Selection of a Novel and Highly Specific Tumor Necrosis Factor α (TNFα) Antagonist

INSIGHT FROM THE CRYSTAL STRUCTURE OF THE ANTAGONIST-TNFα COMPLEX

Povilas Byla, Mikkel H. Andersen, Thor L. Holtet, Helle Jacobsen, Mette Munch, Hans Henrik Gad, Hans Christian Thøgersen, and Rune Hartmann

Inhibition of tumor necrosis factor α (TNFα) is a favorable way of treating several important diseases such as rheumatoid arthritis, Crohn disease, and psoriasis. Therefore, an extensive range of TNFα inhibitory proteins, most of them based upon an antibody scaffold, has been developed and used with variable success as therapeutics. We have developed a novel technology platform using C-type lectins as a vehicle for the creation of novel trimeric therapeutic proteins with increased avidity and unique properties as compared with current protein therapeutics. We chose human TNFα as a test target to validate this new technology because of the extensive experience available with protein-based TNFα antagonists. Here, we present a novel and highly specific TNFα antagonist developed using this technology. Furthermore, we have solved the three-dimensional structure of the antagonist-TNFα complex by x-ray crystallography, and this structure is presented here. The structure has given us a unique insight into how the selection procedure works at a molecular level. Surprisingly little change is observed in the C-type lectin-like domain structure outside of the randomized regions, whereas a substantial change is observed within the randomized loops. Thus, the overall integrity of the C-type lectin-like domain is maintained, whereas specificity and binding affinity are changed by the introduction of a number of specific contacts with TNFα.

Tetranectin belongs to the large class of C-type lectins characterized by a common fold known as the C-type lectin-like domain (CTLD) (1). Tetranectin is a homotrimeric human protein found in both plasma and tissue. This protein binds the lysine-binding kringle domains from apolipoprotein A (2), plasminogen (3), and angiostatin (4). Tetranectin is a 60-kDa protein built from a structural unit composed of three identical chains, each with a CTLD domain located C-terminally to a trimerizing coil-coil region (5). The CTLD domains retain their structural integrity as separate protein domains, (6, 7) and, moreover, it was shown that their binding to the known tetranectin ligand, plasminogen kringle-4, exhibits the same thermodynamic parameters, irrespective of whether it was analyzed in the form of free monomeric domains or as tethered domains in the complete homotrimeric protein (3). In addition, the thermodynamic analysis showed that the formation of the trimer led to an apparent 100-fold affinity increase, which most likely is due to the avidity effect caused by the three-fold clustering of CTLD domains in the complete protein. Comparison of ensembles of natural CTLD domains for which the known structure and ligand specificity are known shows that the ligand-binding site can accommodate a diverse range of ligands. We therefore concluded that the tetranectin CTLD might be a useful scaffold for designing novel protein therapeutics. We could change the sequence of loops within the CTLD scaffold in the monomeric version without perturbing the overall structure. Thus, CTLD serves as an efficient starting point for the in vitro selection of a high affinity antagonist with a low immunogenicity. In this procedure, we use the monomeric CTLD domain during the in vitro selection procedure but use the naturally occurring trimeric version in downstream applications.

Borean Pharma established an extended and coherent technology platform to use C-type lectins as a vehicle for the creation of novel trimeric therapeutic proteins with increased avidity and unique properties as compared with current protein therapeutics, such as antibodies and small protein scaffolds. Human tetranectin may be readily tailored to meet specific therapeutic needs by "reprogramming" CTLD. Each CTLD has five loop regions, each 6–9 amino acids in length, which determine the binding specificities. Reprogramming is performed by creating phage libraries displaying CTLD, where specific loops are randomized, followed by selection. Randomization can be repeated either sequentially or iteratively. Furthermore, the use of the CTLD platform ensures selected protein candidates, which are highly homologous to a native human secreted protein and thus of low immunogenicity. Initial validation of this novel scaffold technology was achieved by selecting an antagonist of hTNFα, as described herein. Subsequently, the platform has been effectively used on a number of diverse and therapeutically relevant targets. Current potency of the tetranectin-derived hTNFα antagonist was obtained through carefully man-
**Structure of a Novel TNFα Antagonist**

**Experimental Procedures**

Development and Construction of a Truncated TNFα Antagonist Gene—TN-2-B-1-C31 was developed by the use of phage display selection technology. A phage library displaying the monomeric scaffold structure of the C-type lectin domain of human tetranectin containing random amino acid residues in positions 116–122 (loop 1) and 145–150 (loop 4) was constructed and used for the selection of hTNF-binding CTLDs. A candidate from the primary selection, TN-2, was taken through three rounds of affinity maturation using general phage selection techniques. A construct expressing the full-length protein of TN-2-B-1-C31 was created by cloning the coding region of TN-2-B-1-C31 into the BamHI and EcoRI sites of the vector pT7CIH6-AmpR (8).

CTLD Purification—Escherichia coli (strain BL21 DE3) cells were harvested by centrifugation and lysed in 100 ml of lysis buffer by sonication followed by the addition of detergent buffer and inclusion body recovery for refolding in the present first generation procedure. Inclusion bodies were washed with 0.5% Triton X-100, 1 mM EDTA, pH 8, and resuspended in 6 M guanidine, 50 mM diithiothreitol, 50 mM Tris-HCl, pH 8. After inclusion body solubilization, the buffer was changed to 8 M urea, 1 M NaCl, 5 mM β-mercaptoethanol, 50 mM Tris-HCl, pH 8, and the fusion CTLD was captured on a nickel-nitrilotriacetic acid column (Qiagen). After elution with 8 M urea, 1 M NaCl, 5 mM β-mercaptoethanol, 20 mM EDTA, 50 mM Tris-HCl, pH 8, the fusion CTLD was refolded by dilution with 3 M guanidine, 50 mM dithiothreitol, 50 mM Tris-HCl, pH 8, the fusion CTLD was further purified on a SOURCE 15S column in 8 M urea, 50 mM NaAc, pH 4.5, 0–1 M NaCl gradient and finally buffer-exchanged into 50 mM NaAc, pH 5.0, buffer.

**hTNFα Purification**—E. coli strain BL-21 (Invitrogen) was transformed with a pT7-CIIH6-TNFα expression vector encoding human TNFα and grown to an A_{600} of 0.5 in standard tryptone-yeast extract medium with 100 μg/ml ampicillin and 10 mM MgSO_{4} before induction with ACE46 phage and rifampicin addition to 200 μg/ml. Cells from 6 liters of culture were harvested by centrifugation, washed (0.1 M Tris-HCl, pH 8, 1 M NaCl), sonicated, and centrifuged. The supernatant was loaded on a nickel-nitritoltriacetic acid column (Qiagen), and the fusion TNFα was captured, eluted, and further purified on an SP-Sepharose FF column (GE Healthcare). Digestion with factor Xa at +5 °C overnight was applied afterward. The protease and cleaved fusion tag were captured on soybean trypsin inhibitor and nickel-nitritoltricic acid columns, respectively. A final Q-Sepharose FF step yielded a pure hTNFα batch.

**Biacore Assay**—Surface plasmon resonance binding analysis was performed on a Biacore 3000 instrument. The hTNFα capture antibody (AHC3712) to be immobilized was dissolved in 10 mM NaAc, pH 5.0, and then immobilized on a CMS Biacore sensor chip using the amine coupling kit (Biacore). Binding analysis was performed at a flow rate of 5 μl/min. Before loading of the protein sample, the chip was equilibrated in 10 mM Tris, pH 8.0, 150 mM NaCl, 2 mM CaCl_2, 50 μM EDTA, and 0.005% surfactant P20. Recombinant human TNFα was dissolved in 10 mM Tris, pH 8.0, 150 mM NaCl, 2 mM CaCl_2 at 10 μg/ml, and 10 μl was injected. Aliquots (20 μl) of CTLD C31 were injected using the KINJECT option. 5 minutes of dissociation were allowed before the chip was regenerated with sequential injection of 0.05% SDS and 10 mM glycine, pH 2.5. Binding of CTLD C31 was analyzed at six different protein concentrations, ranging from 1.5 to 50 nM. Binding data were evaluated using the BIAevaluation program version 3.2 (Biacore).

**Inhibition of hTNFα-induced Apoptosis**—The effect of inhibition of hTNFα by trimeric TN-2-B1-C31 was analyzed in a standard cell assay for the antagonistic effect of hTNFα binders on the cytotoxic effect of hTNFα on the murine L929 cell line as described in Ref. 9.

**Crystallization, Data Collection, and Structure Determination**—The complex was formed by mixing hTNFα with CTLD at a 1:1 molar ratio. Initial crystallization screening was performed with the Crystal Screen™ system (Hampton Research). Optimal crystals were grown by mixing equal volumes of protein solution with reservoir solution containing 0.1 M Tris-HCl, pH 8.0, 0.35 mM MgAc, and 20% 2-propanol. The crystals were frozen directly in the crystallization solution at the 1911-3 beam line at the National Laboratory for Synchrotron Radiation in Lund, Sweden, MAX-lab (10) or the X12 beam line at European Molecular Biology Laboratory (EMBL) Hamburg. The crystals belonged to the P6_{1}2_{1}2_{1} space group with cell dimensions of a = b = 84 Å, c = 150 Å.

The structure of the CTLD-hTNFα complex was solved by molecular replacement using Phaser (11). We used a search model composed of one wild type hTNFα monomer (Protein Data Bank (PDB) code 1TNF) (12) and the wild type CTLD (6) (PDB code 1TN3), excluding the loops, which were changed during the in vitro evolution procedure. The resulting model

---

*Tetranectin*  
Primary lib  
TN-2  
1. mat lib  
TN-2-B  
2. mat lib  
TN-2-B-1  
3. mat lib  
TN-2-B-1-C31  
a  
b  
c

---

*FIGURE 1. Schematic overview of the selection and maturation process.*
was used as a starting point for wARP/ARP (13), which did build 98% of the complex (including the maturation loops). The model was finalized with Coot (14) and refined in REFMAC5 (15). The final model consists of residues 9–157 for hTNFα, residues 46–180 for CTLD excluding the loop region 52–56, for which no electron density was seen, 244 water molecules, and one magnesium ion. 99.1% of the residues are in the allowed regions of the Ramachandran plot, with 0.4% (1 residue) in the generously allowed region and Arg-120 from CTLD in the disallowed region. Figures were made using the program PyMOL (19).

RESULTS AND DISCUSSION

A primary phage display library was generated by randomizing loops 1 and 4 in the CTLD domain of tetranectin, and selection for binding to hTNFα was performed by panning. This resulted in a candidate molecule of high specificity but suboptimal affinity (Clone TN-2). This clone was then subjected to three consecutive steps of affinity maturation. First, loops 1 and 4 were randomized individually to provide full sequence coverage of each loop, and phage display selection was performed to select for higher affinity. This did not result in any changes in loop 1 (the loop 1 sequence was reselected), but 3 out of 4 amino acids were changed in loop 4 (only the 1st proline was conserved and would remain conserved throughout the maturation). An additional unexpected change occurred outside the randomized area, where DCTLD157 was changed to Gly. The significance of this change, although not fully clear, may have helped stabilize the CTLD structure and resulted in substantially improved affinity (Table 1). However, the TN-2-B clone was incapable of inhibiting hTNFα-induced apoptosis. In the second maturation step, loop 1 was randomized in increments of 3 amino acids, and the selection procedure was changed to favor clones with low off-rates. This was done by including prolonged washing steps and competition by free hTNFα. The resulting TN-B-1 clone showed a 300-fold reduction in off-rate and improved bioactivity. During the third and final stage of maturation, loop 3 was randomized together with two positions in loop 4. This only led to a marginal improvement in the binding kinetics (35-fold in affinity and 6-fold in off-rate for the monomeric CTLD). However, the bioactivity improved by almost 200-fold for the trimeric tetranectin derivative.

The improved bioactivity of clones obtained by applying selective pressure for “better” dissociation kinetics demonstrated the potential of this strategy to improve the efficacy of tetranectin-based therapeutics. It should be noted that the selection as well as the binding data originate from the monomeric version of CTLD, whereas the trimeric version was used for measuring the biological activity. As compared with the moderate improvement in binding of the monomeric CTLD after the third round of maturation, the 200-fold improvement in biological activity of the trimeric tetranectin derivative might well be explained by a large increase in avidity of the trimeric tetranectin derivative.

To further understand the three-dimensional interactions responsible for the high affinity binding and to assure that the overall structure of tetranectin was maintained, we determined the crystal structure of the CTLD-hTNFα complex by molecular replacement at 2.1 Å resolution (Fig. 2A). The final model consists of residues 9–157 for hTNFα and residues 46–180 for CTLD, excluding residues 52–56, for which no electron density was observed (Table 2). The secondary structure of hTNFα bound to CTLD closely resembles that of free hTNFα, with a root mean square deviation of 1.2 Å for Ca atoms (12, 16). Likewise, the non-randomized part of the CTLD scaffold exhibited little change in structure (root mean square deviation for Ca atoms with free native CTLD is less than 1 Å), which validates the use of human tetranectin as a scaffold or platform for engineering new biologics. As for the CTLD loops, the structure has changed substantially in the randomized loop 3 and loop 4 (supplemental Fig. 1). In contrast, the randomized loop 1 backbone resembles that of native CTLD despite a complete change of sequence. The side chains of the randomized maturation loops display a systematic directionality toward hTNFα, creating a visibly depressed surface in the previously rather flat hTNFα area. The buried, accessible surface area at the interface between hTNFα and the CTLD is 865 Å², and the interaction surface features a mixture of polar, van der Waals, and hydrophobic interactions. Hydrophobic interactions are often the driving force in tight protein-protein interactions. However, we observed a more diverse set of interactions, which in this case is most likely found because the CTLD must adapt to the hTNFα surface, including neutralizing the charge present.

Residues KCTLD116 and SRYFCTLD-(119–122) in loop 1 are conserved throughout the maturation procedure. We were unable to select new loop 1 sequences in the first maturation round by complete randomization of loop 1. In the second round of maturation, where we performed partial randomization of loop 1, KCTLD116 and SRYFCTLD-(119–122) were reselected, and only VRCTLD-(117–118) was changed to RW. This suggests that these residues play an essential role in the binding of CTLD to hTNFα. The structure shows that they interact with a well organized loop structure in hTNFα consisting of residues 138–141 (Fig. 2B). This loop in hTNFα has multiple internal contacts; R138 hydrogen-bonds with D140 and is further prevented from lateral flexibility by close van der Waals

| Maturation round | Clone   | $K_D^a$ | $K_{on}^b$ | $K_{off}^c$ | $K_i$ | 95% confidence intervals |
|------------------|---------|---------|------------|------------|------|-------------------------|
| Primary          | TN2     | >5.0 × 10^-5 | ND         | ND         | ND   | ND                      |
| First            | TN2-B   | 7.3 × 10^-4  | 1.3 × 10^7 | 9.2 × 10^-1 | 10^-4 | 1440                   |
| Second           | TN2-B1  | 1.2 × 10^-4  | 2.5 × 10^6 | 2.9 × 10^-3 | 10^-4 | 1026–17 |
| Third            | TN2-B1-C31 | 3.4 × 10^-10 | 2.6 × 10^6 | 8.9 × 10^-4 | 2.8  | 1.6–6.6                    |

a Primary, first, and second clones were analyzed with ~500 RU ligand immobilized, and the third clone was analyzed with ~50 RU. $T$-value is defined as parameter value divided by S.E.

b ND, not detectable.
packing with Y_{TNF}141, thus providing a stable binding epitope for the CTLD. R_{CTLD}120 and Y_{CTLD}121 form hydrogen bonds with D_{TNF}140 and R_{TNF}138 of hTNF\(_{\alpha}\), respectively. Considerable strain is put on R_{CTLD}120, making it fit into the tight space at the complex interface, which results in \(\psi/\varphi\) angles outside the allowed regions of the Ramachandran plot. The hydroxyl group of S_{CTLD}119 forms multiple hydrogen bonds with the backbone of K_{CTLD}116 and F_{CTLD}122 and thus locks the conformation of the randomized loop 1. Furthermore, S_{CTLD}119 is situated at the center of the CTLD-TNF\(_{\alpha}\) interaction surface pointing into the core of the CTLD, and thus there is substantial steric restriction on this residue. K_{CTLD}116 is also conserved in the selection procedure but does not contact hTNF\(_{\alpha}\). However, K_{CTLD}116 forms several contacts with both the backbone and the side chain of Q_{CTLD}148 and the side chain of Q_{CTLD}151. Thus, the length and charge of lysine are favorable at position 116, stabilizing the structure of the randomized loops. The R_{CTLD}117 hydrogen binding with hTNF\(_{\alpha}\).

Loop 4 has changed conformation considerably as compared with the native tetranectin CTLD structure. However, the loop makes relatively few contacts with hTNF\(_{\alpha}\) that are predominantly made by W_{CTLD}149. P_{CTLD}146 appears in the first round of selection and is maintained throughout the maturation. We believe that P_{CTLD}146 dictates a change in the conformation of loop 4, which is required to avoid sterical conflict with hTNF\(_{\alpha}\). Thus, in the first round of selection, loop 1 provides the major part of the binding affinity, and loop 4 changes position to allow this interaction. In the later maturation stages, W_{CTLD}149 appears and provides additional hydrophobic interaction with hTNF\(_{\alpha}\) and probably contributes to the lower off-rate.

Loop 3 was randomized during the third and final round of selection. Loop 3 does not make any direct contact with hTNF\(_{\alpha}\). However, the position of the loop has shifted substantially relative to the native CTLD structure. It is likely that the
Structure of a Novel TNFα Antagonist

Table 2

Data collection and refinement statistics

| Parameter                        | Value |
|----------------------------------|-------|
| Space group                      | P6,22 |
| Cell dimensions                  | 84, 84, 149 |
| α, β, γ (°)                      | 90, 90, 120 |
| Resolution (Å)                   | 4.21 (2.15–2.1) |
| R_factor or Rmerge               | 0.08 (0.48) |
| I/σ(I)                           | 55.1 (9.0) |
| Completeness (%)                 | 99.9 (99.8) |
| Redundancy                       | 33 (26.1) |

Refinement

| Parameter                        | Value |
|----------------------------------|-------|
| Resolution (Å)                   | 2.1   |
| No. of reflections               | 17855 |
| R_factor or Rmerge               | 0.17/0.22 |
| No. of atoms                     | 2209  |
| Protein                          | 1     |
| Water                            | 244   |
| B-factors                        | 28.2  |
| Ligand/ion                       | 46.4  |
| Water                            | 39.185 |
| r.m.s. deviations                | 0.016 |
| Bond angles (°)                  | 1.16  |

Acknowledgments—Beamline access was funded partly via the Dan-scott consortium. We are grateful to the staff of beam lines 911 at MAX-lab Lund and X12 at EMBL Hamburg for help in data collection. We are also grateful to the staff at Borean Pharma A/S.

References

1. Zelensky, A. N., and Gready, J. E. (2005) FEBS J. 272, 6179–6217
2. Caterer, N. R., Graversen, J. H., Jacobsen, C., Moestrup, S. K., Sigurskjold, B. W., Etzerodt, M., and Thøgersen, H. C. (2002) Biol. Chem. 383, 1743–1750
3. Graversen, J. H., Lorentsen, R. H., Jacobsen, C., Moestrup, S. K., Sigurskjold, B. W., Thøgersen, H. C., and Etzerodt, M. (1998) J. Biol. Chem. 273, 29241–29246
4. Mogues, T., Etzerodt, M., Hall, C., Engelich, G., Graversen, J. H., and Hartshorn, K. L. (2004) J. Biomed. Biotechnol. 2004, 73–78
5. Nielsen, B. B., Kastrup, J. S., Rasmussen, H., Holtet, T. L., Graversen, J. H., Etzerodt, M., Thøgersen, H. C., and Larsen, I. K. (1997) FEBS Lett. 412, 388–396
6. Kastrup, J. S., Nielsen, B. B., Rasmussen, H., Holtet, T. L., Graversen, J. H., Etzerodt, M., Thøgersen, H. C., and Larsen, I. K. (1999) Acta Crystallogr. D Biol. Crystallogr. 54, 757–766
7. Nielbo, S., Thomsen, J. K., Graversen, J. H., Jensen, P. H., Etzerodt, M., Poulsen, F. M., and Thøgersen, H. C. (2004) Biochemistry 43, 8636–8643
8. Holtet, T. L., Graversen, J. H., Clemmensen, I., Thøgersen, H. C., and Etzerodt, M. (1997) Protein Sci. 6, 1511–1515
9. Hogan, M. M., and Vogel, S. N. (2001) in Current Protocols in Immunology (Coligan, J. E., ed) p. 183, John Wiley & Sons, Inc., New York
10. Ursby, T. C. B. M., Cerenius, Y., Svensson, C., Sommarin, B., Fodje, M. N., Kvick, A., Logan, D. T., Als-Nielsen, J., Thunnissen, M. M. G. M., Larsen, S., and Liljas, A. (2004) in AIP Conference Proceedings, Eight International Conference on Synchrotron Radiation Instrumentation, Vol. 705, pp. 1241–1246, American Institute of Physics, College Park, MD
11. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) J. Appl. Crystallogr. 40, 658–674
12. Eck, M. I., and Sprang, S. R. (1989) J. Biol. Chem. 264, 17595–17605
13. Lamzin, V. S., and Wilson, K. S. (1997) Methods Enzymol. 277, 269–305
14. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132
15. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. D Biol. Crystallogr. 53, 240–255
16. Eck, M. J., Beutler, B., Kuo, G., Merryweather, J. P., and Sprang, S. R. (1988) J. Biol. Chem. 263, 12816–12819
17. Maini, R., St Clair, E. W., Breedveld, F., Furst, D., Kalden, J., Weisman, M., Smolen, J., Emery, P., Harriman, G., Feldmann, M., and Lipsky, P. (1999) Lancet 354, 1932–1939
18. Moreland, L. W., Baumgartner, S. W., Schiff, M. H., Tindall, E. A., Fleischmann, R. M., Weaver, A. L., Ettinger, R. E., Cohen, S., Koopman, W. J., Mohler, K., Widmer, M. B., and Bloch, C. M. (1997) N. Engl. J. Med. 337, 141–147
19. DeLano, W. L. (2002) The PyMOL Molecular Graphics System, DeLano Scientific LLC, San Carlos, CA

Changes in loop 3 help to stabilize the conformation of both loop 3 and loop 4 and provide a more rigid interaction platform. Loop 3 is normally involved in binding of Ca2+, but Mg2+ is found bound in the former Ca2+-binding site, and crystallization is performed in the presence of Mg2+. However, none of the ions are needed for binding to hTNFα, and selections have been performed in the absence of both ions (data not shown).

Several anti-TNFα drugs have been marketed, based either upon antibody technology or upon modified soluble TNF receptor (17, 18). We compared the CTLD clone selected here with the available drugs. In our in vitro bioassays, the TN2-B1-C31 clone compared favorably to these drugs, both in terms of binding affinity and when comparing the ability to inhibit TN-2B-1-C31:hTNFα-induced apoptosis (data not shown).

In summary, the data show that the CTLD scaffold can be “reprogrammed” for binding to novel targets through a short series of directed evolutionary steps while maintaining its core scaffold structure. Furthermore, the crystal structure of the CTLD-TNFα complex provided a unique insight into the workings of directed evolution. Initial binding affinity is provided by loop 1, which is accompanied by a change in the structure of loop 4 removing steric hindrance. During later steps of selection, in which a low off-rate was favored, additional buried hydrophobic contacts appeared, represented by W_{CTLD}18 and W_{CTLD}149. The selection also seems to favor additional rigidity of the antagonist, as exemplified by the changes of structure in loops 3 and 4. The successful selection for low off-rates is of particular importance for development of therapeutically use-