Len_M4L/Y27dD/T94H

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Introduction

While trying to delineate the effects of point mutations in protein Len, an immunoglobulin variable domain (V_L), we found that when some of the single mutations that individually stabilize the protein are put together, their contribution to stability of the protein could be additive. The triple-mutant Len_M4L/Y27dD/T94H is one such case, increasing the thermodynamic stability of native Len from 7.7 kcal/mole to 12.1 kcal/mole. In this study, we have crystallized and determined the structure of this triple mutant.

Methods and Materials

Len_M4L/Y27dD/T94H was prepared as reported earlier [1] and crystallized by the hanging-drop vapor-diffusion method. X-ray diffraction data were collected initially on an R-Axis IIc to a resolution of 2.6 Å and then later to a higher resolution of 1.5 Å at the Structural Biology Center's (SBC-CAT) 19-ID beamline at the Advanced Photon Source (APS).

Results

The triple-mutant Len_M4L/Y27dD/T94H was crystallized from 20% PEG4000, 10% 2-propanol, and 0.1 M HEPES (pH = 7.5). The crystals contained one V_L dimer per asymmetric unit with unit cell dimensions of a = 62.5 Å, b = 105.2 Å, and c = 42.3 Å in space group $P2_12_12$. The structure was determined by molecular replacement method with x-ray diffraction data collected on R-Axis IIc detector. The native protein Len [Protein Data Bank (PDB) code 1lve; dimer generated by crystallographic operation and atoms beyond CB removed for the mutated residues] was used as the search model. The final structure had an R-factor of 21.1% and R-free of 23.8% for 8.0–1.5 Å data with 361 water molecules included. The Ramachandran plot contained 90% of the residues in most-favored regions. The coordinates are deposited in the PDB (code 1EEQ).

The structure of the V_L domain in both the triple-mutant and the native rLen are very similar with rms deviations between α-carbon positions of 0.55 and 0.63 Å, respectively, for each monomer in the triple mutant. The electron density for the mutated residues are well defined in both monomers. Asp27d (OD1) forms hydrogen bonds with the backbone nitrogen and the side chain of Ser27f in both monomers. In monomer one it also forms a hydrogen bond with the peptide nitrogen of residue 28. Within each monomer, the ring of His94 is approximately parallel to the ring of Tyr96 with partial overlap of the two rings. Leu4 occupies the same space as Met4 in the native structure. The CD2 atom of leucine is in approximately the same position as the sulfur atom of the methionine.

The dimer structure in the triple mutant is different compared to that of the native. When one of the domains of the triple mutant is superimposed on the native, a rotation of 11° and a translation of 2.5 Å are required to superimpose the second domains. The difference in the dimer structure is caused by the introduction of extra interdomain hydrogen bonds in the triple mutant. In the triple mutant, Glu55 forms a salt bridge with His94 on both sides of the dimer. In addition, each Glu55 forms a hydrogen bond with peptide nitrogen of Tyr96.

Discussion

We have constructed and determined the structure of a stabilized immunoglobulin variable domain. Three mutations were introduced that were previously found to be stabilizing as single-site mutations. The mutations M4L, Y27dD, and T94H are distant from each other in the three-dimensional structure and their contributions to stability are additive.

For entropic reasons, a methionine-to-leucine substitution in the interior of a protein is expected to be stabilizing. The Asp residue at 27d forms new hydrogen bonds that would not be possible when a Tyr is present at this position. The contribution of His94 to the domain stability is not very clear. The His94 forms stacking interactions with Tyr96 of the same domain; however, it also forms a salt bridge with Glu55 across the dimer interface. The Glu55 is also involved in a hydrogen bond with peptide nitrogen of Tyr96 across the dimer interface. There are also some stacking interactions between His94 of one monomer and Tyr49 of the other monomer. The dimerization constant of the triple mutant is 400 fold higher than that of the native.

In conclusion, single-site mutations in a protein that are individually stabilizing can be put together if they are separated enough in the three-dimensional structure of the protein and result a super stable protein.

Acknowledgments

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References

[1] P. Wilkins Stevens, R. Raffen, D.K. Hanson, Y-L. Deng, M. Berrios-Hammond, F.A.Westholm, M. Eulitz, R. Wetzel, A. Solomon, M. Schiffer, and F.J. Stevens, *Prot. Sci.* **4**, 421–432 (1995).

[2] P.R. Pokkuluri, R. Raffen, L. Dieckman, C. Boogard, F. J. Stevens, and M. Schiffer, manuscript in preparation.