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Convergence of Experimental, Computational and Evolutionary Approaches Predicts the Presence of a Tetrameric Form for CD3-ζ

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Experimental results using multiple site-specific infrared dichroism have shown that, when reconstituted into lipid bilayers, the orientation of the transmembrane domain of CD3-ζ is not compatible with a dimeric right-handed model reported previously. This model, obtained using a computational approach that uses evolutionary data, is in agreement with mutagenesis data and homology modelling. This suggested that, in our experimental conditions, the oligomeric state of CD3-ζ may not be dimeric. We have explored this possibility by performing global searching molecular dynamics simulations assuming different homo-oligomeric sizes (from 2 to 6). In these simulations, the helix tilt was restrained to the average helix tilt obtained experimentally, 12°. Only a left-handed tetrameric model was compatible with the experimentally observed tilt and rotational orientation of the helix, and was also the lowest-energy model amongst the candidate structures obtained. Furthermore, simulations performed using close homologues demonstrate that this model is compatible with evolutionary conservation data. Finally, the pattern of residue conservation in the ζ family of proteins strongly argues in favour of the presence of a left-handed hetero-oligomer with an orientation compatible with the tetramer we present. These results show that both the known dimeric and the so far undetected tetrameric form may be of functional importance in the cell.

Keywords: membrane proteins; molecular dynamics; molecular modelling; site-specific infrared dichroism; CD3-ζ

Introduction

The CD3-ζ chain, one of the invariant subunits of the T-cell receptor (TCR), is known to form disulphide-linked dimers in SDS-PAGE, where the two monomers associate *via* their transmembrane domains.[1] It is thought that CD3-ζ contains a glycine-based dimerization motif similar to that of glycophorin A (gpA).[2] Recently, we have obtained a model consistent with this hypothesis using a new method[3] to select structures that originate from a global searching molecular dynamics simulation.[4,5] The method is based on the hypothesis that silent amino acid substitutions, present in variants identified from evolutionary conservation data or mutagenesis analyses, do not affect the stability of a native structure but may destabilize the non-native structures also found. Modelling CD3-ζ as a homodimer, a right-handed bundle was identified[3] that was consistent with predictions based on mutagenesis, gpA-homology modelling, and the presence of a disulphide bond.[1]

As no experimental data exists to confirm this model, we used (see the accompanying paper[6]) site-specific infrared dichroism,[7] labelling at 11 different residues along the transmembrane helix with a modified peptidic carbonyl 1-$^{13}C{=}^{18}O$. Surprisingly, the experimental data were incompatible with the previously suggested model.[3]

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Abbreviations used: FTIR, Fourier transform infrared spectroscopy; ATR, attenuated total internal reflection; RMSD, root-mean-square deviation; CNS, Crystallography & NMR System; CHI, CNS searching of helix interactions; Fmoc, 9-fluorenylmethoxycarbonyl HFIP, hexafluoroisopropanol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; TFA, trifluoroacetic acid; SSID, site-specific infrared dichroism.

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In this respect, although it is known that CD3-ζ forms disulphide-linked homodimers in SDS-PAGE,[1,8] it is possible that they could be present in other oligomeric states in native conditions, which, as in our work, involve the presence of a lipid bilayer. This suggests two possible explanations for the inconsistency described above. The first is that the data we have obtained from site-specific infrared dichroism (SSID) corresponds to a dimer or a higher-order oligomer that is not present in native conditions. The second is that the data correspond to a higher-order oligomer that is present in native conditions and has not been detected. Note that the presence of a native dimer in our preparation would be in conflict with data presented previously (see above and the accompanying paper).

We have tested the above hypotheses using a combination of methods based on global searching molecular dynamics simulations.[4,5] This computational method produces a number of low-energy structures that may contain the correct structure, which can then be selected based on existing experimental data or evolutionary data. For example, past studies have used mutagenesis, as in dimerizing glycophorin A[9,10] or pentamerizing phospholamban,[11] or orientational data from SSID, as in M2[12] and phospholamban.[13] Recently, we have shown[3] that the correct model can also be selected with a purely computational approach using simulations performed for close homologues (see above). We have observed for M2 and for Plb (unpublished results) that the correct model can be selected using energy criteria, i.e. the correct model is also the lowest-energy model for each one of the homologues used in the simulations. We have observed this to be true (not shown) for the right-handed dimeric CD3-ζ[3] and for the trimeric invariant chain from the MHC class II (unpublished results).

However, none of these strategies would be useful if a model with the correct orientation was not present amongst the low-energy structures found during the simulation. We have observed for M2 (not shown), a homotetramer from the *Influenza A* virus, that this can be ensured by restraining the helix tilt to an experimentally observed value.

Herein, we have used the fact that we have obtained experimental data for the CD3-ζ transmembrane domain,[6] which include the average helix tilt and the local helix tilt at various points along the helix. However, in order to explore the different structures arising from increasing oligomeric sizes, we have not restrained the local helix tilt because in our present set-up, this is not possible without restraining as well the orientation of the helix. This, by definition, would bias the search towards certain rotational orientations and would alter the relative energy of the candidate structures. Therefore, the simulations using either different oligomeric sizes or a given oligomeric size for different homologues were performed while restraining the global, average, helix tilt found previously.[6]

CD3-ζ forms disulphide-linked homodimers in SDS-PAGE.[1,8] In a lipid bilayer, however (our experimental system), any oligomerization state is in theory possible. Consequently, we investigated alternative oligomeric states by performing molecular dynamics global searches assuming increasing oligomeric sizes, from dimer to hexamer. As stated above, however, unless the helix tilt is restrained to an experimentally determined value, this method, which produces a number of low-energy structures, does not always contain the correct model. Also, as we have observed for M2 and Plb (not shown) that this model should be the one with lowest energy. We have used these tools to examine which of the simulations using different oligomeric sizes produces a structure that is in agreement with the data and that is the lowest-energy structure for a particular oligomeric size.

Finally, one should keep in mind when analysing the structure of CD3-ζ that it is part of the large TCR multimeric structure. The implications of this fact are elaborated below.

Results and Discussion

The results for the global searching molecular dynamics simulations using different oligomeric sizes are represented in Figure 1. This Figure (left panel) shows that the results from the dimeric simulation do not contain any clusters with the experimentally observed orientation (see the broken line). The clusters that are closest ($\omega_{V44} \sim 200^\circ$, right-handed) are clearly not the lowest-energy ones (see right panels).

In the simulation performed with a trimeric arrangement, some right-handed structures (filled circles) are in good agreement with the data, although the energy of these structures is not very different from that of the other structures (right panel). One of the tetramers in contrast, has an orientation compatible with the experimentally observed one (see the broken line) and has, at the same time, the lowest energy amongst the candidate structures (see the right-hand panel, structure inside a box). For the simulations performed using pentamers and hexamers, neither of these conditions was met.

We note that, for the dimeric arrangement, because an orientation compatible with the data was not found when restraining the helix tilt to the experimentally observed value, an artifactual dimeric structure is very unlikely. Therefore, based on these results, only tetramers and perhaps trimers could account for the experimental data.

Use of close homologues

In order to assess these possibilities, we used the fact that a native model, unlike an artifact, should not be destabilized by any of the conservative mutations present in close homologues.[3] In addition, this model should be the lowest-energy cluster in each of the simulations performed for each of the close homologues. Therefore, we used the homologous sequences in Figure 9, restraining

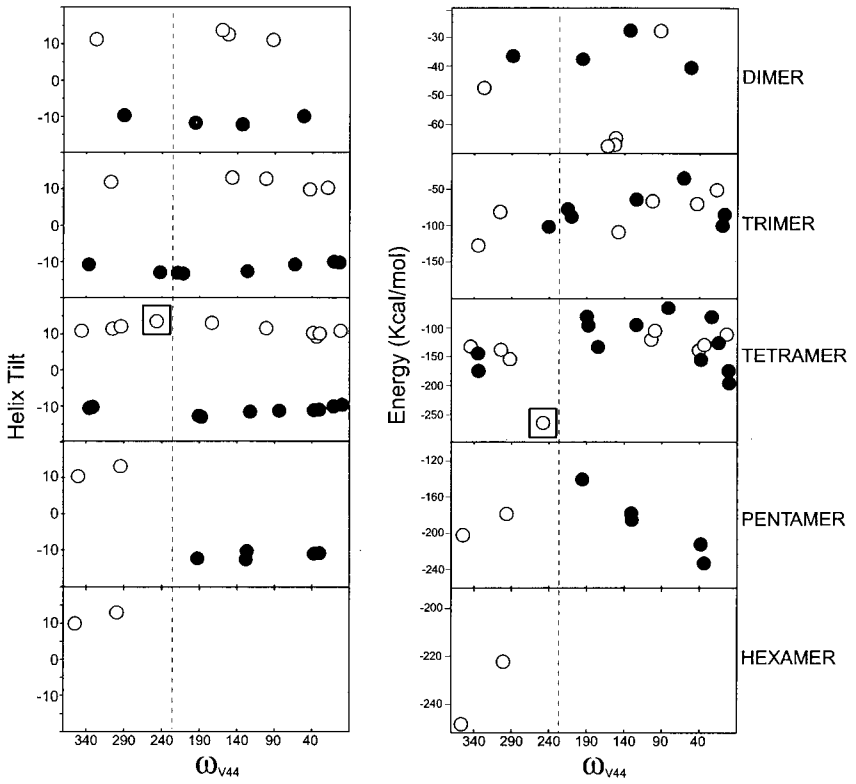

Figure 1. Distribution of clusters from global searches assuming dimers, trimers, tetramers, pentamers or hexamers, restraining the helix tilt to the average experimental helix tilt, 12°. Left panel: clusters are plotted as a function of their orientation, represented by ω_{V44}, and the helix tilt (negative and positive crossing angles represent right and left-handed bundles, respectively). The structures that are left and right-handed are represented by open and filled circles, respectively. Right panel: clusters are plotted as a function of their orientation, represented by ω_{V44}, and their energy. The value for ω_{V44} found experimentally[6] is represented by a broken line (at 224°). The tetrameric structure that is in agreement with the data and also the lowest energy one is indicated inside a box.

the helix tilt to the experimentally determined value, to simulate either trimers or tetramers.

Figure 2 shows the results of these simulations for the trimeric arrangement. In fact, a ''complete set'', i.e. a set of similar structures pertaining to each of the homologues (broken line) exists that is compatible with the experimental data (see crosses). However, the structures that belong to this set are clearly those with highest energy (lower panel). In contrast, for the simulations performed assuming a tetrameric arrangement (see Figure 3), not only was a complete set found at an orientation compatible with the experimental data (ω_{V44} of ca 245°), which is less than 20° away from the experimentally determined value of 224(±11)°, but also the structures in this set are the lowest-energy structures in each of the simulations (lower panel).

We conclude therefore that these are strong indications that CD3-ζ forms a tetramer in our system, and that it is not an artifact of our preparation, as this orientation and oligomeric size are compatible with evolutionary conservation data. We note that a simulation performed without restraining the helix tilt did not produce any complete set for the tetramer or the trimer.

Incorporation of structural restraints into a molecular dynamics simulation

Once a tetrameric model was selected that was in good agreement with both the orientation as well as the evolutionary conservation data, another simulation assuming a tetrameric arrangement, but incorporating all the orientational restraints from SSID obtained in the accompanying paper,[6] was performed in order to obtain a more accurate description of this tetrameric structure. The restraints used (calculated as described in Methods), are represented in Table 1.

Figure 4 shows the result of this simulation for left-handed (left panel) and for right-handed (right panel) structures. Clearly, the left-handed structures, in addition to being confined to a relatively

Table 1. Orientational restraints incorporated in a molecular dynamics simulation obtained from the parameters ω and local β for each labelled residue (see Methods)

Residue	∠C═O/z-axis (deg.)	∠C—C_α/z-axis (deg.)
34L	16	126
35L	8	106
38I	18	123
39L	28	120
41I	16	128
43G	21	125
44V	22	133
45I	10	121
46L	18	117
48A	22	134
49L	-	-

∠C═O/z is the angle between the C═O bond and the z-axis, while ∠C—C_α/z is the angle between the C—C^α bond and the z-axis.

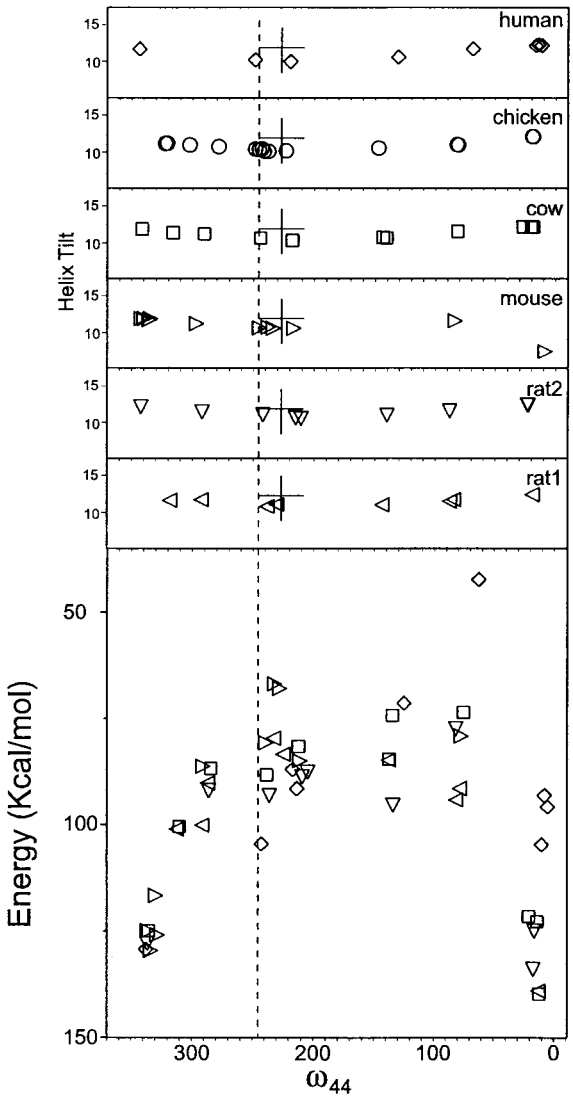

Figure 2. Results from global searches restraining the helix tilt to the experimental 12° (see the accompanying paper[6]), assuming a right handed trimer and using sequences from different species; human (◇), chicken (○), cow (□), mouse (▷), rat 2 (▽) and rat 1 (◁). The cross indicates the average experimentally determined values, i.e. $\omega_{V44} = 224°$ and $\beta = 12°$. The broken line indicates the value predicted from the simulations, i.e. $\omega_{V44} = 242°$.

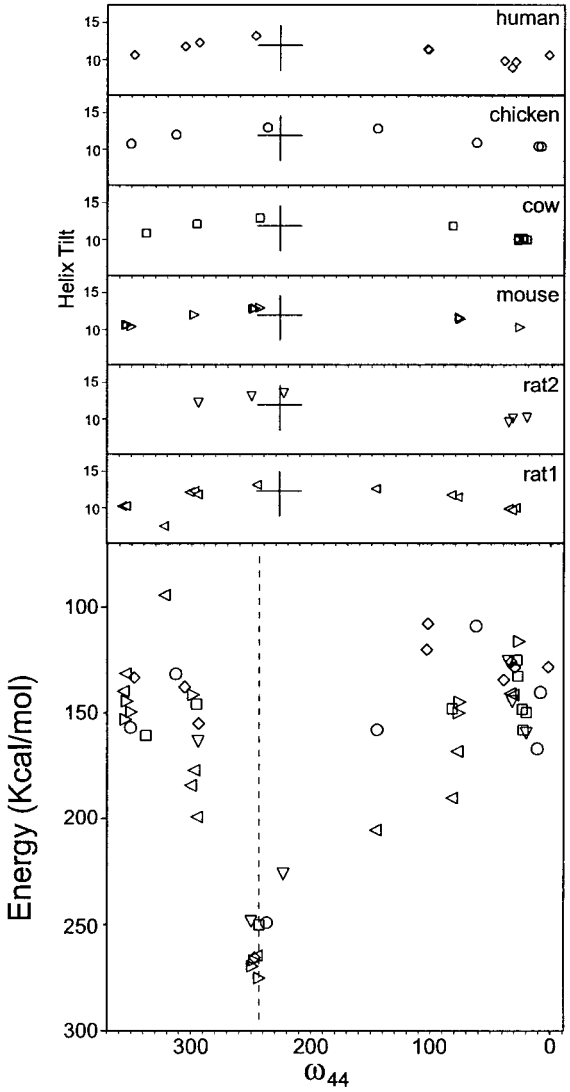

Figure 3. Results from global searches restraining the helix tilt to the experimental 12° (see the accompanying paper[6]), assuming a left-handed tetramer and using sequences from different species; human (◇), chicken (○), cow (□), mouse (▷), rat 2 (▽) and rat 1 (◁). The cross indicates the average experimentally determined values, i.e. $\omega_{V44} = 224°$ and $\beta = 12°$. The broken line indicates the value predicted from the simulations, i.e. $\omega_{V44} = 242°$.

small range of ω angles, have lower energy than the right-handed bundles (right panel). Note that in previous work,[12,13] the correct model was selected amongst a group of low-energy structures that could have any possible orientation ω, according to how compatible they were with the experimental data, and it was difficult to determine if the bundle was right or left-handed. Here, application of these restraints unequivocally points to a left-handed structure and a range of possible orientations (~70°), which is very small.

Table 2 compares the ω angle and local helix tilt for each labelled residue obtained experimentally using multiple site-specific dichroism[6] and those for the lowest-energy, left-handed tetrameric model in Figure 4. The results are very similar, showing that we can effectively restrain both orientation and helix tilt in a transmembrane domain.

Slices through this tetrameric model are shown in Figure 5. The centre of the bundle is lined by residues D36, L9, L16 and L19, and there is no obvious pore. We note that the α carbon atoms at residue G32 in the two monomers are separated by ~7.5 Å, which allows for the formation of a disul-

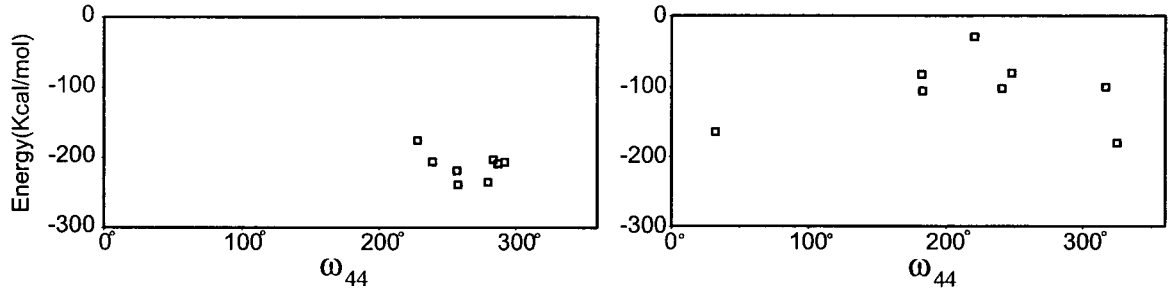

Figure 4. Results from a global searching molecular dynamics simulation for left-handed (left panel) and right-handed (right panel) bundles in which the restraints in Table 1 were incorporated.

phide bridge between any two monomers in the tetramer (G32 was substituted for C32 in the simulations and in the experiment). We also note that the production of an artifactual tetramer due solely to this C32G substitution is extremely unlikely, as little change in surface expression of TCR was observed when C32G CD3-ζ was used.[2]

The residues important for tetramerization are evolutionarily conserved

If CD3-ζ, as these results seem to suggest, can associate both as a homodimer in SDS and as a homotetramer in a lipid bilayer, we should expect that, in general, the non-conserved residues in the homologous sequences used in the simulations (see Figure 9) should not possess a high interaction energy in either the dimeric or the tetrameric forms. Conversely, residues with high interaction energy in the dimer or in the tetramer should be evolutionarily conserved. Figure 6 shows that, in general, the residues with high energy of interaction for (a) the right-handed dimer and the left-handed tetramer and (b) the non-conserved residues do not coincide. Residue 51, however, has a high interaction energy in the dimer, although a change is observed only in the chicken, which is the more divergent. The tetramer shows highly interactive residues at positions 46 and 50 although again, these mutations do not seem particularly disruptive.

We then used the fact that CD3-ζ is known to associate with other members of the TCR ζ family of proteins,[8] constituted by three proteins, ζ, η and the γ subunit of the high-affinity immunoglobulin ε receptor (Fcε-γ). These proteins have a high degree of homology, and ζ and η are identical in the extracellular and transmembrane domain.

To test if these other members of the ζ family associate in a similar way with CD3-ζ, we conducted simulations using either a Fcε-γ homodimer or a Fcε-γ homotetramer (not shown). As expected, it was found for the dimer that possessed the lowest-energy cluster coincided with a right-handed dimer analogous to that reported for CD3-ζ.[3] For the tetramer, the lowest-energy cluster was a left-handed bundle with the same orientation as that of the tetramer reported here.

We then hypothesized that if CD3-ζ and Fcε-γ form homodimers and homotetramers, they may be able to form heterotetramers, i.e. with a ζ and a Fcε-γ homodimer. If this was the case, similarly to CD3-ζ (see above), residues that are non-conserved when comparing the sequences of the two different members of the family, i.e. ζ and Fcε-γ, should not be coincident with highly interactive residues in the tetramer. Only sequences Fcε-γ corresponding to human, rat and mouse are available. This comparison, however, was carried out only between

Table 2. Comparison between the angles ω and β obtained experimentally for each of the labelled residues and the predicted ω in the lowest-energy left-handed tetrameric structure in Figure 4

Residue	$\omega_{experimental}$ (deg.)	$\omega_{tetramer}$ (deg.)	$\beta_{experimental}$ (deg.)	$\beta_{tetramer}$ (deg.)
34	295 ± 6	287	14 ± 2	14
35	22 ± 5	18	20 ± 2	20
38	300 ± 10	340	19 ± 5	19
39	110 ± 5	64	19 ± 6	15
41	280 ± 12	293	7 ± 2	11
43	120 ± 9	109	8 ± 1	8
44	224 ± 11	230	8 ± 1	5
45	330 ± 6	335	8 ± 2	10
46	77 ± 10	58	12 ± 2	13
48	234 ± 14	260	9 ± 2	-
49	342 ± 30	346	-	-

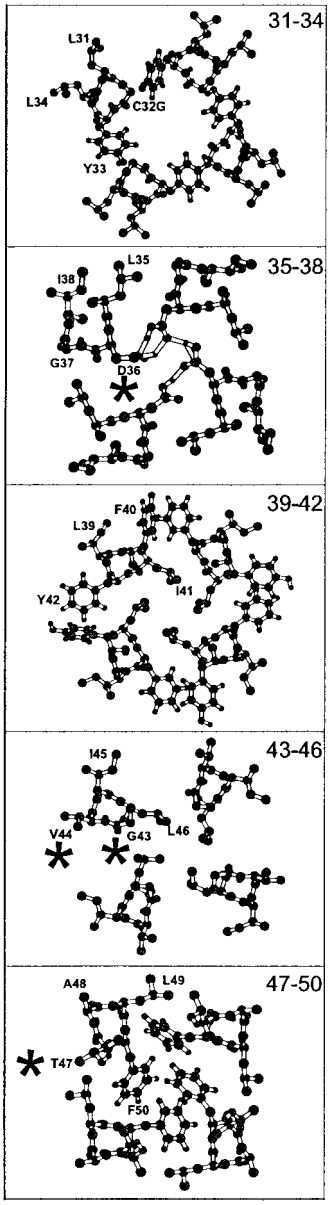

Figure 5. Ball-and-stick representation of slices through the CD3-ζ homotetramer. The atoms are represented at 0.25 of the van der Waals radii. The residue numbers are indicated in the top right-hand corner. For clarity, the bonds connecting the backbone atoms are coloured in grey. Backbone N and C^{α} are black and hydrogen atoms are white. Non-polar hydrogen atoms are not represented. The molecular graphics were generated with MOLSCRIPT.[18]

the sequences of CD3-ζ and Fcε-γ for human and mouse because the sequence corresponding to rat contains similar mutations relative to mouse and did not provide additional information.

Figure 7 shows that none of the residues with high energy of interaction in the tetramer (filled circles) coincides with non-conserved residues (see boxes). Note that although residue 46, which has a high interaction energy in the tetramer, is conserved comparing the human sequences, it presents some variability in the mouse sequence. Similarly, it is not conserved when comparing CD3-ζ sequences (Figure 6(b)), which suggests that at this position some variability is allowed both when the tetramer is formed by the CD3-ζ homotetramer or a CD3-ζ/Fcε-γ heterotetramer. Finally, the pattern of conservation when comparing the CD3-ζ/Fcε-γ sequences is somewhat incompatible with the existence of CD3-ζ/Fcε-γ hetero-dimers analogous to those described for the CD3-ζ right-handed homodimer.[3] Residues 44 and 51, which are clearly non-conserved when comparing these two sequences, possess high interaction energy in the dimeric structure (open circles). As residue 44 has been suggested to be part of a dimerizing motif,[2] this suggests that a putative hetero-oligomer would not possess the same structure as the previously suggested dimer.[3] An alternative explanation, however, is that the change V44I might have been compensated with another change, I45V, which is present in the Fcε-γ sequences in human and mouse, and in the sequence of rat (accession number P20411) with sequence LCYILDAILF LYGIVLTLLYC.

Handedness

If the right-handed dimer[3] and the left-handed tetramer (this work) are both correct, the distribution of the non-conserved residues for CD3-ζ around a helical wheel should be compatible with either hand. The same should happen by looking at the CD3-ζ/Fcε-γ comparison if right-handed heterodimers and heterotetramers are possible. As not enough sequences are available for Fcε-γ, we could not test this hypothesis for putative right-handed Fcε-γ homodimers or putative left-handed Fcε-γ homo-tetramers.

Figure 8 (top) shows that the non-conserved residues (boxes) when comparing CD3-ζ sequences (Figure 6) are located on one side of the helix for a right-handed bundle (top left), with the residues with high interaction energy in the dimer (open circles) on the other side of the helix. Further, the non-conserved residues are not incompatible with a left-handed orientation (top right).

Also, Figure 8 (bottom) shows that when comparing the CD3-ζ and Fcε-γ sequences (Figure 7), the non-conserved residues (boxes) group preferentially according to a left-handed bundle (bottom, right). This suggests that a right-handed CD3-ζ/Fcε-γ heterodimer is not likely, although a left-handed dimer with the same orientation is still possible. Incidentally, such a dimer was identified in Figure 1, which possessed the same inter-helical interacting face as the previously proposed model, i.e. at 160°, and was clearly the lowest-energy cluster in the simulation that used dimers (Figure 1, top panel, right). Further, the residues that have high interaction energy in the tetramer (filled circles) and the residues non-conserved are located

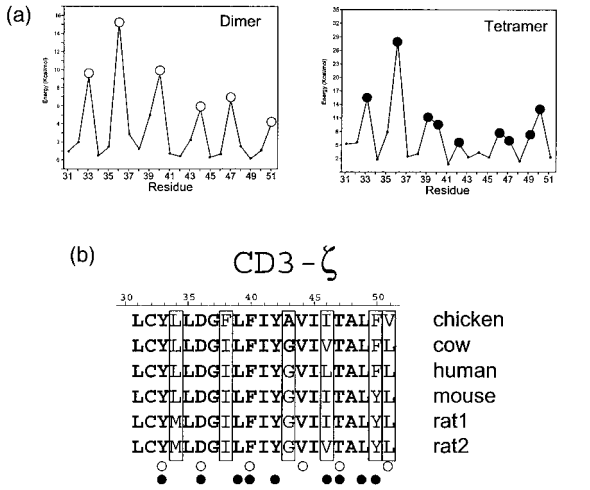

Figure 6. (a) Interaction energy per residue for the the dimeric model reported previously[3] and the tetrameric model reported here. The residues with higher interaction energy are represented with open (dimer) or filled (tetramer) circles. (b) Comparison between the transmembrane sequences CD3-ζ for the homologues used in the simulation. The non-conserved residues are inside a box. The residues with high interaction energy in (a) are indicated.

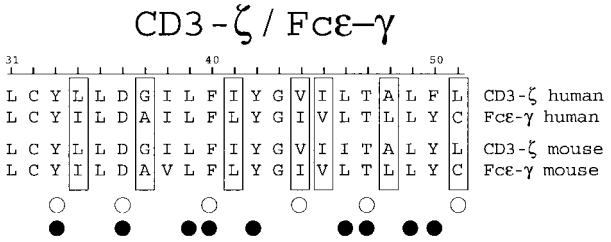

Figure 7. Comparison between the transmembrane sequences of Fcε-γ and ζ (right) for human and mouse. The residues that change for both species are inside a box. Non-conserved residues are inside boxes and residues with higher energy interaction in the dimer and tetramer are represented by open and filled circles, respectively.

in the opposite faces of the helix (bottom, right). This shows that any heterocomplex between CD3-ζ and Fcε-γ is most likely left-handed.

Overall, these results suggest strongly that a left-handed structure, either a dimer or a tetramer, exists when a hetero-oligomer is formed. Also that when homodimers or homo-tetramers exist, they should be right-handed or left-handed, respectively. In any case, a number of possibilities exist when transmembrane domains of the ζ family interact, and we have shown that a left-handed tetramer, either a heterotetrameric CD3-ζ/Fcε-γ or homotetrameric CD3-ζ, is one of them.

It should remain clear that the left-handed tetrameric structure that we propose is in addition to the previously proposed dimeric model.[3] It is possible that, in the absence of interactions with CD3 chains or αβ chains, the predominant oligomer is a tetramer. Association with other TCR subunits or other proteins or even phosphorylation, could trigger a change in conformation, leading to the formation of a gpA-like homodimer, like that suggested previously.[2,3] The cytoplasmic tail of CD3-ζ contains three so-called immunoreceptor tyrosine activation motifs (ITAMs) that are phosphorylated upon TCR engagement.[19] Phosphorylation in turn permits association with proteins containing an Src homology 2 domain and subsequent downstream signalling events. The ability of CD3-ζ to form alternatively dimers and tetramers could be essential in its signal transduction role.

Conclusion

The study described above attempts to consolidate two streams of data into a single model for the transmembrane complex of TCR CD3-ζ. A single left-handed tetrameric structure is found to be compatible with the orientation restraints based on SSID as well as the evolutionary homology data.

Methods

Global search molecular dynamics

All calculations were performed using PCNS, the parallel-processing version of the Crystallography and NMR System (CNS Version 0.3),[14] with the OPLS parameter set and united atom topology,[15] explicitly describing only polar and aromatic hydrogen atoms. A global search was carried out *in vacuo* as described elsewhere, using CHI (CNS searching of helical interactions), assuming a symmetrical interaction between the helices in the homo-oligomer.[5] Calculations were carried out using the following sequences corresponding to the CD3-ζ transmembrane domain (Figure 9): human (p20963), rat1 (L08447), rat2 (D13555), mouse (P24161), chicken (AJ002317) and bovine (jc4664) which consisted of residues 31–51.

The mutation C32G was present in the simulations to facilitate the global search, as this silent mutation does not dramatically reduce cell-surface expression of the TCR.[2] The aspartate residue was modelled in its protonated form, as would be expected based on energy considerations.[16] Trials (see below) were carried out starting from both left and right crossing angles (-25° or $+25^\circ$), with the helices rotating 360° about their helical axes in 20° increments. Four trials were carried out from each starting configuration using different initial random velocities, i.e. 288 trials in total.

Each trial consisted of a molecular dynamics and energy minimization protocol as such: (i) 100 steps of minimization were undertaken with electrostatic interactions turned off and the REPEL function turned on to rapidly remove any steric clashes. (ii) Subsequently, 500 steps of standard minimization were undertaken, followed by (iii) 5 ps of a molecular dynamics simulation at

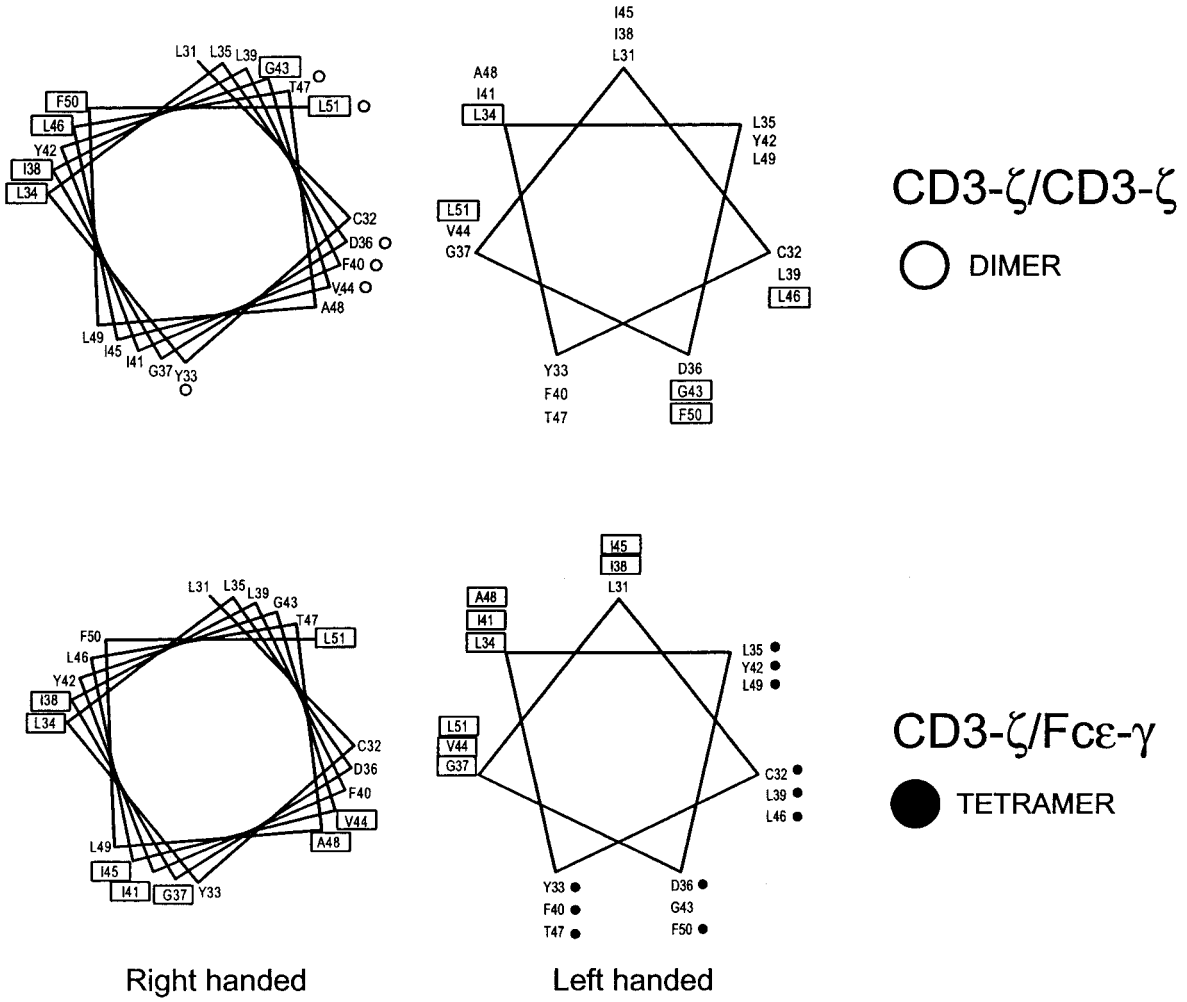

Figure 8. Helical wheel representations for left-handed and right-handed α-helical bundles that depict the non-conserved residues when comparing the CD3-ζ sequences (top) and the CD3-ζ/Fcε-γ sequences. Non-conserved residues are inside boxes and residues with higher energy interaction in the dimer and tetramer are represented by open and filled circles, respectively.

600 K; (iv) and an additional 5 ps at 300 K. (v) Finally the structures were subjected to 250 steps of energy minimization. The energy of each structure was calculated taking into account standard bonded and non-bonded terms, as implemented in CNS.

Clusters of output structures were identified, containing five or more structures within 1.0 Å C^{α} RMSD from any other structure within the cluster. Consequently, some clusters overlap, and output structures may be members of more than one cluster. The output structures in a cluster were averaged and subjected to a further simulated annealing protocol, as in the initial search (see above). This average was taken as representative of the cluster. Simulations assuming a parallel trimeric, tetrameric, pentameric or hexameric oligomerization were conducted in the same way.

The sequences of human and mouse for CD3-ζ were compared to the corresponding sequences of the γ subunit of the high-affinity immunoglobulin ε receptor (Fcε-γ), with OWL accession numbers p30273 and p20491, respectively.

Analysis of the simulations

To analyse the data, the structures identified in the simulations were plotted against the rotational pitch angle ω of an arbitrarily specified residue in CD3-ζ, in

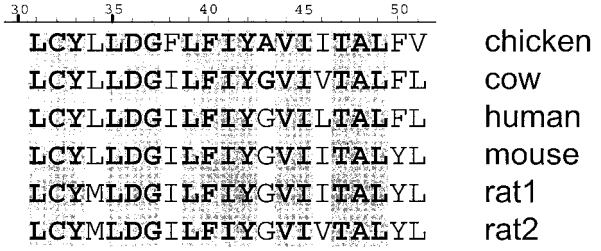

Figure 9. Sequences of the transmembrane domain of CD3-ζ used in the simulations. The accession numbers for each of the sequences are given in Methods. The conserved residues in all homologues are in grey shading.

this case V44. The angle ω (see the accompanying paper[6]) represents the angle between a vector perpendicular to the helix axis, oriented towards the middle of the C═O bond, and a plane that contains both the helical axis and the normal to the bilayer. This angle is defined as 0° in the direction of the helix tilt. The helical axis is calculated as a vector with starting and end points above and below the defined residue. These points correspond to the mean of the coordinates of the five α carbon atoms N-terminal and the five α carbon atoms C-terminal to the defined residue, respectively. In the simulations, the handedness of the bundle is represented by the crossing angle between consecutive helices, i.e. positive or negative for left or right-handed bundles, respectively. The overall helix tilt is computed as the average of the angles between each helix axis and the normal to the bilayer.

Use of experimentally derived restraints

For simulations where the helix tilt was restrained, the angle between the vectors connecting every C^{α} of residue i and C^{α} of residue $i+7$ and the z-axis was restrained to β, the average experimental value obtained for the helix tilt in the accompanying paper, as described.[13] The values for the local β and ω for a particular residue were used to obtain restraints to be introduced in molecular dynamics simulations. These restraints were orientation refinement energy terms that depend on the angle θ between a particular bond and the z-axis, i.e.:

$$E = k_{\text{dichro}}(\theta - \theta_0)^2 \quad (1)$$

where θ represents the actual angle and θ_0 represents the target angle. The overall weight for these orientational constraints was chosen to be $k_{\text{dichro}} = 800$ cal/grad^2, determined empirically.[12]

In this case, we used the carbonyl C═O bond and the bond formed between the carbon in C═O and the α carbon atom of a particular residue. The angles between these two bonds and the z-axis were restrained to values obtained using the local β and ω obtained experimentally for the particular residue, and α and δ for the particular bond (see Figure 1 of the accompanying paper). The angle α and δ used in the calculations were 16° and 0° for the C═O bond, and 135° and 35° for the C—C^{α} bond, respectively. The angles δ and α between the bond C—C^{α} and the C═O bond and the helix axis were calculated using an α-helical model in which the helix axis coincides with the z-axis, using the function CREATE in CHI (CNS, Crystallography and NMR System (CNS Version 0.3)[14]). The angle between the C═O bond and the helix axis is also 16° when calculated from the data reported by Marsh & Pali.[17]

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