# The Interplay of Ligand Binding and Quaternary Structure in the Diverse Interactions of Dynein Light Chain LC8

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Dynein light chain LC8 is a small, dimeric, and very highly conserved globular protein that is an integral part of the dynein and myosin molecular motors but appears to have a broader role in multiple protein complexes unrelated to molecular motors. LC8 binds to two families of targets: those having a KXTQT sequence fingerprint and those having a GIQVD fingerprint. All known LC8 binding partners containing these fingerprints share a common binding site on LC8 that raises the question of what determines binding specificity. Here, we present the crystal structure of apo-LC8 at 1.7-Å resolution, which, when compared with the crystal structures of several LC8 complexes, gives insight into the mechanism underlying the binding diversity of LC8. Peptide binding is associated with a shift in quaternary structure that expands the hydrophobic binding surface available to the ligand, in addition to changes in tertiary structure and ordering of LC8 around the binding groove. The observed quaternary shift suggests a mechanism by which binding at one of the two identical sites can influence binding at the other. NMR

spectra of titrations with peptides from each fingerprint family show evidence of allosteric interaction between the two binding sites, to a differing degree in the two ligand families. Allosteric interaction between the binding sites may be a mechanism to promote simultaneous binding of ligands from the same family, providing a physiological role for the two fingerprints.

## Introduction

LC8 (called DYNLL1 in mammals)[1] is a highly conserved and widely expressed 10-kDa globular protein identified as an essential component of the dynein[2–4] and myosin V[5] molecular motors, binding directly to specific sites on the myosin V heavy chain[6,7] and the dynein intermediate chain IC74.[8,9] Interestingly, as much as 60% of LC8 is not associated with these molecular motors,[10] and LC8 has been identified in interactions with a number of nonmotor proteins. Some of these, such as Swallow, a protein essential for RNA localization,[11] are associated with active transport along microtubules, leading to the widely held view that LC8 is a dynein cargo adaptor.[12,13] But others, such as neuronal nitric oxide synthase,[14] are not clearly associated with active transport, leading to the emerging concept that LC8 is primarily a dimerization hub, binding to disordered regions on diverse partners and inducing them to dimerize and form partially ordered structures, often coiled coils.[8,15,16] Recently, this was illustrated dramatically for the nuclear pore complex protein Nup159, in which multiple copies of LC8 bind like “beads on a string” and induce formation of a coiled coil nearby.[17]

LC8 is a dimer containing a pair of central β-sheets composed of four strands from one subunit and one strand crossed over from the other subunit.[18] One side of each sheet is flanked by two helices, and the other side forms the dimer interface (Fig. 1a). Two identical binding sites are formed in a groove at the dimer interface, such that both apo- and doubly bound LC8 are symmetric dimers. Ligands bind next to β3 as a sixth strand of the β-sheet.[18,19] All known ligands belong to one of two sequence classes, the GIQVD family or the KXTQT family.[9,20] Both families share a conserved glutamine residue whose side chain projects out of the binding cleft and forms an N-terminal cap for helix α2.[19] As before, we adopted a notation in which the conserved glutamine residue is referred to as $Q_0$ and C-terminal and N-terminal residues are referred to as $Q_{+n}$ and $Q_{-n}$, respectively. The side chains of residues $Q_{+1}$, $Q_{-1}$, and $Q_{-3}$ project toward the interior of LC8, into a mostly hydrophobic groove lined by the aromatic side chains of residues

Phe73, Tyr75, and Tyr77 (Fig. 1b). Hydrophilic residues of strand β1 separate this hydrophobic cavity from the solvent. Interestingly, the peptide binding cleft is about 1 Å wider when bound to the bulkier side chains of a GIQVD ligand versus a KXTQT ligand.[19] The width of the cleft in the absence of peptide has not been described, because no crystal structure of apo-LC8 has yet been published and because this was not examined in the NMR structure of apo-LC8.[21]

Here, we present the crystal structure of apo-LC8 at 1.7-Å resolution. The structure reveals an unexpected induced-fit structural change and suggests that the binding of ligands to the two sites in dimeric LC8 is not independent. Using NMR spectroscopy, we monitored titrations of LC8 with ligands from both families to provide evidence in solution of an interaction between the binding sites.

## Results

### Structure solution

The structure of apo-LC8 was solved by rigid-body refinement, using the LC8 chain from the structure of LC8–Swa (peptide consisting of residues 281–297 of Swallow) as the initial model,[19] and refined to 1.7-Å resolution, converging at $R_{cryst}$=17.9% and $R_{free}$= 21.1% (Table 1). All residues were in the most favored Ramachandran region except for Asn51, which, as in all LC8 structures, has a positive φ angle and is part of a turn that also features a cis-peptide bond between Pro52 and Thr53.

The new synchrotron-based structure of LC8–Swa is very similar to the one we reported previously based on home-source X-ray data:[19] main-chain atoms align with an RMSD of 0.22 Å. The LC8–Swa structure also agrees well with the LC8–KXTQT motif complex in the recently solved 2.8-Å crystal structure of the ternary complex LC8–TcTex–IC74:[24] main-chain atoms align with an RMSD of 0.43 Å. For the comparisons that follow, we used the 2.0-Å LC8– Swa structure as the representative of the KXTQT family because it is the highest-resolution example.

### Description of apo-LC8

The apo-LC8 structure has well-defined and continuous density for all but the first three residues of the LC8 main chain, and the peptide binding cleft contains clearly defined bound water molecules, indicating that no complexed LC8 is present in the crystals. The structure also contains one sulfate ion not observed in any of the bound forms. In the following paragraphs, we describe differences between the ligand-bound and apo-LC8 structures in terms of global changes in structure and mobility, local changes in structure and mobility, and the solvent structure in the peptide binding cleft. Also, because the ligand-bound structure is the known reference state, we will describe changes in terms of what happens upon ligand dissociation rather than ligand binding.

## Shear movement

Removal of the bound ligand from LC8 is associated with a shear movement[25] of the LC8 subunits that decreases the width of the peptide binding cleft (Fig. 2; Table 2). Measured as the distance from $63C^{\alpha}$ to $9C^{\alpha}$, the cleft widths are 14.2 Å in LC8–nNOS (a GIQVD ligand), 13.4 Å in LC8–Swa (a KXTQT ligand), and 12.3 Å in apo-LC8. This cleft narrowing reburies 50–100 Å$^2$ of hydrophobic surface exposed by removal of the ligand. The exposed hydrophobic surface area arises mainly from strand β4 (residues Phe73, Tyr75, and Tyr77), with smaller contributions from strand β5 (Ala82 and Leu84). Removing a GIQVD ligand without changing the conformation of the LC8 main chain exposes <200 Å$^2$ to the solvent (Table 2). Removing a KXTQT ligand exposes <150 Å$^2$; in apo-LC8, only <100 Å$^2$ remains exposed.

## Packing at the dimer interface

Both the interior and the dimer interface of LC8 are well packed, and the packing at the interface is affected only slightly by the shear movement. For LC8–Swa, the average occupied volume (see Materials and Methods) for interior residues (those buried in both monomer and dimer) is within 1% of a reference volume derived from a survey of well defined protein crystal structures.[26] The dimer interface, defined as residues that are buried only in the dimer, is even better packed than the interior, with a mean fractional residue volume 2% below the reference volume. A similar pattern is seen for LC8–nNOS (peptide consisting of residues 226–237 of neuronal nitric oxide synthase), in which the ratio of mean observed volume to reference volume is 1.00 at the interface and is 1.03 in the interior. The slightly looser packing in LC8–nNOS may be an artifact of the lower resolution of the structure. Only in apo-LC8 (the highest-resolution structure) is the packing at the interface (mean volume/reference volume = 1.04) less efficient than the packing in the interior (mean volume/reference reference = 1.00); however, the difference in mean occupied volume of 4% is not larger than the per-residue standard deviation observed in a broad sample of well-packed residues.[26] Thus, despite the 2-Å displacement of the dimer interface between LC8–nNOS and apo-LC8, packing efficiency changes only very slightly. This is because the interface is very flat and because the side chains make small adjustments in conformation ($\chi_1$ and $\chi_2$) to avoid collisions, counteracting the movement of the main chain (Fig. 3a). Only one residue (Ile57) converts to a different rotamer to avoid steric clash with its symmetry mate (Fig. 3b).

### Local conformational changes and increased heterogeneity upon ligand dissociation

Removal of the ligand is accompanied by a change in conformation of the C-terminus of the protein as the last two residues (Ser88 and Gly89) move away from the dyad axis and into the peptide binding site (Fig. 4a). The β5–β2 interaction is thus shortened by one residue, and hydrogen bonds joining Ser88 O to Ser882 $O^{\gamma}$ and Ser88 N to Thr53 O are broken. The space created between β2 and Ser88 is filled by a network of ordered water molecules, making contacts with the side chain of His55 and the backbones of Ser88 and Thr53,all of which are buried in the ligand-bound forms. Residue Ser88 can be phosphorylated in vivo, a potential regulatory mechanism for LC8.[27]

A second rearrangement occurs at the opposite end of the binding groove where the ligand is in contact with the turn connecting β2 and β3 (Fig. 4b). This turn is at the dimer interface, such that in the bound form, the backbone of Asn61 makes hydrogen bonds with the backbone of Asn612. Upon removal of the peptide, this loop becomes less ordered as reflected by the crystallographic B-factors and the appearance of alternate conformations in the backbones of residues Arg60 and Asn61 (Fig. 4c). The two symmetry-related loops move apart, disrupting the interaction between Asn61 and Asn612. A sulfate ion, not seen in any of the peptide-bound forms, occupies the resulting space between the subunits, coordinated in two alternate conformations by the backbone amides of Arg60 and Asn61.

### Solvent structure in the ligand binding groove

In apo-LC8, ordered water molecules form hydrogen bonds with all the groove-facing backbone amides and carbonyls of strand β3 (Fig. 5). Some of these water molecules also bind to others to form networks that cover the hydrophobic surface of the groove. One such network covers Phe73 and consists of three water molecules bridging His68 $N^{\delta}$, Val66 O, Val66 N, and Ser64 $O^{\gamma}$. Another covers Tyr75 and consists of four water molecules bridging Asn10 $N^{\delta}$ to Ser64 N and Ser64 O. Upon ligand binding, the water network over Phe73 is replaced by the side chain of the $Q_{-3}$ residue.

### Observation of intermediate states by NMR

To complement the structural information available from crystallography on apo-LC8 and that on doubly occupied LC8, we sought to probe the structure of singly bound forms by using heteronuclear single-quantum coherence (HSQC) spectra to monitor the titration of LC8 with its ligands over a range of 0 equivalent to 1 equivalent. Titrations were performed using peptides derived from two ligands in the KXTQT family [IC (peptide consisting of residues 123–138 of the dynein intermediate chain IC74) and Swa] and one ligand from the GIQVD family (nNOS). As ligand is titrated into LC8, peaks corresponding to apo-LC8 decrease in intensity and peaks corresponding to doubly occupied LC8 appear. Neither peak is appreciably broadened, showing that the free and bound forms are in slow exchange. As noted previously for titration with nNOS,[28] at intermediate stages of titration, for some residues, a third, and sometimes fourth, peak grows and then shrinks in intensity during the titration (Fig. 6). Such intermediate peaks representing the singly bound forms can also be observed during titration with Swa and IC, although the peaks occur for fewer residues and tend to be less well resolved from the peaks corresponding to either the apo form or the doubly occupied form. For all three peptides, the intermediate peaks occur only for residues at the dimer interface or at the peptide binding site (Fig. 7).

## Discussion

### Folding coupled to binding

In protein–protein interactions, the extent to which folding and binding are coupled spans a broad spectrum, from the traditional “lock-and-key” model in which two preorganized interaction surfaces fit together to form a mutually complementary and well packed interface to the other extreme in which one partner has no intrinsic structure and adopts an ordered fold only in the presence of its partner.[29] The latter case, which is common among regulatory proteins especially in eukaryotes, is exemplified by the ligands of LC8, which tend to be natively disordered segments of proteins that fold into a β-strand at the recognition site and into a stable coiled coil at a distant site in the presence of LC8.[16]

LC8, unlike its ligands, has a recognizable binding groove even in the apo form and thus at first glance resembles a protein in the lock-and-key model. However, on closer examination, the binding groove in apo-LC8 is significantly more disordered than LC8 overall, becoming fully ordered only upon formation of the complex: temperature factors decrease, amide protons are better protected from the solvent,[19] and fewer $R_{ex}$ terms are required to model $^{15}$N relaxation.[30] Through steric constraints, the ligand promotes conformational order and homogeneity: the β2–β5 interaction is extended by one residue, and the β2–β3 turn collapses to a single ordered conformation. In the apo-LC8 crystal structure, this turn is partly stabilized by a backbone-coordinated sulfate ion, which very likely arises from the high concentration of ammonium sulfate used in crystallization. In vivo, this sulfate ion may be replaced by a more prevalent ligand, such as phosphate, or may be absent altogether, possibly resulting in an even greater degree of flexibility for this loop than that seen in the crystal structure.

The structural differences between the apo-LC8 and ligand-bound LC8 crystal structures are corroborated by chemical shift perturbations in the NMR spectra of these complexes in solution.[19] Such perturbations are sensitive indicators of changes in conformation or contact with a ligand. For LC8, as expected, the largest perturbations occur in strand β3 and the N-terminal of helix α2, the residues lining the peptide binding cleft. However, there are also significant perturbations for the last few residues of strand β5 that are not in contact with the peptide. This is now explained by the conformational change for these residues in apo-LC8 versus bound LC8 (Fig. 4a).

## Binding and quaternary structure

The major conformational change seen in ligand-bound versus apo-LC8 is a widening of the binding groove by <1 Å for KXTQT ligands and that by <2 Å for GIQVD ligands. The width of the cleft seems to be determined by the steric contact between the $Q_{+1}$ residue of the ligand and Tyr77 of LC8 and that between the $Q_{-1}$ residue and Tyr75. The expansion of the cleft in complexes exposes considerable hydrophobic surface area to the solvent, 50 $Å^2$ for KXTQT ligands and 100 $Å^2$ for GIQVD ligands, which is then covered by the bound ligand. The favorable burial of this hydrophobic surface is presumably what drives the narrowing of the cleft upon release of the ligand. It also implies that the net driving force for ligand binding is less than what would exist for a preformed binding site.

The quaternary shift of LC8 is an example of shear motion at a domain interface.[25] Shear motions are sliding movements perpendicular to the interface in which the surfaces remain in close contact, there is no qualitative change in packing (such as a different interdigitation of side chains) and there is little or no change in main-chain conformation, and there are few or no rotameric conversions. Good packing density is maintained by small adjustments in side-chain conformation within a single rotameric well. In LC8, the shear movement seems to be made possible by the rather flat (noninterdigitated) and nonpolar nature of the interface (Fig. 3). The difference between apo-LC8 and LC8–nNOS of 2 Å is apparently the upper limit for shear movement.[25] Between these extremes, it appears that the flat interface allows a near-continuum of association geometries, such as the 1-Å shift associated with KXTQT ligands. Fine adjustment of the size of the ligand binding groove through shear motion is one mechanism by which LC8 binds a variety of peptide sequences.

The shear movement provides a rationale for the design of the LC8 dimer interface as adjacent β-sheets with a swapped-over strand. A common model of protein association is that burial of hydrophobic surface contributes a large free energy change but little specificity, and short-range interactions, such as hydrogen bonds and Van der Waals contacts, provide specificity and determine the precise final conformation.[31] LC8 dimerization is both moderately tight[32] and very specific (the LC8 dimerization interface is not known to bind any other protein). The design of the LC8 dimer interface—two flat β-sheet surfaces with one swapped-over strand—is effective in providing high affinity and specificity while still allowing a large quaternary shear: affinity is provided by the substantial (1000 Å ) hydrophobic surface buried at

the flat interface (see Fig. 3), and specificity is provided by the crossed-over β-strand (intersubunit backbone hydrogen bonds).

The shear motion in LC8, albeit a subtle change in conformation (1–2 Å), is supported by several lines of evidence. Our observation was based on a comparison of the crystal structures of apo-LC8, LC8–Swa, and LC8–nNOS (Table 2). Although it was not reported at the time of publication, in retrospect, the same observation can be made by a comparison of the NMR structures of apo-LC8, LC8–Bim, and LC8–nNOS.[21] The agreement on this feature between the solution structures and the crystal structures is generally very good, ruling out the possibility that the shear motion is an artifact due to crystal packing effects (a point that is also reinforced by the agreement between KXTQT crystal structures in different space groups). Where there is minor disagreement between the methods (e.g., the 15-Å cleft in the NMR structure of LC8–nNOS versus 14 Å in the crystal structure), we took the crystal structures to be more convincing and accurate, for several reasons. First, the crystal structures are generally of higher quality (75%–80% of residues in the most favored Ramachandran region for the NMR structures versus 98% for the crystal structures) and higher precision (RMSD=0.92 Å for the ensemble of LC8–Bim NMR structures versus 0.18 Å of coordinate error based on $R_{free}$ for the crystal structure of LC8–Swa). Also, the NMR structures are based primarily on distance restraints, but their precision (1–2 Å) is as large as the change in quaternary structure we are trying to measure, and in the NMR experiments used, intrasubunit distances are indistinguishable from intersubunit distances. Finally, the NMR structures are determined by a molecular dynamics protocol in which good van der Waals contacts are maintained; thus, in the calculation of the LC8–Bim and LC8–nNOS NMR structures, the peptide binding cleft is forced open by the presence of the ligand, whereas in the calculation of the apo-LC8 structure, the cleft is free to collapse. In contrast, the crystal structures do not suffer from this somewhat circular reasoning: the electron density very clearly demonstrates the change in quaternary structure even at the molecular replacement stage when no ligand is present in the model.

Other pieces of evidence also support the existence of the shear motion in solution. The residues in the turns flanking strand β3 are mobile in apo-LC8 but not in the complex with the KXTQT ligand Bim.[30] Since the swapped-over strand β3 is part of the βsheet of the opposite subunit, these residues are precisely the "hinge" that must bend to accommodate the shear movement. Also, a global conformational change coupled to binding is suggested by the observation of intermediate peaks during titration, as described below.

## Allostery

LC8 is a symmetric dimer with two identical ligand binding sites. Forming the doubly bound complex (as observed in the crystal structures of LC8– Swa, LC8–IC, and LC8–nNOS) involves two binding steps as shown in Scheme 1.

Are the two binding steps independent? The answer depends on the nature of the singly bound intermediate. Three possible models are outlined in Fig. 8. In a two-state model, it is assumed that the effect of ligand binding to LC8 is restricted to small and local conformational changes in the vicinity of the binding site. Binding to one site does not influence binding at the other (i.e., $K_d^1 = K_d^2$ ). In a three-state model, binding of the first ligand is linked to a global change in protein conformation (the shear motion) that preorganizes the second binding site. This type of model—originally proposed by Monod, Wyman, and Changeux—matches the behavior of many oligomeric proteins that can undergo a shift in quaternary structure.[33] The most general is a four-state model in which binding of the first ligand is linked to a global change in conformation but, unlike in the three-state model, there is no assumption that the conformation in the singly bound state resembles the conformation in the doubly bound state.

Titration monitored by NMR is a useful method for distinguishing between different allosteric models because the number of unique conformational environments is reflected in the number of distinct chemical shifts that can be observed for each residue.[34] Thus, the two-, three-, and four-state models predict two, three, and four distinct peaks per backbone amide, respectively, in $^1H$–$^{15}N$ HSQC spectra. A two-state model is effectively ruled out for LC8 by the observation of intermediate peaks during titration with all three ligands tested. During titration with KXTQT ligands, residues giving one intermediate peak are most common, and just a few (two in LC8–Swa) give two intermediate peaks. During titration with GIQVD ligands, many more residues give two intermediate peaks. Therefore, binding of KXTQT ligands is largely consistent with the three-state model and binding of GIQVD ligands is most consistent with the four-state model. This difference in behavior may be explained by the difference in size between the two families. In the three-state model, binding the first ligand is linked to the full shear movement, which exposes additional hydrophobic surface area in the other unoccupied binding site. This may be tenable for KXTQT ligands that require a 1-Å shear movement, albeit not for GIQVD ligands that require a 2-Å shear movement. Thus, the global conformational change associated with binding of the first GIQVD ligand may be different from the shear movement observed between the apo and doubly bound crystal structures.

An intriguing consequence of LC8 adopting a three- or four-state model for ligand binding is that the first and second binding constants $K_d^1$ and $K_d^2$ can be different. At the titration midpoint, the relative populations of free, singly bound, and doubly bound LC8 are predicted to be 1:2:1 if independent binding sites are assumed (i.e., $K_d^1 = K_d^2$ ). The observed population of the singly bound form is significantly lower than this prediction (Fig. 6), which can be explained by allowing the second association step to be of higher affinity than the first. Conservatively choosing the most similar values that still fit the titration data well results in a $K_d^1 / K_d^2$ ratio of 2.5 for nNOS and that of 6.0 for IC and Swa.

## Overall implications for LC8 function

Several physiological roles for LC8 have been proposed as more binding partners are discovered. LC8 binds to both dynein and putative dynein cargo molecules, leading to the hypothesis that LC8 is a cargo adaptor.[11,13,35,36] However, in the known structures of LC8–ligand complexes, both dynein and non-dynein ligands compete for the same two identical binding grooves on LC8.[18,19,21] Therefore, models in which LC8 acts as a cargo adaptor are untenable unless LC8 binds one each of two ligands. Arguing against this heterologous binding model is the observation that known LC8 targets tend to be dimeric when bound to LC8 and to have dimerization domains distant from the LC8 binding site.[15,24,37,38] Despite the existence of these distant dimerization domains, in cases in which quantitative measurements are available, intrinsic dimerization of the targets in the absence of LC8 is either greatly weakened or entirely absent.[7,8,15] This has led to the view that LC8 is a hub protein and that its primary function is to promote dimerization in its partners, both in dynein and in other systems.[16]

The allosteric behavior of LC8 reported here may discourage heterologous binding and thus further support the hub model. The shear motion of the LC8 dimer interface serves as a mechanism to finely adjust the width of the peptide binding cleft to match the size of the ligand and optimize its affinity. Because the conformational change mainly affects the quaternary structure, it will happen to the same extent at both sites and thus may allow LC8 to discriminate against simultaneously binding a KXTQT ligand and a GIQVD ligand: a 1-Å shifted dimer will bind a GIQVD ligand suboptimally, a 2-Å shifted dimer will bind a KXTQT ligand suboptimally, and a 1.5-Å shifted dimer would bind both ligands suboptimally.

The role of LC8 as a hub further raises the question as to how its diverse collection of targets can share the same interaction site yet bind with high affinity and specificity. The NMR titration experiments reported here have demonstrated that the first binding event influences the second ($K_d^1/K_d^2 = 6$ for KXTQT peptide ligands). Such cooperative interactions are a common mechanism to enhance both affinity and specificity in biological systems.[39] We propose that allostery in LC8 allows it to act like a switch, preferentially existing either in the apo form or doubly bound to two of the same ligand and disfavoring heterologous states and singly bound states. The distant dimerization domains in various targets can serve to further stabilize doubly bound homodimeric states. With the growing number of known LC8 binding partners, the allosteric interaction between its binding sites, and the structural evidence that the fully occupied form is also the only fully ordered one, we speculate that LC8 present in vivo is not free but is largely bound to homodimers of its many partner proteins.

## Materials and Methods

### Purification and crystallization

During the structure determination of the LC8–Swa complex that we recently reported,[19] the electron density

maps from some of the crystals grown from mixtures of LC8 and Swa showed no evidence for the bound peptide. The ligand-free crystals were identical in appearance but had slightly different unit cell dimensions (a = 44.9 ± 0.1 Å versus 44.9±0.1 Å; c=201.6±0.3 Å versus 204.0±0.4 Å).

All crystals were grown at room temperature in hanging drops made by a 1:1 mixture of the reservoir with a stock solution of 1 mM protein and 2 mM peptide in 20 mM Tris– HCl, pH 8.0. The reservoir was composed of 0.2 M potassium sodium tartrate, pH 5.5, 0.1 M sodium citrate, and 2.0 M ammonium sulfate. The crystals were flash frozen in liquid nitrogen following transfer to a cryoprotectant consisting of reservoir solution plus 10% (v/v) glycerol.

X-ray data collection

For apo-LC8, diffraction data were collected on beamline 5.0.3 at Berkeley National Lab's Advanced Light Source ($\lambda$ = 1.0 Å; $\Delta\varphi$ = 1°; high-resolution pass, 120 10-s images; low-resolution pass, 100 3-s images). Data sets were processed using the HKL suite of programs.[40] Crystals belong to space group $P6_122$ with a = b = 44.97 Å and c = 202.11 Å, with one molecule in the asymmetric unit and a solvent content of 57%.

Diffraction data for LC8–Swa were collected on Advanced Light Source beamline 8.2.1 ($\lambda$ = 0.98 Å; $\Delta\varphi$ = 1°; 130 4-s images). Images were integrated with MOSFLM,[41] and reflections were merged using SCALA.[42] Crystals belong to space group $P6_122$ with a=b=44.15 Å and c=203.71 Å, with one molecule in the asymmetric unit and a solvent content of 49%.

Structure determination and refinement

The structure of apo-LC8 was solved by using the LC8 chain from the previously reported LC8–Swa structure [Protein Data Bank (PDB) entry 2p1k[19]] as an initial model. Before refinement, 10% of the reflection data were set aside for cross-validation. The test set comprised the same reflections used in the LC8–Swa structure plus new randomly selected reflections beyond 2-Å resolution. Molecular replacement at 3.5-Å resolution using MOLREP[43] followed by rigid-body refinement using REFMAC[44] resulted in $R_{cryst}$ and $R_{free}$ values of 38% and 41%, respectively. The structure was iteratively refined using REFMAC and Coot†, including TLS refinement,[45] to final $R_{cryst}$ and $R_{free}$ values of 17.9% and 21.1%, respectively. During refinement, ordered water molecules were added or removed by the criterion of having reasonable hydrogen-bonding partners and a peak in the $2F_o-F_c$ electron density map of at least 1$\sigma$. Water molecules were numbered on the basis of final peak electron density from 1 (the highest) to 109 (the weakest).

The new structure of LC8–Swa was solved similarly. Following rigid-body refinement, $R_{cryst}$ and $R_{free}$ were 40% and 42%, respectively, and there was strong density for the bound peptide. A model for Swa was built into this density. The structure was refined iteratively to $R_{cryst}$ and $R_{free}$ values of 21.5% and 26.5%, respectively.

Per-atom contributions to solvent-accessible surface area were calculated using VOLBL.[46] Structure diagrams were produced with PyMOL‡. Occupied volumes of residues were calculated by the Voronoi method[47] using the tools available online from the Database of Molecular Movements§. The fractional volume of each residue was defined as the ratio of its calculated volume to a reference volume derived from a set of high-resolution structures selected from the PDB.[26] Buried residues were defined as those having less than 5 $Å^2$ of solvent-accessible surface area. Interface residues were defined as residues exposed in the monomer that lose at least 50% of their solvent-accessible surface upon dimerization.

## NMR spectroscopy

NMR samples of $^{15}N$-labeled LC8 were prepared as described previously.[19] $^{1}H$–$^{15}N$ HSQC spectra were recorded at 303 K with 256 scans per increment (for LC8–Swa and LC8–nNOS) or at 298 K with 64 scans per increment (for LC8–IC) on a 600-MHz Bruker DRX spectrometer. Spectra were processed with NMRPipe.[48] Plots of spectra were prepared with burrow-owl.[49] For quantitative measurement of peak intensities, peaks were converted to a Gaussian line shape using a Lorentzian-to-Gaussian transform, and then the frequency-domain data were fit by least-squares minimization to Gaussian line shapes using in-house software. Chemical shifts and line widths were constrained to be equal throughout a titration series. Reported intensities were corrected for dilution of the sample due to the addition of peptide stock solution, which was 5 to 10 times the protein concentration.

Theoretical curves for the relative populations of apo, singly bound, and doubly bound forms were derived by solving the following set of equations:

$$\frac{[LC8] \times [X - LC8 - X]}{[X - LC8] \times [LC8 - X]} = \frac{K_d^1}{K_d^2}$$

$$[X - LC8] = [LC8 - X]$$

$$[LC8] + [X - LC8] + [LC8 - X] + [X - LC8 - X] = 1$$

## PDB accession codes

The coordinates of apo-LC8 and LC8–Swa have been deposited in the Research Collaboratory for Structural Bioinformatics PDB with accession codes 3bri and 3e2b, respectively.

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Fig.1. Overview of theLC8–Swa complex:LC8 chain A is shown in blue; LC8 chain B, in dark blue; and Swa peptide, in yellow. (a) View along the 2-fold symmetry axis. (b) Detail of the peptide binding cleft using the $Q_n$ notation, where $Q_0$ refers to the conserved glutamine residue of the ligand.

Table 1. Data collection and refinement statistics

Fig.2. The changing width of the peptide binding cleft. The surface of LC8, looking into the peptide binding groove, is shown for (a) LC8–nNOS, (b) LC8–Swa, and (c) apo-LC8. The surfaces of strands β4 and β5 are shaded red.

Table 2. Induced-fit structural changes in the peptide binding cleft of LC8 due to shear movement

Fig. 3. Dimer interface features allowing the shear movement. LC8–nNOS (red/green) is overlaid on apo-LC8 (purple), with alignment based on the lower subunits. The view is along the 2-fold symmetry axis, parallel with the β- strands, in the layer containing (a) Phe86 and (b) Ile57. Note the rotameric interconversion of Ile57. The fluid shear movement of 2 Å is evident in the upper subunits and is facilitated by close residue packing yet lack of side-chain interdigitation.

Fig.4. Conformational changes at the exits of the peptide binding groove.(a)Stereoview looking along the 2-fold axis toward the N-terminal exit of the peptide binding groove showing the LC8–Swa complex (blue/yellow) and apo-LC8 (purple), with chain B shaded darker than chain A. Alignment is based on chain A (right side of the figure), excluding the interleaved strand β3. Dashed lines indicate hydrogen bonds. (b) Stereoview along the 2-fold axis toward the C-terminal exit showing the β2–β3 loop. Coloring and alignment are as in (a). In apo-LC8, a sulfate ion sits at the dimer interface, coordinated by the backbone amides of residues 60 and 61. (c) Same view as in (b) showing $2F_o - F_c$ density for apo-LC8 contoured at 1.0σ. Nitrogen atoms are shown in blue, and oxygen atoms are shown in red. Both alternate conformations are shown overlaid for the backbone in the β2–β3 loop (arrows).

Fig.5. Solvent structure in the apo-LC8 ligand binding groove. (a) $2F_o-F_c$ density contoured at 1.2σ. Water molecules are shown as red spheres. (b) Ribbon diagram and bound water molecules (red) are shown for apo-LC8 (purple) overlaid with the peptide from LC8–Swa (ghostly yellow). Water 20 (bridging Tyr75 OH and Phe62 O) has an interesting analog in LC8–Swa, where water 1 bridges the same two residues plus Swa Thr293 $O^{\gamma}$ and is the only buried water molecule in the structure as well as the most ordered one. An analogous water molecule is missing from LC8–nNOS, due to the different peptide main-chain conformation that leaves insufficient room between the $Q_{-1}$ and $Q_{+1}$ residues.

Fig.6. Titration of LC8 with peptide ligands monitored by NMR:(a and d) LC8–IC; (b and e) LC8–Swa; (c and f) LC8– nNOS. (a–c) Excerpts of HSQC spectra with (left to right) 0 equivalent, 0.4 equivalent, and 1 equivalent of ligand added. Peaks for free LC8 (apo) and bound LC8 (doubly occupied) are labeled (f) and (b) respectively. Red dots indicate new peaks arising from singly bound LC8, which are present only in the middle of the titration curve. (d–f) Titration curves for the resonances shown in (a) to (c). Crosses and squares represent relative intensities of peaks corresponding to free and doubly-bound forms, respectively. Circles represent the sum of relative intensities of intermediate peaks. Curves represent populations

predicted by the two-site binding model (see Discussion), with $K^1_d/K^2_d = 6.0$ for Swa and IC and $K^1_d/K^2_d = 2.5$ for nNOS.

Fig. 7. Spatial distribution of residues showing intermediate peaks. (a) nNOS. (b) Swa. (c) IC. The peptide (yellow) shows the location of the occupied binding groove and that of the unoccupied binding groove (faded yellow). On the sequence schematic diagram, upward bars indicate residues for which an intermediate peak is observed near the apo peak and downward bars indicate residues for which an intermediate peak is observed near the doubly bound peak. These same residues are indicated by spheres on the structure.

Scheme 1. Two-step formation of LC8-ligand complexes, where X is the ligand and the dissociation constants are defined as $K^1_d = [LC8][X]/[LC8 - X]$ and $K^2_d = [LC8 - X][X]/[X - LC8 - X]$. The binding sites and ligands are identical, so $[LC8-X]=[X-LC8]$.

Fig. 8. Models for allostery in LC8 binding. Different polygonal shapes represent different conformations of LC8, and a black dot indicates an occupied binding site. (a) Two independent binding sites. Binding at one site does not affect the conformation at the other site. There are only two possible chemical environments, corresponding to the occupied and unoccupied states. (b) Binding coupled to a global conformational change, such as the shear motion. There are three possible chemical environments: the occupied state, the distinct unoccupied state of the free form, and the distinct unoccupied state of the singly bound form. (c) Unique conformation for the singly bound state. There are four possible chemical environments—two corresponding to the same apo and bound states from (a) and (b) and two more from the singly bound state that, lacking symmetry, provides two environments, neither of which is equivalent to the apo or bound state. Only for model (a) are the affinities necessarily equal for the first and second binding steps.