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The cytoplasmic domain of the T-cell receptor zeta subunit does not form disordered dimers

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Abstract

Intrinsically disordered regions in proteins play active roles in recognition, signaling and molecular sorting. They often undergo coupled folding and binding giving rise to largely ordered interfaces with their binding partners. The cytoplasmic region of the T-cell receptor zeta subunit (ζ_{cyt}) has previously been proposed to specifically dimerize in the absence of a disorder-to-order transition, suggesting an intriguing dimerization mechanism that may involve multiple transient interfaces. We show here using analytical ultracentrifugation, NMR, size-exclusion chromatography and multi-angle light scattering that neither ζ_{cyt} nor the cytoplasmic region of CD3 ϵ significantly populate a dimeric state, but that they are mostly monomers in solution up to millimolar concentrations. They experience a salt- and concentration-dependent shift of their elution volume in size exclusion chromatography previously interpreted as dimerization. Our data shows that ζ_{cyt} does not form a highly disordered protein complex and leaves open the question as to whether completely disordered dimers (or other oligomers) exist in nature.

Keywords

Intrinsically disordered protein; fuzzy complex; immune receptor; analytical ultracentrifugation; size-exclusion chromatography

Intrinsically disordered proteins (IDPs) and intrinsically disordered protein regions (IDRs) are common in eukaryotic proteins^{1, 2} and are often more than simple linkers between domains. They play active roles in recognition, signaling and molecular sorting.³⁻⁵ IDRs are generally accepted to interact with their binding partners through coupled binding and folding.^{6, 7} Their relative lack of hydrophobic residues and intramolecularly stabilizing interactions prevents them from folding on their own⁸ but they fold efficiently on complementary surfaces of specific binding partners.⁹ Recent work has demonstrated that some IDPs retain functional disorder even in complexes.¹⁰ What is the functional advantage of these so-called ‘fuzzy’ complexes? (The term ‘fuzzy’ complex derives from ‘fuzzy’ logic and is supposed to indicate the possibility of signaling outputs between 0 and 1 made possible by the conformational complexity in the complex. As this concept does not necessarily apply to all such complexes, we use ‘fuzziness’ to describe conformational disorder in the bound state.) In the most striking examples, disorder is not restricted to

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regions flanking binding motifs but extends into the binding interface through fraying of the ends of bipartite motif¹¹, dynamic interactions of several motifs in an IDP with a given binding partner¹²⁻¹⁴, or self-organization of elastomeric proteins into highly hydrated, largely disordered aggregates.¹⁵ Well-described examples are the dynamic interactions of the regulatory region of the cystic fibrosis transmembrane regulator (CFTR) with other domains of the protein¹² and of the yeast cyclin-dependent kinase inhibitor Sic1 with the substrate recognition subunit of its ubiquitin ligase, Cdc4.^{13; 16} Sic1 harbors nine phosphorylation-dependent binding motifs for Cdc4, each of which interacts with a single binding pocket in Cdc4, one at a time, in a dynamic equilibrium.¹³ Although each motif is ordered when engaged, each motif is disordered in the complex a significant part of the time while other motifs are engaged. The resulting disorder and conformational averaging in the complex presumably allows Cdc4 to sense the phosphorylation state of Sic1 through polyelectrostatic interactions¹⁷ and read out the cell cycle status of the cell in an ultrasensitive manner.^{18; 19} In contrast, a single binding motif and a stable interaction generated through coupled folding and binding would be expected to produce a graded response. In the case of the Sic1/Cdc4 complex, the function of disorder in the complex is well understood but in other cases may be less obvious and difficult to demonstrate directly.

An interesting related but more general question is: How disordered can a biologically functional ‘fuzzy’ complex be? An often cited example^{4; 10; 20-26} for a highly ‘fuzzy’ complex is the putative dimer formed by the cytoplasmic domain of the T-cell receptor zeta subunit (ζ_{cyt}).²⁷ Indeed, Sigalov *et al.* reported that the intracellular domains of several immune receptor subunits, including Ig α , Ig β , IGE, CD3 γ , CD3 δ , CD3 ϵ and T-cell receptor zeta dimerize in solution *in vitro*.²⁷ Of these immune receptor domains, ζ_{cyt} was investigated in most detail and was reported to have a monomer-dimer dissociation constant of $\sim 10 \mu\text{M}$ and a dimer-tetramer dissociation constant of $\sim 1\text{mM}$. Affinities at least in the micromolar range may have biological relevance because clustering of T-cell receptor subunits through extracellular ligands²⁸ and pre-clustering even in their absence is suggested to lead to high local concentrations of intracellular domains.²⁹

NMR spectra of ζ_{cyt} at low and high micromolar concentrations, at which ζ_{cyt} is expected to exist mostly as either monomer or dimer, respectively, were reported to be identical.³⁰ This result was taken as evidence that the dimer remains fully disordered.³⁰ A stable dimer interface would result in differences in chemical shift and line width between monomeric and dimeric ζ_{cyt} . Speculation into the mechanism of dimerization led to a model in which several different interfaces are sampled in a dynamic equilibrium, giving rise only to small chemical shift or intensity differences that may only be observed in NMR spectra upon close inspection.²³ We used complimentary biophysical techniques to test the hypothesis that ζ_{cyt} forms disordered dimers through multiple interfaces.

ζ_{cyt} is a monomer in solution

We first acquired ^1H , ^{15}N HSQC spectra of ζ_{cyt} at 10 and 400 μM concentrations (Figure 1a). Indeed, the spectra are nearly identical to each other and to the spectra reported by Sigalov *et al.*³⁰ Comparison of resonance intensities under the two conditions did not reveal localized broadening or candidate dimerization interfaces (Figure 1b; assignments reported by Orekhov and colleagues³¹). To increase the sensitivity of NMR experiments towards the detection of transient interactions³², we spin-labeled ζ_{cyt} with *S*-(2,2,5,5-tetramethyl-2,5-dihydro-1 *H*-pyrrol-3-yl)methyl methanesulfonothioate (MTSL) on a cysteine residue introduced to the N-terminus and measured the paramagnetic relaxation enhancement (PRE) of amide protons along the sequence (Figure 1c and Figure S1). The PRE profile shows a strong PRE effect close to the labeling position where signals are broadened beyond detection, and the effect decreases gradually with increasing distance in primary sequence

from the labeling position. The profile is close to that expected for a random coil chain (red lines) with additional weak PRE effects observed around position 60. To distinguish between intra- and intermolecular interactions as a source of these additional PRE effects, we measured PRE effects as a function of protein concentration. The close similarity of the profiles at different concentrations demonstrates that the PRE effects of residues far away from the labeling position in primary sequence are caused by intra- rather than by intermolecular interactions. Thus, ζ_{cyt} exhibits transient structure, but we did not observe signs of even transient dimerization.

To test the possibility of a much weaker self-association than previously reported, we increased the local concentration of ζ_{cyt} by disulfide crosslinking the N-termini, which usually connect to the transmembrane domain. We chose this orientation to mimic the condition of clustered T cell receptor at the membrane, where a ζ_{cyt} dimer may have biological relevance. We crosslinked two molecules through an N-terminal cysteine attached via a glycine/serine-rich linker (denoted Cys- ζ_{cyt}). The NMR spectra of Cys- ζ_{cyt} and disulfide-linked Cys- ζ_{cyt} differ only in the glycine and serine resonances adjacent to the cysteine/cystine (Figure 1d). These data demonstrate that ζ_{cyt} does not self-associate when spatial constraints at the membrane are mimicked, neither via an ordered nor a disordered dimer.

Next, we investigated the oligomerization state of ζ_{cyt} by sedimentation velocity (SV) analytical ultracentrifugation (AUC), a technique that does not require calibration relative to proteins of known molecular weight (Mw).^{33; 34} SV AUC demonstrated that most of the protein existed as a monomer (Figure 2a,b) as evident from a single peak in a continuous sedimentation coefficient distribution model $c(s)$ (Figure 2a) as well as in a two-dimensional size-and-shape distribution analysis³⁵ (Figure 2b). The experimental Mw was slightly larger than the theoretical (Mw=13,157 Da and confirmed by ESI mass spectrometry, see Supplemental Figure S2). This indicated the possibility of weak self-association which we probed via sedimentation equilibrium (SE) AUC. SE AUC is independent of protein shape and the classical method to rigorously characterize biomolecular oligomerization.^{33; 34} The centrifugal forces generate a concentration gradient of the loading material in the cell and therefore probe oligomerization in a concentration range much broader than the loading concentrations. Even weak oligomerization can thus be observed with ease. In contrast to Sigalov *et al.*²⁷, we observed sedimentation behavior indicative of mostly monomers (Figure 2c). A single species model fit well but resulted in a Mw that was slightly larger than the theoretical (Table 1). The data did not fit to a monomer/dimer/tetramer self-association model, excluding the possibility of tetramerization. A global fit of SE data from three different protein concentrations to a reversible monomer-dimer self-association model resulted in a dissociation constant of 6.2 mM (Figure 2c, Table 1 and Supplemental Figure S3), i.e. nearly three orders of magnitude weaker than reported by Sigalov *et al.*²⁷ At a micromolar ζ_{cyt} concentration, e.g. 10 μM , only a fraction of 0.35% of ζ_{cyt} would be dimeric at a K_D of 6mM (compared to a fraction of 50% with a K_D of 10 μM , see Supplemental Figure S4). The data can alternatively be fit equally well to a two-discrete-species model in which the first species' Mw was fixed to 13,157 Da and the second was floated and fit to ~28,000 Da at a fraction of ~0.5% (Table 1). Although even weak self-association may be biologically relevant in clustered TCRs, the lack of interactions between protomers in disulfide-linked Cys- ζ_{cyt} demonstrates that ζ_{cyt} does not dimerize even at extremely high local concentrations and the AUC data likely report on a low concentration contaminant. We consistently observed mainly ζ_{cyt} monomers up to millimolar concentrations with SE AUC at 277 and 298K and in 10mM phosphate buffer with 10mM or 150mM added NaCl.

As dimerization had been reported also for other immune receptor cytoplasmic domains²⁷, we next probed the oligomeric state of CD3 ϵ with AUC. We again observed monomers in

SV and SE AUC experiments even at high concentrations, and a dissociation constant of 8.3 mM when we forced a fit to a reversible monomer-dimer self-association model (Figures 2d-f, Table 1) suggesting that there was at most very weak, if any, dimerization. Hence, NMR and AUC data demonstrated that both ζ_{cyt} and CD3 ϵ are mostly monomers in solution even up to millimolar concentrations.

ζ_{cyt} exhibits a concentration-dependent shift of its elution volume in SEC

Next, we investigated the hydrodynamic properties of ζ_{cyt} as a function of concentration by size exclusion chromatography (SEC) and the absolute molar mass of the eluting species by coupled multi-angle light scattering (MALS)³⁶. ζ_{cyt} consistently elutes as a monomeric species. Surprisingly, the elution volume (V_{el}) changes as a function of concentration (Figure 3a). Importantly, the polydispersity was between 1.000 and 1.002 confirming that ζ_{cyt} elutes as a homogenous species, not a mixture of different oligomeric states. As MALS measures the weight-average molar mass, even a small amount of dimer, oligomer or aggregate would increase the measured molar mass significantly. At the lowest protein concentration (70 μM), the molar mass is higher in the slopes of the peak in the MALS profile (Figure 3a). This is often indicative of a small amount of sticky aggregate that elutes in a long tail extending into later peaks in the chromatogram. At higher concentrations, ζ_{cyt} elutes in a single, narrow peak. These data demonstrate unequivocally that ζ_{cyt} is a monomer in the concentration range covered (70-700 μM) but that it may surprisingly be prone to aggregation at low protein concentration. The shift of V_{el} as a function of protein concentration was puzzling and as it may previously have been interpreted as a sign of self-association, we investigated it further.

Sigalov *et al.* reported that ζ_{cyt} , at a high loading concentration of above 400 μM , elutes at a retention time typical for a ~27 kDa folded protein, i.e. at approximately the M_{w} of a ζ_{cyt} dimer.²⁷ Lowering the loading concentration progressively shifted the elution volume to larger values until reaching the M_{w} of the monomer. Although IDPs usually have larger hydrodynamic radii than folded proteins, the authors used folded proteins as standards to calibrate the molecular weight to the retention volume of the column. We took a similar approach and also observed a shift of the elution volume to higher values, i.e. smaller apparent molecular weights ($M_{\text{w}}^{\text{app}}$) upon lowering the loading concentration on a Superdex 75 10/300 gel filtration column (GE Healthcare) (Figure 3b). The shift was not as large as reported by Sigalov *et al.* but the data could be fit to a monomer-dimer equilibrium with a dissociation constant of 1 μM . The $M_{\text{w}}^{\text{app}}$ of the monomer and the dimer obtained by the fit were 21,000 and 28,000Da, respectively, requiring a considerable compaction upon dimerization if the shift was indeed caused by self-association. CD3 ϵ showed a similar concentration dependence of its elution volume and its $M_{\text{w}}^{\text{app}}$ (Figure 3b).

We considered the following causes for the apparent disagreement between SEC and other biophysical data: (1) Confinement-induced dimerization of ζ_{cyt} and CD3 ϵ in the gel filtration matrix but not free in solution, (2) overloading of the gel filtration column accompanied by exclusion of protein molecules from appropriate pore sizes and premature elution, (3) interaction with the gel filtration resin or other parts of the column, and (4) non-ideal protein behavior that leads to drag through disordered regions. We went on to systematically test above hypotheses.

The concentration-dependent shift in SEC is resin- and salt-dependent

First, we acquired an HSQC spectrum of ζ_{cyt} in P-6DG gel filtration resin (BioRad) to mimic SEC conditions for NMR spectroscopy (Figure 3c). The line width of resonances were broad reflecting long rotational correlation times due to spatial confinement but the

chemical shifts and relative intensities of resonances were nearly identical to the ones free in solution. We were thus able to eliminate the possibility of crowding-induced dimerization under SEC conditions.

Second, in case the concentration-dependent shift of the elution volume we observed for ζ_{cyt} and CD3 ϵ was caused by overloading of the gel filtration column at high concentrations (100 μ l of 400 μ M protein, i.e. $\sim$ 0.5mg ζ_{cyt} under our conditions), the same shift should be observable for other proteins as well. We tested this hypothesis with Gli3-90, a 90 amino acid fragment of the largely intrinsically disordered protein Gli3, which is disordered in solution. Its elution volume did not shift as a function of loading concentration (Figure 3b) suggesting that a shift is not typical for disordered proteins in general but specific for ζ_{cyt} and CD3 ϵ .

Third, to test whether an interaction with the resin or other parts of the column lead to the observed shift, we switched from the cross-linked agarose and dextran resin of the Superdex 75 column to a Shodex KW-803 column with a silica matrix. Unexpectedly, the M_w^{app} at low micromolar concentration of ζ_{cyt} was close to the theoretical monomer M_w of 13,157Da in 20mM potassium phosphate buffer pH 7.0 at 150mM NaCl (Figure 3d). IDPs usually have larger hydrodynamic radii than folded proteins of the same M_w and therefore are expected to elute at higher M_w^{app} .³⁶ Between 50 and 100 μ M loading concentration the M_w^{app} plateaued at $\sim$ 20,000Da, i.e. too small for a ζ_{cyt} dimer (theoretical M_w of 26,314 Da). These data indicated that the shift was not caused by dimerization, in agreement with NMR, AUC and MALS data.

Fourth, because we had observed the concentration-dependent shift with two types of resins, we considered the possibility of non-ideal protein behavior at higher protein concentrations. As such behavior strongly depends on solution conditions, we varied the salt concentration and investigated the concentration-dependent elution volume at low and high salt concentrations (20mM potassium phosphate pH 7 with 0M or 1M NaCl, respectively, Figure 3d). In low salt, the M_w^{app} varied between 4,200 and 26,000Da, which are inconsistent with a monomer-dimer equilibrium, as the lower M_w^{app} is well below the theoretical M_w of ζ_{cyt} . These data demonstrate that the elution volume at low concentration is shifted, while it may be normal at high concentration. High salt was able to quench the concentration dependent shift nearly completely. High salt also reduced the shift for CD3 ϵ (Figure 3e). In 1M NaCl, the MALS profile is consistently indicative of a monomeric state (Figure 3f). These SEC-MALS data at different salt concentrations together with AUC and NMR clearly demonstrate that ζ_{cyt} does not dimerize with low micromolar dissociation constant but is mostly a monomer at physiologically relevant concentrations and that the behavior in SEC is not caused by dimerization but rather by non-standard behavior of the protein at low concentration.

Sigalov *et al.* reported the absence of a concentration-dependent shift in 6M GdmCl and a monomer-like M_w^{app} . Our data suggest that the high ionic strength would have been enough to quench the shift and the denaturant not required. Also, we found it was important to calibrate the column at different salt concentrations, or the M_w^{app} at high salt would have been close to the theoretical monomer M_w . ζ_{cyt} and CD3 ϵ are enriched in charged residues (see Supplemental Material, Table S1) and their hydrodynamic radii are thus expected to be highly salt-dependent as net charge per residue is the strongest determinant of the compactness of IDPs.³⁷⁻³⁹ A M_w^{app} of 25 kDa as observed for ζ_{cyt} at high salt is in agreement with an extended conformation enabled via screening of electrostatic interactions.

We considered whether non-ideal attractive or repulsive interactions could explain the unusual behavior in SEC. AUC is a powerful method for revealing non-ideality and allows

the extraction of second virial coefficients and non-ideality coefficients for sedimentation and diffusion. We did not see strong indications for non-ideality in our data; all coefficients were relatively small. We thus conclude that ζ_{cyt} and CD3 ϵ interact weakly with gel filtration resin in a salt- and concentration-dependent manner and may also behave slightly non-ideally. Interestingly, the interaction is only obvious at low protein concentrations suggesting that few interaction sites are available on the column. Upon their saturation the rest of the protein seems to behave like a normal IDP. As there seem to be only few sites, they may not be on the resin but e.g. on a minor resin byproduct. This may explain why we observed a V_{el} shift for two different resins. An interaction with few available sites or a weak interaction may be obscured in NMR experiments because of ensemble averaging or by the broad line width of resonances in resin, respectively, and thus not obvious from these experiments.

The biophysical model that emerged from the previous reports of a largely disordered dimer^{27; 30} suggested multiple transient interfaces, each sampled only part of the time and therefore limiting order in the dimer.²³ Such forces would likely not lead to specific dimerization but rather higher-order oligomers or even liquid-like protein assemblies that have been suggested to be important for controlling signaling events and spatial organization in the cell.^{14; 40; 41} Importantly, weak inter-particle interactions do not lead to discrete dimerization or in fact any discrete oligomerization but should rather be viewed as an attractive potential leading to a large number of polymeric states under certain solution conditions and can indeed cause protein interactions that favor liquid-liquid demixing or crystallization.⁴² Specific dimerization of an IDP is expected to be accompanied by a disorder-to-order transition of its interface leading to differences in NMR spectra for monomer and dimer states of IDPs. Our data demonstrate that ζ_{cyt} and CD3 ϵ do not form highly disordered complexes but are monomers in solution up to millimolar concentrations. Although many protein complexes contain functionally important disorder, this leaves the question open whether completely disordered protein dimers or any completely disordered complexes exist in nature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The cytoplasmic domain of the T-cell receptor ζ chain (ζ_{cyt}) is disordered.
- ζ_{cyt} does not significantly populate a dimer as tested by NMR, AUC and SEC-MALS.
- We observed a concentration dependent shift in the elution volume during SEC.
- This is caused by non-physiological interactions with the chromatography resins.
- ζ_{cyt} does not dimerize through transient interactions without ordering.

Figure 1. ζ_{cyt} ^1H - ^{15}N HSQC spectra under different conditions report on a monomeric state (a) ^1H - ^{15}N HSQC spectra of 10 μM (blue) and 400 μM (red) ζ_{cyt} are highly similar. (b) Comparison of scaled resonance intensities at 10 and 400 μM ζ_{cyt} as a function of residue number show no sequence specific broadening. (c) Paramagnetic relaxation enhancement profiles of spin-labeled ζ_{cyt} show transient intra- rather than inter-molecular contacts. Γ_2 for ζ_{cyt} MTSL-spin-labeled at position -7 (marked by an asterisk) as a function of protein concentration (150 μM , top; 50 μM , middle; 15 μM , bottom) was calculated from intensity ratios of a paramagnetic and diamagnetic sample pair⁴³, the latter obtained by reduction with 10fold excess of ascorbic acid. The red solid lines represent the expected PRE profile for a random coil model of the protein calculated from a 100,000 conformer ensemble generated

with flexible-meccano.⁴⁴ (d) Disulfide-linked ζ_{cyt} shows no evidence of a dimerization interface because HSQC spectra of reduced (cyan) and disulfide-linked ζ_{cyt} (red) are identical except for linker region signals. Resonances unique to Cys- ζ_{cyt} and absent from wildtype ζ_{cyt} are marked by asterisks; one linker serine resonance with chemical shift perturbation upon Cys crosslinking is marked with an arrow. Disulfide-linked ζ_{cyt} was obtained by oxidation with tetrathionate⁴⁵ and subsequent preparative SEC. NMR assignments are taken from Jaravine *et al.*³¹. ζ_{cyt} was in 20mM potassium phosphate pH 6.2, 1mM EDTA, 0.5mM benzamidine, 90% H₂O/10% D₂O. Gradient selected sensitivity enhanced HSQC spectra were recorded at 288K on a Bruker Avance 600 MHz NMR spectrometer with a TCI triple resonance cryogenic probe with 1024x128 complex data points and varying number of scans to account for the different protein concentrations and sensitivity. Data processing was performed in TopSpin (Bruker BioSpin) and spectral analysis in Sparky (Goddard, T.D. and Kneller, D.G. University of California, San Francisco).

Figure 2. ζ_{cyt} and CD3 ϵ are mostly monomeric

AUC sedimentation velocity (a,b,d,e) and sedimentation equilibrium (c,f) experiments were carried out in PBS pH 7.0, 1mM EDTA, 1mM benzamidine for ζ_{cyt} (left) and in PBS pH 7.0 for CD3 ϵ (right). (a,d) Continuous sedimentation coefficient distribution model $c(s)$ and (b,e) two-dimensional size-and-shape distribution analyses of sedimentation velocity data of (a,b) 340 μM ζ_{cyt} and (d,e) 290 μM CD3 ϵ . Shown are contour plots of $c(M, f/f_0)$ of the transformed velocity data with 0 fringes/S (white) to maximum value fringes/S (red), with increasing color temperature indicating higher values. Velocity data were acquired at 20 °C at a rotor speed of 50,000 rpm and indicate only one species in solution close to the monomer M_w .^{34; 35} (c,f). Fringe displacement profiles at equilibrium for 140 μM , 80 μM and

20 μ M and absorbance scans at 280nm for (c) 20 μ M ζ_{cyt} were analyzed globally (for the profiles at all concentrations see Supplemental Figure S2) and absorbance scans at 280 nm for (f) 290 μ M CD3 ϵ are plotted *versus* the distance from the axis of rotation (radius). ζ_{cyt} was centrifuged at 20 °C at increasing speeds of 22,000 (black, 32 hours) and 38,000 rpm (red, 23 hours), CD3 ϵ was centrifuged at 4 °C at increasing speeds of 18,800 (black, 46 hours), 31,800 (red, 38 hours) and 54,000 rpm (blue, 22 hours). The *solid lines* represent the global nonlinear least squares best-fit of all the data sets to a reversible monomer-dimer self-association model yielding K_{Ds} of 6.2 and 8.3 mM, respectively. (See Supplemental Methods for more detailed information.)

Figure 3. The SEC concentration-dependency of ζ_{cyt} and CD3 ϵ is not caused by dimerization
 (a) SEC-MALS profiles demonstrate the monomeric state of ζ_{cyt} despite the concentration-dependency of V_{el} . 100 μl of ζ_{cyt} at 700 μM (red), 140 μM (blue) and 70 μM (green) concentration was injected with an autosampler onto a Shodex KW-803 gel filtration column (with guard column) equilibrated in 20 mM potassium phosphate buffer pH 7, 1 mM EDTA, with 150 mM NaCl and the protein in the eluent detected by UV and a Wyatt DAWN-HELEOS MALS detector with a laser at 658 nm wavelength. SEC-MALS data plotted as a molar mass distribution superimposed on the chromatogram of absorbance at 280 nm as a function of elution volume. (b) 100 μl sample of ζ_{cyt} , CD3 ϵ or Gli3 1-90 at a given concentration from a dilution series was injected onto a Superdex 75 10/300 gel

filtration column in PBS pH 7, 1mM EDTA at 0.7ml/min, its elution volume (V_{el}) monitored and converted into an apparent molecular weight (Mw^{app}) based on calibration with globular protein standards (BioRad Gel Filtration Standard) under given buffer conditions. The solid lines serve to guide the eye. The injection loops were overfilled with 1.5 times their volume to prevent a change in V_{el} due to inconsistent injection. (c) 1H - ^{15}N HSQC spectra of 400 μ M (red) ζ_{cyt} in buffer and (purple) in P-6DG gel filtration resin have nearly identical chemical shifts. Resonances of ζ_{cyt} in resin are broad due to motional restriction under spatial confinement. ζ_{cyt} was in 20mM potassium phosphate pH 6.2, 1mM EDTA, 0.5mM benzamidine, 90% H_2O /10% D_2O . In case of P6-DG, 50mM NaCl was added to prevent non-specific interactions with the resin. The amount of resin required to pack a 500 μ l gel filtration column was hydrated in an excess of ζ_{cyt} solution for several hours, then transferred to a 5mm NMR tube which was centrifuged to pack the resin and remove trapped air bubbles. The supernatant was removed. (d,e) The proteins' apparent Mws were investigated under different salt concentrations. 50 μ l of (c) ζ_{cyt} or (d) CD3 ϵ were injected onto a Shodex KW-803 gel filtration column, the Mw^{app} calculated based on careful calibration under given buffer conditions. Buffers were 20mM potassium phosphate pH 7, 1mM EDTA with NaCl varying between 0 and 1000mM as indicated. (f) SECMAALS profiles as in (a) with 1M NaCl in the buffer.

Table 1

Sedimentation equilibrium analysis of ζ_{cyt} and CD3 ϵ according to a single species, a two discrete species or a monomer-dimer self-association model.

Sample	concentration [μM] ^a	Mw ^b [kDa]	rmsd ^c	Mw ^d [kDa]	rmsd ^c	K _D [mM] ^e	rmsd ^c
ζ_{cyt}	20, 80, 140	14.712	0.0065	28.013	0.0051	6.2	0.0061
CD3 ϵ	290	6.689	0.0037	17.090	0.0033	8.3	0.0043

^aTotal protein loading concentrations in μM , proteins in PBS pH 7, 1mM EDTA, 1mM benzamidine.

^bSingle species model with mass the average molecular mass.

^cRoot mean square deviation with units in interference or absorbance and for the global analysis the average rmsd of all the datasets.

^dMolecular weight of the second species in a two-discrete-species model, first species fixed to 13.157 kDa; the fraction of the second species is $\sim 0.5\%$.

^eDissociation equilibrium constant K_D for the reversible monomer-dimer self-association model; the M_w is fixed to the theoretical value.