were analyzed as described.[11]

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