An investigation of the structure of tumour necrosis factor α in *Cyprinus carpio*

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SUBMISSION OF THESIS FOR A RESEARCH DEGREE

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Abstract

Tumour necrosis factor α (TNF-α) is a soluble cytokine that has a number of different roles in the immune system. It carries out these functions by binding to one of its receptors TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). *Cyprinus carpio* (common carp) is an economically important fish found in Europe and East Asia. Although TNF-α has been found to affect the immune system, its exact structure and function is not known. Knowledge of the structure could help in research into the function of TNF-α in common carp and fish in general.

The four isoforms of TNF-α in common carp are all highly conserved in amino acid sequence when aligned with each other, suggesting a very similar structure and function. TNF-α4 had an extended protein sequence at the C-terminal end, however, this did not affect the alignment. When aligned with TNF-α from humans and mice, several conserved residues were found to affect structure and function in human TNF-α. This was also seen in the alignment of TNF-α from common carp with other species of fish and mammals. A clustering of conserved residues, where structural elements of TNF-α of humans existed, was also observed. The prediction of secondary structure in TNF-α of common carp was very similar to that of TNF-α in humans and mice. The size, position and number of β-strands in the predicted structure was similar to the known structures. However, no α-helices had been predicted, other than in TNF-α4. These results may contribute to the determination of the structure of TNF-α in common carp, which can lead to further research into its function in fish.

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Acknowledgements

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Chapter 1: An introduction to TNF and the TNF Superfamily

1.1 Introduction

Tumour necrosis factor α (TNF-α) is a soluble cytokine that is produced as a response to various stimuli in the immune system. It was originally discovered in humans by Carswell *et al.* (1975) who had found TNF-α, then called TNF, was induced by endotoxin. It was found that when endotoxin was injected into mice infected with *Bacillus Calmette-Guerin* (BCG), TNF-α was produced in all cases and selectively caused the necrosis of malignant cells. Endotoxin is a lipopolysaccharide which is found in the membrane of Gram-negative bacteria. The TNF cytokine was also found to have different effects on other tumours; having increased sensitivity against some while others were very resistant to the cytokine suggesting that TNF was selective against the cells it affected. It is produced primarily within immune cells such as macrophages, but it can also be produced in lymphoid cells, mast cells, endothelial cells, fibroblasts, and neuronal cells. The reason it is given the "α" denomination is due to the original discovery of Lymphotoxin-α. When first discovered, Lymphotoxin-α was denoted as TNF-β due to the similarities it had with TNF-α. However, after it was discovered that it was a different cytokine, but part of the same protein family, the name was changed to Lymphotoxin with TNF-α retaining its name.

TNF-α exists in two forms; one of these forms is a type II 26-kDa trans-membrane protein (tmTNF), arranged in stable homotrimers and found on TNF-α producing cells (Tang *et al*. 1996). A type II trans-membrane protein is a structure where multiple α-helices pass through the lipid bilayer. TmTNF exists with the N- terminus situated within the cytoplasm of the cell and the C-terminus outside of the cell. Once activated, it is cleaved from the cell surface by the metalloproteinase TNF-α converting enzyme (TACE) (Black *et al*. 1997) between the alanine (-1) and the valine (+1) in the extra cellular domain. A metalloproteinase is a

proteolytic enzyme which uses a metal for its catalytic mechanism. TNF-α is released into the outside environment where it exists as a soluble cytokine (sTNF). Although TNF-α interacts with cells in its soluble form, it is also able interact with other cells while in its trans-membrane form. If tmTNF is juxtaposed with the effector cell it can form cell-cell contacts with its receptor (Grell *et al*. 1995).

TNF-α can exert effects on various cells through binding to specific receptors on the cell surface. These receptors are known as tumour necrosis factor binding receptors (TNFR) and, as with TNF-α, are a part of a large family of receptors corresponding to various members of the TNF superfamily. These receptors are characterised by one to six cysteine rich repeats which are found in their extracellular domains, with each repeat having three disulphide bridges (Naismith and Sprang, 1998). Cysteine rich repeats are a structural motif whereby a stretch of amino acids have an unusually high number of cysteine residues. TNF-α binds to one of two receptors: TNFR1 and TNFR2. These receptors are characterised by having four cysteine rich repeats and an elongated shape that allows them to interact with TNF-α (Banner *et al*. 1993). Although TNF-α interacts with both, it has a higher affinity for TNFR1 in the soluble form (Grell *et al*. 1998). It has also been found that the trans-membrane form of TNF-α is better at activating TNFR2 for certain processes such as T-cell activation (Grell *et al*. 1995). The exact reason for this distinction is not currently known, but it is thought it may be due to the differing stabilities of the ligand/receptor complexes. TNFR2 is also the receptor in which Lymphotoxin (LT), a structurally similar cytokine, interacts.

TNF-α is involved in a large number of cellular processes. When the cytokine binds to either of its receptors, it results in the activation of a number of signalling pathways that lead to a host of specific responses. A number of these processes are associated with the immune system. It is considered to be a major mediator of the inflammatory response. Tracey *et al*.

(1988) had discovered that animals administered with TNF-α were found to have organs with significant inflammation, such as the omentum. The omentum is a layer of peritoneum that surrounds adnominal organs. TNFR1 contains a region within its structure known as a death domain. This death domain can recruit various proteins to death domain receptors and couple them to caspase activation, which can eventually lead to cell apoptosis (Tartaglia *et al*. 1993). This implies TNF-α is associated with programmed cell death. TNFR1 is also able to indirectly recruit TNF receptor associated factors (TRAF). TRAFs are a family of signal transducers that couple to TNFR and other proteins to activate cellular processes, signal pathways and gene expression (Bradley and Pober, 2001). TNFR2 is also able to induce gene expression, but recruits TRAF directly and communicates with TNFR1 (Wajant *et al*. 2003). Roles in other processes include activation of human dendritic cells (Caux, *et al*. 1994).

TNF-α and its receptors are part of a large family of proteins known as the TNF superfamily. The TNF superfamily consists of a number of cytokines and receptors which are characterized by their similarities in sequence, function, and structure. Each ligand is specific to a set of receptors. Receptors can have multiple ligands, but each ligand will have an affinity to the receptor that is specific to that cytokine; such is the case for TNF-α and Lymphotoxin. Virtually all of the ligands are expressed by cells in the immune system, while the receptors have been found in most of the cell types within the body (Aggarwal, 2003).

TNF-α has also been studied in a number of other species. As well as in humans, the structure of TNF-α has also been solved in mice. The TNF-α structure of mice was determined by Baeyens *et al*. (1999), and was found to have a number of similarities. Each subunit has a jelly roll topology, which is a formation of β-strands that are mostly antiparallel. It also forms a trimer like human TNF-α. It also shares some similarities in its receptors and function. Human TNF-α is able to bind to mouse TNFR1 but cannot bind to TNFR2, showing some

structural similarities (Vandenabeele *et al*, 1995). An investigation by Pasparakis *et al*. (1996) showed that mice that had a mutated TNF-α gene and were infected with the bacterium *Listeria monocytogene* would exhibit a 10,000 to 100,000 fold higher concentration of the bacterium in their liver and spleen. This is in contrast to mice who produced TNF-α normally, which had a much lower concentration of the bacterium when infected. This showed that TNF-α in mice plays a significant role in the immune system.

Cyprinus carpio (common carp) is a breed of freshwater fish that is part of the *Cyprinidae* family of fish. Geographically, it is typically found in Europe and Asia. The ancestral wild type carp was originally found in the Black, Caspian and Aral Sea and dispersed into the east (Balon, 1995). It is commonly thought there is two sub species, a European variant *Cyprinus carpio* and an East Asian variant *Corythoichthys haematopterus*. Common carp is one of the most farmed freshwater fishes in the world, with the fish being found in all countries in some economic areas, such as the Mediterranean (Vilizzi, 2012; FAO , 2015).

TNF-α and its roles have been researched mainly in mammalian cells such as humans, rabbits and mice. However, there has been some research into TNF-α in vertebrates and other genus such as *Oncorhynchus* and *Cyprinidae*. For the purposes of this review, I will be focusing on current research of TNF-α in humans and common carp while making comparisons to other forms of TNF-α in different species. This is because the large majority of research has been performed on human TNF-α. Also, as I will be focussing on the secondary structure of TNF-α in common carp it is important to review what research has been performed on the cytokine.

1.2 The Immune System

The immune system is a collection of cellular processes that work together with the purpose of protecting an organism against invading pathogens. Without the immune system, pathogens would likely kill the host organism after a short time. In humans, various cytokines play a role in the immune system. TNF-α in particular plays a large role in the immune system, directly and indirectly activating various immune processes. The immune system of humans is well documented with numerous pathways and cytokines having been identified. However, very little is known about the immune system of fish, especially in regards to certain proteins such as TNF-α. Given the conserved nature of TNF-α in evolution, similar immune processes may correspond to those seen in fish.

1.2.1 Immunity in Humans

In humans, the immune system is a large, complex network of cellular processes that together protect cells from foreign pathogens that could potentially cause damage to the body. It is composed of two distinct forms of immunity that, while essentially carrying out the same function, protect the body in different ways. Innate immunity as a whole refers to the system that is used against foreign pathogens that first enter the body. It is considered the first line of defence and acts indiscriminately against contaminants. Adaptive immunity is very specific and will only act on certain pathogens through the recognition of various factors that apply to that group. As the name suggests, adaptive immunity can acquire its specificity for various pathogens that have not been seen before. Once a pathogen has been encountered for the first time, the body can achieve specificity so if that particular pathogen is seen again the adaptive immune system will be able to complement the innate immune system so that the response is greatly accelerated.

There are a number of cells that are specifically associated with the immune system. The adaptive and innate immune systems both have cells and processes that are specifically associated with them. White blood cells (leukocytes) are a group of cells involved with the immune system whose function is to defend against infectious diseases and foreign materials. These cells include neutrophils, basophils, monocytes, eosinophils, and lymphocytes. Neutrophils are cells that kill bacteria using a process called phagocytosis, where the foreign material is engulfed and degraded by proteolytic enzymes released by the cell (Brinkmann *et al*. 2004). It responds to inflammatory stimuli and migrates to infected cells and tissues. Basophils secrete histamine and are involved in mediating the inflammatory response. Monocytes give rise to a number of different cell types such as macrophages and dendritic cells. Monocytes mature into macrophages once they leave the bloodstream and carry out a similar role to neutrophils by phagocytosing microorganisms. However, they are much larger and live longer than neutrophils (Alberts *et al*. 2002). They are also able to secrete cytokines, TNF-α being one of these. Eosinophils are cells that have a number of different functions such as playing a role in the allergic response or in inflammation. It is also able to produce cytokines, such as TNF-α.

There are five types of lymphocyte: Natural Killer (NK) cells, cytotoxic T cells, effector helper T cells, B cells and γδT cells. NK cells are associated with innate immunity and mediate high levels of cytotoxicity while also producing cytokines and chemokines. They can be split into two categories: classical NK cells and T NK cells. The main difference between the two is that classical NK cells do not express T cell antigen receptors and are CD3 negative, while T NK cells are essentially a population of T cells that share characteristics with classical NK cells (Biron *et al*. 1999). Cytotoxic T cells are responsible for killing cells that have been infected with foreign pathogens. By contrast, effector helper T cells are responsible for secreting a variety of cytokines, TNF-α being one of them, that

can act as local mediators for other white blood cells (Alberts *et al*. 2002). γδT cells are a branch of T cells, which have T-cell antigen receptors, and are composed of γ and δ chains as opposed to α and β chains. They represent a very small fraction of the total T cell population but are enriched at certain locations such as the epithelia of certain organs (Kabelitz and Wesch, 2003). They also have antitumor and immunoregulatory activities (Girardi, 2006). B cells are responsible for synthesizing large groups of small protein components known as antibodies. Antibodies are produced as both cell surface receptors and as secreted molecules. Antibodies are small molecules that are able to both bind to antigens and activate cellular processes that cause bacteriolysis (lysis of the bacterium) or are able to present a specific antigen to other immune cells such as cytotoxic T cells. B cells are able to produce a specific antibody for a specific antigen. B cells are required in order for the adaptive immune system to be functional.

Innate immunity is the first response the human body has towards invading pathogens. It was originally thought that innate immunity was completely indiscriminate towards foreign pathogens and materials. However, it has been found that cellular processes activated in the innate immune response can discriminate between cells from the body and a number of different pathogens. The method in which pathogens are recognised is through the use of germline encoded pattern recognition receptors (PRRs). PRRs possess a set of characteristics that allow them to work. PRRs are able to recognise components that are essential for the life of the pathogen, called pathogen associated molecular patterns (PAMPs). These PAMPs are components of the bacterial or fungal cell wall. PRRs are expressed by host cells and are able to recognise pathogens at all stages of the life cycle. The final thing is that PRRs are expressed on all cells of a given type, regardless of any immunity achieved from the adaptive immune system (Akira *et al*. 2006). Toll-like receptors are a class of PRRs that are able to recognise a number of different aspects of bacteria such as lipopolysaccharides in the cell

walls of bacteria, bacterial DNA (Krieg, 2002), and double stranded RNA (Alexopoulou *et al*. 2002). Other examples of PRRs include Nucleotide oligomerization domain proteins (NODs), peptidoglycan recognition proteins (PGRPs), and C-type lectin receptors (CLRs) (Sukhithasri *et al*. 2013).

The adaptive immune response acts usually towards the end of an infection. It is only able to act on foreign pathogens if innate immunity has already been activated. Lymphocytes are required in order for adaptive immunity to be possible. In adaptive immunity cytotoxic T cells, helper T cells and B cells are the lymphocytes primarily involved. The way in which the adaptive immune system is able to function is described by the clonal selection theory. It describes that, in a mammal, a large amount of lymphocytes with a high degree of diversity are randomly generated. As each of these lymphocytes develops in a lymphoid organ it becomes specific in reacting to only a single antigen. This is done before the lymphocyte ever comes into contact with the antigen it is committed to. Once a lymphocyte does come into contact with the antigen it is specific to, it will become active and rapidly begin to proliferate and differentiate into an effector cell (Burnet, 1959). This method implies that the immune system, in theory, has a lymphocyte that can act against most foreign pathogens. One antigen can activate a number of different lymphocytes due to having a number of different antigenic determinants.

1.2.2 Immunity in Fish

In mammals, the adaptive immune system has been shown to play a very important role in the defence against bacteria and viruses. The ability to recognise certain pathogens and selectively destroy them, while also preventing against further infections by the same pathogen, is an important ability that has been vital to the survival of various mammals. The innate immune system, while indiscriminate in recognising pathogens, is the only line of

defence the body has before the adaptive immune system can respond. It is through the combination of these two systems that the human body can withstand most forms of bacterial or viral infection. Fish have both an adaptive and innate immune system much like mammals. However, due to the environment fish live in, the innate immune system plays a much larger role and is even more vital in pathogen defence.

In mammals, the innate and adaptive immune systems work cooperatively in the defence against pathogens. In fish, the adaptive and innate system is present, but the innate immune system is used in the majority of cases of invading pathogens. This is in large part due to the limitations of the adaptive immune system in various fish species. The innate immune system in fish is very quick to act, requiring very little time to respond, much like in mammals. However, the adaptive immune system is very slow and can take at least four to six weeks in response to a foreign pathogen for many species of fish (Ellis, 2001). The environment in which fish reside contains a large number of bacteria and viruses. Many of these bacteria are able to kill fish in a matter of days (Ellis, 2001). In these cases, the adaptive immune response would be too slow to respond. As a result, innate immunity is effectively the most important defence fish have against foreign pathogens.

There are many similarities between the innate immune systems of multicellular organisms. They share many of the same cells and methods of actions. In the immune system, cells associated with innate immunity are able to recognise pathogens by use of PRRs. There are different classes of the PRRs which activate different immune responses such as phagocytosis and opsonization (Pasare and Medzhitov, 2004). Some of these classes include Toll-like receptors, NOD-like receptors (NLRs) and C-type lectins (Pellitero, 2008). In the case of NLRs, in mammals they are responsible for detecting microbial components in the cell and inducing NF-κB (nuclear factor kappa beta) and MAPK (mitogen-activated protein

kinase) signalling. By contrast, the functions of Toll-like receptors and NLRs are not very well known in fish. It has been discovered that Toll-like receptors do not have the same ligands as their mammalian counter parts (Palti, 2011).

There are cells known for their role in innate immunity in general, such as non-cytotoxic dendritic cells (Bassity and Clark, 2012) or cytotoxic cells such as macrophages (Mulero *et al*. 2008). There are also examples of where certain cells involved in innate immunity while not found in other species may still be related. Catfish were found to contain cells similar to Natural killer cells in mammals known as non-specific cytotoxic cells (NCC). They were able to cause the lysis of transformed mouse and human cell lines, through direct cell-cell contact, in a process similar to Natural Killer cells in mammals (Evans *et al*. 1984). It has also been suggested that NCC cells are the evolutionary ancestor of natural killer cells in mammals (Evans *et al*. 1984).

1.3 Structure and Phylogeny of TNF-α

1.3.1 Structure of Human TNF-α

The three-dimensional structure of human TNF-α has been determined and extensively analysed. The soluble form of TNF-α was determined by Eck and Sprang (1989) at a resolution of 2.6 Å. Human TNF-α is made up of three identical subunits. These subunits are then clumped together to form a trimer structure. Each of the three subunits are closely packed together to form a trimer with a 3-fold axis of symmetry. The subunits are arranged in such a way that the edge of each subunit is packed towards the interior face of the adjacent subunit. Figure 1.1 shows a three dimensional image of the structure.

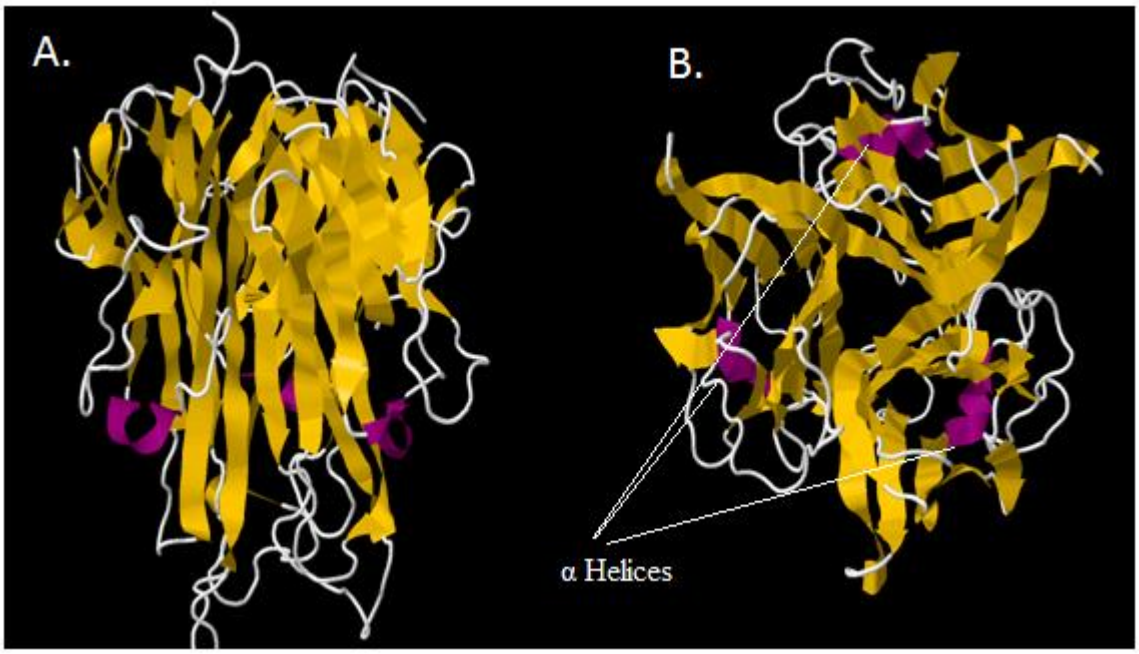


Figure 1.1: The three dimensional structure of TNF-α shown from the side and top of the cytokine. A. displays the side of the cytokine. B. displays the top of the cytokine. The β-sheets are coloured yellow while the α-helix in each subunit is coloured purple. The structure was originally determined by Eck and Sprang (1989). The three dimensional image was produced using PyMol version 1.7.4, using the pdb file 1TNF, obtained from www.rcsb.org.

Every subunit is composed of a number of β-sheets that have an antiparallel formation. An α-helix is also situated at the outer edge of each subunit. The antiparallel β-sheets form a sandwich structure called a β barrel or a "jelly-roll". Figure 1.2 shows a diagram of the antiparallel β-sheet structure seen in each subunit.

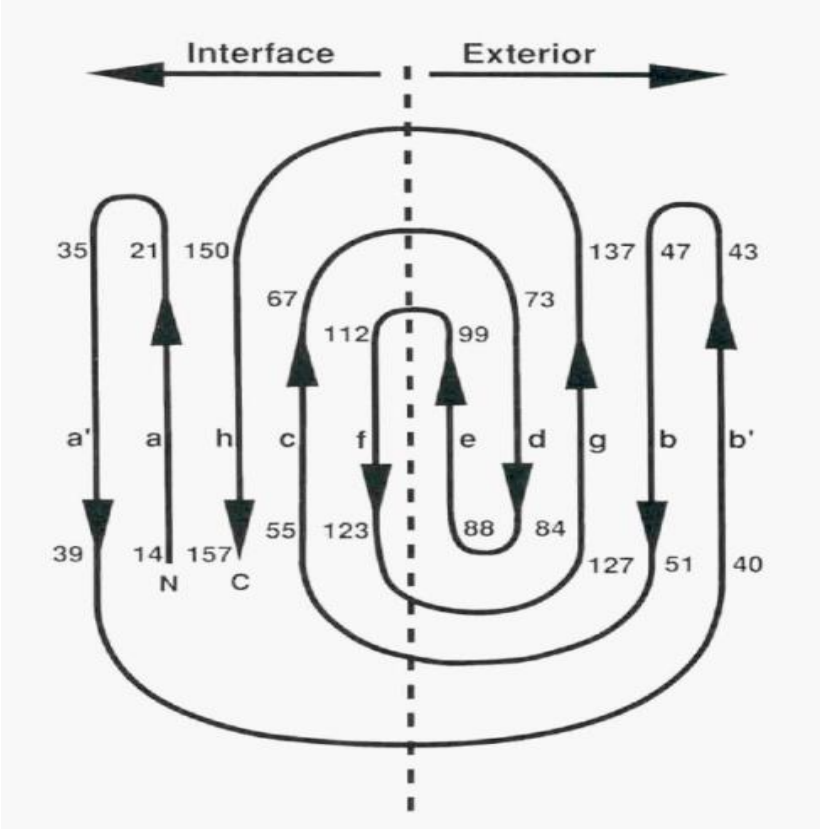


Figure 1.2: A diagram showing the topology of individual β-strands that form the jelly-roll formation in a TNF-α subunit. Each strand is labelled in the order they follow in the polypeptide sequence (Figure was taken from Eck and Sprang, 1989).

The jelly roll has two β-sheets that form a flat inner and curved outer surface in the subunit. Each sheet is composed of five β-strands. As shown in Figure 1.2, the interior sheet is composed of strands a, h, c, and f. It is called such as it faces the trimer axis of TNF-α. The remaining strands e, d, g, and b form the exterior surface of the jelly roll. The exterior surface faces the outside of the molecule and, in comparison to the interior surface which is relatively flat, is highly curved. Strands h and a form the amino and the carboxyl-terminal strands of the molecule and are packed closely together. Residues 8-12 of these strands are involved in interactions the trimer makes with other molecules. Strand b in the exterior surface is interrupted at residue 26, which results in a fold that forms the remaining two strands a' and b'. These strands flank both the interior and exterior surface of the subunit and stabilize the amino terminus. Going from the amino terminal to the carboxyl terminal, the β strands get

progressively longer; this gives the subunit a step like appearance. When looking along an axis that is parallel to the β sheet, each of the sheets is twisted by 60˚ with respect to the overall subunit. Each sheet is also approximately 25 Å across and 20 Å from sheet to sheet. Two polypeptide loops exist, that links strands c – d and e – f respectively. These loops are linked together by a disulphide bridge. Although each subunit is made up of mostly β-strands, an α-helix can be found at residues 138-142 in each subunit respectively. These α-helices are very small and are only comprised of one turn. The interior of the subunit is closely packed together, mostly with the side chains of hydrophobic amino acids. At the area where the amino and carboxyl terminals ends of the subunit are in close contact, there is a small depression which is surrounded by the residues 11-13, 38-40, 51-55, and 155-157. These residues all correspond to either the polypeptide loops that connect β-strands a to b and b to c or to extensions of the β-strands. The amino acid side chain of Y-56 is found in this depression and is lined predominantly with the polar and hydrophobic side chains: K-11, P-12, V-13, A-14, A-38, N-39, G-40, S-52, E-53, G-54, and A-156. Y-56 has a hydroxyl group which is thought to play a role in receptor binding (Figure 1.3).

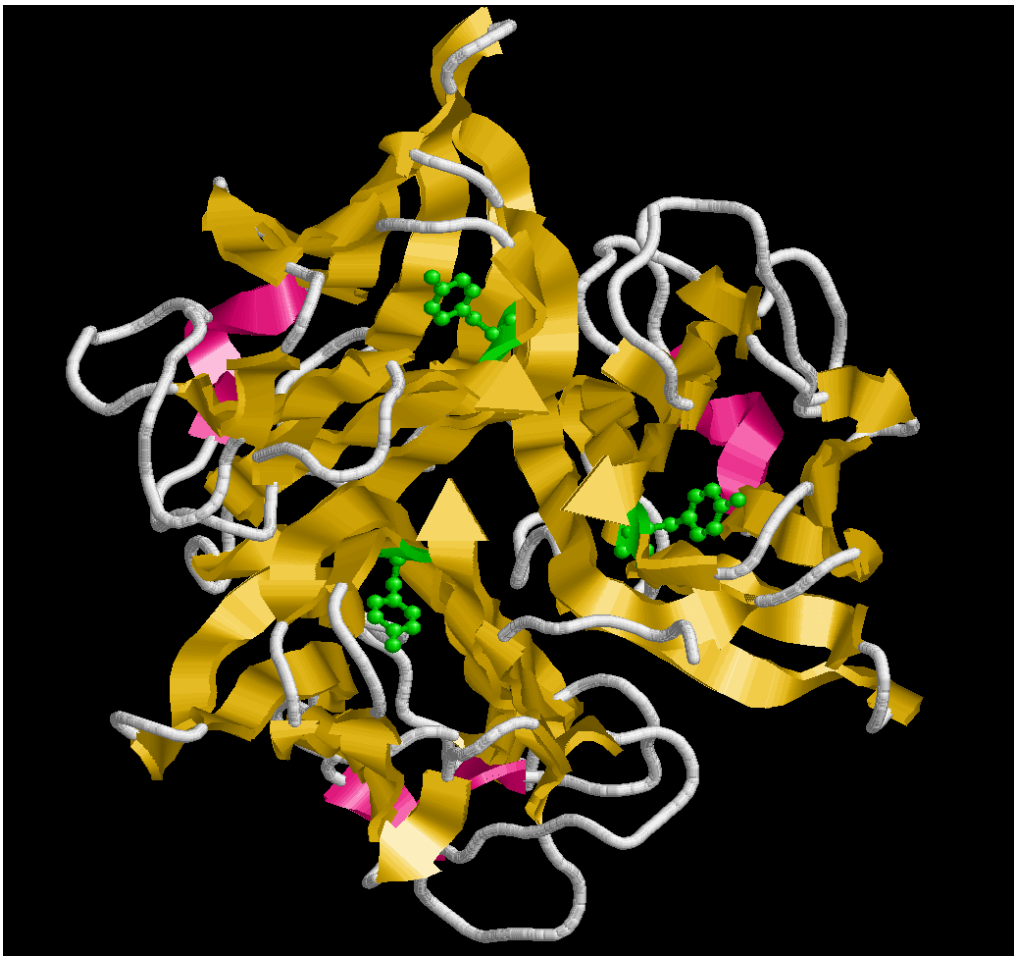


Figure 1.3: The three-dimensional structure of human TNF-α with the Y-56 amino acid highlighted in green. Y-56 is thought to play a role in receptor binding. The structure was originally determined by Eck and Sprang (1989). The three dimensional image was produced using PyMol version 1.7.4, using the pdb file 1TNF, obtained from www.rcsb.org.

The trimer does not have regular 3-fold symmetry, with the axis being tilted 40° from the y-axis of the crystallographic unit cell. It also forms a 140° angle along the crystallographic x and z planes. This is thought to be the result of each subunit having non-equivalent packing environments (Eck and Sprang, 1989). It has also been suggested that possible non symmetric interactions occur between the TNF-α trimer and its receptor due to the non-equivalent packing, from what was observed in crystals of TNF-α grown by Eck and Sprang (1988). The β-sheets of adjacent subunits form a joint, composed of the carboxyl-terminal edge of one subunit and the inner face of the adjacent subunit. The carboxyl-terminal edge

of each subunit also crosses with the inner face of the adjacent subunit at a 30˚ angle. This allows for extensive interactions between residues. A large portion of the residues found on strands e and f of the crossing faces are involved with trimer contacts. Interactions between the subunits mostly involve hydrophobic residues. There are forty residues that are packed into each of the subunit interfaces. An open polar channel is found at the top of the TNF-α trimer near the 69-101 disulphide bridge (Figure 1.4). The channel is lined by carbonyl, oxygen and amide NH groups found on strands f and e of the subunit. It is through this channel that the 3-fold axis of the trimer passes. The two residues K-98 and E-116 are found along the triad axis and are going into the interior of the trimer. These residues form an intra-subunit pair of ions, which are part of the interactions between the subunits. The residues could also form an inter-subunit salt bridge between the same residues in the adjacent subunit if the K side chain were to rotate. (Figure 1.4).

Figure 1.4: The three-dimensional structure of human TNF-α with the residues K-98 and E-116 residues and the disulphide bridge formed between C-69 and C-101 highlighted. K-98 and E-116 have the ability to form a salt bridge with the same residues on adjacent subunits. Both residues are highlighted in red and blue respectively. The individual subunits are coloured green, magenta, and orange respectively. The bonds coloured in yellow are disulphide bridges between C-69 and C-101 in each subunit. The structure was originally determined by Eck and Sprang (1989). The three dimensional image was produced using PyMol version 1.7.4, using the pdb file 1TNF, obtained from www.rcsb.org.

The ring structure of Y-119 forms a mutual contact among the three subunits about the 3-fold axis. L-57 and 157 also circle the 3-fold axis near the base of TNF-α. Together with the

hydrophobic residues that are found midway through the trimer, this forms the hydrophobic core of the molecule (Figure 1.5).

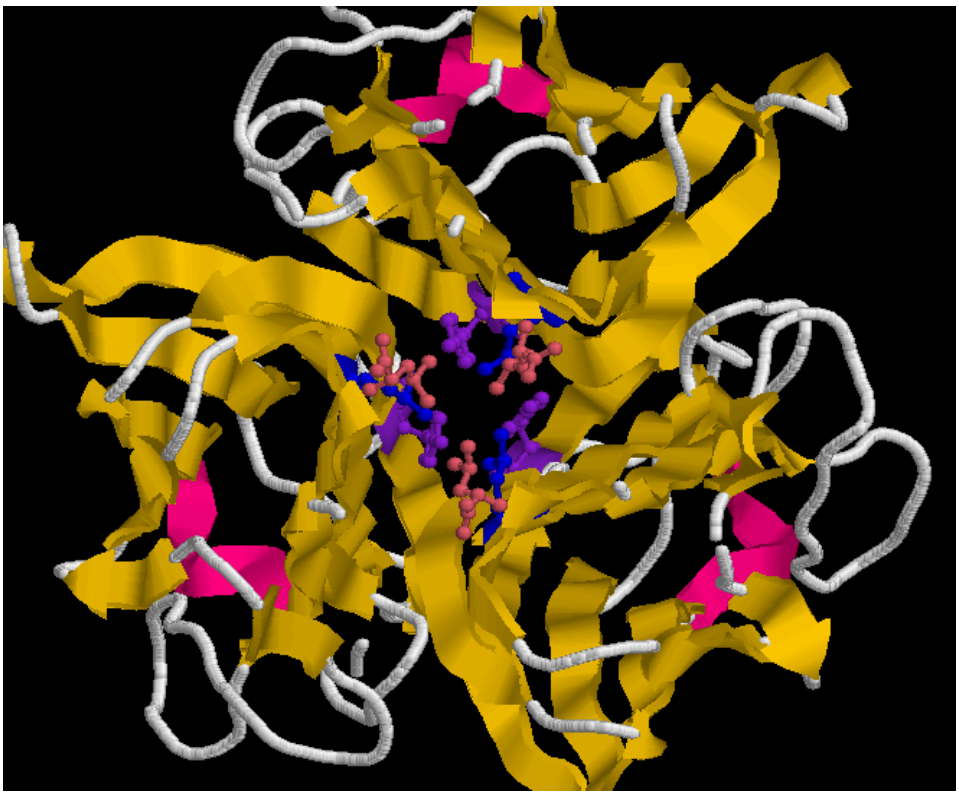


Figure 1.5: The three-dimensional structure of human TNF-α with the residues Y-119, L-57 and L-157 highlighted. Y-119 (coloured purple), L-57 and L-157 (coloured blue and pink respectively) form the hydrophobic core of the cytokine. The structure was originally determined by Eck and Sprang (1989). The three dimensional image was produced using PyMol version 1.7.4, using the pdb file 1TNF, obtained from www.rcsb.org.

The crystallographic structure of TNF-α has been produced, when bound with various other molecules. He *et al*. (2005) has produced the structure of TNF-α when interacting with a small molecule inhibitor composed of trifluoromethylphenyl indole and dimethyl chromone moieties linked by a dimethylamine spacer. By binding to TNF-α, the compound was found to displace one of the subunits in the TNF-α molecule from its formation in the trimer structure. This caused a conformational change in the molecule from a trimer to a dimer. The inhibitor was also able to inhibit the activity of TNF-α in biochemical and cell based assays.

The inhibitor makes contact with several residues on TNF-α on each of the subunits. These residues are L-57, Y-59, S-60, Q-61, Y-119, L-120, G-121, G-122, and Y-151. The fact that the binding of these residues inhibits the activity of TNF-α shows that they play a role in maintaining the quaternary structure of the cytokine. A three dimensional structural diagram of the inhibitor bound to human TNF-α is shown in Figure 1.6.

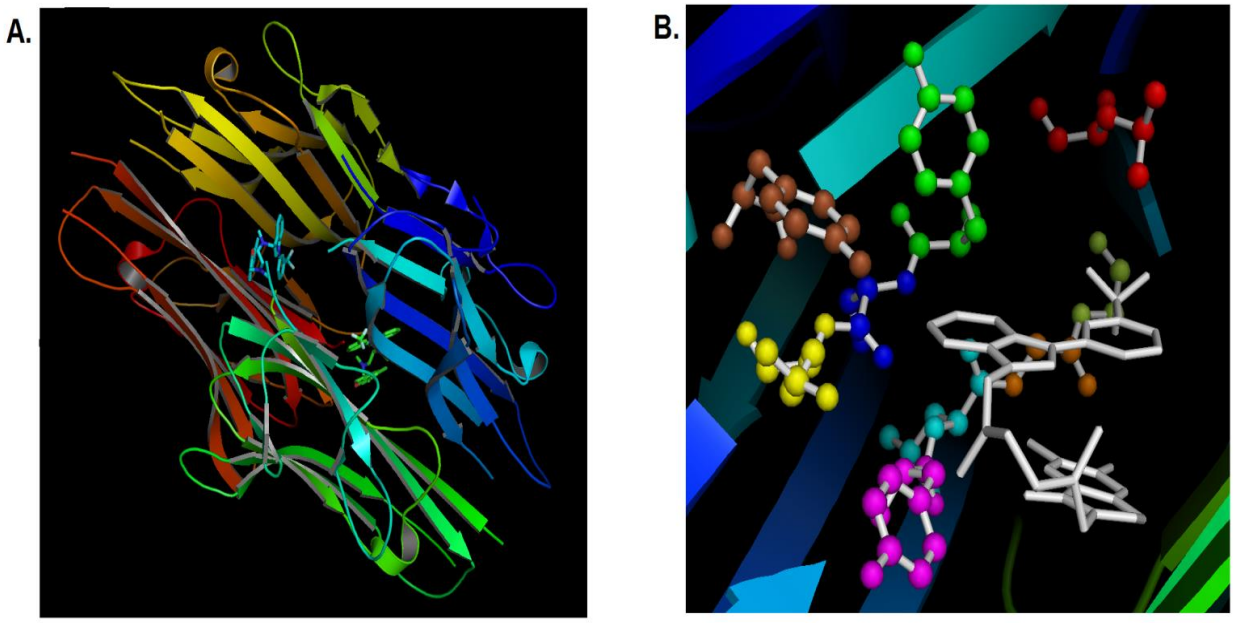


Figure 1.6: The three dimensional structure of TNF-α with a small molecule inhibitor. A. shows the overall structure of the small molecule inhibitor bound to TNF-α. B. shows a close-up of an area of the structure where the inhibitor makes contact with the residues on TNF-α. On TNF-α the residues K-57 (red), Y-59 (green), S-60 (blue), Q-61(yellow), Y-119 (purple), L-120 (light blue), G-121 (orange), G-122 (lime green), and Y-151 (brown). The grey structure is the small molecule inhibitor. The structure was originally determined by He *et al.* (2005). The three dimensional image was produced using PyMol version 1.7.4, using the pdb file 2AZ5, obtained from www.rcsb.org.

Mutagenesis experiments on TNF-α and its receptors TNFR1 and TNFR2 have revealed certain amino acids may play a very significant role in binding. An investigation by Zhang *et al*. (1992) involved performing site directed mutagenesis on human TNF-α and showed that the mutation of several residues had different effects. Changes at the surface residues H-15, W-28, Y-56, Y-59, H-78, Y-119, and possibly S-60 and Y-115 caused impairment of

trimer formation in TNF-α. This resulted in a loss of biological activity. Furthermore, changes in Y-87, S-95, S-133 and S-147 did not affect the formation of the trimer but caused a reduction in the cytotoxicity of the cytokine. It should also be noted that Y-59 and Y-119 were two of the residues that a small molecule inhibitor, as described by He *et al*. (2005), interacted with which affected the biological activity of TNF-α. Other mutagenesis experiments performed by Loetscher *et al*. (1993) showed that, when mutated, Y-87 caused a complete loss in binding activity of either TNF-α receptors, demonstrating its importance. It was also found by Shibata *et al*. (2008) that when TNF-α binds to TNFR1, the residue sits in a hydrophobic pocket where it interacts with L-67, L-71, A-62, and S-63. It is thought to maintain the complex between TNF-α and TNFR1. Another residue of interest on TNF-α is R-31 which had been found to strongly interact with the residues D-54, E-57, and E-70 on the binding surface of TNFR2 (Mukai *et al*. 2010).

With the discovery of TNF-α and documentation of its effects on various cellular processes, a number of autoimmune disorders have been associated with the cytokine, such as Crohn disease and rheumatoid arthritis. Infliximab is a chimeric antibody composed of an IgG1 constant region from humans and an antigen binding variable region from mice (Knight *et al.* 1993). Infliximab is able to neutralize human TNF-α by binding to either the transmembrane or soluble form of the cytokine. It is thought that binding to either of these forms blocks the ability of TNF-α to bind to one of its receptors. It has also been shown that, when binding to the transmembrane form of TNF-α, it can lead to lysis of the cell (Scallon *et al*. 1995). Liang *et al*. (2013) solved the three dimensional structure of human TNF-α in complex with Infliximab Fab fragment. The structure reveals a TNF-α trimer bound by three systematically arranged infliximab Fab molecules. This three dimensional structure is shown in Figure 1.7.

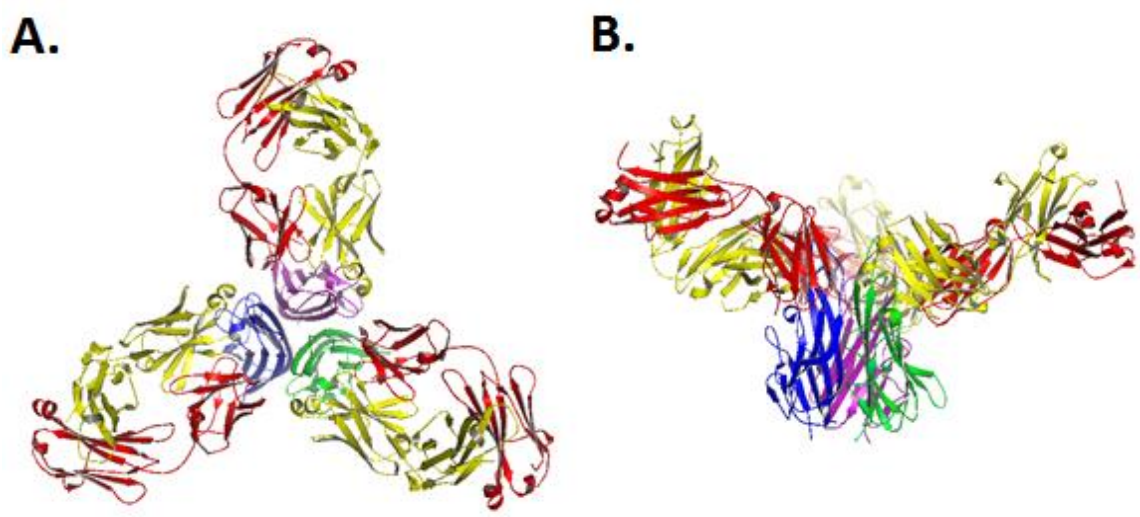


Figure 1.7: The three dimensional crystallographic 3-fold structure of a human TNF-α molecule when in complex with infliximab Fab molecules. A. shows the structure when viewed from the top and B. shows the structure when viewed from the side. The green, blue and purple structures represent the subunits of human TNF-α. The red and yellow structures represent the light and heavy chains of infliximab Fab respectively. The structure originally determined by Liang *et al*. (2013). The three dimensional image was produced using PyMol version 1.7.4, using the pdb file 4G3Y, obtained from www.rcsb.org.

One molecule of infliximab Fab will interact with only one subunit of the TNF-α cytokine. The interface of TNF-α is composed of two loops, C-D and E-F. The key residues: I-28–W-33, R-52–N-57, and Y-102–S-105 in the heavy chain of infliximab Fab form contacts with these loops. The G-H and C-D loop also binds to several residues in the light chain of infliximab, including H-92–W-94. Liang *et al*. (2013) also superimposed the TNF-α-infliximab Fab complex over a TNF-α-TNFR2 complex and found several differences. In the C-D and E-F loops the residues E-67–H-73 and T-105–K-112 mostly contribute to the interaction between TNF-α and infliximab Fab. The E-F loop was found to not be a part of the three dimensional structure of TNF-α-TNFR2. While the loop is essential to the binding of Infliximab, the author's state there is no evidence to suggest it has a biological purpose in TNF-α. As the loop plays a significant role in binding to Infliximab it may be the case that it once played a role which diminished over time due to evolution. Although its function in

humans is unknown, it still may play a role in other species so it is worth knowing about the loop to see if its role is different in TNF-α in common carp.

1.3.2 Structure of Human TNFR1 and TNFR2

TNF-α can exert its effects on cells by binding to one of two receptors, TNFR1 and TNFR2. Both receptors are associated with distinct signalling pathways and, as a result, contribute to the activation of different processes within the cell. TNFR1 is expressed in the majority of cells throughout the body. It is associated with apoptosis within the cell. This is achieved through the formation of a protein complex using TNFR-associated death domain (TRADD) and Fas-associated protein with death domain (FADD) to enact a signalling cascade which eventually leads to apoptosis. TNFR2, meanwhile, is only expressed on certain cells within the immune system such as T-cells (Ware *et al.* 1991). TNFR2 is generally associated with cell survival, relating to functions in immunity to infections, cancer, or inflammation (Faustman and Davis, 2010). Figure 1.8 shows the three dimensional structure of a TNF-α - TNFR2 complex along with a single TNFR2 molecule.

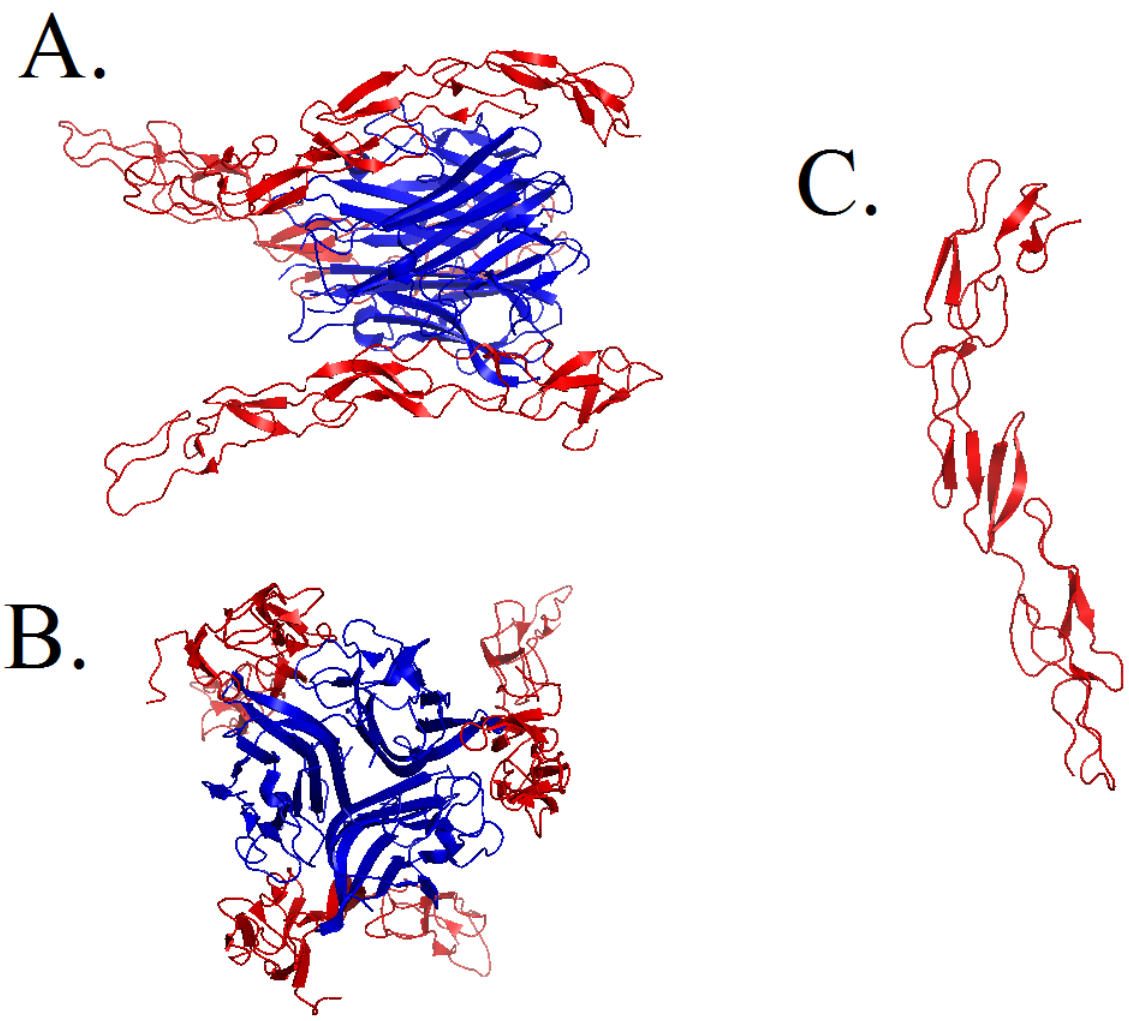


Figure 1.8: The three dimensional crystallographic 3-fold structure of TNF-α when in a complex with TNFR2. A. shows the overall structure when viewed from the side. B. shows the structure when viewed from the top. C shows a single TNFR2 molecule when not in a complex with TNF-α. The structure was originally determined by Mukai *et al*. (2010). The three dimensional image was produced using PyMol version 1.7.4, using the pdb file 3ALQ, obtained from www.rcsb.org.

The structure of TNFR2 is composed of four cysteine rich domains called CRD1 through to CRD4. Each of these domains has a specific function within TNFR2. CRD1 is used for forming the TNFR self-complex on the cell. The self-complex is a complex of TNFR molecules which pre-assemble before making contact with the cell surface and TNF-α (Chan *et al*. 2000). CRD1 and CRD2 are involved in TNF-α binding. It is currently unknown what function the remaining domain CRD4 has within TNFR2 (Mukai *et al*. 2010). The CRDs

also contain modules that are self-contained units within the TNFR2 structure. TNFR2 is composed of three modules, A1, A2, and B2, the number denotes the amount of disulphide bonds in each of the modules. The modules have been found in other members of the TNF superfamily such as TNFR1 (Naismith and Sprang, 1998). The TNF-α cytokine forms a central homotrimer where three molecules of TNFR2 are bound around it. There are four regions in the complex; the core of the interface between the TNF-α and the TNFR2 molecules can be separated into two regions called region 3 and region 4. In region 3 is the A1 module of CRD2, while the B2 module of CRD2 and the A2 module of CRD3 are in region 4. On each of the CRD3 modules, there is an R-113 and R-77 residue. These residues form close contacts with D-143, Q-149, and E-23 on TNF-α. Because of this it is thought that they play an important role in TNF-α binding. (Figure 1.9) (Mukai *et al*. 2010). Region 1 is an area comprised of the amino acids 143 to 149, which have been found to be essential for binding to a loop from TNF-α. Region 2 consists of a loop structure composes of the residues S-79 to D-81.

Figure 1.9: The three dimensional structure of a subunit of TNF-α interacting with TNFR2. TNF-α interacts via the R-113/R-77 (blue) residue within TNFR2 and the D-143 (magenta), Q-149 (cyan), and E-23 (orange) residues within TNF-α of the TNF-α- TNFR2 complex. It is thought that these residues play a role in TNF-α binding due to close contacts formed between the residues. The structure was originally determined by Mukai *et al*. (2010). The three dimensional image was produced using PyMol version 1.7.4, using the pdb file 3ALQ, obtained from www.rcsb.org.

TNFR1 shares some similarities with TNFR2, but also has some differences. One of the main differences is in the modules found within each of the CRDs. Within TNFR1 there is a C2 module in addition to the A1 and B2 modules, and there is no A2 module within TNFR1. It is thought that the residue R-77 in CRD2 is essential to TNF-α binding (Banner *et al*. 1993) in comparison to the addition of R-113 in TNFR2. Despite both receptors binding to TNF-α, there is a difference in electrostatic potential. There are three amino acid residues, D-54, E-57, and E-70, which are found on the interface of TNFR2, in region 3, and are clustered together to form a more negatively charged surface than that of TNFR1.

1.3.3 Structure of Lymphotoxin

As TNF-α is part of a large superfamily, it shares many similarities with other members. The cytokine Lymphotoxin (LT) (originally considered to be TNF-β) was discovered in lymphocytes and later found to share structural motifs and functions with TNF-α (Granger *et al*. 1969; Eck *et al*. 1992). Its primary sequence is 32% identical to TNF-α (Paul and Ruddle, 1988) and it has been shown to exhibit many of the same cytotoxic effects. The structure of LT has been shown to be very similar to TNF-α with a few small differences (Eck *et al*. 1992). Each subunit of LT contains two antiparallel β-sheets which are arranged in a "jelly roll" formation. Figure 1.10 shows the crystal structure of an LT subunit.

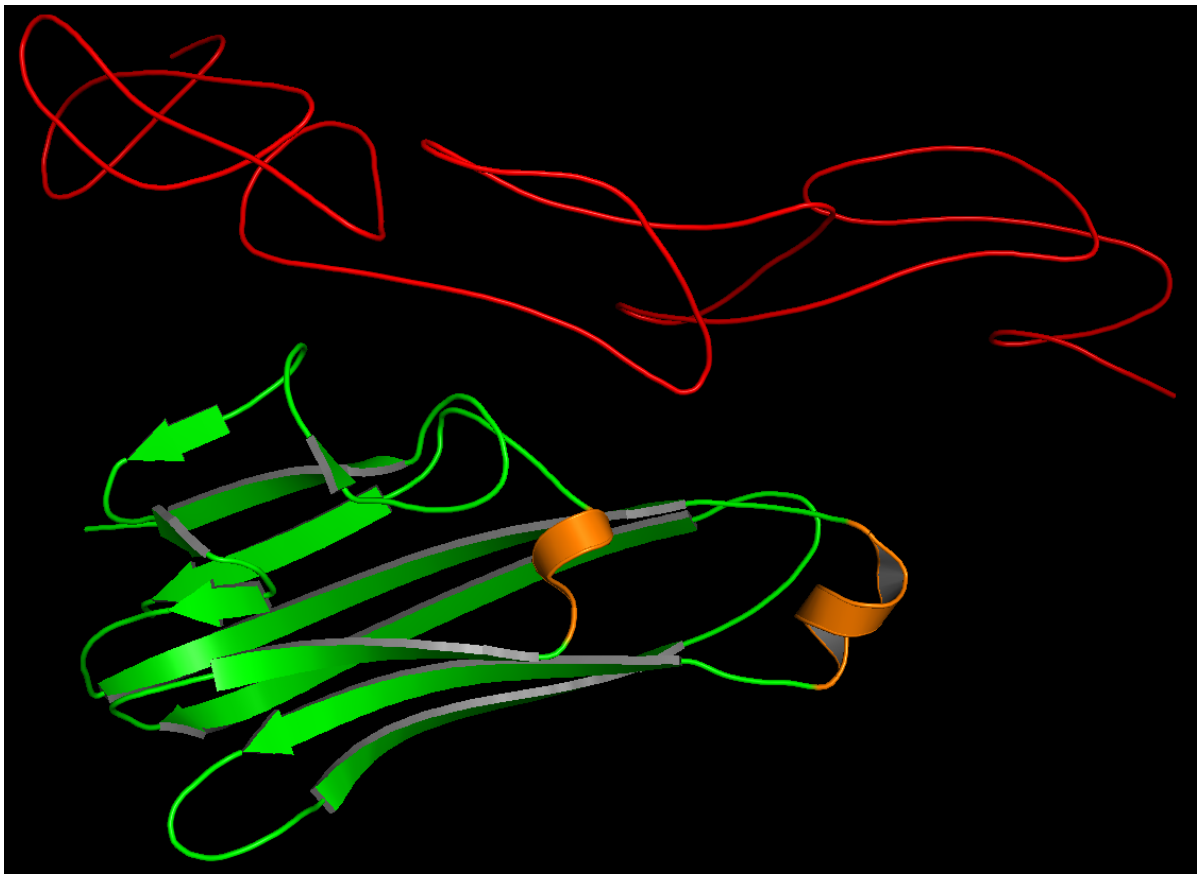


Figure 1.10: The three dimensional structure of a Lymphotoxin subunit bound to a fragment of TNFR1. The structure currently shows only a single subunit bound to a TNFR1 receptor. The subunit contains the jelly roll topology found in TNF-α along with the two α-helices which are coloured orange. The structure was originally determined by Banner *et al*. (1993). The three dimensional image was produced using PyMol version 1.7.4, using the pdb file 1TNR, obtained from www.rcsb.org.

The subunits are capable of forming the same tertiary structure as TNF-α with a similar 3-fold axis. In the hydrophobic core of the trimer, twenty out of the thirty residues are identical to that of TNF-α, five of which are non-conservative replacements. The polypeptide loops that connect strands c–d and e–f in TNF-α are present; however in LT the c – d loop is four residues longer while the e–f loops contain five fewer residues. The disulphide bridge, between residues 69-101, that connects the two loops in TNF-α is not present in LT and is instead replaced by tyrosine.

1.4 Function of TNF-α

Human TNF-α is part of a large complex network of signalling pathways. TNF-α can be produced by a number of different cells including macrophages, T-lymphocytes, granulocytes, and natural killer cells (NK). TNF-α has been produced by cells involved directly in immune and non-immune cell types. It is important to know the function of TNF-α in species which have already been studied extensively. This is because it can help in determining the function for another species such as common carp. In this section, the function of TNF-α in humans will be discussed as it has been the most extensively studied. Also, since TNF-α is an evolutionarily conserved cytokine, it is likely to have a similar function in other species.

1.4.1 TNF-α Mediated Cell Death

Apoptosis is a cell mediated process that results in the death of a cell. Cell death caused by infection or stress related injury would normally result in the cell rupturing and spilling its contents into the surrounding environment, a process called cell necrosis. This would likely result in damage to neighbouring cells due to the presence of various enzymes and proteases within the cytoplasm of a cell. By contrast, Apoptosis is a very clean process that causes a cell to break down its internal components by activating a number of proteases. This causes the cell to break down and mark itself for phagocytosis by cells in the immune system, causing no damage to neighbouring cells. Apoptosis is required in order to keep the amount of cells within the body at a constant level. Mutation of genes related to apoptosis often leads to the development of a number of conditions, one of the most prominent being the various forms of cancer. Apoptosis is an evolutionarily conserved process, having been found to play a significant role in numerous other species such as insects (Clem, 2005), fish (Weyts *et al*. 1998), and mice (Chen *et al*. 2001).

Apoptosis of a cell is carried out by a set of signal cascades that are activated when certain conditions have been met. A number of different proteins and enzymes are required for the activation and execution of apoptosis. One of the most prominent is the caspase-cascade signalling system. Caspases form a large protein family of which 14 members have currently been discovered (Fan *et al*. 2005). Apoptosis can be activated from one of two ways. The first way is activation of a procaspase through a mitochondrion mediated process. When cellular stress occurs, apoptosis related proteins are activated and will induce mitochondrion to release cytochrome *c* from its permeability transition pores. The presence of deoxyadenosine triphosphate (dATP) or ATP in the cell cytoplasm will cause apoptotic protease activation factor-1 (Apaf-1) to oligomerize. The released cytochrome *c*, cytosolic procaspase-9, dATP, and Apaf-1 will now form a large complex known as the apoptosome.

This complex is then able to activate other procaspases such as procaspase-3 and procaspase-7 which in turn results in the signalling cascade, causing apoptosis (Fan *et al*. 2005). The other method in which apoptosis is activated is through death receptors. A number of receptors situated in the membrane of most cells have a binding domain known as the death domain. The death domain is a region of 60-70 amino acids (Micheau and Tschopp, 2003). The death domain is a motif that is commonly found in the receptors of the TNF superfamily. The receptors of TNF-α, TNFR1 and TNFR2, both have this domain. It is through this domain that TNF-α can induce apoptosis within a cell. The TNF-α cytokine binds to TNFR1 and causes trimerization of the receptor. This causes an adaptor protein TNFR-associated death domain (TRADD) to bind to its own death domain on the receptor. TRADD then acts as a platform and recruits the signalling molecules: TNFR-associated factor 2 (TRAF2), receptor-interacting protein (RIP), and Fas-associated protein with death domain (FADD) (Ashkenzai and Dixit, 1998). TRAF2 and RIP both go on to activate NF-κB signalling while FADD goes on to mediate apoptosis by recruiting several caspase-8 and caspase-10 molecules (Wajant *et al*. 2003). The recruitment of these molecules facilitates their activation by autoprotolysis that in turn will lead to the signalling cascade and eventual apoptosis of the cell. Despite TNF-α having the ability to induce apoptosis within a cell it is a rarely activated process, usually only triggered when protein synthesis has been blocked (Ashkenzai and Dixit, 1998).

1.4.2 NF-κB Signalling

Nuclear factor kappa beta (NF-κB) is a group of dimeric transcription factors that are comprised of members of the NF-κB/Rel family. It was originally discovered in B lymphocytes in which it bound to a specific decameric oligonucleotide sequence found in the enhancer of κ light chain immunoglobulin, with high specificity (Sen and Baltimore, 1986). Since then it has been found in many other cell types. NF-κB is involved with a large

number of different processes and plays a large role within the immune system. They are often induced in response to cytokines, such as TNF-α, foreign pathogens, and problems caused by the outside environment. NF-κB comes in two forms when in an activated state, p65 (Rel-A) and p50. Both of these forms, as well as all members of the NF-κB/Rel family share a three hundred amino acid Rel homology domain which mediates dimerization, DNA binding, and nuclear localization (Verna *et al*. 1995) which is also the site of interaction for members of the I-κB protein family (Miyamoto and Verma, 1995). When not active, NF-κB exists in the cytoplasm of the cell and complexes with an IκB protein. The IκB proteins are members of a family consisting of IκBα, IκBβ, IκBε, IκBγ, and Bcl3 in mammals (Perkins, 2000). Of the IκB proteins, IκBα, IκBβ, IκBε can complex with the NF-κB dimer and mask their nuclear translocation sequence. This retains a ternary complex of NF-κB and the IκB protein and prevents NF-κB from entering the cell nucleus (Barnes and Larin, 1997). In order for NF-κB to be induced, two conserved serine residues found on the N-terminal regulatory domain of IκBs need to be phosphorylated. This targets IκB for polyubiquitination and degradation by the 26S proteasome (Karin, 1999). The IκB kinase that phosphorylates IκBα and β is a 700kD complex (known as the IKK complex) consisting of the proteins IκB kinase α, β, and γ (NEMO) (Karin, 1999). IκB kinase α and β are required for encoding catalytic kinase subunits and γ is needed for activation of the complex by upstream signalling pathways. Once free of the IκBs, before it can be fully activated NF-κB is further phosphorylated by several kinases such as mitogen activated protein kinases (MAPK) and protein kinase C (PKC) (Wajant *et al*, 2003). Once fully activated NF-κB will be able to travel into the nucleus of the cell where it will bind to the promoter regions of various target genes. The phosphorylation and degradation of the IκBs by the IKK complex can be induced by a number of cytokines, such as TNF-α.

NF-κB can regulate the expression of a number of genes involved in the immune system and, specifically, the inflammatory response. It acts on genes for proinflammatory cytokines, chemokines, inflammatory enzymes, adhesion molecules and receptors. One of the proinflammatory cytokines it acts on is TNF-α. This results in a feedback loop where TNF-α can stimulate the activation of NF-κB while simultaneously being regulated by it, as well. This is the same case for another proinflammatory cytokine Interleukin-1β (Barnes and Larin, 1997).

As previously mentioned one of the functions of TNF-α is to induce apoptosis through binding to TNFR1. NF-κB plays a role in preventing this process from occurring. An investigation by Li *et al*. (1999) showed that mice that were deficient in IKKβ would die due to uncontrolled liver apoptosis, despite the presence of IKKα. It could be gathered from this that IKKβ is required for activation of NF-κB and that the lack of NF-κB has caused unregulated apoptosis to occur.

1.4.3 Mediated Activation of JNKs

Mitogen activated protein kinases (MAPK) are a group of kinases that can affect a large number of factors in cell regulation. They are activated and respond to various forms of chemical and physical stresses. They are also unique in that they can only be found in eukaryotes. The MAPKs form a very large group, each of which can be activated through a number of different ways. Within this group are the c-Jun N-terminal kinases (JNKs). C-Jun is a putative transforming gene of avian sarcoma virus 17. It is a proto-oncogene gene that is associated with gene regulation and contains no introns (Hattori *et al*. 1988). It is part of a group of related basic region-leucine zipper proteins that can dimerize to form transcription factors which are usually designated as activator protein-1 (AP-1) (Chang and Karin, 2001). It encodes a protein that has an 80% similarity to the viral protein (Bohmann

et al. 1987) which interacts with specific DNA sequences to regulate their expression. The purpose of JNKs is to phosphorylate the amino acid domains of c-Jun proteins and enhance their ability to affect DNA sequences, while not affecting DNA binding (Kallunki *et al*. 1996).

JNK can be activated through a number of different pathways. TNF-α can mediate activation of JNK through the use of a TRAF-2 dependent non-apoptotic pathway (Natoli *et al*. 1997; Reinhard *et al*. 1997). TNF-α can act through TRAF2 by binding to its receptor TNFR1. MAP kinase kinase 7 (MKK7) and MAP kinase kinase 4 (MKK4) are a pair of enzymes that are encoded by the MAP2K7 gene and form part of the MAPK family. These kinases are able to activate JNK by phosphorylating the residue threonine 180 in the case of MKK7 and tyrosine 182 in the case of MKK4. Targeted gene disruption in mice, performed by Tournier *et al*. (2001), showed that disruption of MKK7 genes was able to prevent JNK activation when caused by proinflammatory cytokines. However, disruption of both MKK7 and MKK4 genes was required to block JNK activation when caused in cells undergoing environmental stress. TNF-α can strongly activate MKK7 but does not significantly activate MKK4. This would suggest that MKK4 is not necessary for activation of JNK, this is also supported by Tournier *et al*. (2001) who found that MKK4 cannot activate JNK alone.

Germinal Center Kinases (GCKs) are a subfamily of enzymes that are part of the larger Sterile 20 (Ste20) family of serine/threonine kinases. They participate in a number of cellular processes that are involved in the determination of cell fate and regulation of its functions. The GCKs can be split up into one of several groups based on their structural and functional properties. The first group, GCK-I, have a highly conserved C terminal region consisting of 350 amino acids. This can then be further split up into two domains, a leucine rich domain of 140 – 150 amino acids and a C terminal domain (Katz *et al*. 1994). The Group I GCKs

have been shown to have a role in JNK activation through the use of the cytokine TNF-α. The second group, GCK-II, share catalytic homology with group I but their C terminal domains differ significantly (Kyriakis, 1999). The third group of GCKs, GCK-III, consists of three members, MST3, MST4, and STK25. MST3 has been found to be involved in apoptosis (Huang *et al*. 2002) and STK25 in cell migration and adhesion (Matsuki *et al*. 2010). The fourth group of GCKs, GCK-IV, include members, such as SPAK, that have been found to play a role in the activation of activator protein 1 (AP-1) transcription factor (Li *et al*, 2004). The way GCK-1 proteins are able to activate JNK, using TNF-α, is by way of association. TNF-α has been found to be able to activate various GCK-1 proteins such as GCKR (Shi and Kehrl, 1997) and HGK (Yao *et al*. 1999). GCKR in particular also interacts and is activated by TRAF2 (Shi *et al*. 1999). GCK is then able to associate with TRAF2 and activate MEK Kinase 1 (MEKK1), a member of the mitogen-activated protein (MAP) kinase kinase (MAPKK) family (Chadee *et al*. 2002). MEKK1 is then able to phosphorylate, thereby activating MKK4 and MKK7. They then in turn activate JNK by phosphorylation. Based on this mechanism, GCK is only able to activate JNK due to association rather than direct interaction.

1.5 TNF-α in Health

TNF-α has been shown to play a number of roles in various cellular signalling pathways or processes. As a result it has also been implicated in a number of health conditions in humans. Some of these conditions are associated with the immune system, while others are linked to apoptosis and cell death.

Autoimmunity is a collective term for a number of disorders that occur as a result of the immune system functioning abnormally. Normally the immune system will work to remove

or destroy invading pathogens. However, in autoimmunity the immune system attacks cells and organs which are normally found within an organism. This often causes a number of conditions which can lead to permanent injury or death of the organism.

Rheumatoid arthritis is an autoimmune disorder that typically affects joints and is associated with inflammation. It is typically characterised by how it affects the synovial joint, specifically the synovial membrane. Normally the synovial membrane is composed of a layer of cells that is roughly one to two cells thick. However, during rheumatoid arthritis various cells such as neutrophils, macrophages and T lymphocytes are recruited from the blood to synovial fluid, which is found in the joint, and the membrane. This causes the membrane to become inflamed due to the large amount of cells that have been recruited, resulting in the membrane becoming six to eight cells thick instead of what is normal. This inflammation can eventually lead to the loss of cartridge bone within the joint due to the erosion. This erosion is caused by a number of metalloproteinases produced by macrophages in response to various cytokines (Feldmann *et al*. 1996). TNF-α is one of the cytokines that has been found in the synovial fluid (Hopkins and Meager, 1998). TNF-α has also been found to induce the production of Interlukin-6, another proinflammatory cytokine (Matsuno *et al*. 2002). Interleukin 6 is a cytokine that is able to activate or prevent various cellular processes or recruit immune cells, such as T-cells (Scheller *et al*. 2011).

Cancer is the term given to a large group of diseases that can occur in the body and spread to other parts. The disease occurs due to cell growth that is uncontrolled, thus leading to the development of a tumour. This abnormal growth is caused by defective genomes in a cell which can lead to cell processes which would normally prevent cancer becoming deactivated, such as cell apoptosis. When first discovered, TNF-α was found to cause the necrosis of malignant cells in mice, hence its namesake. However, TNF-α has also been

found to be expressed in a number of human tumours such as ovarian cancer (Naylor *et al*. 1993) and breast cancer (Miles *et al*. 1994). In other cancers, such as prostate cancer, serum TNF-α levels have correlated with the progression of the disease (Michalaki *et al*. 2004).

As mentioned previously, NF-κB regulates the genes of many proinflammatory enzymes. It is involved in a number of inflammatory related conditions in which TNF-α plays a significant role. It has been found that NF-κB can lead to the progression of tumour. Hepatocellular Carcinoma (HCC) is a leading cause of cancer and is associated with patients with a chronic hepatitis background (Block *et al*. 2003). Pikarsky *et al*. (2004), found in a study that NF-κB activation had been found at the parenchyma of *Mdr2*-knockout mice, mostly adjacent to inflamed portal tracts suggesting inflammation caused by NF-κB. It was found that NF-κB had contributed to neoplastic growth in *Mdr2*-knockout mice. It was also discovered that NF-κB was essential for tumour progression, however, played very little part in tumour initiation. Conversely, ablation of NF-kB activity resulted in a decrease, in tumour progression. In these findings, TNF-α was the primary mediator in NF-kB activation and a major driver of anti-apoptosis of NF-κB.

1.6 TNF-α in Fish

TNF-α has been found and characterised in a number of different species of fish such as zebrafish (Pressley *et al*. 2005) and rainbow trout (Laing *et al*. 2002). One of the currently known differences between TNF-α in fish and in other mammals such as humans is the presence of multiple isoforms. In humans, there is only one form of TNF-α, whereas in fish multiple isoforms have been found. In common carp four different isoforms have been discovered (Zhao *et al*. 2012). In carp, Saeij *et al*. (2003) had found that there were multiple isoforms of TNF-α. TNF-α 1 and 2 are two isoforms which had similarities to TNF-α in *Homo sapiens,* in sequence identity. The further two isoforms TNF-α3 and TNF-α4 had been

discovered by Savan and Sakai. (2004) and Zhao *et al*. (2012) This has been seen in other fish such as in rainbow trout where three forms of TNF-α have been discovered (Hong *et al.* 2013).

Other differences have been found which seem to show that TNF-α can have a different effect depending on the species of fish. This is shown in *Oncorhynchus mykiss* (rainbow trout), *Carassius auratus* (goldfish) and *Sparus aurata* (gilthead seabream), in how TNF-α can activate phagocytes. In rainbow trout and goldfish, TNF-α has been shown to greatly enhance phagocyte activity (Grayfer *et al.* 2008; Zou *et al.* 2003). Conversely, in gilthead seabream it was found that TNF-α was able to activate endothelial cells but not phagocytes directly (Roca *et al.* 2008). TNF-α has also been found to be poorly expressed in the phagocyte, acidophilic granulocytes (Sepulcre *et al.* 2007), when it comes to seabream. This is in comparison to mammals where the presence of PRRs greatly increased the expression of TNF-α in certain phagocytic cells (Jiang *et al*. 2005). Roca *et al*. (2008) had found a number of differences in the TNF-α cytokine from zebrafish and gilthead seabream. It was found that the zebrafish form of TNF-α increased the susceptibility of the fish to bacterial and viral infections. It was also found that the seabream form of TNF-α was able to activate endothelial cells and promote the recruitment of leucocytes while also inducing expression of proteins involved in innate immunity, such as E-selectin.

1.7 Conclusion

TNF-α is a soluble cytokine that is produced as a response to various stimuli in the immune system. Two forms of the protein exist, the trans-membrane form and the soluble form. Although both forms are capable of interacting with cells, the soluble form or the external portion of the transmembrane form, is what primarily interacts with cells. This has been

shown by the presence of residues that can affect the structure of the protein or binding to its receptors. These residues are all located in the protein that becomes the soluble form. It is this portion that also binds with either of the proteins receptors, TNFR1 and TNFR2. As TNF-α in common carp is part of the same superfamily then it can be theorised that this will also be the case for that cytokine.

The function of TNF-α in humans has been shown to affect a number of different systems in the immune system such as apoptosis, inflammation, and various cells signalling pathways. While the exact function of TNF-α in fish is not known, it has been shown to affect the immune system, some of which is different to what has been found in humans. What this does show is that there are at least some similarities between TNF-α in humans and fish and that it would be worth comparing the two to see if they share any functions or structure. The prediction of any functions can benefit the understanding of TNF-α in common carp by revealing what the role of the protein is in fish and what differences there are between TNF-α in humans.

Currently there is a large amount of information on TNF-α in humans. Conversely, in fish and common carp in particular, there is very little information on its structure or function. There are some questions that need to be answered about TNF-α in common carp. Some of these include; what is the role of the cytokine in fish immunity, why are there multiple isoforms, do these isoforms have any specific roles, and how do they differ to each other and TNF-α in other species?

Predicting the structure of one of the four different isoforms of TNF-α in common carp may be able to answer some of these questions and help further research of the cytokine. As the structure of human TNF-α has already been solved, comparing it to the predicted structures

of TNF-α in common carp can reveal the possible locations of specific structural features such as active sites or areas in which the protein binds to its substrate. This understanding can, in turn, contribute to determining the actual structure of TNF-α in common carp, which can then be used to determine the functions of the protein.

1.8 Aims and Objectives

Tumour necrosis factor alpha (TNF-α) is a cytokine that plays a large role in the immune system of vertebrates. The three dimensional structure and function has been well characterised in some vertebrate species, such as in humans and mice. However, very little is known about TNF-α in fish, in regards to both the structure and the specific function in fish immunity.

This investigation will focus on predicting the secondary structure of TNF-α in common carp. This will enhance the knowledge base of the structure of TNF-α in carp and other fish. This can lead to further knowledge in the treatment of TNF-α related diseases in fish, and the production of drugs. This is particularly important in common carp, an economically significant fish that is farmed in numerous parts of the world.

To predict the secondary structure of TNF-α in common carp, the protein sequence of TNF-α, from common carp, will be analysed, compared with the sequences of TNF-α from other species and also those with known structures. This will be done to determine whether there are any links that could reveal the location of functional sites within the protein sequence of TNF-α in common carp. This could also reveal elements of the secondary structure. To supplement this, using prediction software, the secondary structure of TNF-α in common carp will be predicted and compared with the known structures of TNF-α in humans and mice. These data will be combined with the analysis to give overall prediction.

Chapter 2: Materials and Methods

2.1 Identification of the Protein Sequences

The protein sequences for TNF-α in common carp were found by searching the protein database on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov), using the search term "Tumour necrosis factor *Cyprinus carpio*". The protein sequences and their respective accession numbers are shown in Table 2.1.

Protein	**Accession Number**	**Protein Sequence**
Cyprinus carpio TNF-α1	CAC84641	MMDLESQLLEEGGLLPLPQVMVSRRKSGSSKSG VWRVCGVLLAVALCAAAAVCFTLNKSQNNQEGG NALRLTLRDHLSKANVTSKAAIHLIGAYEPKVS TETLDWKKNQDQAFTSGGLKLVEREIIIPTDGI YFVYSQVSFHINCKTNMTEDHDLVHMSHTVLRY SDSYGRYMPLFSAIRTACAQASNTDDLWYNTIY LGAAFKLRAGDRLRTETTEELLPSVETGDGKTF FGVFAL
Cyprinus carpio TNF-α2	CAC84642	MMDLENQFLEEGALPLPQVMVSRRKSGVWRVCG VLLAVALCAAAAVCFTLNKSQNNQEGGNALRLT LRDHLSKENVTSKVAIHLTGAYDPDVCKDNLDW KQNQDQAFVSGGLELVDREIIIPNDGIYFVYSQ VSFHISCKHDMTEDQDVVHMSHAVLRYSESYGS YKPLFSAIRSACVHASDSEDLWYNTIYLGAAFN LRARDRLRTETTKELLPRVESENGKTFFGVFAL
Cyprinus carpio TNF-α3	BAC77690	MMDLESQPLPQEMVSRRNASSSKSAVWWVCGVL LAVALCAAAAVCFTLNKNNQEGGNEQRLTLKDN LSKENVTSKVAIHLSGAYEPDVSKNNIDWKQNQ DGAFVSGGLKLVDREIIIPNDGIYFIYSQVSFH ISCKNDMTEDQEVMHVSHAVFHYSDFFGIYKPL IRAARSACVHASNTEDVWYDTIYLGAAFSLRAG DKLCTKTTTELLPRVETDNAKTFFGVFAL
Cyprinus carpio TNF-α4	AFQ20281	MMDLESQLLEEGGLLPLPQVMVSRRKSGSSKSG VWRVCGVLLAVALCAAAAVCFTLNKSQNNQEGG NALRLTLRDHLSKANVTSKAAIHLTGAYESEVS TETLDWKKNQDQAFTSGGLKLVEREIIIPTDGI YFVYSQVSFHISCKTGMPEEHDIVHMSHTVLRY SDSYSSYKPLFSAIRSACAQASNTEDLWYNTIY LGAAFKLRAGDRLCTKTTKELLPSVETDNGKTF FGGVCFMMCTLKKSVKIGHNVLYL

Table 2.1: A table to show the accession number and protein sequence (FASTA format) of TNF-α from common carp, used in subsequent analysis.

Protein sequences, for use in the multiple sequence alignment were obtained using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*. 1990), also found on the NCBI website (http://blast.ncbi.nlm.nih.gov). The search algorithm Protein blast was used. The following parameters were applied to the search. The query sequences used were that of *Cyprinus carpio* TNF-α1, TNF-α2, TNF-α3, and TNF-α4 (Table 2.1). The protein database used in the search was the Non-redundant protein sequences (nr). The organisms that were searched were fish (taxid: 7898) and mammals (taxid: 40674). The maximum number of target sequences displayed was set to 20000. The remaining algorithm parameters were set as follows: Expect threshold was set to 10, maximum matches in a query ranger was set to 0, the scoring matrix used was BLOSUM62, and the gap costs were Existence 8 and Extension 2. The sequence similarity of each result, compared to the original query protein sequences, were calculated by the Blast search program. When selecting the sequences the following parameters were used. Only full sequences were selected and partial or incomplete sequences were discarded. The protein sequence had to be of a roughly equal size to the TNF sequences of common carp, used in the query as sequences that were significantly different were found to be partial or incomplete. The cut off for the E-value was set to 9e-04.

Table 2.2 and 2.3 show the sequences that were selected for use in this investigation.

Common name	**Latin name**	**Protein**	**Accession Number**	**Database**
Common carp	*Cyprinus carpio*	TNFα1	CAC84641	NCBI
Common carp	*Cyprinus carpio*	TNFα2	CAC84642	NCBI
Common carp	*Cyprinus carpio*	TNFα3	BAC77690	NCBI
Common carp	*Cyprinus carpio*	TNFα4	AFQ20281	NCBI
Crucian carp	*Carassius carassius*	TNFα	AGU42191	NCBI
Crucian carp	*Carassius carassius*	TNFα2	AIL81468	NCBI
Goldfish	*Carassius auratus*	TNFα2	ABU50127	NCBI
Zebrafish	*Danio rerio*	TNFα	AAR06286	NCBI
Grass Carp	*Ctenopharyngodon idella*	TNFα	ADY80577	NCBI

Channel catfish	*Ictalurus punctatus*	TNFα	NP_001187101	NCBI
Atlantic salmon	*Salmo salar*	TNFα	NP_001117089	NCBI
Rainbow trout	*Oncorhynchus mykiss*	TNFα	NP_001117846	NCBI
Rainbow trout	*Oncorhynchus mykiss*	TNFα3	CCH10518	NCBI
Japanese seabass	*Lateolabrax japonicus*	TNFα	AAR02413	NCBI
Turbot	*Scophthalmus maximus*	TNFα	ACN41911	NCBI
Striped trumpeter	*Latris lineata*	TNFα	ACQ99509.1	NCBI
Croceine croaker	*Larimichthys crocea*	TNFα	ABK62876	NCBI
European seabass	*Dicentrarchus labrax*	TNFα	AAZ20770	NCBI
Orange-spotted grouper	*Epinephelus coioides*	TNFα1	AEH59794	NCBI
Orange-spotted grouper	*Epinephelus coioides*	TNFα2	AEH59795	NCBI
Yellow grouper	*Epinephelus awoara*	TNFα	AAV80240	NCBI
Gilt-head (sea) bream	*Sparus aurata*	TNFα	Q8JFG3	NCBI
Striped beakfish	*Oplegnathus fasciatus*	TNFα	ACM69339	NCBI
Green chromide	*Etroplus suratensis*	TNFα	AEM59514	NCBI
Pacific bluefin tuna	*Thunnus orientalis*	TNFα2	BAG72142	NCBI
Southern bluefin tuna	*Thunnus maccoyii*	TNFα1	AGH24761	NCBI
Black Porgy	*Acanthopagrus schlegelii*	TNFα	AAP94278	NCBI
Olive flounder	*Paralichthys olivaceus*	TNFα	BAA94969	NCBI
Brook Trout	*Salvelinus fontinalis*	TNFα	AAF86331	NCBI
Nile tilapia	*Oreochromis niloticus*	TNFα	NP_001266462	NCBI
Japanese puffer	Takifugu rubripes	TNFα	NP_001033074	NCBI

Table 2.2: A table which shows the selected TNF sequences of fish, obtained from doing a search using Protein BLAST.

Common name	**Latin name**	**Protein**	**Accession Number**	**Database**
Human	*Homo sapiens*	TNFα	AAA61198.1	NCBI
Mouse	*Mus musculus*	TNFα	AAB65593.1	NCBI
Groundhog	*Marmota monax*	TNFα	AAP80576	NCBI
Mongolian gerbil	*Meriones unguiculatus*	TNFα	BAD67163	NCBI
Rat	*Rattus norvegicus*	TNFα	NP_036807.1	NCBI
White-footed mouse	*Peromyscus leucopus*	TNFα	P36939	NCBI
Leschenault's rousette	*Rousettus leschenaultii*	TNFα	BAH02563	NCBI
Domestic Cat	*Felis catus*	TNFα	AAA30818	NCBI
Guinea pig	*Cavia porcellus*	TNFα	NP_001166496	NCBI
Dog	*Canis familiaris*	TNFα	P51742	NCBI
Northern elephant seal	*Mirounga angustirostris*	TNFα	AGI56042	NCBI

West Indian manatee	*Trichechus manatus latirostris*	TNFα	NP_001266188	NCBI
Horse	*Equus caballus*	TNFα	P29553	NCBI
Chacma baboon	*Papio hamadryas ursinus*	TNFα	O77510	NCBI
Large tree shrew	*Tupaia tana*	TNFα	Q539C2	NCBI
Pig	*Sus scrofa*	TNFα	P23563	NCBI
Bactrian camel	*Camelus bactrianus*	TNFα	Q75N23	NCBI
Ferret	*Mustela putorius furo*	TNFα	AGN95898	NCBI
Rhesus macaque	*Macaca mulatta*	TNFα	P48094	NCBI
Green monkey	*Chlorocebus sabaeus*	TNFα	ACI28908	NCBI
Common squirrel monkey	*Saimiri sciureus*	TNFα	Q8MKG8	NCBI
Tammar wallaby	*Macropus eugenii*	TNFα	O77764	NCBI
White-tufted-ear marmoset	*Callithrix jacchus*	TNFα	Q19LH4	NCBI
Koala	*Phascolarctos cinereus*	TNFα	AFY22676	NCBI
Sooty mangabey	*Cercocebus atys*	TNFα	ABI18975	NCBI
Bighorn sheep	*Ovis canadensis*	TNFα	ADG21120	NCBI
Baboon	*Papio sp*	TNFα	P33620	NCBI
Brush-tailed possum	*Trichosurus vulpecula*	TNFα	P79374	NCBI
Red deer	*Cervus elaphus*	TNFα	P51743	NCBI
Domestic water buffalo	*Bubalus bubalis*	TNFα	P59693	NCBI
Nilgai	*Boselaphus tragocamelus*	TNFα	Q1G1A2	NCBI
Beluga whale	*Delphinapterus leucas*	TNFα	Q8WNR1	NCBI

Table 2.3: A table which shows the selected TNF sequences of mammals, obtained from doing a search using Protein BLAST.

In the case of the protein sequences for TNF-α from humans and mice, the full protein sequence was used rather than just the portion the represents the soluble protein. This is because the selected sequences all use the full TNF-α sequence rather than the soluble form of the cytokine. Table 2.4 shows the protein sequence of TNF-α in humans and mice with the soluble region highlighted. As much of the literature will refer to the soluble form of TNF-α in humans and mice (as the structure was produced from the soluble form), the difference in the position of specific residues will be accounted for by adding the remaining number of residues in the full sequence to the number.

Human TNF-α	MSTESMIRDVELAEEALPKKTGGPQGSRRCLFLSLFSFLIVAGATTLFCLLHFGVIGPQREEFPR DLSLISPLAQAVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEG LYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIY LGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL
Mouse TNF-α	MSTESMIRDVELAEEALPQKMGGFQNSRRCLCLSLFSFLLVAGATTLFCLLNFGVIGPQRDEKFP NGLPLISSMAQTLTLRSSSQNSSDKPVAHVVANHQVEEQLEWLSQRANALLANGMDLKDNQLVVP ADGLYLVYSQVLFKGQGCPDYVLLTHTVSRFAISYQEKVNLLSAVKSPCPKDTPEGAELKPWYEP IYLGGVFQLEKGDQLSAEVNLPKYLDFAESGQVYFGVIAL

Table 2.4: A table which shows the protein sequence of TNF-α from humans and mice. The area with the yellow background indicates the portion of the sequence that represents the soluble form of the cytokine.

2.2 Global Sequence alignment

A global alignment of the sequences selected from the BLAST search and the TNF-α sequences from common carp was produced using Clustal Omega (Sievers *et al.* 2011; Groujon *et al*. 2010) from http://www.ebi.ac.uk/. The following settings were used to produce the global alignment. The option to dealign input sequences was set to no, mbed-like clustering guide tree was set to yes, mbed-like clustering iteration was set to yes, number of combined iterations was set to default, max guide tree iterations was set to default, max hmm iterations was set to default, and the order of the sequences was set to input.

The results of the alignment were then transferred to Boxshade (http://www.ch.embnet.org/software/BOX_form.html) to format the alignment. Any other modifications were performed in Microsoft Word.

2.3 Secondary Structure Prediction

Prediction of the secondary structure was performed by using two different software methods. The first software, Predict Protein (Yachdav *et al.* 2014), uses several methods that predict different aspects of the secondary structure. The method PROFphd was used in this particular prediction. PROFphd predicts the secondary structure and solvent accessibility of protein sequences. The second software used was PSIPRED (Jones, 1999) found at http://bioinf.cs.ucl.ac.uk/psipred/. Only the PSIPRED v3.3 prediction method was used. In

this prediction PSIPRED gives the possible location of an α-helix or a β-strand along with a rating of how confident that prediction is. This is visualised in the form of a diagram where the location of any α-helices and β-strands are highlighted. The predictions were then compared with the crystal structure of TNF-α in humans (Eck and Sprang, 1989) and mice (Baeyens *et al.* 1999).

2.4 Three Dimensional Structure Prediction

Prediction of the tertiary structure was performed by using software provided by the Phyre2 server (Kelley *et al*. 2015). The software uses several methods to build a 3D model of the protein based on its amino acid sequence. The predictions were then compared with the 3D model produced from the crystal structure of TNF-α in humans (Eck and Sprang, 1989) and mice (Baeyens *et al.* 1999).

Chapter 3: Results and discussion of Phylogenetic and structural analysis of *Cyprinus carpio* TNF

3.1 Sequence alignment of the TNF isoforms from *Cyprinus carpio*

The sequences of the four isoforms of TNF-α in common carp were aligned with each other. The alignment was then analysed in order to determine if there were any homology between the three isoforms. Figure 3.1 shows the protein sequences of the four isoforms of TNF from common carp. Approximately 149 residues, in each protein, were conserved in the alignment. Out of the remaining residues in the alignment, approximately 34 of the residues had a conservative substitution and 18 had semi conservative substitutions. In total, this means that 62% of the alignment is conserved, 14% has a conservative substitution and 7% has a semi conservative substitution. The largest difference can be seen in TNFα-4, which has a significantly longer sequence than the other isoforms. This can be seen at the end of the alignment starting at residue M-238 of TNF-α4.

The sequence similarity of the isoforms is exhibited as follows. TNF-a1 has an 80%, 72% and 92% sequence similarity to TNF-α2, TNF-α3 and TNF-α4 respectively. TNF-α2 has a 76% and an 82% sequence similarity to TNF-α3 and TNF-α4 respectively. TNF-α3 has a 72% sequence similarity to TNF-α4. The sequence identity of the aligned sequences are as follows. TNF-a1 has an 83%, 74% and 91% sequence identity to TNF-α2, TNF-α3 and TNF-α4 respectively. TNF-α2 has an 80% and an 82% sequence identity to TNF-α3 and TNF-α4 respectively. TNF-α3 has a 74% sequence identity to TNF-α4. Due to the large number of conserved residues this would suggest that the isoforms all have a very similar structure and function. TNF-α4 has a longer sequence, but the majority of the cytokine is still conserved. TNF-α4 also had the highest sequence similarity with TNF-α1, with a score of 92%. This

suggests that the longer protein sequence may just be an extension of the C-terminus and does not have a major effect on the function or overall structure of the cytokine.

Figure 3.1: A global alignment of the isoforms of TNF-α from common carp. The black box indicates residues that are conserved and the grey box indicates residues that are conservative substitutions. The "*" symbol indicates every residue in that column is fully conserved. The ":" symbol indicates that at least one residue in the column will be a conservative substitution, meaning that it has been substituted for one with similar chemical properties. The "." symbol indicates that at least one residue in the column will be a semi conservative substitution where the substituted residue has a similar conformation but different chemical properties

3.2 Global alignment of *Cyprinus carpio* TNF-α with the known structures of TNF-α from humans and mice.

The secondary and three dimensional structure of TNF-α in humans and mice has been previously solved and are well-studied (Eck and Sprang, 1989; Baeyens *et al*. 1999). Figure 3.2 shows the alignment of TNF-α in humans and mice. This alignment was then analysed in order to observe the homology between the two sequences, before they were compared with the isoforms of TNF-α in common carp. Approximately 185 residues in each protein were conserved in the alignment. Out of the remaining residues, 25 had a conservative substitution and 7 had a semi conservative substitution. In total this means that approximately 80% of the alignment is conserved, 11% had a conservative substitution and 3% had a semi conservative substitution. It is clear that there is a large amount of homology between the two proteins. The positions of the or α-helix and β sheets found within the three dimensional structure are almost the same in both proteins with only minor differences in size. This shows that the TNF-α in humans and mice would both be very similar in structure. The large number of conserved residues would also suggest that the proteins both a have very similar functions as well.

Figure 3.2: A multiple sequence alignment of TNF-α from humans and mice. The regions with a red and blue outline indicate the positions of β-strands and α-helices respectively. The yellow line indicates the region in which the membrane form of TNF-α from humans and mice is cut to produce the soluble version of the cytokine. The "*" symbol indicates that every residue in that column is conserved. The ":" symbol indicates that at least one residue in that column will be a conservative substitution, meaning that it has been substituted for one with similar chemical properties. The "." symbol indicates that at least one residue in the column is a semi conservative substitution where the substituted residue has a similar conformation but different chemical properties.

A global alignment of the isoforms of TNF-α from common carp and TNF-α from humans and mice was performed. A global alignment involves aligning every residue in the selected protein sequences; this is shown in Figure 3.3. This was done to see if any conserved residues or ones with conservative substitutions could indicate the location of secondary structural features or protein functions. In TNF-α of humans, the protein is cleaved by TACE between residues 76 (Alanine) and 77 (Valine) respectively (Moss *et al.* 1997). This has been pointed out in Figure 3.3 by a yellow line. The remaining portion of the protein makes up the fragment used in the determination of the protein structure.

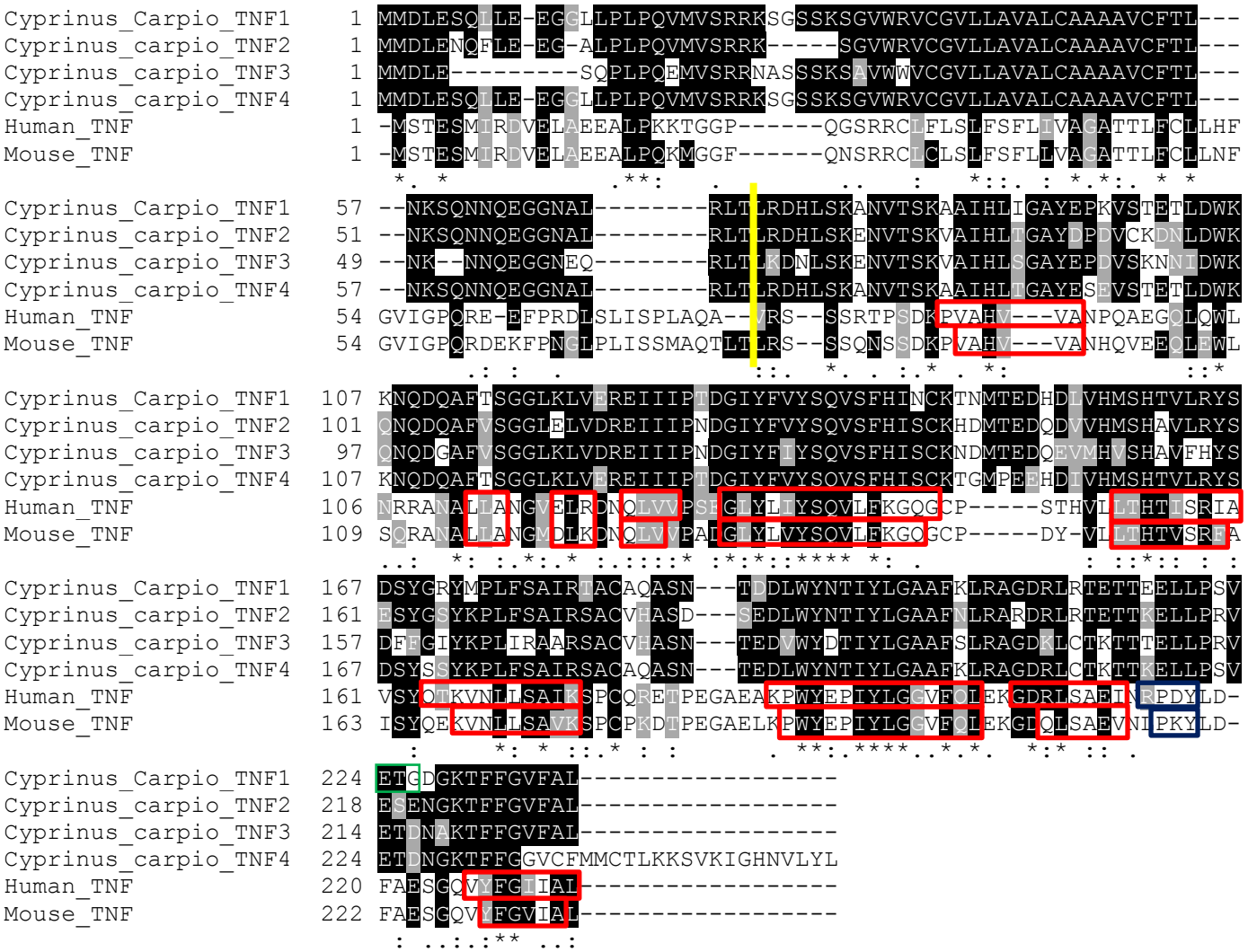


Figure 3.3: A multiple sequence alignment of TNF-α from common carp, humans and mice. The regions with a red and blue outline indicate the positions of β-strands and α-helices respectively. The yellow line indicates the region in which the membrane form of TNF-α from humans and mice is cut to produce the soluble version of the cytokine. The "*" symbol indicates that every residue in that column is conserved. The ":" symbol indicates that at least one residue in that column will be a conservative substitution, meaning that it has been substituted for one with similar chemical properties. The "." symbol indicates that at least one residue in the column is a semi conservative substitution where the substituted residue has a similar conformation but different chemical properties.

Across the whole alignment, in each of the protein sequences, there are a total of 40 conserved residues, 49 residues that have conservative substitutions and 34 residues that have semi conservative substitutions. Of the residues that are conserved, 9 residues in each proteins are situated in what would be the transmembrane region of TNF-α in humans and mice. The remaining conserved residues are in the soluble portion of TNF-α in humans and mice. This is also true for the conservative substitutions where the majority are found in the soluble portion of TNF-α in humans and mice. Each of the isoforms of TNF-α in common carp has the following percentage of residues conserved: 21% in TNF-α1 and TNF-α2, 22% in TNF-α3, and 19% in TNF-α4. The sequence similarity of TNF-α in humans and mice is 32% towards TNF-α1, TNF-α2, and TNF-α4, and is 27% towards TNF-α4 of common carp. As with the alignment shown in Figure 3.1 the most noticeable difference occurs with TNF-α4. Possibly due to having a significantly longer sequence the cytokine does not have a similar C-terminal end as the remaining TNF-α isoforms in common carp or TNF-α from humans and mice. The last two residues, a V and an L residue, are conserved in the remaining three isoforms of TNF-α in common carp and in TNF-α of humans and mice. The corresponding residues in TNF-α4 for common carp are a C and an F residue which are a semi conservative and a conservative substitution respectively. The amount of conserved residues is much lower than previously seen in Figure 3.1 However, the presence of conserved residues would suggest that there are similarities in structure and function between TNF-α from humans and mice, and TNF-α from common carp.

The red and blue boxes in Figure 3.3 indicate the locations of β-strands and α-helices respectively in TNF-α of humans and mice. Humans and mice have the same amount of β-strands and α-helices with 10 strands and 1 helix between them. All of the β-strands and α-helices are roughly the same size and in similar positions with the largest difference in size being two residues. When comparing the protein sequence of TNF-α in just humans and

mice most of the residues are conserved. When TNF-α in humans and mice are compared with the sequences for common carp, all of the 10 β-strands seen in TNF-α of humans and mice have at least a conservative substitution, with most having at least one conserved residue. Only two β-strands have no conserved residues and only conservative substitutions. These are situated between residues L-112 and 114, and Q-123 and V-126 in humans. In the case of the β-strand situated between Q-123 and V-126, the corresponding residues in TNF-α of common carp are all conserved between the isoforms. The remaining β-strands have a mixture of conserved residues and conservative substitutions. The fifth and eighth β-strands situated between G-130 – G-144 and K-188 – I-202 in humans, have the largest amount of conserved residues with 7 and 8 conserved residues respectively. They also have 4 and 1 conservative substitutions respectively as well. In the case of the α-helixes in TNF-α of humans and mice, there were no conserved residues or ones with conservative or semi conservative substitutions. The presence of conserved residues and semi conservative substitutions in positions where there were β-strands would suggest the possible locations of β-strands within the isoforms of TNF-α in common carp. This is particularly in the two largest strands situated between G-130 – G-144 and K-188 – I-202 in human TNF-α as they have the largest amount of conserved residues and conservative substitutions. The lack of any conserved residues or ones with conservative substitutions in the position of the α-helix could indicate one of two things. The first is that there are no α-helices in any of the TNF-α isoforms or if there are it is located in a position that is different to what is seen in TNF-α of humans and mice. The α-helix has been found in other members of the TNF superfamily, such as in human Lymphotoxin which has two α-helices so it may just be case of the α-helix being in a different location (Banner *et al*. 1993). However, the three residues, an E and two L residues, situated in this position in TNF-α in common carp are conserved between the isoforms. So these sets of residues may have another purpose.

Several of the conserved residues, and those with conservative mutations, have been found to be involved with various functions in human TNF-α. The first conserved residue is a Serine located at position 78 of the multiple sequence alignment (using the protein sequence of common carp TNFα-1 as a counting base). In humans, this residue was found to be the site of glycosylation when O-glycosylated TNF-α was produced by human B-cell lymphoblastic leukemia cells (Takakura-Yamamoto *et.al*. 1996). As the residue is conserved it indicates that S-78 in TNF-α from common carp might also be a site of glycosylation However, in human TNF-α only a small amount of the protein that had become glycosylated were produced. Although the residue is conserved in TNF-α from mice it has not been shown to undergo glycosylation as of yet. The significance of glycosylation in humans is not currently known. TNF-α in humans is only able to act on various cells by binding to one of two receptors, TNFR1 and TNFR2. The crystal structure of TNFR2 bound to TNF-α in humans has been determined by Mukai *et al*. (2010). It was found that several residues in TNF-α directly interacted with certain residues in TNFR2. These were the residues R-108, D-219, Q-225, and E-99. Although none of these residues are conserved in the sequence alignment, the corresponding residues at R-108 and Q-225 have conservative substitutions in the isoforms of TNF-α in common carp. This shows that they have similar biochemical properties so this could be indicative of a possible binding site for the isoforms of TNF-α in common carp. Several site directed mutagenesis experiments were performed by Loetscher *et al.* (1993) to determine how human TNF-α bound to one of the two receptors, TNFR1 and TNFR2. Several residues within the human TNF-α sequence were mutated to see if binding was affected. The residue Y-163 was found to be crucial for interactions with both receptors, as mutations at this point caused almost a complete loss of binding activity. This would suggest that the residue may be important structurally as binding to both receptors are affected. An investigation by Mukai *et.al.* (2009) also suggested this residue was important as TNFR1-selective mutants produced in a library all retained Y-163. While the residues at

this position, within the alignment, are not completely conserved there is a high degree of similarity. Only TNF-α3 from common carp has a residue that is different by having an F residue instead of a Y, which is a conservative substitution. Y and F residues both share a similar molecular structure, with the key difference being a hydroxyl group found on Y. This indicates that this particular residue may play some role in binding within the isoforms of TNF-α in common carp, if not participating in the binding then perhaps maintenance of the structure. An inhibitor of human TNF-α was identified by He *et al.* (2005). This inhibitor was a small molecule which, when bound to TNF-α, would cause inhibition of receptor binding. X-ray crystallography studies showed that the compound caused a conformational change in the TNF-α trimer, causing one of the subunits to become displaced and results in a TNF-α dimer. This inactivates the cytokine, thus causing a loss of function. This would suggest that the residues which form contacts with the small molecule inhibitor play a role in formation of the TNF-α trimer. Of the residues in human TNF-α, which form contacts with the inhibitor several are conserved in the sequence alignment between TNF-α of common carp, humans and mice. These residues are L-133, S-136, Q-137, Y-195, L-196, G-197 and G-198. Aside from G-198 and L-133 which are both conservative substitutions and semi conservative substitutions respectively, all of the residues are conserved within the alignment. As these residues have been conserved this would suggest that these residues also play a role formation or maintenance of the TNF-α structure in common carp. It could also indicate they specifically are involved with trimer formation given that these residues are involved with maintenance of the trimer in humans. This in turn indicates that the tertiary structure of TNF-α in common carp is possibly a trimer as well. Several of these residues were also found to be conserved in several other trimeric TNF-α superfamily members such as LIGHT, FasL, and Lymphotoxin (He *et al,* 2005) indicating that they may be a common feature in the TNF-α superfamily. The residue Y-132, in human TNF-α is a residue that is thought to play a role in receptor binding due to the presence of a hydroxyl group (Eck and

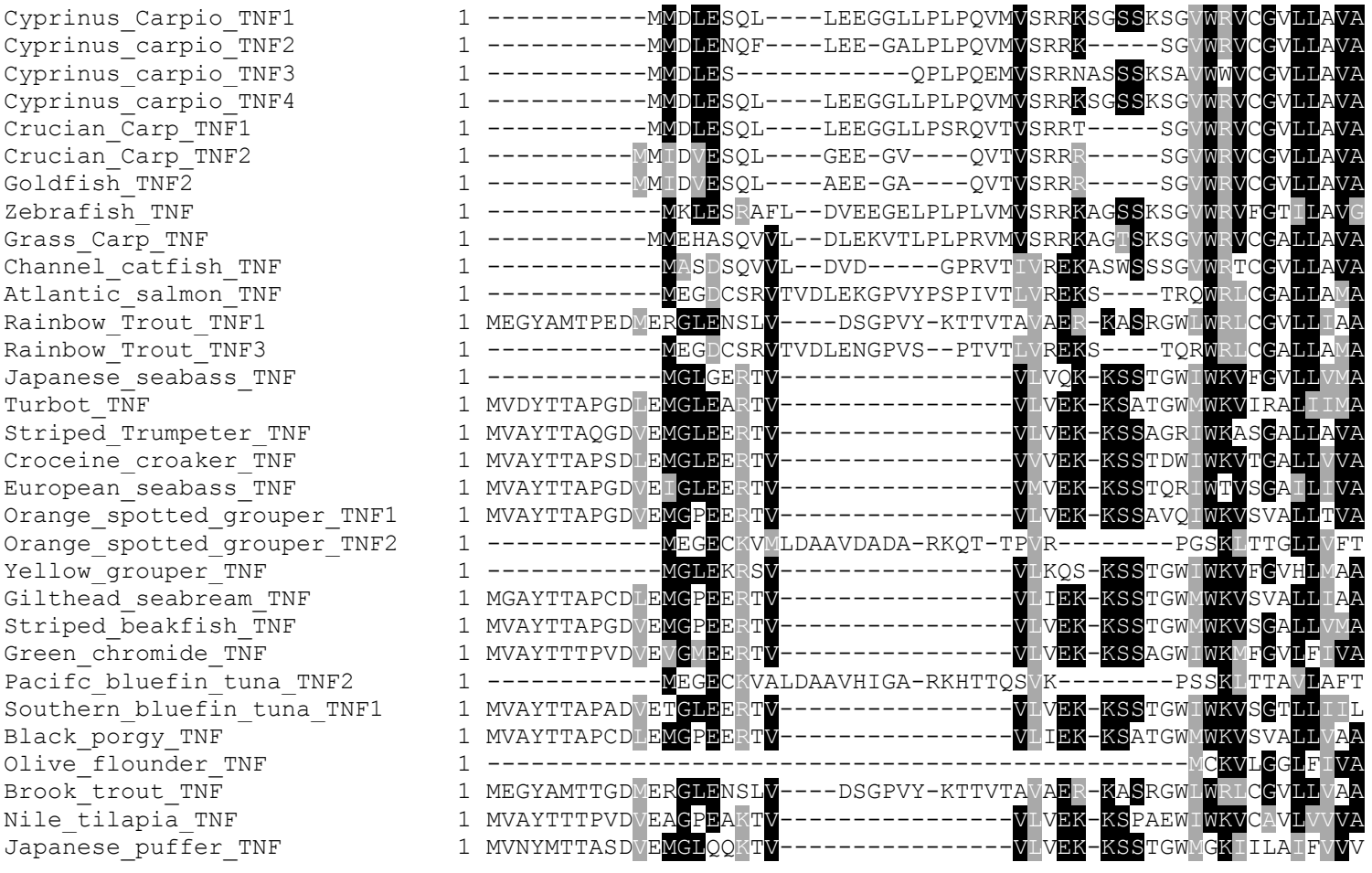
Sprang, 1990). Changes to the residue causes the impairment of trimer formation and resulted in a loss of biological activity (Zhang *et al.* 1992). This indicates that it is important for the formation of the TNF-α trimer. Y-132 was found to be conserved in the alignment, suggesting it plays a similar role in TNF-α of common carp. Zhang *et al* (1992) had also found a mutation at this residue resulted in impaired or reduced receptor binding. Despite these findings Y-132 was not found to be directly involved in receptor binding when TNF-α is in complex with TNFR2, but it may be that it still affects receptor binding by maintaining the trimer structure. This effect also occurred when the residues Y-195, Y-191, H-154, S-136, and W-104 were mutated in human TNF-α. This caused a loss in receptor binding and in cytotoxicity. All of the residues are conserved in the sequence alignment, which may imply they have a similar function in receptor binding, in TNF-α of common carp. Of these residues Y-195 and S-136 were also affected by the inhibitor which inhibited receptor binding (He *et al*, 2005). This further indicates that this area may be a functional site for receptor binding in the TNF-α isoforms of common carp in the corresponding residues between L-133 and G-198 of human TNF-α. Y-195, in humans, forms part of the hydrophobic core of the TNF-α trimer. The other two residues which form this hydrophobic core, L-133 and L-233, are not conserved but do have conservative substitutions at these positions, with an F residue in common carp at L-133 and another F residue in TNF-α4 of common carp at L-233. In the case of L-233, isoforms TNF-α1, TNF-α2, and TNF-α3 also have an L residue at this position, with TNF-α4 being the only exception.

As Y-195, Y-191, H-154, S-136, and W-104 all affect receptor binding in human TNF-α, they may all be a part of the receptor binding interface of the protein. As they are all conserved this interface may also be similar in common carp. Infliximab, an inhibitor of TNF-α in humans, is able to affect the cytokine by preventing its ability to bind to one of the TNF-α receptors. Inflixmab binds to TNF-α in humans through the C-D and E-F loops,

situated between residues Q-143 - H-149 and S-175- K-188 respectively, within the cytokine subunits (Liang *et al*. 2013). Out of the residues in these loops, only C-177 is fully conserved between TNF-α of humans, mice, and the isoforms in common carp. This indicates that this particular residue may have some significance in binding either to the TNF-α receptors for common carp or to an undiscovered inhibitor. In the crystal structure of TNF-α in humans it has been shown that K-174 and E-192 are able to form salt bridges between subunits in the tertiary structure (Eck and Sprang, 1989). Neither of these residues are fully conserved in the sequence alignment of TNF-α between humans, mice and the isoforms of TNF-α common carp. However, in these positions within TNF-α of common carp, there are conservative substitutions with an R and an N (or a D in the case of TNF-α3 of common carp) respectively. The residues Y-195, L-133 and 233, in humans, all form the hydrophobic core of the TNF-α tertiary structure. Of these residues, only Y-195 is fully conserved, however the other two residues have conservative substitutions. In the case of L-233, all the isoforms have the same C terminal residue, aside from TNF-α4 due to have a significantly longer protein sequence. As the substitutions are conservative it is likely they share the same biochemical properties and so may have a similar function. In sharing a similar function, these residues may still form the hydrophobic core in the tertiary structure of TNF-α in common carp.

3.3 Sequence alignment of *Cyprinus carpio* TNF and other species of fish

Figure 3.3 shows an alignment of the isoforms of TNF-α from common carp and TNF-α from other species of fish. This was done to see if there were any evolutionarily conserved regions which might suggest areas of important features within the protein sequence.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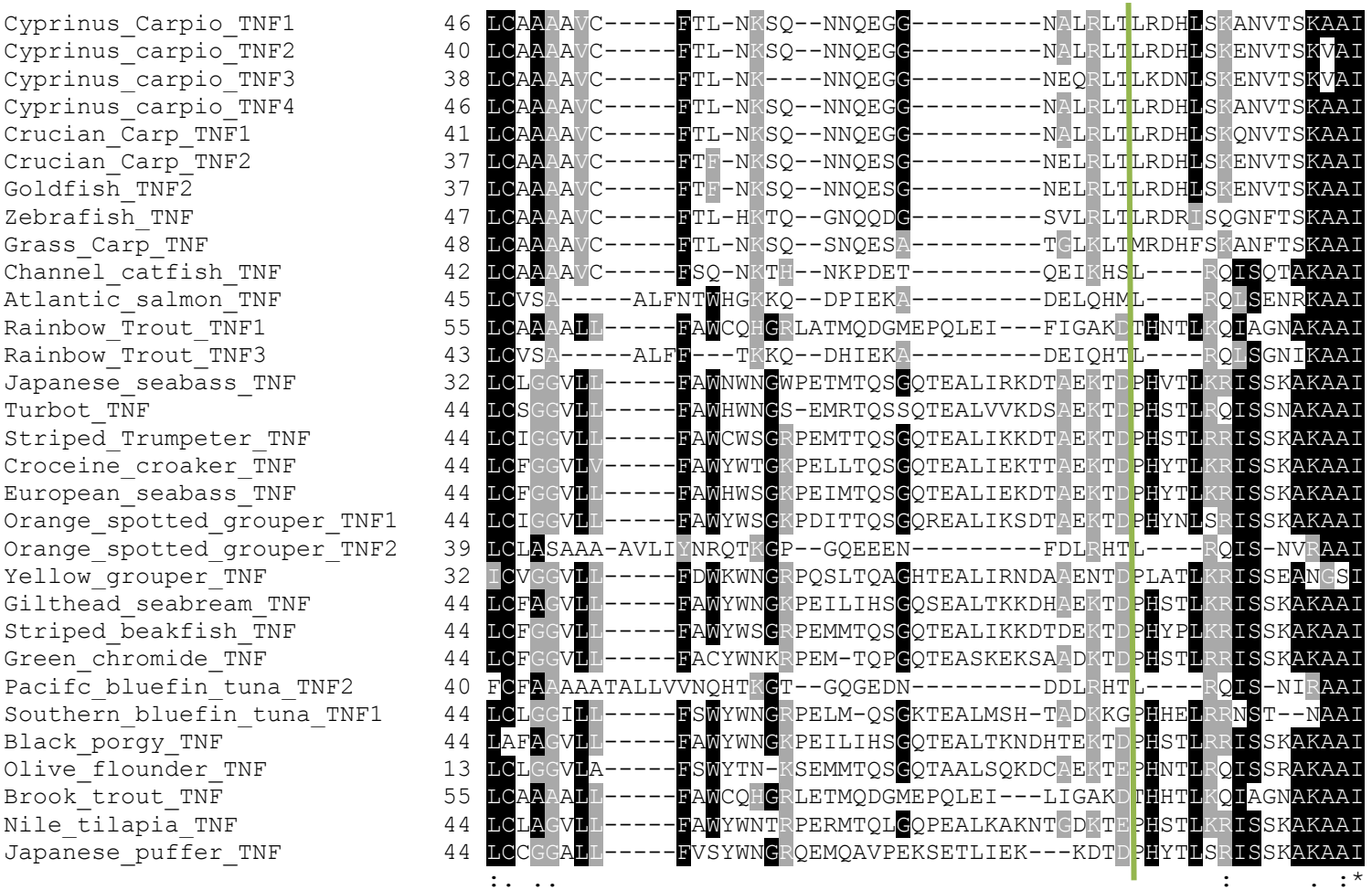
Cyprinus_carpio_TNF3 1 ----------MMDLES----------QPLPQEMVSRRNASSSKSAVWWVCGVLLAVA
Cyprinus_carpio_TNF4 1 ----------MMDLESQL----LEEGGLLPLPQVMVSRRKSGSSKSGVWRVCGVLLAVA
Crucian_Carp_TNF1 1 ----------MMDLESQL----LEEGGLLPSRQVTVSRRT-----SGVWRVCGVLLAVA
Crucian_Carp_TNF2 1 ----------MMIDVESQL----GEE-GV----QVTVSRRR-----SGVWRVCGVLLAVA
Goldfish_TNF2 1 ----------MMIDVESQL----AEE-GA----QVTVSRRR-----SGVWRVCGVLLAVA
Zebrafish_TNF 1 ----------MKLESRAFL--DVEEGELPLPLVMVSRRKAGSSKSGVWRVFGTILAVG
Grass_Carp_TNF 1 ----------MMEHASQVVL--DLEKVTLPLPRVMVSRRKAGTSKSGVWRVCGALLAVA
Channel_catfish_TNF 1 ----------MASDSQVVL--DVD-----GPRVTIVREKASWSSSGVWRTCGVLLAVA
Atlantic_salmon_TNF 1 ----------MEGDCSRVTVDLEKGPVYPSPIVTLVREKS----TRQWRLCGALLAMA
Rainbow_Trout_TNF1 1 MEGYAMTPEDMERGLENSLV----DSGPVY-KTTVTAVAER-KASRGWIWRLCGVLLIAA
Rainbow_Trout_TNF3 1 ----------MEGDCSRVTVDLENGPVS--PTVTLVREKS----TQRWRLCGALLAMA
Japanese_seabass_TNF 1 ----------MGLGERTV----------VLVQK-KSSTGWIWKVFGVLLVMA
Turbot_TNF 1 MVDYTTAPGDLEMGLEARTV----------VLVEK-KSATGWMWKVIRALIIMA
Striped_Trumpeter_TNF 1 MVAYTTAQGDVEMGLEERTV----------VLVEK-KSSAGRIWKASGALLAVA
Croceine_croaker_TNF 1 MVAYTTAPSDLEMGLEERTV----------VVVEK-KSSTDWIWKVTGALLVVA
European_seabass_TNF 1 MVAYTTAPGDVEIGLEERTV----------VMVEK-KSSTQRIWTVSGAILIVA
Orange_spotted_grouper_TNF1 1 MVAYTTAPGDVEMGPEERTV----------VLVEK-KSSAVQIWKVSVALLTVA
Orange_spotted_grouper_TNF2 1 ----------MEGECKVMLDAAVDADA-RKQT-TPVR--------PGSKLTTGLLVFT
Yellow_grouper_TNF 1 ----------MGLEKRSV----------VLKQS-KSSTGWIWKVFGVHLMAA
Gilthead_seabream_TNF 1 MGAYTTAPCDLEMGPEERTV----------VLIEK-KSSTGWMWKVSVALLIAA
Striped_beakfish_TNF 1 MVAYTTAPGDVEMGPEERTV----------VLVEK-KSSTGWMWKVSGALLVMA
Green_chromide_TNF 1 MVAYTTTPVDVEVGMEERTV----------VLVEK-KSSAGWIWKMFGVLFIVA
Pacifc_bluefin_tuna_TNF2 1 ----------MEGECKVALDAAVHIGA-RKHTTQSVK--------PSSKLTTAVLAFT
Southern_bluefin_tuna_TNF1 1 MVAYTTAPADVETGLEERTV----------VLVEK-KSSTGWIWKVSGTLLIIL
Black_porgy_TNF 1 MVAYTTAPCDLEMGPEERTV----------VLIEK-KSATGWMWKVSVALLVAA
Olive_flounder_TNF 1 ----------MCKVLGGLFIVA
Brook_trout_TNF 1 MEGYAMTTGDMERGLENSLV----DSGPVY-KTTVTAVAER-KASRGWIWRLCGVLLVAA
Nile_tilapia_TNF 1 MVAYTTTPVDVEAGPEAKTV----------VLVEK-KSPAEWIWKVCAVLVVA
Japanese_puffer_TNF 1 MVNYMTTASDVEMGLQQKTV----------VLVEK-KSSTGWMGKIILAIFVVV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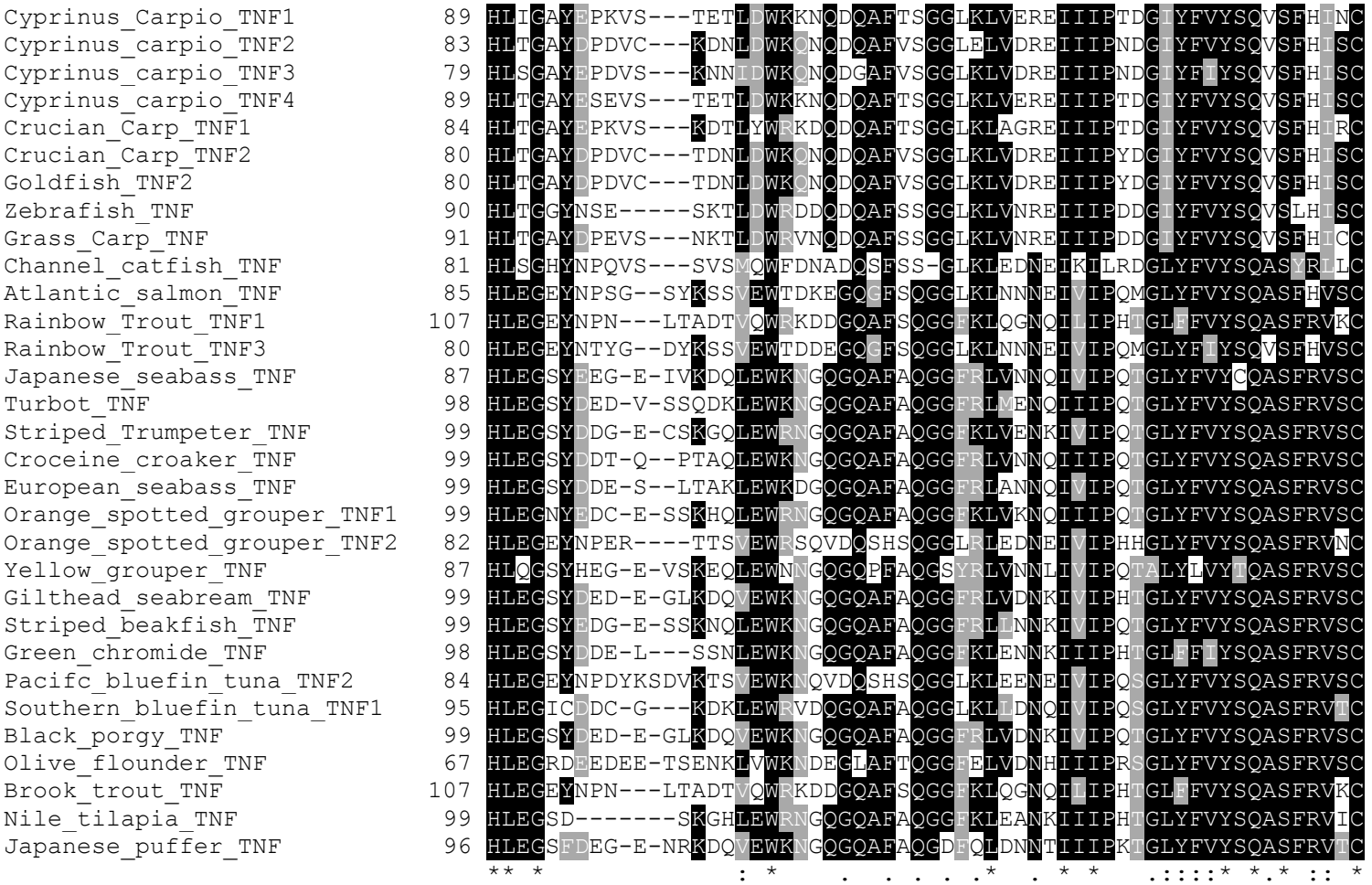
Cyprinus_carpio_TNF2 40
Cyprinus_carpio_TNF3 38
Cyprinus_carpio_TNF4 46
Crucian_Carp_TNF1 41
Crucian_Carp_TNF2 37
Goldfish_TNF2 37
Zebrafish_TNF 47
Grass_Carp_TNF 48
Channel_catfish_TNF 42
Atlantic_salmon_TNF 45
Rainbow_Trout_TNF1 55
Rainbow_Trout_TNF3 43
Japanese_seabass_TNF 32
Turbot_TNF 44
Striped_Trumpeter_TNF 44
Croceine_croaker_TNF 44
European_seabass_TNF 44
Orange_spotted_grouper_TNF1 44
Orange_spotted_grouper_TNF2 39
Yellow_grouper_TNF 32
Gilthead_seabream_TNF 44
Striped_beakfish_TNF 44
Green_chromide_TNF 44
Pacifc_bluefin_tuna_TNF2 40
Southern_bluefin_tuna_TNF1 44
Black_porgy_TNF 44
Olive_flounder_TNF 13
Brook_trout_TNF 55
Nile_tilapia_TNF 44
Japanese_puffer_TNF 44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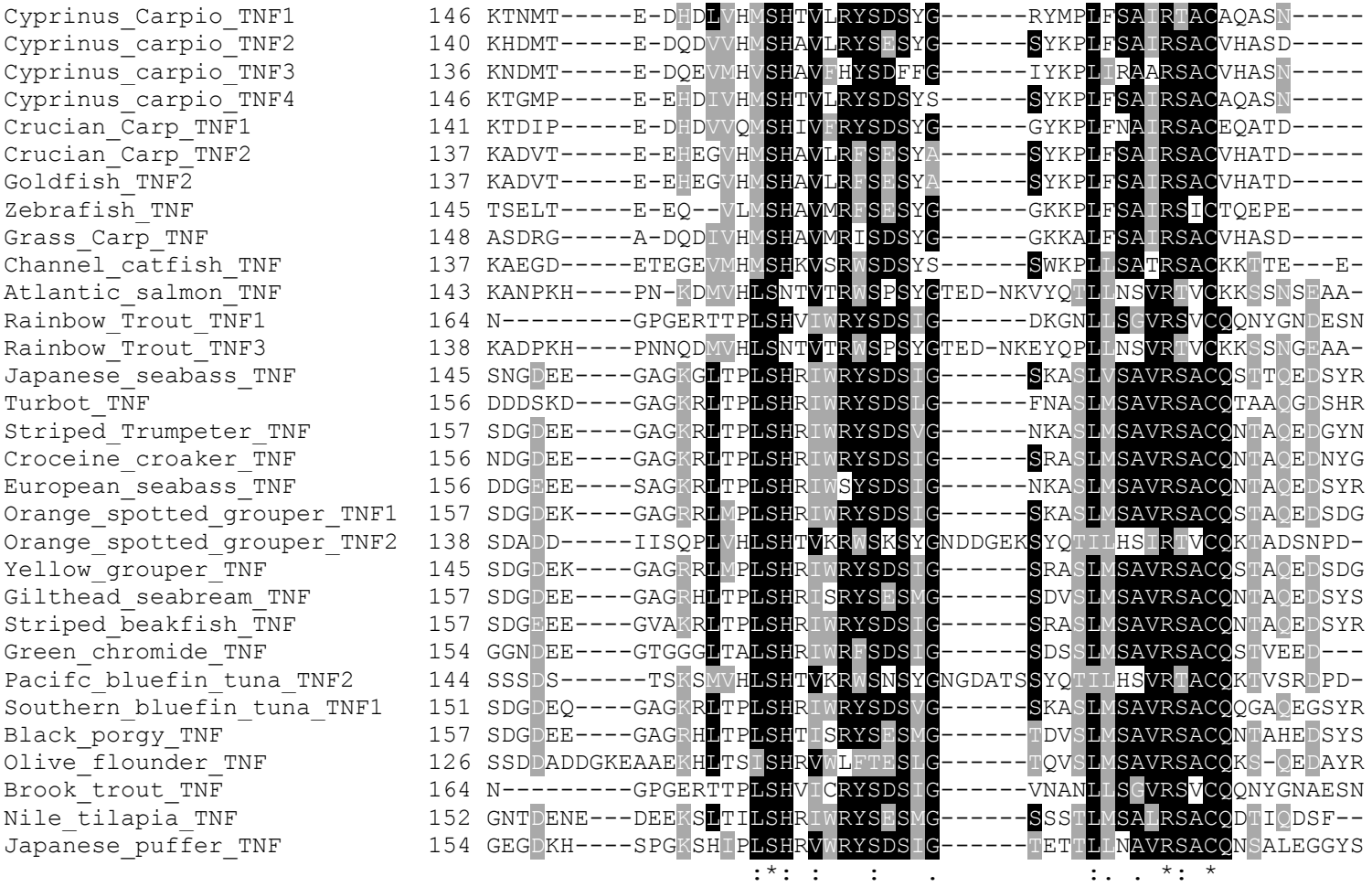
Cyprinus_carpio_TNF2 83
Cyprinus_carpio_TNF3 79
Cyprinus_carpio_TNF4 89
Crucian_Carp_TNF1 84
Crucian_Carp_TNF2 80
Goldfish_TNF2 80
Zebrafish_TNF 90
Grass_Carp_TNF 91
Channel_catfish_TNF 81
Atlantic_salmon_TNF 85
Rainbow_Trout_TNF1 107
Rainbow_Trout_TNF3 80
Japanese_seabass_TNF 87
Turbot_TNF 98
Striped_Trumpeter_TNF 99
Croceine_croaker_TNF 99
European_seabass_TNF 99
Orange_spotted_grouper_TNF1 99
Orange_spotted_grouper_TNF2 82
Yellow_grouper_TNF 87
Gilthead_seabream_TNF 99
Striped_beakfish_TNF 99
Green_chromide_TNF 98
Pacifc_bluefin_tuna_TNF2 84
Southern_bluefin_tuna_TNF1 95
Black_porgy_TNF 99
Olive_flounder_TNF 67
Brook_trout_TNF 107
Nile_tilapia_TNF 99
Japanese_puffer_TNF 96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Cyprinus_carpio_TNF2 140 KHDMT-----E-DQDVVHMSHAVLRYSESYG------SYKPLFSAIRSACVHASD-----
Cyprinus_carpio_TNF3 136 KNDMT-----E-DQEVMHVSHAVFHYSDFFG------IYKPLIRAARSACVHASN-----
Cyprinus_carpio_TNF4 146 KTGMP-----E-EHDIVHMSHTVLRYSDSYS------SYKPLFSAIRSACAQASN-----
Crucian_Carp_TNF1 141 KTDIP-----E-DHDVVQMSHIVFRYSDSYG------GYKPLFNAIRSACEQATD-----
Crucian_Carp_TNF2 137 KADVT-----E-EHEGVHMSHAVLRFSESYA------SYKPLFSAIRSACVHATD-----
Goldfish_TNF2 137 KADVT-----E-EHEGVHMSHAVLRFSESYA------SYKPLFSAIRSACVHATD-----
Zebrafish_TNF 145 TSELT-----E-EQ--VLMSHAVMRFSESYG------GKKPLFSAIRSICTQEPE-----
Grass_Carp_TNF 148 ASDRG-----A-DQDIVHMSHAVMRISDSYG------GKKALFSAIRSACVHASD-----
Channel_catfish_TNF 137 KAEGD-----ETEGEVMHMSHKVSRWSDSYS------SWKPLLSATRSACKKTTE---E-
Atlantic_salmon_TNF 143 KANPKH----PN-KDMVHLSNTVTRWSPSYGTED-NKVYQILLNSVRIVCKKSSNSEAA-
Rainbow_Trout_TNF1 164 N---------GPGERTTPLSHVIWRYSDSIG------DKGNLLSGVRSVCQQNYGNDESN
Rainbow_Trout_TNF3 138 KADPKH----PNNQDMVHLSNTVTRWSPSYGTED-NKEYQPLLNSVRTVCKKSSNGEAA-
Japanese_seabass_TNF 145 SNGDEE----GAGKGLTPLSHRIWRYSDSIG------SKASLVSAVRSACQSTTQEDSYR
Turbot_TNF 156 DDDSKD----GAGKRLTPLSHRIWRYSDSLG------FNASLMSAVRSACQTAAQGDSHR
Striped_Trumpeter_TNF 157 SDGDEE----GAGKRLTPLSHRIWRYSDSVG------NKASLMSAVRSACQNTAQEDGYN
Croceine_croaker_TNF 156 NDGDEE----GAGKRLTPLSHRIWRYSDSIG------SRASLMSAVRSACQNTAQEDNYG
European_seabass_TNF 156 DDGEEE----SAGKRLTPLSHRIWSYSDSIG------NKASLMSAVRSACQNTAQEDSYR
Orange_spotted_grouper_TNF1 157 SDGDEK----GAGRRLMPLSHRIWRYSDSIG------SKASLMSAVRSACQSTAQEDSDG
Orange_spotted_grouper_TNF2 138 SDADD-----IISQPLVHLSHTVKRWSKSYGNDDGEKSYQTILHSIRTVCQKTADSNPD-
Yellow_grouper_TNF 145 SDGDEK----GAGRRLMPLSHRIWRYSDSIG------SRASLMSAVRSACQSTAQEDSDG
Gilthead_seabream_TNF 157 SDGDEE----GAGRHLTPLSHRISRYSESMG------SDVSLMSAVRSACQNTAQEDSYS
Striped_beakfish_TNF 157 SDGEEE----GVAKRLTPLSHRIWRYSDSIG------SRASLMSAVRSACQNTAQEDSYR
Green_chromide_TNF 154 GGNDEE----GTGGGLTALSHRIWRFSDSIG------SDSSLMSAVRSACQSTVEED---
Pacifc_bluefin_tuna_TNF2 144 SSSDS------TSKSMVHLSHTVKRWSNSYGNGDATSSYQTILHSVRTACQKTVSRDPD-
Southern_bluefin_tuna_TNF1 151 SDGDEQ----GAGKRLTPLSHRIWRYSDSVG------SKASLMSAVRSACQQGAQEGSYR
Black_porgy_TNF 157 SDGDEE----GAGRHLTPLSHTISRYSESMG------TDVSLMSAVRSACQNTAHEDSYS
Olive_flounder_TNF 126 SSDDADDGKEAAEKHLTSISHRVWLFTESLG------TQVSLMSAVRSACQKS-QEDAYR
Brook_trout_TNF 164 N---------GPGERTTPLSHVICRYSDSIG------VNANLLSGVRSVCQQNYGNAESN
Nile_tilapia_TNF 152 GNTDENE---DEEKSLTILSHRIWRYSESMG------SSSTLMSALRSACQDTIQDSF--
Japanese_puffer_TNF 154 GEGDKH----SPGKSHIPLSHRVWRYSDSIG------TETTLLNAVRSACQNSALEGGYS

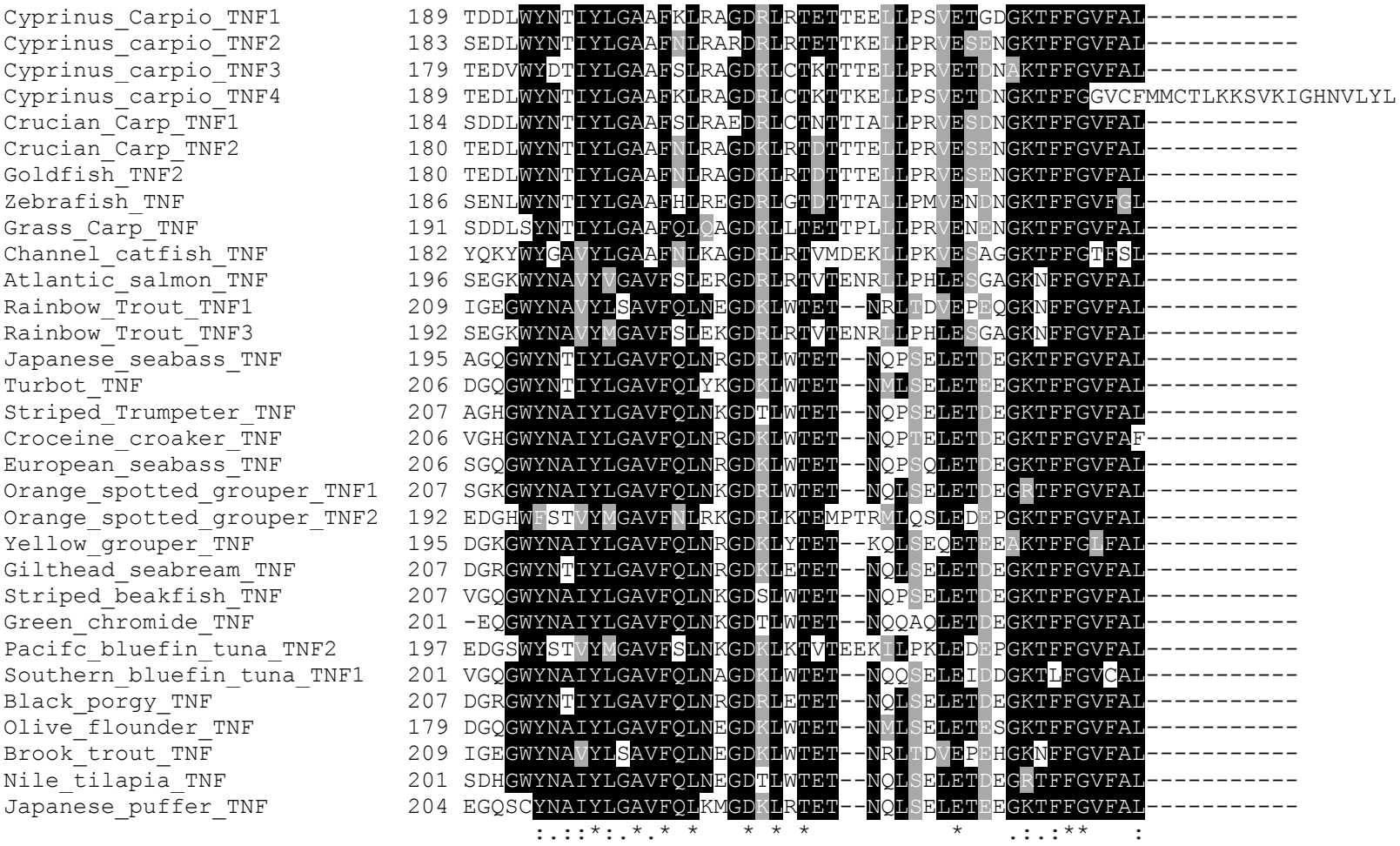


Figure 3.4: A multiple sequence alignment of TNF-α from the various species of fish shown in Table 2.2. The green line indicates a putative site in which the membrane bound form of TNF-α is cut to produce the soluble form. This site was based on the site TACE cleaves TNF-α in humans. Residues with a black background indicate areas of conservation and residues with a grey background indicate areas of conservative substitutions. The "*" symbol indicates that every residue in the corresponding column is conserved. The ":" symbol indicates that at least one residue in the corresponding column will be a conservative substitution, meaning that it has been substituted for one with similar chemical properties. The "." symbol indicates that at least one residue in the corresponding column is a semi conservative substitution where the substituted residue has a similar conformation but different chemical properties.

Common Name	Latin Name	Protein	Order	Sequence Similarity
Crucian carp	*Carassius carassius*	TNFα	*Cypriniformes*	81%
Crucian carp	*Carassius carassius*	TNFα 2	*Cypriniformes*	76%
Goldfish	*Carassius auratus*	TNFα 2	*Cypriniformes*	76%
Grass Carp	*Ctenopharyngodon idella*	TNFα	*Cypriniformes*	72%
Zebrafish	*Danio rerio*	TNFα	*Cypriniformes*	67%
Channel catfish	*Ictalurus punctatus*	TNFα	*Siluriformes*	52%
Nile tilapia	*Oreochromis niloticus*	TNFα	*Perciformes*	48%
Rainbow trout	*Oncorhynchus mykiss*	TNFα 3	*Salmoniformes*	47%
Atlantic salmon	*Salmo salar*	TNFα	*Salmoniformes*	46%
Black Porgy	*Acanthopagrus schlegelii*	TNFα	*Perciformes*	46%
Southern bluefin tuna	*Thunnus maccoyii*	TNFα 1	*Perciformes*	45%
Pacific bluefin tuna	*Thunnus orientalis*	TNFα 2	*Perciformes*	44%
Striped trumpeter	*Latris lineata*	TNFα	*Perciformes*	42%
Orange-spotted grouper	*Epinephelus coioides*	TNFα 1	*Perciformes*	42%
Japanese seabass	*Lateolabrax japonicus*	TNFα	*Perciformes*	42%
Turbot	*Scophthalmus maximus*	TNFα	*Pleuronectiformes*	41%
Croceine croaker	*Larimichthys crocea*	TNFα	*Perciformes*	41%
Gilt-head (sea) bream	*Sparus aurata*	TNFα	*Perciformes*	41%
Striped beakfish	*Oplegnathus fasciatus*	TNFα	*Perciformes*	41%
Olive flounder	*Paralichthys olivaceus*	TNFα	*Pleuronectiformes*	41%
Rainbow trout	*Oncorhynchus mykiss*	TNFα	*Salmoniformes*	40%
European seabass	*Dicentrarchus labrax*	TNFα	*Perciformes*	40%
Orange-spotted grouper	*Epinephelus coioides*	TNFα 2	*Perciformes*	40%
Brook Trout	*Salvelinus fontinalis*	TNFα	*Salmoniformes*	40%
Green chromide	*Etroplus suratensis*	TNFα	*Perciformes*	39%
Japanese puffer	Takifugu rubripes	TNFα	*Tetraodontiformes*	38%
Yellow grouper	*Epinephelus awoara*	TNFα	*Perciformes*	36%

Table 3.1: A table of the sequence similarities calculated for the protein sequence of TNF-α, in the different species of fish used in Figure 3.4, when compared with TNF-α from common carp.

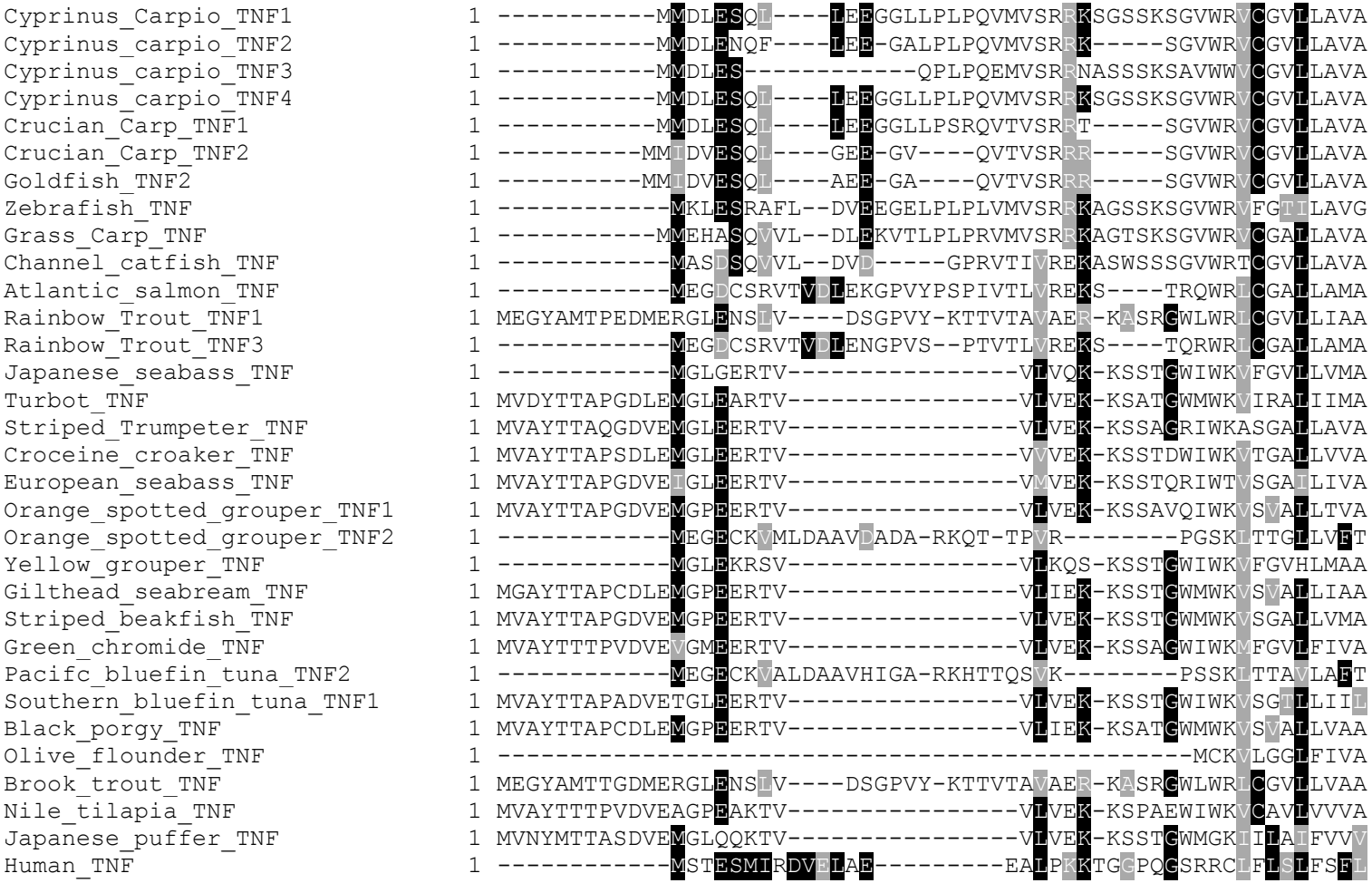
There are less conserved residues between TNF-α and the species of fish than what was shown in Figure 3.4. There are 25 conserved residues, 23 residues with conservative substitutions and 21 residues with semi conservative substitutions. The green line represents the area in which the soluble portion of the protein may start from. This position was based on looking at where the soluble version of TNF-α in humans would have started in the alignment shown in Figure 3.4. However, unlike the alignment shown if Figure 3.4, there were no residues that were conserved or had conservative substitutions in the same position. This indicates that the possible site in which the protein is cleaved to make the soluble version may be at another residue. There are also no conserved residues before this position and only one conservative substitution at L-61 of the alignment. The first set of conserved residue exists at I-88, H-89, L-90, and G-92 of the alignment (when using TNF-α1 of common carp as a base sequence for counting). Their conservation in the alignment between the large numbers of fish species would suggest that they do play a significant role in TNF-α of fish. Of these residues, H-89 was conserved in the alignment with TNF-α from humans and mice (Figure 3.4). They were also positioned in the first β-strand of the protein. This may indicate the position of a β-strand within TNF-α across the different species of fish. Other residues are also conserved in the previous alignment (Figure 3.4) between TNF-α from common carp, humans, and mice. Counting from TNF-α1 in common carp, the residues W-105, L-120, Y-136, Q-138, S-140, C-145, S-159, R-180, C-183, Y-198, F-203, L-205, D-209, L-211, F-232, and G-233 were all conserved in the alignment with TNF-α from humans. Out of these residues, W-105 and Y-198 were both found to be involved in receptor binding. This indicates that this may be a possible area where protein-protein interactions may occur. Out of the all of the conserved residues, I-125, I-127, R-180, and T-213 where previously conservative substitutions in Figure 3.4.

There are three areas in the alignment where there are a number of conserved residues that are relatively close together. These are between positions 131 -145, 193-205 and 228-237 when using TNF-α1 of common carp as the counting sequence. The cluster suggests an area that is important between the different species of fish. In the alignment shown in Figure 3.4 a large β-strand was found in TNF-α of humans and mice in this area. The end of the alignment, between residues 228-237 in TNF-α1 of common carp shows the most consistency, with the majority of residues in this area conserved between species. The biggest difference is in TNF-α4 of common carp where there is no conservation from G-234 onwards. However, as seen in previous alignments shown in Figure 3.1 and 3.3, this does not seem to affect the alignment in any major way.

The sequence similarity between common carp and the other species of fish is shown in Table 3.1. The fish with the highest sequence similarity are Crucian carp, Goldfish, Grass carp, and Zebrafish which have a sequence similarity of 81%, 76%, 72%, and 67% respectively. These fish, as well as common carp, are all part of the *Cypriniformes* order. The alignment in figure 3.4 shows a similar pattern of conservation (indicated by the black boxes) so there would likely be more similarities in function and structure between fish in the *Cypriniformes* order. The next fish, Channel catfish, has a sequence similarity of 52%. The majority of the proteins fall between 48% and 40% sequence similarity. This is primarily composed of fish from the *Perciforms* order of species. The only fish species that have a sequence similarity of less than 40% are Green chromide, Japanese puffer, and Yellow grouper which have a similarity of 39%, 38%, and 36% respectively.

3.4 Sequence alignment of *Cyprinus carpio* TNF and other species of fish and mammals

Figure 3.5 shows an alignment of the isoforms of TNF-α from common carp, TNF-α from some species of fish, and TNF-α from various mammals, including humans and mice. This was done to see if any residues have been evolutionarily conserved between the different species and to reveal any structural and functional characteristics.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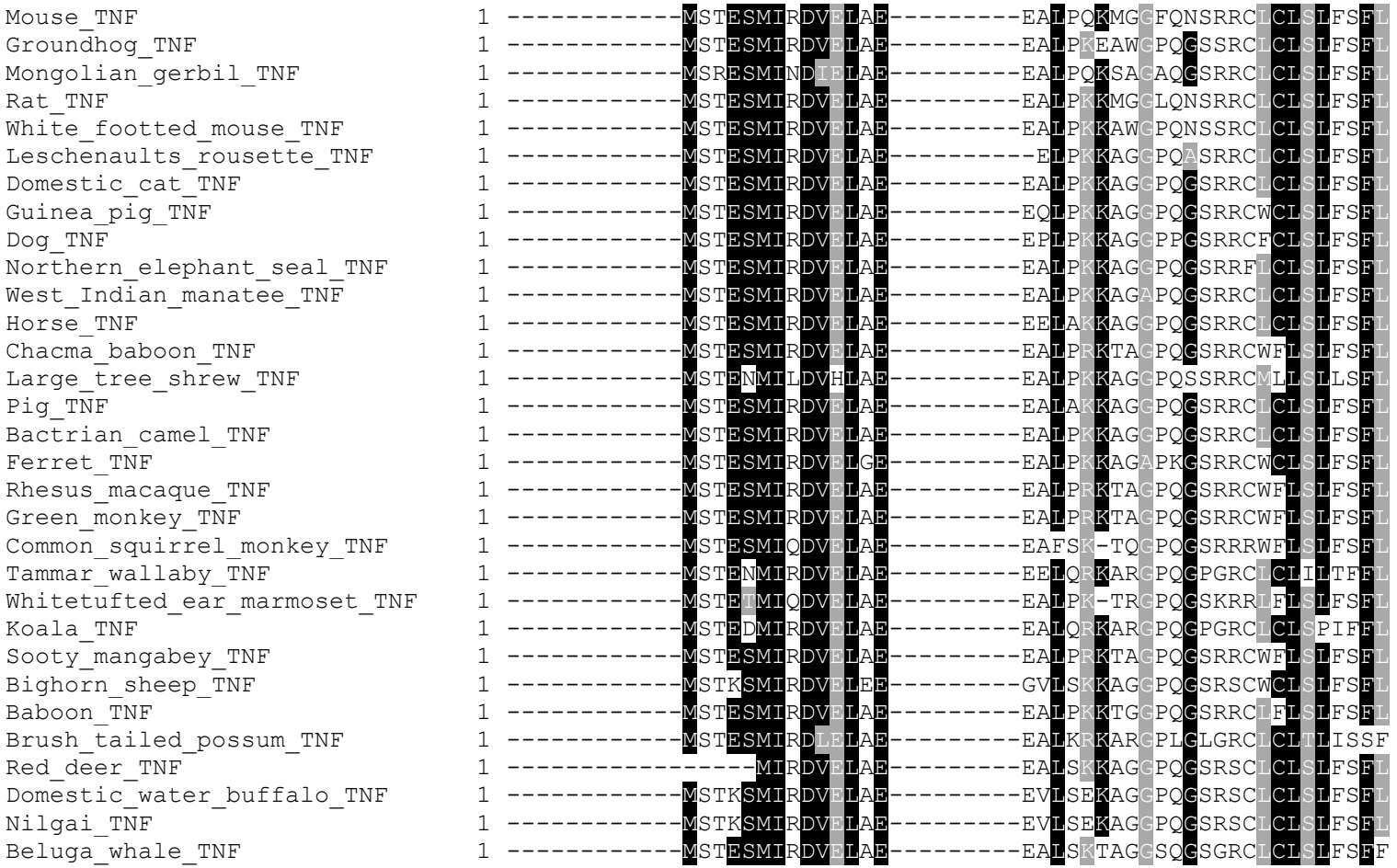
Cyprinus_carpio_TNF2 1 ----------MMDLENQF----LEE-GALPLPQVMVSRRK-----SGVWRVCGVLLAVA
Cyprinus_carpio_TNF3 1 ----------MMDLES----------QPLPQEMVSRRNASSSKSAVWWVCGVLLAVA
Cyprinus_carpio_TNF4 1 ----------MMDLESQL----LEEGGLLPLPQVMVSRRKSGSSKSGVWRVCGVLLAVA
Crucian_Carp_TNF1 1 ----------MMDLESQL----LEEGGLLPSRQVTVSRRT-----SGVWRVCGVLLAVA
Crucian_Carp_TNF2 1 ----------MMIDVESQL----GEE-GV----QVTVSRRR-----SGVWRVCGVLLAVA
Goldfish_TNF2 1 ----------MMIDVESQL----AEE-GA----QVTVSRRR-----SGVWRVCGVLLAVA
Zebrafish_TNF 1 ----------MKLESRAFL--DVEEGELPLPLVMVSRRKAGSSKSGVWRVFGTILAVG
Grass_Carp_TNF 1 ----------MMEHASQVVL--DLEKVTLPLPRVMVSRRKAGTSKSGVWRVCGALLAVA
Channel_catfish_TNF 1 ----------MASDSQVVL--DVD-----GPRVTIVREKASWSSSGVWRTCGVLLAVA
Atlantic_salmon_TNF 1 ----------MEGDCSRVTVDLEKGPVYPSPIVTLVREKS----TRQWRLCGALLAMA
Rainbow_Trout_TNF1 1 MEGYAMTPEDMERGLENSLV----DSGPVY-KTTVTAVAERR-KASRGWLWRLCGVLLIAA
Rainbow_Trout_TNF3 1 ----------MEGDCSRVTVDLENGPVS--PTVTLVREKS----TQRWRLCGALLAMA
Japanese_seabass_TNF 1 ----------MGLGERTV----------VLVQK-KSSTGWIWKVFGVLLVMA
Turbot_TNF 1 MVDYTTAPGDLEMGLEARTV----------VLVEK-KSATGWMWKVIRALIIMA
Striped_Trumpeter_TNF 1 MVAYTTAQGDVEMGLEERTV----------VLVEK-KSSAGRIWKASGALLAVA
Croceine_croaker_TNF 1 MVAYTTAPSDLEMGLEERTV----------VVVEK-KSSTDWIWKVTGALLVVA
European_seabass_TNF 1 MVAYTTAPGDVEIGLEERTV----------VMVEK-KSSTQRIWTVSGAILIVA
Orange_spotted_grouper_TNF1 1 MVAYTTAPGDVEMGPEERTV----------VLVEK-KSSAVQIWKVSVALLTVA
Orange_spotted_grouper_TNF2 1 ----------MEGECKVMLDAAVDADA-RKQT-TPVR--------PGSKLTTGLLVFT
Yellow_grouper_TNF 1 ----------MGLEKRSV----------VLKQS-KSSTGWIWKVFGVHLMAA
Gilthead_seabream_TNF 1 MGAYTTAPCDLEMGPEERTV----------VLIEK-KSSTGWMWKVSVALLIAA
Striped_beakfish_TNF 1 MVAYTTAPGDVEMGPEERTV----------VLVEK-KSSTGWMWKVSGALLVMA
Green_chromide_TNF 1 MVAYTTTPVDVEVGMEERTV----------VLVEK-KSSAGWIWKMFGVLFIVA
Pacifc_bluefin_tuna_TNF2 1 ----------MEGECKVALDAAVHIGA-RKHTTQSVK--------PSSKLTTAVLAFT
Southern_bluefin_tuna_TNF1 1 MVAYTTAPADVETGLEERTV----------VLVEK-KSSTGWIWKVSGILLIIL
Black_porgy_TNF 1 MVAYTTAPCDLEMGPEERTV----------VLIEK-KSATGWMWKVSVALLVAA
Olive_flounder_TNF 1 ----------MCKVLGGLFIVA
Brook_trout_TNF 1 MEGYAMTTGDMERGLENSLV----DSGPVY-KTTVTAVAERR-KASRGWLWRLCGVLLVAA
Nile_tilapia_TNF 1 MVAYTTTPVDVEAGPEAKTV----------VLVEK-KSPAEWIWKVCAVLVVVA
Japanese_puffer_TNF 1 MVNYMTTASDVEMGLQQKTV----------VLVEK-KSSTGWMGKIILAIFVVV
Human_TNF 1 ----------MSTESMIRDVELAE---------EALPKKTGGPQGSRRCLFLSLFSFL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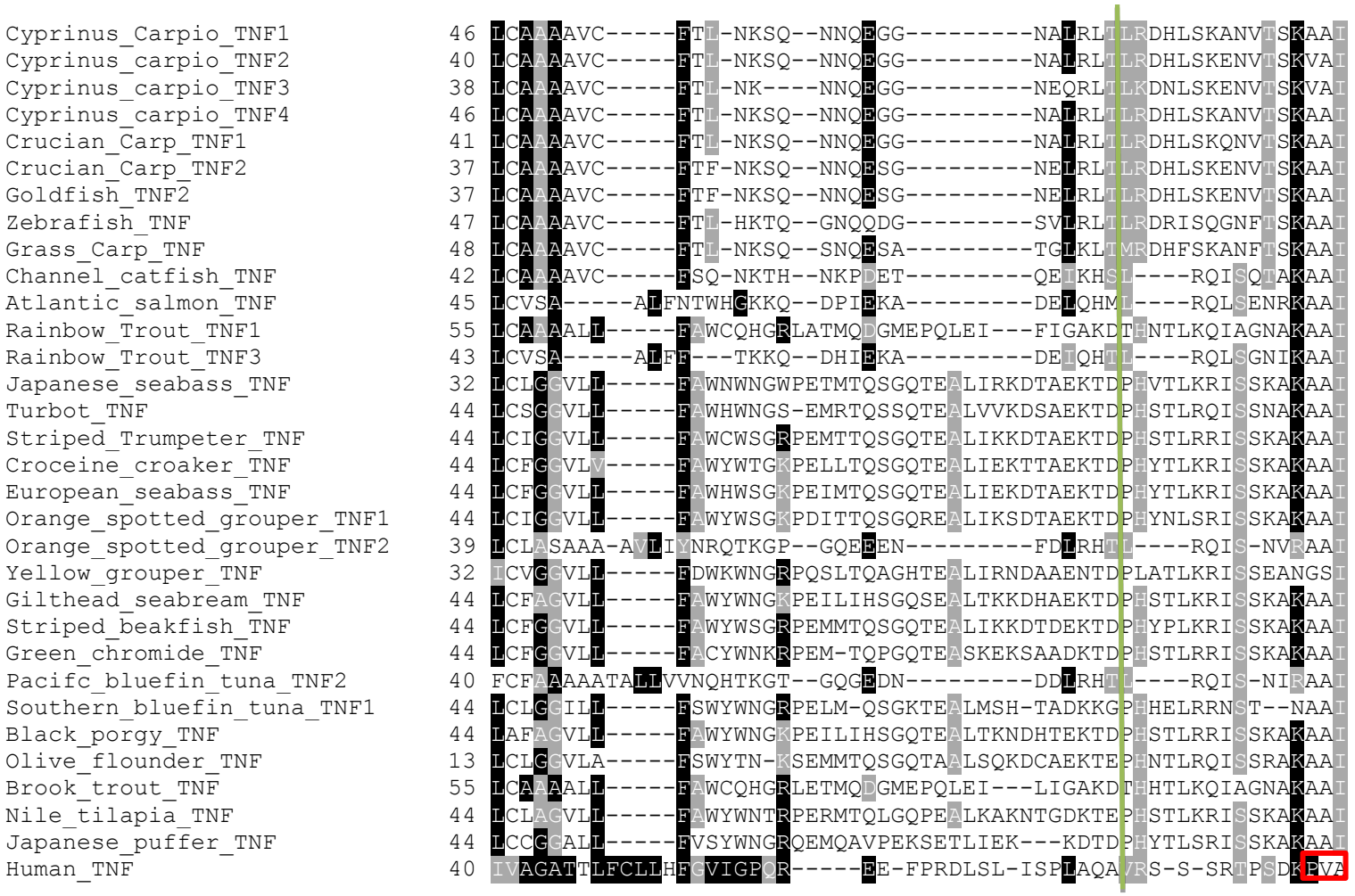
Groundhog_TNF 1 ----------MSTESMIRDVELAE---------EALPKEAWGPQGSSRCLCLSLFSFL
Mongolian_gerbil_TNF 1 ----------MSRESMINDIELAE---------EALPQKSAGAQGSRRCLCLSLFSFL
Rat_TNF 1 ----------MSTESMIRDVELAE---------EALPKKMGGLQNSRRCLCLSLFSFL
White_footted_mouse_TNF 1 ----------MSTESMIRDVELAE---------EALPKKAWGPQNSSRCLCLSLFSFL
Leschenaults_rousette_TNF 1 ----------MSTESMIRDVELAE----------ELPKKAGGPQASRRCLCLSLFSFL
Domestic_cat_TNF 1 ----------MSTESMIRDVELAE---------EALPKKAGGPQGSRRCLCLSLFSFL
Guinea_pig_TNF 1 ----------MSTESMIRDVELAE---------EQLPKKAGGPQGSRRCWCLSLFSFL
Dog_TNF 1 ----------MSTESMIRDVELAE---------EPLPKKAGGPPGSRRCFCLSLFSFL
Northern_elephant_seal_TNF 1 ----------MSTESMIRDVELAE---------EALPKKAGGPQGSRRFLCLSLFSFL
West_Indian_manatee_TNF 1 ----------MSTESMIRDVELAE---------EALPKKAGAPQGSRRCLCLSLFSFL
Horse_TNF 1 ----------MSTESMIRDVELAE---------EELAKKAGGPQGSRRCLCLSLFSFL
Chacma_baboon_TNF 1 ----------MSTESMIRDVELAE---------EALPRKTAGPQGSRRCWFLSLFSFL
Large_tree_shrew_TNF 1 ----------MSTENMILDVHLAE---------EALPKKAGGPQSSRRCMLLSLLSFL
Pig_TNF 1 ----------MSTESMIRDVELAE---------EALAKKAGGPQGSRRCLCLSLFSFL
Bactrian_camel_TNF 1 ----------MSTESMIRDVELAE---------EALPKKAGGPQGSRRCLCLSLFSFL
Ferret_TNF 1 ----------MSTESMIRDVELGE---------EALPKKAGAPKGSRRCWCLSLFSFL
Rhesus_macaque_TNF 1 ----------MSTESMIRDVELAE---------EALPRKTAGPQGSRRCWFLSLFSFL
Green_monkey_TNF 1 ----------MSTESMIRDVELAE---------EALPRKTAGPQGSRRCWFLSLFSFL
Common_squirrel_monkey_TNF 1 ----------MSTESMIQDVELAE---------EAFSK-TQGPQGSRRRWFLSLFSFL
Tammar_wallaby_TNF 1 ----------MSTENMIRDVELAE---------EELQRKARGPQGPGRCLCLILTFFL
Whitetufted_ear_marmoset_TNF 1 ----------MSTETMIQDVELAE---------EALPK-TRGPQGSKRRLFLSLFSFL
Koala_TNF 1 ----------MSTEDMIRDVELAE---------EALQRKARGPQGPGRCLCLSPIFFL
Sooty_mangabey_TNF 1 ----------MSTESMIRDVELAE---------EALPRKTAGPQGSRRCWFLSLFSFL
Bighorn_sheep_TNF 1 ----------MSTKSMIRDVELEE---------GVLSKKAGGPQGSRSCWCLSLFSFL
Baboon_TNF 1 ----------MSTESMIRDVELAE---------EALPKKTGGPQGSRRCLFLSLFSFL
Brush_tailed_possum_TNF 1 ----------MSTESMIRDLELAE---------EALKRKARGPLGLGRCLCLTLISSF
Red_deer_TNF 1 ----------MIRDVELAE---------EALSKKAGGPQGSRSCLCLSLFSFL
Domestic_water_buffalo_TNF 1 ----------MSTKSMIRDVELAE---------EVLSEKAGGPQGSRSCLCLSLFSFL
Nilgai_TNF 1 ----------MSTKSMIRDVELAE---------EVLSEKAGGPQGSRSCLCLSLFSFL
Beluga_whale_TNF 1 ----------MSTESMIRDVELAE---------EALSKTAGGSQGSGRCLCLSLFSFF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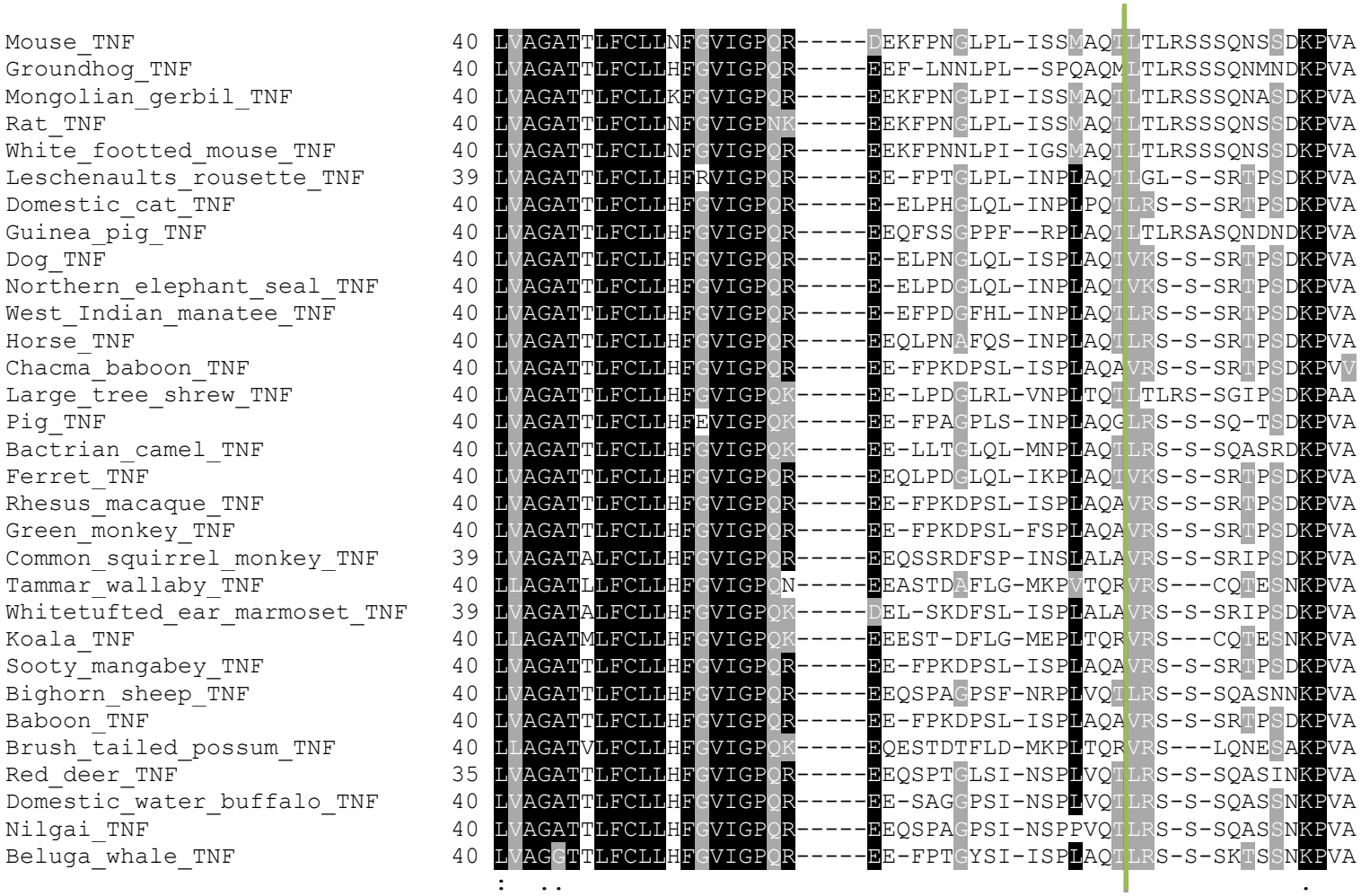
Cyprinus_carpio_TNF2 40
Cyprinus_carpio_TNF3 38
Cyprinus_carpio_TNF4 46
Crucian_Carp_TNF1 41
Crucian_Carp_TNF2 37
Goldfish_TNF2 37
Zebrafish_TNF 47
Grass_Carp_TNF 48
Channel_catfish_TNF 42
Atlantic_salmon_TNF 45
Rainbow_Trout_TNF1 55
Rainbow_Trout_TNF3 43
Japanese_seabass_TNF 32
Turbot_TNF 44
Striped_Trumpeter_TNF 44
Croceine_croaker_TNF 44
European_seabass_TNF 44
Orange_spotted_grouper_TNF1 44
Orange_spotted_grouper_TNF2 39
Yellow_grouper_TNF 32
Gilthead_seabream_TNF 44
Striped_beakfish_TNF 44
Green_chromide_TNF 44
Pacifc_bluefin_tuna_TNF2 40
Southern_bluefin_tuna_TNF1 44
Black_porgy_TNF 44
Olive_flounder_TNF 13
Brook_trout_TNF 55
Nile_tilapia_TNF 44
Japanese_puffer_TNF 44
Human_TNF 40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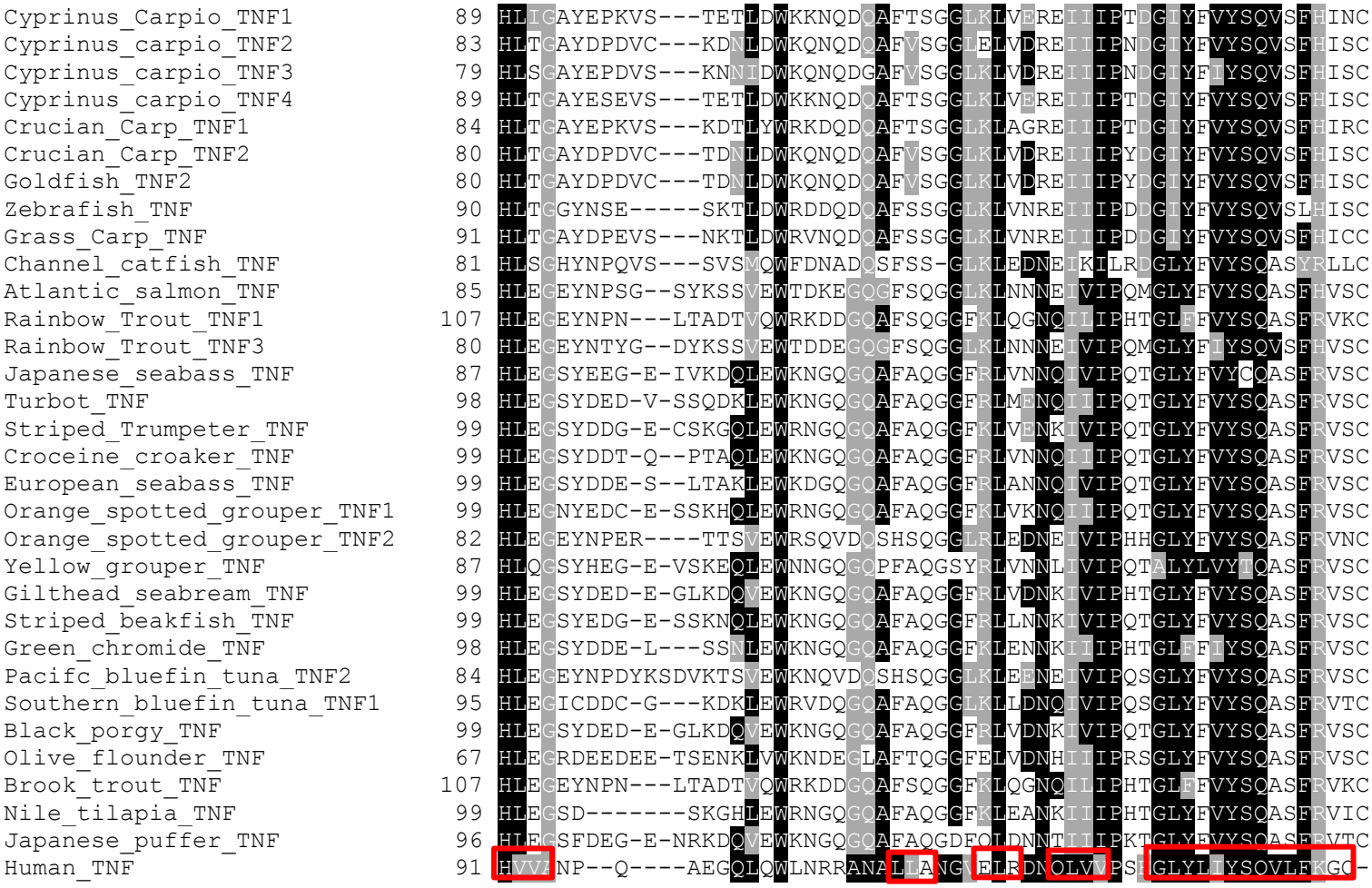
Groundhog_TNF 40 LVAGATTLFCLLHFGVIGPQR-----EEF-LNNLPL--SPQAQMLTLRSSSQNMNDKPVA
Mongolian_gerbil_TNF 40 LVAGATTLFCLLKFGVIGPQR-----EEKFPNGLPI-ISSMAQILTLRSSSQNASDKPVA
Rat_TNF 40 LVAGATTLFCLLNFGVIGPNK-----EEKFPNGLPL-ISSMAQILTLRSSSQNSSDKPVA
White_footted_mouse_TNF 40 LVAGATTLFCLLNFGVIGPQR-----EEKFPNNLPI-IGSMAQILTLRSSSQNSSDKPVA
Leschenaults_rousette_TNF 39 LVAGATTLFCLLHFRVIGPQR-----EE-FPTGLPL-INPLAQILGL-S-SRTPSDKPVA
Domestic_cat_TNF 40 LVAGATTLFCLLHFGVIGPQR-----E-ELPHGLQL-INPLPQILRS-S-SRTPSDKPVA
Guinea_pig_TNF 40 LVAGATTLFCLLHFGVIGPQR-----EEQFSSGPPF--RPLAQILTLRSASQNDNDKPVA
Dog_TNF 40 LVAGATTLFCLLHFGVIGPQR-----E-ELPNGLQL-ISPLAQIVKS-S-SRTPSDKPVA
Northern_elephant_seal_TNF 40 LVAGATTLFCLLHFGVIGPQR-----E-ELPDGLQL-INPLAQIVKS-S-SRTPSDKPVA
West_Indian_manatee_TNF 40 LVAGATTLFCLLHFGVIGPQR-----E-EFPDGFHL-INPLAQILRS-S-SRTPSDKPVA
Horse_TNF 40 LVAGATTLFCLLHFGVIGPQR-----EEQLPNAFQS-INPLAQILRS-S-SRTPSDKPVA
Chacma_baboon_TNF 40 LVAGATTLFCLLHFGVIGPQR-----EE-FPKDPSL-ISPLAQAVRS-S-SRTPSDKPVV
Large_tree_shrew_TNF 40 LVAGATTLFCLLHFGVIGPQK-----EE-LPDGLRL-VNPLTQILTLRS-SGIPSDKPAA
Pig_TNF 40 LVAGATTLFCLLHFEVIGPQK-----EE-FPAGPLS-INPLAQGLRS-S-SQ-TSDKPVA
Bactrian_camel_TNF 40 LVAGATTLFCLLHFGVIGPQK-----EE-LLTGLQL-MNPLAQILRS-S-SQASRDKPVA
Ferret_TNF 40 LVAGATTLFCLLHFGVIGPQR-----EEQLPDGLQL-IKPLAQIVKS-S-SRTPSDKPVA
Rhesus_macaque_TNF 40 LVAGATTLFCLLHFGVIGPQR-----EE-FPKDPSL-ISPLAQAVRS-S-SRTPSDKPVA
Green_monkey_TNF 40 LVAGATTLFCLLHFGVIGPQR-----EE-FPKDPSL-FSPLAQAVRS-S-SRTPSDKPVA
Common_squirrel_monkey_TNF 39 LVAGATALFCLLHFGVIGPQR-----EEQSSRDFSP-INSLALAVRS-S-SRIPSDKPVA
Tammar_wallaby_TNF 40 LLAGATLLFCLLHFGVIGPQN-----EEASTDAFLG-MKPVTQRVRS---CQTESNKPVA
Whitetufted_ear_marmoset_TNF 39 LVAGATALFCLLHFGVIGPQK-----DEL-SKDFSL-ISPLALAVRS-S-SRIPSDKPVA
Koala_TNF 40 LLAGATMLFCLLHFGVIGPQK-----EEEST-DFLG-MEPLTQRVRS---CQTESNKPVA
Sooty_mangabey_TNF 40 LVAGATTLFCLLHFGVIGPQR-----EE-FPKDPSL-ISPLAQAVRS-S-SRTPSDKPVA
Bighorn_sheep_TNF 40 LVAGATTLFCLLHFGVIGPQR-----EEQSPAGPSF-NRPLVQILRS-S-SQASNNKPVA
Baboon_TNF 40 LVAGATTLFCLLHFGVIGPQR-----EE-FPKDPSL-ISPLAQAVRS-S-SRTPSDKPVA
Brush_tailed_possum_TNF 40 LLAGATVLFCLLHFGVIGPQK-----EQESTDTFLD-MKPLTQRVRS---LQNESAKPVA
Red_deer_TNF 35 LVAGATTLFCLLHFGVIGPQR-----EEQSPTGLSI-NSPLVQTLRS-S-SQASINKPVA
Domestic_water_buffalo_TNF 40 LVAGATTLFCLLHFGVIGPQR-----EE-SAGGPSI-NSPLVQILRS-S-SQASSNKPVA
Nilgai_TNF 40 LVAGATTLFCLLHFGVIGPQR-----EEQSPAGPSI-NSPPVQILRS-S-SQASSNKPVA
Beluga_whale_TNF 40 LVAGGTTLFCLLHFGVIGPQR-----EE-FPTGYSI-ISPLAQILRS-S-SKTSSNKPVA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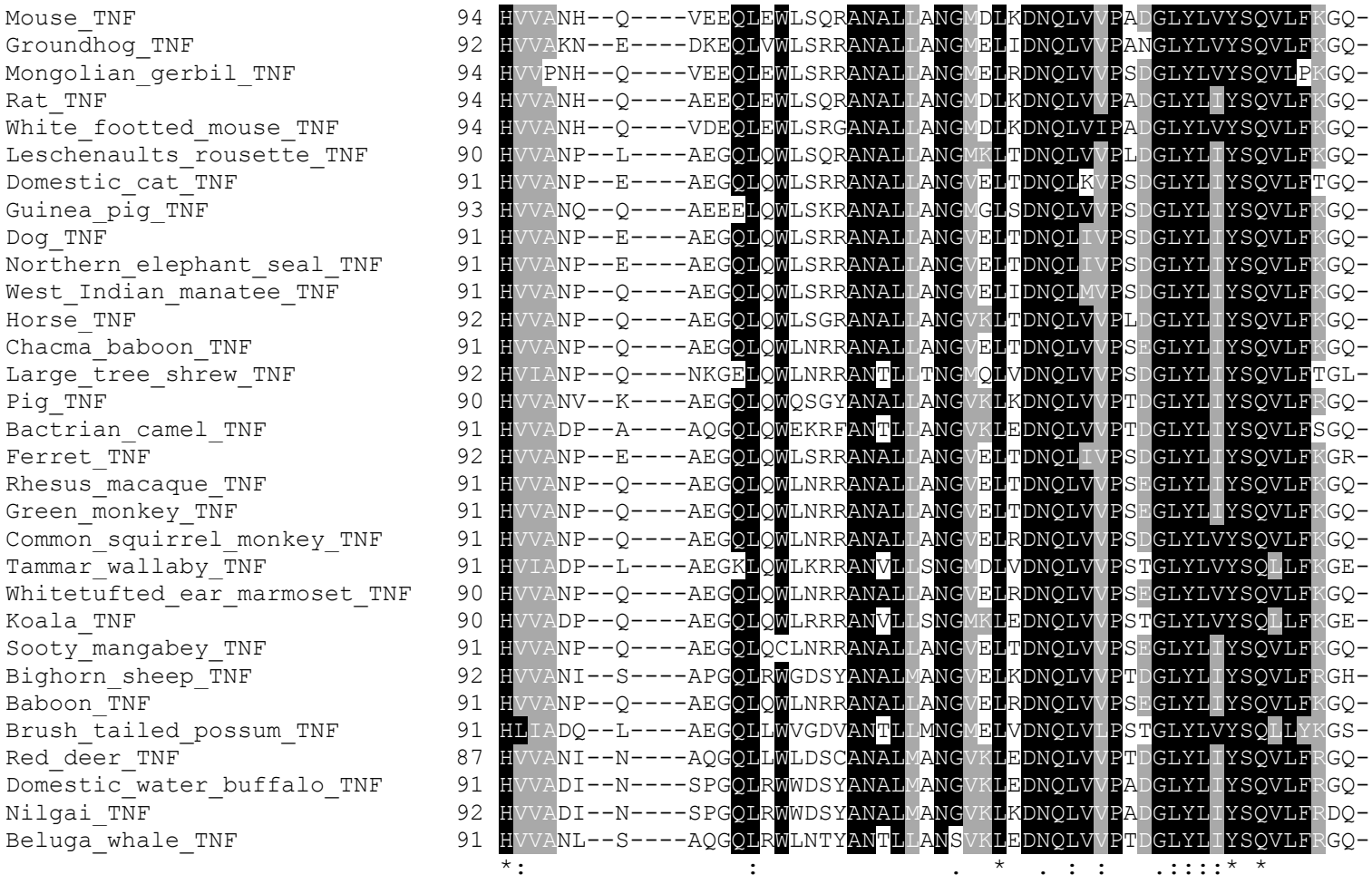
Cyprinus_carpio_TNF2 83
Cyprinus_carpio_TNF3 79
Cyprinus_carpio_TNF4 89
Crucian_Carp_TNF1 84
Crucian_Carp_TNF2 80
Goldfish_TNF2 80
Zebrafish_TNF 90
Grass_Carp_TNF 91
Channel_catfish_TNF 81
Atlantic_salmon_TNF 85
Rainbow_Trout_TNF1 107
Rainbow_Trout_TNF3 80
Japanese_seabass_TNF 87
Turbot_TNF 98
Striped_Trumpeter_TNF 99
Croceine_croaker_TNF 99
European_seabass_TNF 99
Orange_spotted_grouper_TNF1 99
Orange_spotted_grouper_TNF2 82
Yellow_grouper_TNF 87
Gilthead_seabream_TNF 99
Striped_beakfish_TNF 99
Green_chromide_TNF 98
Pacifc_bluefin_tuna_TNF2 84
Southern_bluefin_tuna_TNF1 95
Black_porgy_TNF 99
Olive_flounder_TNF 67
Brook_trout_TNF 107
Nile_tilapia_TNF 99
Japanese_puffer_TNF 96
Human_TNF 91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Groundhog_TNF 92 HVVAKN--E----DKEQLVWLSRRANALLANGMELIDNQLVVPANGLYLVYSQVLFKGQ-
Mongolian_gerbil_TNF 94 HVVPNH--Q----VEEQLEWLSRRANALLANGMELRDNQLVVPSDGLYLVYSQVLPKGQ-
Rat_TNF 94 HVVANH--Q----AEEQLEWLSQRANALLANGMDLKDNQLVVPADGLYLIYSQVLFKGQ-
White_footted_mouse_TNF 94 HVVANH--Q----VDEQLEWLSRGANALLANGMDLKDNQLVIPADGLYLVYSQVLFKGQ-
Leschenaults_rousette_TNF 90 HVVANP--L----AEGQLQWLSQRANALLANGMKLTDNQLVVPLDGLYLIYSQVLFKGQ-
Domestic_cat_TNF 91 HVVANP--E----AEGQLQWLSRRANALLANGVELTDNQLKVPSDGLYLIYSQVLFTGQ-
Guinea_pig_TNF 93 HVVANQ--Q----AEEELQWLSKRANALLANGMGLSDNQLVVPSDGLYLIYSQVLFKGQ-
Dog_TNF 91 HVVANP--E----AEGQLQWLSRRANALLANGVELTDNQLIVPSDGLYLIYSQVLFKGQ-
Northern_elephant_seal_TNF 91 HVVANP--E----AEGQLQWLSRRANALLANGVELTDNQLIVPSDGLYLIYSQVLFKGQ-
West_Indian_manatee_TNF 91 HVVANP--Q----AEGQLQWLSRRANALLANGVELIDNQLMVPSDGLYLIYSQVLFKGQ-
Horse_TNF 92 HVVANP--Q----AEGQLQWLSGRANALLANGVKLTDNQLVVPLDGLYLIYSQVLFKGQ-
Chacma_baboon_TNF 91 HVVANP--Q----AEGQLQWLNRRANALLANGVELTDNQLVVPSEGLYLIYSQVLFKGQ-
Large_tree_shrew_TNF 92 HVIANP--Q----NKGELQWLNRRANTLLTNGMQLVDNQLVVPSDGLYLIYSQVLFTGL-
Pig_TNF 90 HVVANV--K----AEGQLQWQSGYANALLANGVKLKDNQLVVPTDGLYLIYSQVLFRGQ-
Bactrian_camel_TNF 91 HVVADP--A----AQGQLQWEKRFANTLLANGVKLEDNQLVVPTDGLYLIYSQVLFSGQ-
Ferret_TNF 92 HVVANP--E----AEGQLQWLSRRANALLANGVELTDNQLIVPSDGLYLIYSQVLFKGR-
Rhesus_macaque_TNF 91 HVVANP--Q----AEGQLQWLNRRANALLANGVELTDNQLVVPSEGLYLIYSQVLFKGQ-
Green_monkey_TNF 91 HVVANP--Q----AEGQLQWLNRRANALLANGVELTDNQLVVPSEGLYLIYSQVLFKGQ-
Common_squirrel_monkey_TNF 91 HVVANP--Q----AEGQLQWLNRRANALLANGVELRDNQLVVPSDGLYLVYSQVLFKGQ-
Tammar_wallaby_TNF 91 HVIADP--L----AEGKLQWLKRRANVLLSNGMDLVDNQLVVPSTGLYLVYSQLLFKGE-
Whitetufted_ear_marmoset_TNF 90 HVVANP--Q----AEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLVYSQVLFKGQ-
Koala_TNF 90 HVVADP--Q----AEGQLQWLRRRANVLLSNGMKLEDNQLVVPSTGLYLVYSQLLFKGE-
Sooty_mangabey_TNF 91 HVVANP--Q----AEGQLQCLNRRANALLANGVELTDNQLVVPSEGLYLIYSQVLFKGQ-
Bighorn_sheep_TNF 92 HVVANI--S----APGQLRWGDSYANALMANGVELKDNQLVVPTDGLYLIYSQVLFRGH-
Baboon_TNF 91 HVVANP--Q----AEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQ-
Brush_tailed_possum_TNF 91 HLIADQ--L----AEGQLLWVGDVANTLIMNGMELVDNQLVLPSTGLYLVYSQLLYKGS-
Red_deer_TNF 87 HVVANI--N----AQGQLLWLDSCANALMANGVKLEDNQLVVPTDGLYLIYSQVLFRGQ-
Domestic_water_buffalo_TNF 91 HVVADI--N----SPGQLRWWDSYANALMANGVKLEDNQLVVPADGLYLIYSQVLFRGQ-
Nilgai_TNF 92 HVVADI--N----SPGQLRWWDSYANALMANGVKLKDNQLVVPADGLYLIYSQVLFRDQ-
Beluga_whale_TNF 91 HVVANL--S----AQGQLRWLNTYANTLLANSVKLEDNQLVVPTDGLYLIYSQVLFRGQ-
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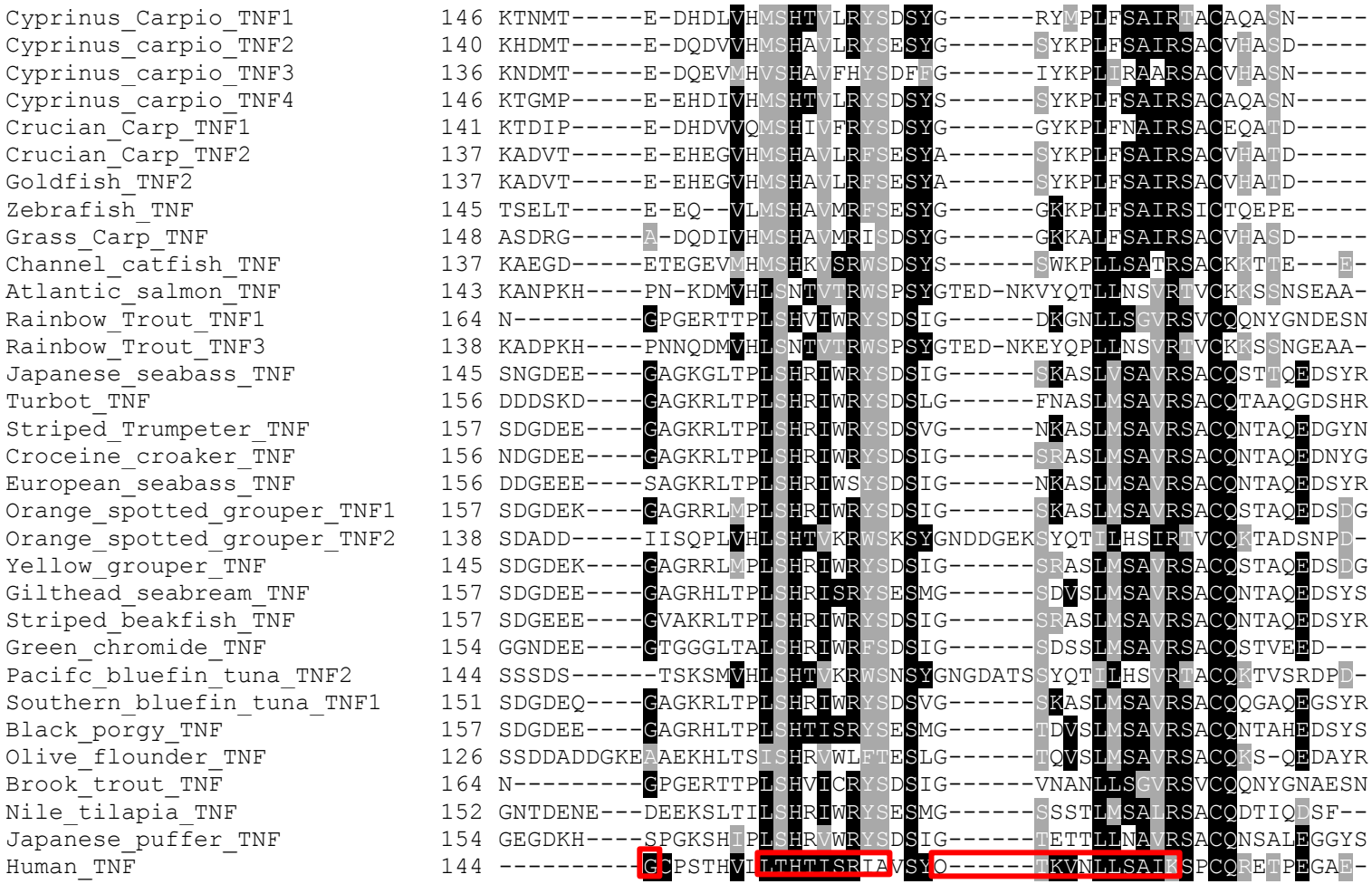
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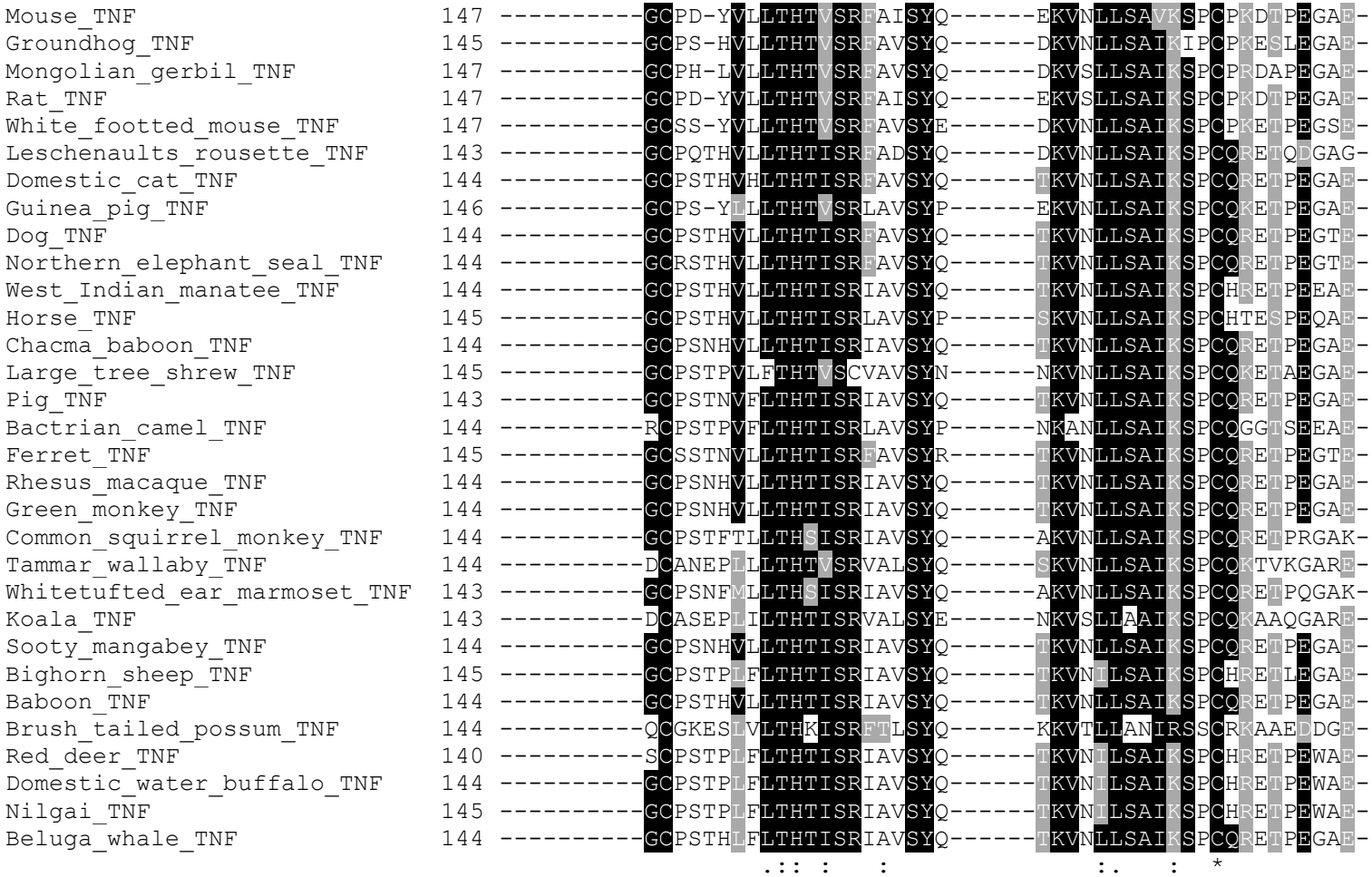
Cyprinus_carpio_TNF3 136 KNDMT-----E-DQEVMHVSHAVFHYSDFFG------IYKPLIRAARSACVHASN-----
Cyprinus_carpio_TNF4 146 KTGMP-----E-EHDIVHMSHTVLRYSDSYS------SYKPLFSAIRSACAQASN-----
Crucian_Carp_TNF1 141 KTDIP-----E-DHDVVQMSHIVFRYSDSYG------GYKPLFNAIRSACEQATD-----
Crucian_Carp_TNF2 137 KADVT-----E-EHEGVHMSHAVLRFSESYA------SYKPLFSAIRSACVHATD-----
Goldfish_TNF2 137 KADVT-----E-EHEGVHMSHAVLRFSESYA------SYKPLFSAIRSACVHATD-----
Zebrafish_TNF 145 TSELT-----E-EQ--VLMSHAVMRFSESYG------GKKPLFSAIRSICTQEPE-----
Grass_Carp_TNF 148 ASDRG-----A-DQDIVHMSHAVMRISDSYG------GKKALFSAIRSACVHASD-----
Channel_catfish_TNF 137 KAEGD-----ETEGEVMHMSHKVSRWSDSYS------SWKPLLSATRSACKKTTE---E-
Atlantic_salmon_TNF 143 KANPKH----PN-KDMVHLSNTVTRWSPSYGTED-NKVYQTLLNSVRIVCKKSSNSEAA-
Rainbow_Trout_TNF1 164 N---------GPGERTTPLSHVIWRYSDSIG------DKGNLLSGVRSVCQQNYGNDESN
Rainbow_Trout_TNF3 138 KADPKH----PNNQDMVHLSNTVTRWSPSYGTED-NKEYQPLLNSVRIVCKKSSNGEAA-
Japanese_seabass_TNF 145 SNGDEE----GAGKGLTPLSHRIWRYSDSIG------SKASLVSAVRSACQSTTQEDSYR
Turbot_TNF 156 DDDSKD----GAGKRLTPLSHRIWRYSDSLG------FNASLMSAVRSACQTAAQGDSHR
Striped_Trumpeter_TNF 157 SDGDEE----GAGKRLTPLSHRIWRYSDSVG------NKASLMSAVRSACQNTAQEDGYN
Croceine_croaker_TNF 156 NDGDEE----GAGKRLTPLSHRIWRYSDSIG------SRASLMSAVRSACQNTAQEDNYG
European_seabass_TNF 156 DDGEEE----SAGKRLTPLSHRIWSYSDSIG------NKASLMSAVRSACQNTAQEDSYR
Orange_spotted_grouper_TNF1 157 SDGDEK----GAGRRLMPLSHRIWRYSDSIG------SKASLMSAVRSACQSTAQEDSDG
Orange_spotted_grouper_TNF2 138 SDADD-----IISQPLVHLSHTVKRWSKSYGNDDGEKSYQTILHSIRTVCQKTADSNPD-
Yellow_grouper_TNF 145 SDGDEK----GAGRRLMPLSHRIWRYSDSIG------SRASLMSAVRSACQSTAQEDSDG
Gilthead_seabream_TNF 157 SDGDEE----GAGRHLTPLSHRISRYSESMG------SDVSLMSAVRSACQNTAQEDSYS
Striped_beakfish_TNF 157 SDGEEE----GVAKRLTPLSHRIWRYSDSIG------SRASLMSAVRSACQNTAQEDSYR
Green_chromide_TNF 154 GGNDEE----GTGGGLTALSHRIWRFSDSIG------SDSSLMSAVRSACQSTVEED---
Pacifc_bluefin_tuna_TNF2 144 SSSDS------TSKSMVHLSHTVKRWSNSYGNGDATSSYQTILHSVRTACQKTVSRDPD-
Southern_bluefin_tuna_TNF1 151 SDGDEQ----GAGKRLTPLSHRIWRYSDSVG------SKASLMSAVRSACQQGAQEGSYR
Black_porgy_TNF 157 SDGDEE----GAGRHLTPLSHTISRYSESMG------TDVSLMSAVRSACQNTAHEDSYS
Olive_flounder_TNF 126 SSDDADDGKEAAEKHLTSISHRVWLFTESLG------TQVSLMSAVRSACQKS-QEDAYR
Brook_trout_TNF 164 N---------GPGERTTPLSHVICRYSDSIG------VNANLLSGVRSVCQQNYGNAESN
Nile_tilapia_TNF 152 GNTDENE---DEEKSLTILSHRIWRYSESMG------SSSTLMSALRSACQDTIQDSF--
Japanese_puffer_TNF 154 GEGDKH----SPGKSHIPLSHRVWRYSDSIG------TETTLLNAVRSACQNSALEGGYS
Human_TNF 144 ----------GCPSTHVLLTHTISRIAVSYQ------TKVNLLSAIKSPCQRETPEGAE-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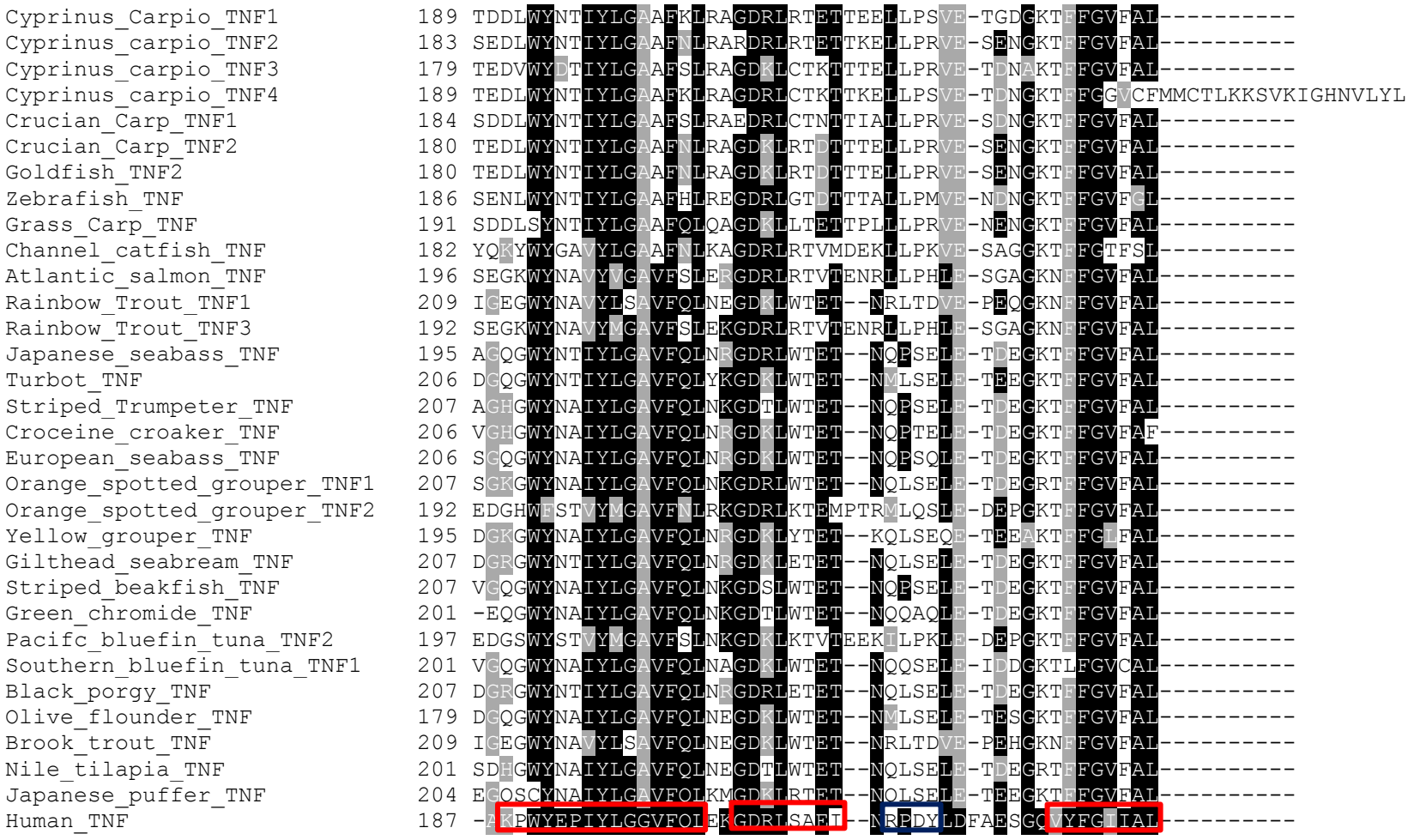
Groundhog_TNF 145 ----------GCPS-HVLLTHTVSRFAVSYQ------DKVNLLSAIKIPCPKESLEGAE-
Mongolian_gerbil_TNF 147 ----------GCPH-LVLLTHTVSRFAVSYQ------DKVSLLSAIKSPCPRDAPEGAE-
Rat_TNF 147 ----------GCPD-YVLLTHTVSRFAISYQ------EKVSLLSAIKSPCPKDTPEGAE-
White_footted_mouse_TNF 147 ----------GCSS-YVLLTHTVSRFAVSYE------DKVNLLSAIKSPCPKETPEGSE-
Leschenaults_rousette_TNF 143 ----------GCPQTHVLLTHTISRFADSYQ------DKVNLLSAIKSPCQRETQDGAG-
Domestic_cat_TNF 144 ----------GCPSTHVHLTHTISRFAVSYQ------TKVNLLSAIKSPCQRETPEGAE-
Guinea_pig_TNF 146 ----------GCPS-YLLLTHTVSRLAVSYP------EKVNLLSAIKSPCQKETPEGAE-
Dog_TNF 144 ----------GCPSTHVLLTHTISRFAVSYQ------TKVNLLSAIKSPCQRETPEGTE-
Northern_elephant_seal_TNF 144 ----------GCRSTHVLLTHTISRFAVSYQ------TKVNLLSAIKSPCQRETPEGTE-
West_Indian_manatee_TNF 144 ----------GCPSTHVLLTHTISRIAVSYQ------TKVNLLSAIKSPCHRETPEEAE-
Horse_TNF 145 ----------GCPSTHVLLTHTISRLAVSYP------SKVNLLSAIKSPCHTESPEQAE-
Chacma_baboon_TNF 144 ----------GCPSNHVLLTHTISRIAVSYQ------TKVNLLSAIKSPCQRETPEGAE-
Large_tree_shrew_TNF 145 ----------GCPSTPVLFTHTVSCVAVSYN------NKVNLLSAIKSPCQKETAEGAE-
Pig_TNF 143 ----------GCPSTNVFLTHTISRIAVSYQ------TKVNLLSAIKSPCQRETPEGAE-
Bactrian_camel_TNF 144 ----------RCPSTPVFLTHTISRLAVSYP------NKANLLSAIKSPCQGGTSEEAE-
Ferret_TNF 145 ----------GCSSTNVLLTHTISRFAVSYR------TKVNLLSAIKSPCQRETPEGTE-
Rhesus_macaque_TNF 144 ----------GCPSNHVLLTHTISRIAVSYQ------TKVNLLSAIKSPCQRETPEGAE-
Green_monkey_TNF 144 ----------GCPSNHVLLTHTISRIAVSYQ------TKVNLLSAIKSPCQRETPEGAE-
Common_squirrel_monkey_TNF 144 ----------GCPSTFTLLTHSISRIAVSYQ------AKVNLLSAIKSPCQRETPRGAK-
Tammar_wallaby_TNF 144 ----------DCANEPLLLTHTVSRVALSYQ------SKVNLLSAIKSPCQKTVKGARE-
Whitetufted_ear_marmoset_TNF 143 ----------GCPSNFMLLTHSISRIAVSYQ------AKVNLLSAIKSPCQRETPQGAK-
Koala_TNF 143 ----------DCASEPLILTHTISRVALSYE------NKVSLLAAIKSPCQKAAQGARE-
Sooty_mangabey_TNF 144 ----------GCPSNHVLLTHTISRIAVSYQ------TKVNLLSAIKSPCQRETPEGAE-
Bighorn_sheep_TNF 145 ----------GCPSTPLFLTHTISRIAVSYQ------TKVNILSAIKSPCHRETLEGAE-
Baboon_TNF 144 ----------GCPSTHVLLTHTISRIAVSYQ------TKVNLLSAIKSPCQRETPEGAE-
Brush_tailed_possum_TNF 144 ----------QCGKESLVLTHKISRFTLSYQ------KKVTLLANIRSSCRKAAEDDGE-
Red_deer_TNF 140 ----------SCPSTPLFLTHTISRIAVSYQ------TKVNILSAIKSPCHRETPEWAE-
Domestic_water_buffalo_TNF 144 ----------GCPSTPLFLTHTISRIAVSYQ------TKVNILSAIKSPCHRETPEWAE-
Nilgai_TNF 145 ----------GCPSTPLFLTHTISRIAVSYQ------TKVNILSAIKSPCHRETPEWAE-
Beluga_whale_TNF 144 ----------GCPSTHLFLTHTISRIAVSYQ------TKVNLLSAIKSPCQRETPEGAE-


Cyprinus_Carpio_TNF1 189 TDDLWYNTIYLGAAFKLRAGDRLRTETTEELLPSVE-TGDGKTFFGVFAL----------
Cyprinus_carpio_TNF2 183 SEDLWYNTIYLGAAFNLRARDRLRTETTKELLPRVE-SENGKTFFGVFAL----------
Cyprinus_carpio_TNF3 179 TEDVWYDTIYLGAAFSLRAGDKLCTKTTTELLPRVE-TDNAKTFFGVFAL----------
Cyprinus_carpio_TNF4 189 TEDLWYNTIYLGAAFKLRAGDRLCTKTTKELLPSVE-TDNGKTFFGGVCFMMCTLKKSVKIGHNVLYL
Crucian_Carp_TNF1 184 SDDLWYNTIYLGAAFSLRAEDRLCTNTTIALLPRVE-SDNGKTFFGVFAL----------
Crucian_Carp_TNF2 180 TEDLWYNTIYLGAAFNLRAGDKLRTDTTTELLPRVE-SENGKTFFGVFAL----------
Goldfish_TNF2 180 TEDLWYNTIYLGAAFNLRAGDKLRTDTTTELLPRVE-SENGKTFFGVFAL----------
Zebrafish_TNF 186 SENLWYNTIYLGAAFHLREGDRLGTDTTTALLPMVE-NDNGKTFFGVFGL----------
Grass_Carp_TNF 191 SDDLSYNTIYLGAAFQLQAGDKLLTETTPLLLPRVE-NENGKTFFGVFAL----------
Channel_catfish_TNF 182 YQKYWYGAVYLGAAFNLKAGDRLRTVMDEKLLPKVE-SAGGKTFFGTFSL----------
Atlantic_salmon_TNF 196 SEGKWYNAVYVGAVFSLERGDRLRTVTENRLLPHLE-SGACKNFFGVFAL----------
Rainbow_Trout_TNF1 209 IGEGWYNAVYLSAVFQLNEGDKLWTET--NRLTDVE-PEQGKNFFGVFAL----------
Rainbow_Trout_TNF3 192 SEGKWYNAVYMGAVFSLEKGDRLRTVTENRLLPHLE-SGACKNFFGVFAL----------
Japanese_seabass_TNF 195 AGQGWYNTIYLGAVFQLNRGDRLWTET--NQPSELE-TDEGKTFFGVFAL----------
Turbot_TNF 206 DGQGWYNTIYLGAVFQLYKGDKLWTET--NMLSELE-TEEGKTFFGVFAL----------
Striped_Trumpeter_TNF 207 AGHGWYNAIYLGAVFQLNKGDTLWTET--NQPSELE-TDEGKTFFGVFAL----------
Croceine_croaker_TNF 206 VGHGWYNAIYLGAVFQLNRGDKLWTET--NQPTELE-TDEGKTFFGVFAF----------
European_seabass_TNF 206 SGQGWYNAIYLGAVFQLNRGDKLWTET--NQPSQLE-TDEGKTFFGVFAL----------
Orange_spotted_grouper_TNF1 207 SGKGWYNAIYLGAVFQLNKGDRLWTET--NQLSELE-TDEGRTFFGVFAL----------
Orange_spotted_grouper_TNF2 192 EDGHWFSTVYMGAVFNLRKGDRLKTEMPTRMLQSLE-DEPGKTFFGVFAL----------
Yellow_grouper_TNF 195 DGKGWYNAIYLGAVFQLNRGDKLYTET--KQLSEQE-TEEAKTFFGLFAL----------
Gilthead_seabream_TNF 207 DGRGWYNTIYLGAVFQLNRGDKLETET--NQLSELE-TDEGKTFFGVFAL----------
Striped_beakfish_TNF 207 VGQGWYNAIYLGAVFQLNKGDSLWTET--NQPSELE-TDEGKTFFGVFAL----------
Green_chromide_TNF 201 -EQGWYNAIYLGAVFQLNKGDTLWTET--NQQAQLE-TDEGKTFFGVFAL----------
Pacifc_bluefin_tuna_TNF2 197 EDGSWYSTVYMGAVFSLNKGDKLKTVTEEKILPKLE-DEPGKTFFGVFAL----------
Southern_bluefin_tuna_TNF1 201 VGQGWYNAIYLGAVFQLNAGDKLWTET--NQQSELE-IDDGKTLFGVCAL----------
Black_porgy_TNF 207 DGRGWYNTIYLGAVFQLNRGDRLETET--NQLSELE-TDEGKTFFGVFAL----------
Olive_flounder_TNF 179 DGQGWYNAIYLGAVFQLNEGDKLWTET--NMLSELE-TESGKTFFGVFAL----------
Brook_trout_TNF 209 IGEGWYNAVYLSAVFQLNEGDKLWTET--NRLTDVE-PEHGKNFFGVFAL----------
Nile_tilapia_TNF 201 SDHGWYNAIYLGAVFQLNEGDTLWTET--NQLSELE-TDEGRTFFGVFAL----------
Japanese_puffer_TNF 204 EGQSGYNAIYLGAVFQLKMGDKLRTET--NQLSELE-TEEGKTFFGVFAL----------
Human_TNF 187 -AKPWYEPIYLGGVFQLEKGDRLSAEI--NRPDYLDFAESGQVYFGIIAL----------

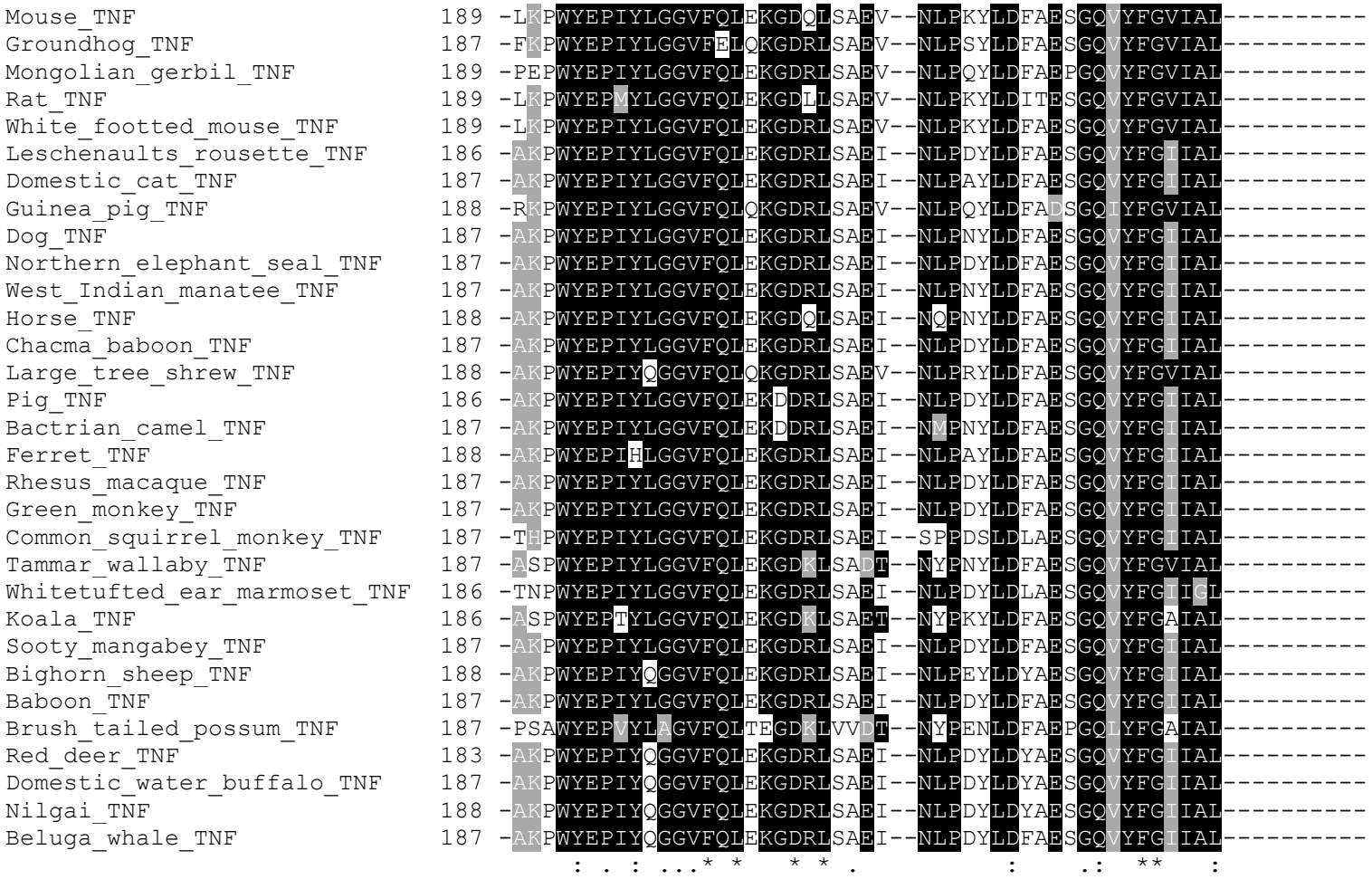


Figure 3.5: A multiple sequence alignment of TNF-α from the various species of fish shown in Table 2.2 and Table 2.3. The boxes with the red and blue outlines indicate the location of β-strands and α-helices in TNF-α of humans respectively. The green line indicates a putative site in which the membrane bound form of TNF-α is cut to produce the soluble form. Residues with a black background indicate areas of conservation and residues with a grey background indicate areas of conservative substitutions. The "*" symbol indicates that every residue in that column is conserved. The ":" symbol indicates that at least one residue in that column will be a conservative substitution, meaning that it has been substituted for one with similar chemical properties. The "." symbol indicates that a residue in the column is a semi conservative substitution where the substituted residue has a similar conformation but different chemical properties.

There are a total of 11 conserved residues, 19 residues with conservative substitutions, and 14 residues with semi conservative substitutions. There is a clear difference in conservation between the different species of fish and mammals. When using TNF-α from humans as a base, between residues 40 and 60 there is a high degree of similarity between the mammals with most of the residue in that area being conserved. By contrast the residues found in the different species of fish have relatively few conserved regions with only L, A, F, and G amino acids showing any conservation. This could perhaps be due to fish having different isoforms of TNF-α as opposed to only one form seen in mammals. It is also likely to be because of the difference in species. Areas of the alignment that have a number of conserved regions between the different species of fish and mammals are between residues 122 – 141, 193 – 211, and 231 – 237 (when using TNF-α1 from common carp as a base sequence for counting).

Figure 3.3 showed that there a several conserved residues that have been shown to play an important role in the structure and function of TNF-α in humans. Several of these residues are conserved and some have conservative substitutions, in the alignment shown in Figure 3.5. The residues L-133, S-136, Q-137, Y-195, L-196, G-197, and G-198 were shown to affect the structure of TNF-α, causing the trimer to become a dimer (He *et al.*, 2005). Other than L-133, the remaining residues are either conserved or have conservative substitutions in the other corresponding sequences of TNF-α. In the case of S-136, TNF-α from Japanese seabass and Yellow grouper had a conservative substitution with a C and T residue respectively. The residue L-196 in human TNF-α had four conservative substitutions at Atlantic salmon which had a V residue and Rainbow Trout TNF-α3, Orange spotted grouper TNF-α2 and Pacific Bluefin tuna TNF-α2 which all had an M residue. The residue L-133 in human TNF-α was only conserved in Yellow grouper for the species of fish. However, L-133 was conserved in all the mammalian species. As the residues have been conserved in

the majority of species, it suggests that they have a very significant role in TNF-α. It is already known that a mutation of these residues effects the TNF-α trimer structure in humans. Their conservation also suggests they may have the same, if not a similar role in other species as well. It also suggests that TNF-α is able to form a trimer in other species. This is also seen in other residues, such as H-154, Y-191, and W-104 which were found to affect the ability of TNF-α to bind to TNFR1 and TNFR2 when mutated in humans. Residue H-154, from human TNF-α, is conserved in all species apart from Atlantic salmon and Japanese seabass which both have non conservative substitutions. A non conservative substitution is where a residue in an alignment is not similar in conformation or chemical properties. The residue W-104 has been conserved in all species except for the Sooty mangabey which also has a non-conservative substitution. This would suggest that these residues have a similar function in all species where there is conservation.

The positions of the β-strands and α-helices, in TNF-α of humans, are shown in the alignment (see Figure 3.5). Looking at the placement of conserved residues and ones with conservative substitutions (shown using a black and grey background respectively), when comparing TNF-α from all the different mammalian species a clear pattern can be seen. In the position of the β-strands, for mammals, most of the species have a conserved residue or a conservative substitution in that area. This is most clearly seen between residues G-130 – G-144, K-188 – L-202, and V-226 – L-233. In these β-strands most of the residues were conserved between the different mammals, indicating the likely position of the β-strand in TNF-α of the respective species. When comparing the protein sequence of TNF-α in the different species of fish, including common carp, there are far less conserved residues between the different species. However, a pattern can still be seen. For example, in the first β-strand between residues P-88 and A-94 of human TNF-α, H-91 is conserved while V-92 and A-94 have been conservatively substituted for an L and a G residue respectively. In the β-strand situated

between residues Q-123 and V-126, L-124, V-125, and V-126 all have a conservative substitution in all of the TNF-α sequences in the fish species, with the exception of Channel catfish. In the case of the β-strands situated between the residues: G-130 – G-144, K-188 – L-202 and V-226 – L-233, most of the corresponding residues in the TNF-α sequence of the fish species have either a conserved residue or a conservative substitution. This would suggest that there are similarities in the structure of TNF-α in different species.

As seen in the previous alignments, shown in Figure 3.3 and 3.4, TNF-α4 of common carp stands out from the other species by having a sequence that is significantly longer than the others. While the C-terminal end, an L residue, for all species was conserved in all cases, the exception being European seabass, TNF-α4 of common carp is different. However, in all of the alignments, TNF-α4 shows similar conservation as the other TNF-α isoforms of common carp. The longer sequence does not seem to have any major effects on the alignments. This would suggest that the longer sequence of TNF-α4 does not have a major effect of the structure or function of the cytokine and may just be an extension of the C-terminal end.

The sequence similarity between common carp and the other mammalian species is shown in Table 3.2. The sequence similarity for TNF-α in mammals, when compared to TNF-α in common carp, is lower than what was seen in the different species of fish, as shown in Table 3.1. The species with the highest sequence similarity was Groundhogs with 36%. The lowest was the Domestic water buffalo with 27%. Humans and mice both have a sequence similarity of 32% when compared to TNF-α in common carp.

Common name	Latin name	Protein	Sequence similarity
Human	*Homo sapiens*	TNFα	32%
Mouse	*Mus musculus*	TNFα	32%
Groundhog	*Marmota monax*	TNFα	36%
Mongolian gerbil	*Meriones unguiculatus*	TNFα	34%
Rat	*Rattus norvegicus*	TNFα	33%
White-footed mouse	*Peromyscus leucopus*	TNFα	34%
Leschenault's rousette	*Rousettus leschenaultii*	TNFα	32%
Domestic Cat	*Felis catus*	TNFα	32%
Guinea pig	*Cavia porcellus*	TNFα	29%
Dog	*Canis familiaris*	TNFα	29%
Northern elephant seal	*Mirounga angustirostris*	TNFα	31%
West Indian manatee	*Trichechus manatus latirostris*	TNFα	31%
Horse	*Equus caballus*	TNFα	32%
Chacma baboon	*Papio hamadryas ursinus*	TNFα	30%
Large tree shrew	*Tupaia tana*	TNFα	33%
Pig	*Sus scrofa*	TNFα	32%
Bactrian camel	*Camelus bactrianus*	TNFα	32%
Ferret	*Mustela putorius furo*	TNFα	28%
Rhesus macaque	*Macaca mulatta*	TNFα	33%
Green monkey	*Chlorocebus sabaeus*	TNFα	33%
Common squirrel monkey	*Saimiri sciureus*	TNFα	30%
Tammar wallaby	*Macropus eugenii*	TNFα	32%
White-tufted-ear marmoset	*Callithrix jacchus*	TNFα	33%
Koala	*Phascolarctos cinereus*	TNFα	31%
Sooty mangabey	*Cercocebus atys*	TNFα	30%
Bighorn sheep	*Ovis canadensis*	TNFα	28%
Baboon	*Papio sp*	TNFα	32%
Brush-tailed possum	*Trichosurus vulpecula*	TNFα	29%
Red deer	*Cervus elaphus*	TNFα	32%
Domestic water buffalo	*Bubalus bubalis*	TNFα	27%
Nilgai	*Boselaphus tragocamelus*	TNFα	31%
Beluga whale	*Delphinapterus leucas*	TNFα	31%

Table 3.2: A table of the sequence similarities calculated for the protein sequence of TNF-α, in the different mammals used in Figure 3.5, when compared with TNF-α from common carp.

3.5 Prediction of the Secondary Structure of TNF-α in *Cyprinus carpio*

In the following section the secondary structure of TNF-α from common carp was predicted using structure prediction software. These predictions were then compared and aligned with the known structures of TNF-α from humans and mice. This was done to see if the predictions were similar to the known structures and whether they matched up the previous alignment (Figure 3.3).

In order to determine the accuracy of the prediction software, the secondary structure of TNF-α from humans and mice was predicted and compared with the known structures. Figure 3.6 shows the secondary structure prediction obtained using PSIPRED and Figure 3.7 shows a diagram of the secondary structure of TNF-α in humans and mice compared with the predicted secondary structure produced by Protein Predict and PSIPRED respectively.

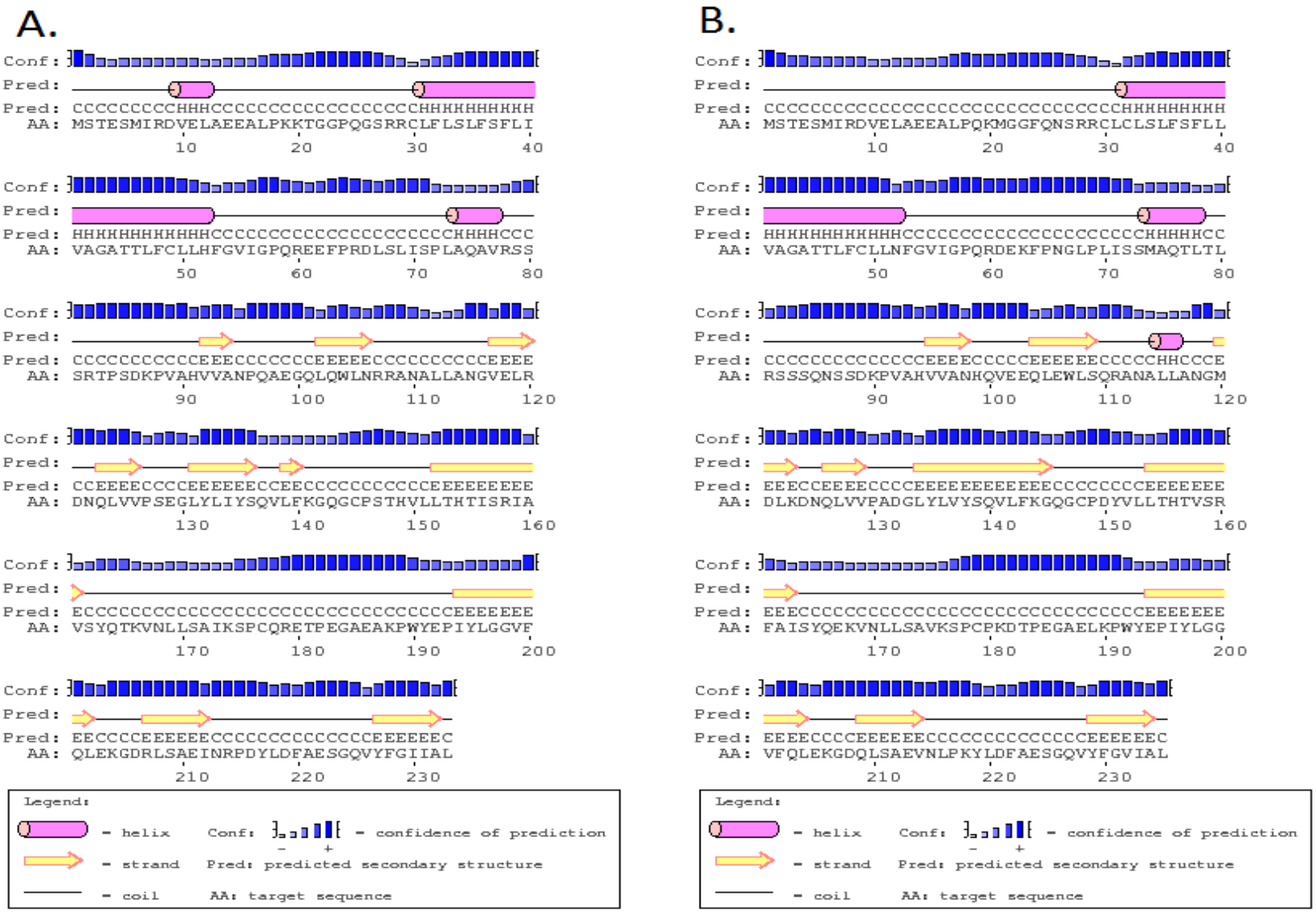


Figure 3.6 A diagram to show the predicted secondary structure of TNF-α from mice and humans, from PSIPRED. A. represents the results obtained for TNF-α from humans and B. represents the results obtained for TNF-α from mice. The pink cylinder represents the prediction of an α-helix, the yellow arrow represents the prediction of a β-strand, while the black line represents the prediction of a coil. The blue rectangles are a visual representation of the prediction confidence, a value generated by PSIRED that displays how confident the program is in its prediction. The larger the rectangle the more confident the prediction.

A.

	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
Human Known TNF	E	E	F	P	R	D	L	S	L	I	S	P	L	A	Q	A	V	R	S	S	S	R	T	P	S	D	K	P	V	A	H	V	V	A	N	P	Q	A	E	G	Q	L	Q	W	L	N	R	R	A	N	A	L	L	A	N	G	V	E	L	R
Protein Predict	E	E	F	P	R	D	L	S	L	I	S	P	L	A	Q	A	V	R	S	S	S	R	T	P	S	D	K	P	V	A	H	V	V	A	N	P	Q	A	E	G	Q	L	Q	W	L	N	R	R	A	N	A	L	L	A	N	G	V	E	L	R
PSIPRED	E	E	F	P	R	D	L	S	L	I	S	P	L	A	Q	A	V	R	S	S	S	R	T	P	S	D	K	P	V	A	H	V	V	A	N	P	Q	A	E	G	Q	L	Q	W	L	N	R	R	A	N	A	L	L	A	N	G	V	E	L	R

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Human Known TNF	D	N	Q	L	V	V	P	S	E	G	L	Y	L	I	Y	S	Q	V	L	F	K	G	Q	G	C	P	S	T	H	V	L	L	T	H	T	I	S	R	I	A	V	S	Y	Q	T	K	V	N	L	L	S	A	I	K	S	P	C	Q	R	E
Protein Predict	D	N	Q	L	V	V	P	S	E	G	L	Y	L	I	Y	S	Q	V	L	F	K	G	Q	G	C	P	S	T	H	V	L	L	T	H	T	I	S	R	I	A	V	S	Y	Q	T	K	V	N	L	L	S	A	I	K	S	P	C	Q	R	E
PSIPRED	D	N	Q	L	V	V	P	S	E	G	L	Y	L	I	Y	S	Q	V	L	F	K	G	Q	G	C	P	S	T	H	V	L	L	T	H	T	I	S	R	I	A	V	S	Y	Q	T	K	V	N	L	L	S	A	I	K	S	P	C	Q	R	E

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Human Known TNF	T	P	E	G	A	E	A	K	P	W	Y	E	P	I	Y	L	G	G	V	F	Q	L	E	K	G	D	R	L	S	A	E	I	N	R	P	D	Y	L	D	F	A	E	S	G	Q	V	Y	F	G	I	I	A	L
Protein Predict	T	P	E	G	A	E	A	K	P	W	Y	E	P	I	Y	L	G	G	V	F	Q	L	E	K	G	D	R	L	S	A	E	I	N	R	P	D	Y	L	D	F	A	E	S	G	Q	V	Y	F	G	I	I	A	L
PSIPRED	T	P	E	G	A	E	A	K	P	W	Y	E	P	I	Y	L	G	G	V	F	Q	L	E	K	G	D	R	L	S	A	E	I	N	R	P	D	Y	L	D	F	A	E	S	G	Q	V	Y	F	G	I	I	A	L

B.

	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
Mouse Known TNF	D	E	K	F	P	N	G	L	P	L	I	S	S	M	A	Q	T	L	T	L	R	S	S	S	Q	N	S	S	D	K	P	V	A	H	V	V	A	N	H	Q	V	E	E	Q	L	E	W	L	S	Q	R	A	N	A	L	L	A	N	G	M
Protein Predict	D	E	K	F	P	N	G	L	P	L	I	S	S	M	A	Q	T	L	T	L	R	S	S	S	Q	N	S	S	D	K	P	V	A	H	V	V	A	N	H	Q	V	E	E	Q	L	E	W	L	S	Q	R	A	N	A	L	L	A	N	G	M
PSIPRED	D	E	K	F	P	N	G	L	P	L	I	S	S	M	A	Q	T	L	T	L	R	S	S	S	Q	N	S	S	D	K	P	V	A	H	V	V	A	N	H	Q	V	E	E	Q	L	E	W	L	S	Q	R	A	N	A	L	L	A	N	G	M

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Mouse Known TNF	D	L	K	D	N	Q	L	V	V	P	A	D	G	L	Y	L	V	Y	S	Q	V	L	F	K	G	Q	G	C	P	D	Y	V	L	L	T	H	T	V	S	R	F	A	I	S	Y	Q	E	K	V	N	L	L	S	A	V	K	S	P	C	P
Protein Predict	D	L	K	D	N	Q	L	V	V	P	A	D	G	L	Y	L	V	Y	S	Q	V	L	F	K	G	Q	G	C	P	D	Y	V	L	L	T	H	T	V	S	R	F	A	I	S	Y	Q	E	K	V	N	L	L	S	A	V	K	S	P	C	P
PSIPRED	D	L	K	D	N	Q	L	V	V	P	A	D	G	L	Y	L	V	Y	S	Q	V	L	F	K	G	Q	G	C	P	D	Y	V	L	L	T	H	T	V	S	R	F	A	I	S	Y	Q	E	K	V	N	L	L	S	A	V	K	S	P	C	P

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Mouse Known TNF	K	D	T	P	E	G	A	E	L	K	P	W	Y	E	P	I	Y	L	G	G	V	F	Q	L	E	K	G	D	Q	L	S	A	E	V	N	L	P	K	Y	L	D	F	A	E	S	G	Q	V	Y	F	G	V	I	A	L
Protein Predict	K	D	T	P	E	G	A	E	L	K	P	W	Y	E	P	I	Y	L	G	G	V	F	Q	L	E	K	G	D	Q	L	S	A	E	V	N	L	P	K	Y	L	D	F	A	E	S	G	Q	V	Y	F	G	V	I	A	L
PSIPRED	K	D	T	P	E	G	A	E	L	K	P	W	Y	E	P	I	Y	L	G	G	V	F	Q	L	E	K	G	D	Q	L	S	A	E	V	N	L	P	K	Y	L	D	F	A	E	S	G	Q	V	Y	F	G	V	I	A	L

Figure 3.7 A diagram to show the known secondary structure of TNF-α from mice and humans compared against the predicted secondary structure. A. shows the results obtained for TNF-α from humans and B. shows the results obtained for TNF-α from mice. In each row, the top section displays the known secondary structure, the middle section displays the prediction made by Protein Predict and the lower section displays the prediction made by PSIPRED. The dark green squares indicate the presence of β-strands in the known structures. The light green squares indicate the presence of β-strands in the predicted structures. The light blue squares indicate the presence of an α-helix in the known structures and the dark blue squares indicate the presence of any predicted α-helices. The column that is colored yellow points out the position in which the TNF-α structure is cleaved to produce the soluble cytokine. As the three dimensional structure of only the soluble version of TNF-α has been determined, the majority of the corresponding protein sequence which was not part of the soluble cytokine has been cut out. The numbers at the top of each section represent the position of the corresponding residue in the protein sequence.

The known structure of human TNF-α (Eck and Sprang, 1989) has ten β-strands and one α-helix. The secondary structure produced by Protein Predict showed that twelve β-strands and no α-helices had been predicted. The β-strand predicted to be located between Q-101 and W-104 does not have a corresponding β-strand in the known structure. The remaining predicted β-strands have a corresponding β-strand in the known structure. However, the predictions are not completely accurate, with many of the predicted β-strands being either a different size or placed in a slightly different position. This can be seen at the β-strands predicted to be located at Y-191 – E-192 and I-194 – L-202. These strands did have a corresponding β-strand in the known structure of TNF-α in humans. However, instead of there being two smaller β-strands, as seen in the prediction, there is one large β-strand between K-188 and L-202 instead.

The secondary structure produced by PSIPRED was similar to the one produced by Protein predict. No α-helix had been predicted in the section of the protein corresponding to the known structure. The known structure has a β-strand between residues L-112 – A-114 and Q-164 – K-174. However, no β-strands have been predicted to be at these locations by PSIPRED. In the same prediction a β-strand was predicted to be between L-102 and N-106. However there is no corresponding β-strand, in the known structure, in that area. The remaining predictions all have corresponding β-strands in the known structure. However, the only strand that was accurately predicted was between Q-123 and V-126. The remaining predictions vary in accuracy with the result being either a different size to the known structure or in a slightly different position. However the general area of the prediction remains constant.

The prediction of the secondary structure of TNF-α in mice seems to be more accurate than it was in humans for both Protein Predict and PSIPRED. Using Protein Predict, the β-strands

situated between residues L-115 - A-117, Q-209 - V-214, and Y-229 - A-234 were accurately predicted with the correct size and position. The remaining predictions are similar to the predictions made for TNF-α in humans, where the predictions had corresponding β-strands in the known structure but were of a different size or position. The one exception to this is the prediction made between residues Q-104 and W-107, where there is no corresponding β-strand in the known structure. No α-helices had been predicted despite one being present in the known structure of TNF-α in mice. Using PSIPRED only two strands had been accurately predicted in terms of both exact position and size. These strands were situated between residues Q-209 - V-214 and Y-229 - A-234. The β-strand predicted to be between residues Q-104 and S-109 has no corresponding β-strand in the known structure. Conversely, the β-strand located between residues K-168 and K-176 in the known structure has no corresponding prediction in the structure produced by PSIPRED. An α-helix had been predicted by PSIPRED to be located between residues L-115 and L-116, however, there is a β-strand in this location in the known structure. The location of the α-helix in the known structure was between residues P-217 and Y-219. However no prediction had been made by PSIPRED at this location.

The soluble version of TNF-α in humans is 157 amino acids in length. In total there are ten β-strands and one α-helix in this structure. There is a total of 87 residues in the β-strands and the α-helix. Protein Predict had predicted there to be eleven β-strands and no α-helices. Out of the eleven β-strands predicted, 63 residues had been correctly predicted. This means the prediction was approximately 85% accurate. In the case of PSIPRED, 10 β-strands and no α-helices had been predicted. Of these 10 β-strands, 49 of the residues had been predicted correctly. This means that the prediction was 76% accurate.

The soluble version of TNF-α in mice is 156 amino acids in length. In total there are a ten β-strands and one α-helix in this structure. There is a total of 75 residues in the β-strands and the α-helix. Protein Predict had predicted there to be eleven β-strands and no α-helices. Out of the eleven β-strands predicted, 63 residues had been correctly predicted. This means the prediction was approximately 92% accurate. In the case of PSIPRED, 9 β-strands and 1 α-helix had been predicted. Of these, 52 of the residues had been predicted correctly. This means that the prediction was 85% accurate.

PSIPRED also produces a result which shows confidence in each prediction. This is a visual representation of how likely the prediction is to be correct. The results of this is shown in Figure 3.6. Taking this into account, several of the predictions have been deemed unlikely to be correct by PSIPRED. The α-helix predicted to be between residue L-115 and L-116 of TNF-α in mice was shown to likely be incorrect as the confidence in the prediction is depicted as being very small. However, the area in which an α-helix is known to be within the protein sequence, between residues P-217 and Y-219, was shown to be have a high confidence in the prediction, despite being incorrect. This also occurred in the prediction of the secondary structure in TNF-α of humans. In the known structure, an α-helix is found between the residues R-214 and Y-217. Yet, PSIPRED has predicted that there is only a coil in this area, with a high confidence in the prediction, despite being incorrect.

Based on the accuracy percentage calculated using the results of Protein Predict and PSIPRED, Protein Predict was more accurate by approximately 9% in human TNF-α and 7 % in mice TNF-α. However, both methods produced fairly accurate results and the difference in accuracy was fairly small. Based on Protein Predict being more accurate, the program was used to predict the secondary structure of TNF-α in common carp. Also, while PSIPRED also determines how likely a prediction is to be correct, this value has been shown

to be incorrect at times. The confidence in the prediction cannot be quantitatively analysed as well. What is clear from the results is that both predictions methods can be used to predict the general area or be used as a guide to where certain secondary structural features may be within the protein sequence. The predictions methods cannot accurately predict the exact size or position of any secondary structural elements. With that in mind the prediction can still be useful in the investigation as it will be able give a general idea of the secondary structure and what to expect.

3.6 Secondary structure prediction of the TNF isoforms in *Cyprinus carpio*

The predicted secondary structures of TNF-α from common carp are shown in Figure 3.8. The sequence alignment in Figure 3.1 showed that the majority of the protein sequences between the four isoforms of TNF-α were conserved, suggesting they would all have a very similar structure and function. Overall the prediction suggests that the secondary structure of the TNF-α isoforms in common carp are very similar. Four of the predicted β-strands in each isoform have the same size and position. These are located between residues situated at position 127 – 129, 132 – 136, 138 – 142, 146 – 158, and 227 – 232. The remaining predicted β-strands are in a similar position but with varying sizes. The biggest differences are seen between the residue positions 195 – 197 and 248 – 258. In this position no β-strand has been predicted for TNF-α1 in Common carp. However TNF-α2, TNF-α3, and TNF-α4 all have a β-strand in this position. TNF-α2 in particular has been predicted to have a β-strand which is only one residue in length at position 194. However, this is clearly an incorrect prediction as it is not possible for a β-strand to only be one residue in length. The other biggest difference, between residue position 248 – 258 shows that a large α-helix has been predicted in TNF-α4 of common carp. The isoforms TNF-α1, TNF-α2, and TNF-α3 all have been predicted to have a β-strand at this position, between the residues 248 and 253. As TNF-α4 has an extended C-terminal end this prediction could be attributed to that.

However, the alignment shown in Figure 3.1 shows that there is little conservation in that area so it may be that the prediction is not accurate or if it is then the α-helix does not play a major role in the cytokine. A small β-strand has also been predicted to be between residues positions 270 and 271 in TNF-α4.

When the prediction is aligned and compared with the secondary structure of TNF-α in humans and mice, a similarity in the structures are shown. Out of all the β-strands predicted, only three have no corresponding strands in TNF-α of humans and mice. These are situated between the residues at positions 117 – 120, 195 – 197, and in the case of TNF-α4 270 – 271 of the alignment. All of the remaining predicted β-strands align with the known secondary structure of TNF-α in humans and mice. Neither of the α-helices in TNF-α of humans and mice had been predicted in any of the isoforms of TNF-α in common carp. There is one α-helix that was predicted to be in only TNF-α4, located between the residues in the positions 248 and 258,. This α-helix is not seen in the other isoforms of TNF-α in common carp nor is it in humans or mice as there is a β-strand in that position. However, as the majority of TNF-α4 aligns with the other isoforms in common carp, the α-helix may just be part of the C-terminal extension.

Based on the results of Figure 3.8, the exact positions and size of the β-strands cannot be accurately determined. However, the prediction can give a general idea of where certain secondary structural motifs might be in the protein sequence. The results shown in Figure 3.8 suggest that the secondary structure of the isoforms of TNF-α in common carp are very similar to TNF-α in humans and mice.

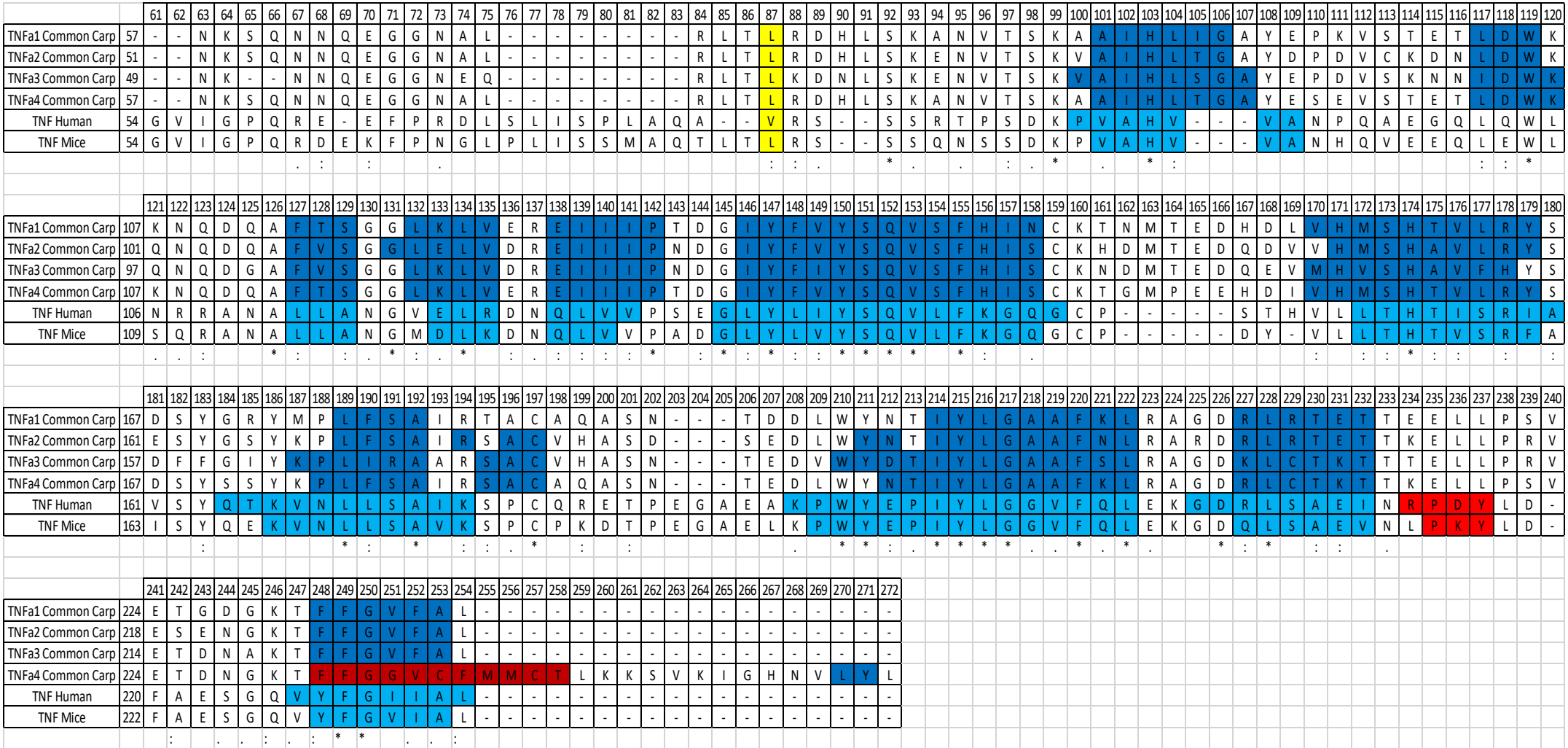


Figure 3.8: A diagram to show the predicted secondary structure of TNF-α from common carp aligned with the known structures of TNF-α from humans and mice. The dark blue squares indicate the presence of β-strands in the predicted structures. The light blue squares indicate the presence of β-strands in the known structures. The dark red squares indicates the presence of an α-helix in the predicted structures. The light red squares indicates the presence of an α-helix in the in the known structures. The column that is coloured yellow indicates the point at which TNF-α in humans and mice would become a soluble protein. As the three dimensional structure of only the soluble version of TNF-α has been determined, the majority of the corresponding protein sequence which was not part of the soluble cytokine has been cut out. The number on the side of each row indicates the position, in the protein sequence, the first residue is in, in that row. The symbols at the bottom of each row correspond to the alignment. The "*" symbol indicates that the residues in the column are completely conserved. The ":" symbol indicates the residues have conservative substitutions and the "." symbol indicates the residues have semi conservative substitutions. The numbers at the top of each row indicate the column number.

3.7 Three dimensional structure prediction of the TNF isoforms in *Cyprinus carpio*

In the following section the three dimensional structure of TNF-α from common carp was predicted. These predictions were then compared with the three dimensional structure of TNF-α in humans and mice to see if there are any similarities or differences.

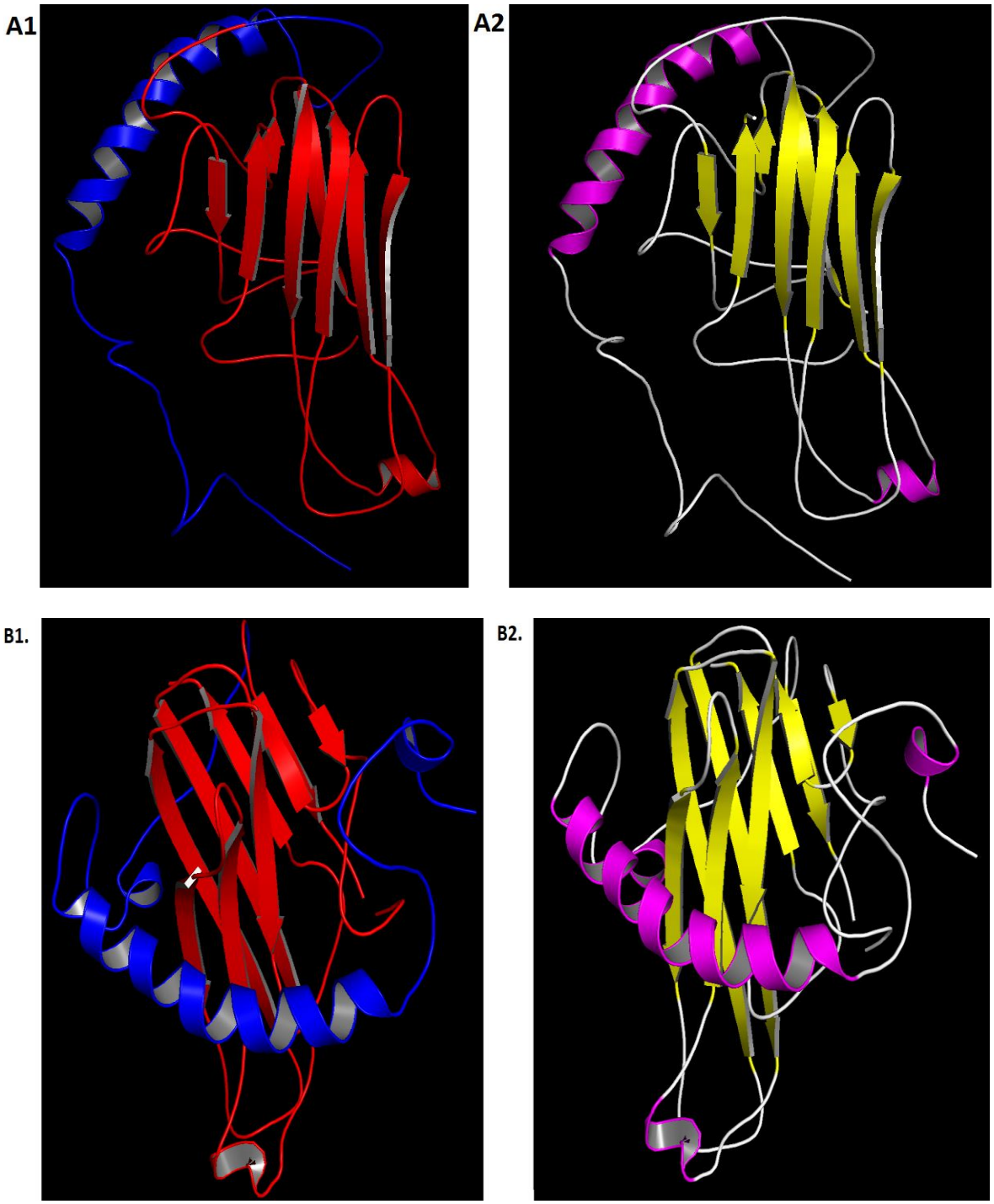
The predicted three dimensional structures of TNF-α from common carp are shown in Figure 3.9. In the predictions for each of the isoforms, a group of β-strands, forming a topology similar to the jelly-roll formation seen in human TNF-α is present. Each of the isoforms have been predicted to have 8 β-strands, with the exception of TNF-α2 which has been predicted to have 9 β-strands. Each of the isoforms have also been predicted to have 2 α-helices in the structure, again with the exception being TNF-α2 which is predicted to have 4 α-helices.

The Phyre2 server also produces a result which shows the confidence in each of the predictions. This is a visual representation of how correct the prediction is likely to be. This result is displayed in figure 3.9 A1, B1, C1 and D1. In the case of every isoform, the large α-helices have been to classified as being likely to be incorrect. In TNF-α1 and TNF-α2 a small α-helix situated between the residues at position 148-152 and 145-148 respectively have been classified as being likely to be correct. The predicted β-strands in all isoforms were classified as having a high confidence, so deemed likely to be correct. The only exception was in the prediction of TNF-α4 where two strands situated between the residues at position 5-8 and 19-22.

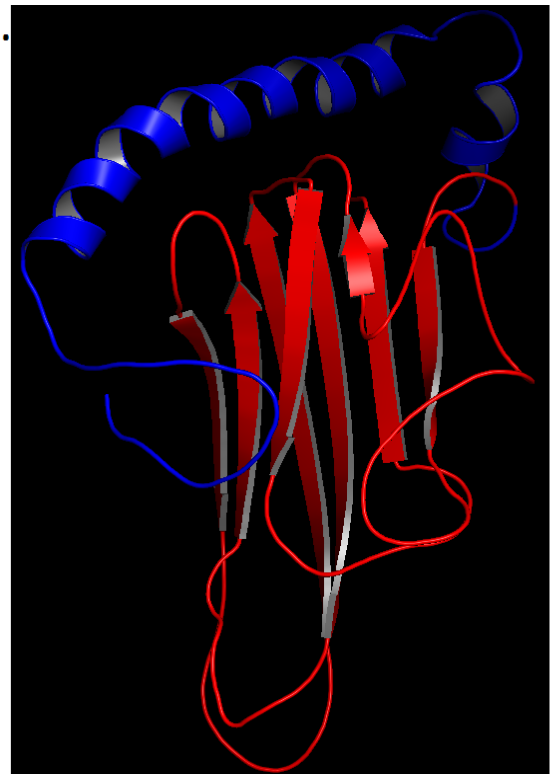
When the predicted structures are compared with the soluble versions of TNF-α in humans and mice (Figure 3.9 E1 and E2) there are a number of similarities. The predicted β-strands

in each isoform have a similar topology. The small α-helix found in TNF-α of humans and mice is only seen in the isoforms TNF-α1 and TNF-α2. The large α-helix that has been predicted in each of the isoforms is not present in TNF-α of humans and mice. However, as the three dimensional structure of TNF-α in humans and mice is of the soluble version of the protein, the predicted version may be a part of the transmembrane portion of the protein.

The predictions shown in figure 3.9 would suggest that the three dimensional structure of TNF-α in common cap would be very similar to TNF-α in humans and mice. This result is similar to the prediction of the secondary structure in figure 3.8.

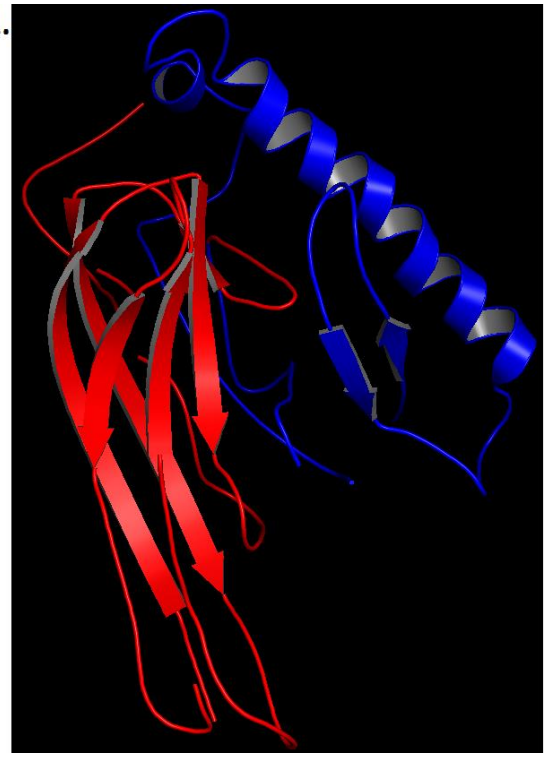

A1
A2
B1.
B2.

C1.

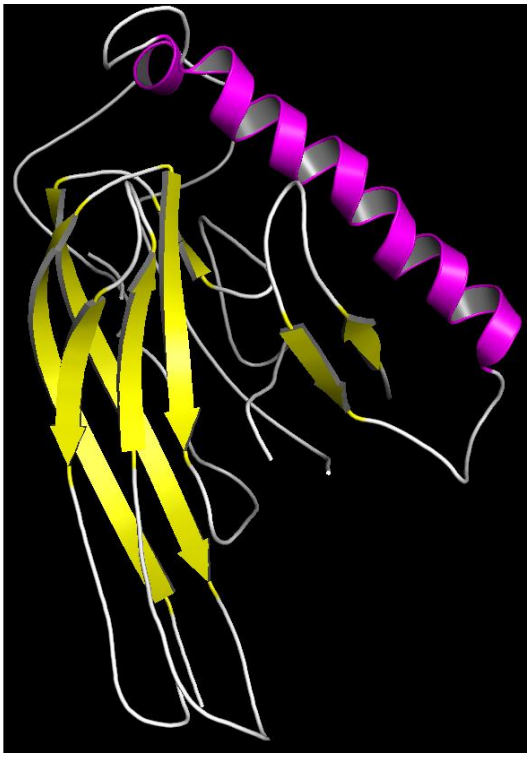


C2.

D1.



D2.



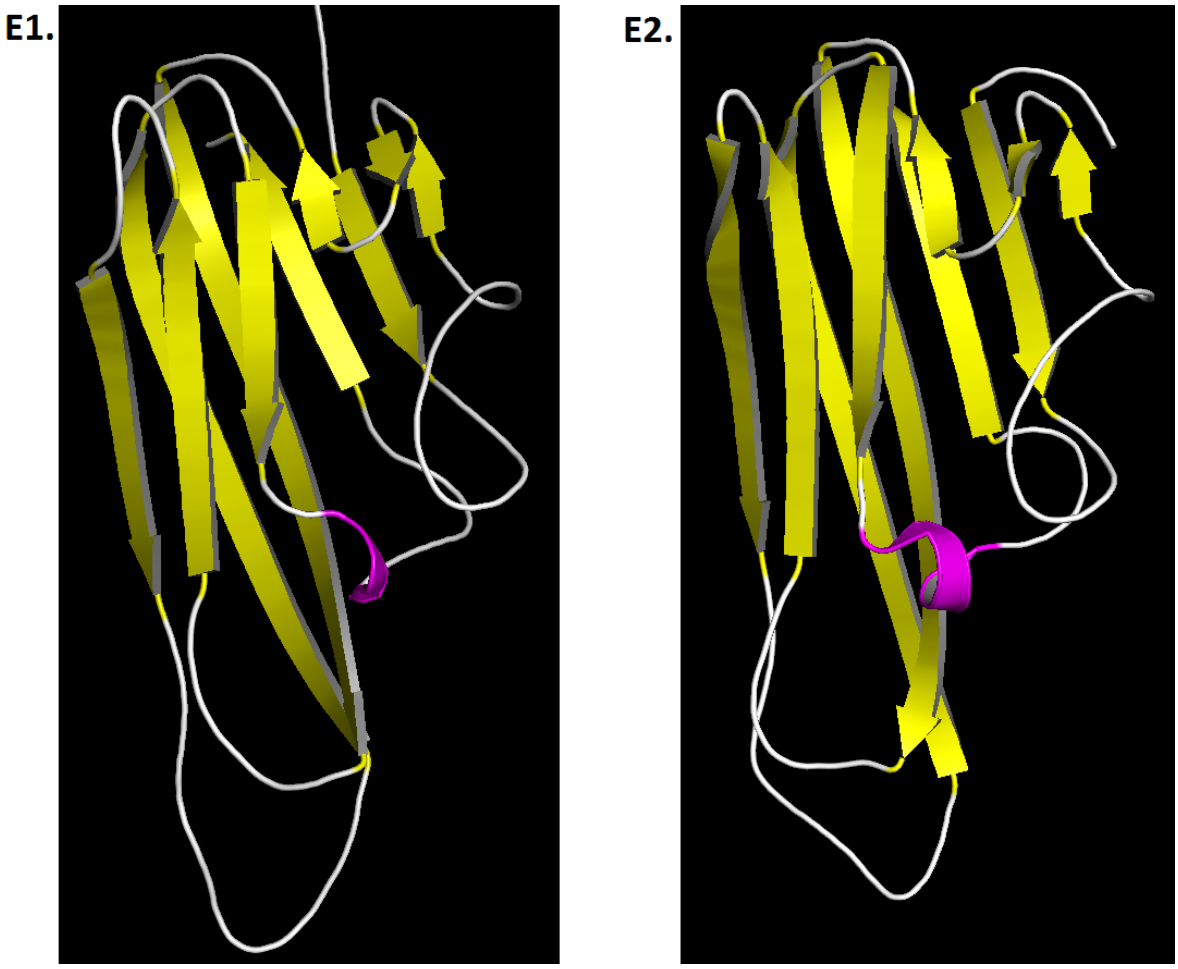


Figure 3.9: A set of diagrams to show the three dimensional structure predictions of the isoforms of TNF-α in common carp and the known structures of TNF-α in humans and mice. A1, B1, C1 and D1 show the predicted three dimensional structure of TNF-α 1, 2, 3 and 4 respectively, with the areas of high confidence and low confidence coloured red and blue. A2, B2, C2 and D2 show the predicted three dimensional structure of TNF-α 1, 2, 3 and 4 respectively, with α-helices coloured purple and β-strands coloured yellow. E1 and E2 show the known three dimensional structure of TNF-α from humans and mice respectively, with α-helices coloured purple and β-strands coloured yellow. The predicted structures were produced using PyMol version 1.7.4, using data produced from the Phyre2 server. The three dimensional structures of TNF-α in humans and mice were produced PyMol version 1.7.4, using the pdb file 1TNF and 2TNF, obtained from www.rcsb.org. The structure of TNF-α in humans and mice was originally determined by Eck and Sprang (1989) and Baeyens *et al* (1999).

Chapter 4. Conclusion

The alignment of the isoforms of TNF-α showed that all four isoforms were very similar. There were a total of 149 conserved residues, 34 residues had a conservative substitution and 18 had a semi conservative substitution. The sequence similarity between the isoforms was also very high with TNF-α2, TNF-α3, and TNF-α4 having an 81%, 72%, and 92% similarity with TNF-α1 respectively. Based on the high amount of conservation and the high sequence similarity it can be confidently predicted that the structure and function of all four isoforms would be very similar, if not identical in some areas. The isoform TNF-α4 had a significantly different terminal sequence, starting at G-234 and ending L-255. The remaining three isoforms all have conserved residues in this area. Although the difference is significant, the majority of the protein sequence of TNF-α4 is still conserved with the remaining isoforms. The isoform TNF-α4 also had the highest sequence similarity at 92%. This would suggest that TNF-α4 would still retain a very similar structure and function to the other isoforms of TNF-α4. It may be that the extended sequence in TNF-α4 is an extension of the C-terminal end and does not affect the structure and function of the isoform in a great way. This difference is also pronounced in the other alignments of TNF-α of common carp and TNF-α from other species. While the exact function of TNF-α in common carp isn't yet known studies have shown that isoforms do play a role in immunity. The isoforms TNF-α1 and TNF-α2 have been shown to promote the expression of several proinflammatory genes and adhesion molecules in endothelial cells. However, TNF-α2 was found to be more potent in promoting expression than TNF-α1 (Forlenza *et al*. 2009). This would suggest that while the isoforms may have the same function they could be expressed differently.

The alignment of TNF-α from humans and mice with the isoforms of TNF-α from common carp had far less conserved residues than what was seen in the previous alignment. However, areas of conserved residues and conservative substitutions seem to cluster where there were

β-strands in the protein sequence of TNF-α in humans and mice. For example, the β-strands situated between the residues G-130 – G-144 and K-188 – L-202, in human TNF-α, both had the largest combined amount of conserved residues and conservative substitutions. This clustering is also seen in the alignment of TNF-α from fish and mammals. Although it is much more clearly seen in TNF-α from mammals, many conserved residues can be seen to form groups where β-strands are located in TNF-α of humans, in TNF-α from fish. Based on this it can be suggested that the β-strands in TNF-α of common carp and other species of fish are in very similar locations to what is seen in TNF-α of humans. However, this is a different case for the α-helix that is found in TNF-α of humans and mice. Unlike the β-strands where clusters of conserved residues and conservative substitution could be seen in the corresponding locations, no conservation could be seen in the case of the α-helix. In the alignment of TNF-α from common carp with TNF-α from humans and mice there were no conserved residues or any conservative substitutions. In the alignment of TNF-α from the different species of fish and mammals, P-215 was conserved in the majority of mammalian TNF-α. However only Japanese seabass, Striped Trumpeter, Croceine croaker, European seabass and Striped beakfish had the same residue. The remaining species of fish, TNF-α from common carp included, did not have any conservation. This would suggest that there are not any α-helices in TNF-α of fish or that it is located in a position different to mammals.

Some of the residues which had been conserved or were conservative substitutions had been found to play a role within human TNF-α. In the alignment of TNF-α from humans and mice several of the residues such as L-133, S-136, Q-137, Y-195, L-196, G-197 and G-198 were found to affect the formation of the trimer in human TNF-α when an inhibitor was bound to them (He *et al.,* 2005). Since all of these residues were either conserved or conservative substitutions, it would suggest that they play a similar role in the isoforms of TNF-α in common carp. This would further suggest that TNF-α in common carp is able to form a

trimer. In human TNF-α, the residue C-177 and C-145 are able to form a disulphide bridge which is involved in intersubunit interactions about the trimer (Eck and Sprang, 1989). The alignment showed that C-177 is conserved in all the isoforms of TNF-α in common carp. C-145 is not conserved in any of the isoforms, however the adjacent residue C-145 in TNF-α1 is conserved amongst the other isoforms. A disulphide bridge could still be formed between these two cysteine residues so this may indicate TNF-α in common carp having the ability to form a trimer since the corresponding disulphide bond in human TNF-α is involved in trimer interactions. C-177 in human TNF-α was also found to be conserved in the alignment involving TNF-α from different species of fish and mammals. This shows that the residue is evolutionarily conserved so may have a significant role. The residue C-145 in TNF-α1 is also conserved among all the other species of fish used in the alignment. The three dimensional structure of TNF-α from common carp was predicted by Forlenza *et al*. (2009) using TNF-α from mice as a base protein sequence. The resulting prediction showed a tertiary structure consisting of three subunits and forming a trimer. Although this is only a prediction it still suggests that TNF-α of common carp is able to form a trimer. The investigation by He *et al.* (2005) also showed that TNF-α in humans was not able to function as a dimer. As TNF-α in common carp is homologous with TNF-α in humans and has a number of conserved residues which inactivate the cytokine when mutated, this suggests that TNF-α in common carp may not function properly as a dimer so a trimer formation is more likely.

Some conserved residues were shown to affect binding of TNF-α to its receptors. The residues R-108 and Q-225 in human TNF-α form interactions with TNFR2. Both of these residues have conservative substitutions in TNF-α of common carp, suggesting a similar function in that cytokine. This may therefore be the location or part of the locations of the binding site for TNFR2 of TNF-α in common carp. Several other conserved residues, Y-195,

Y-191, H-154, S-136, and W-104 in human TNF-α caused impairment of receptor binding when mutated (Zhang *et al*. 1992). This shows they may play some role in receptor binding, hence also playing a similar role in TNF-α of common carp. It could be suggested that the region of TNF-α in common carp is the location of a functional site.

The prediction of the secondary structure of TNF-α showed that the location of the β-strands would be very similar, and in some cases identical, to what is seen in TNF-α of humans and mice. No α-helices had been predicted in any of the isoforms aside from in TNF-α4, which had been predicted to contain an α-helix between F-231 and T-241. Based on the prediction of the known structures of TNF-α in humans and mice, the exact location and size of any secondary structural elements cannot be predicted but a general area and overall structure can be suggested. However, the secondary structure of TNF-α in common carp would be almost identical to that of TNF-α in humans and mice. This would also be supported by what was seen in the alignment of TNF-α from different species of mammals and fish, where conserved residues would cluster around the locations of the β-strands in human TNF-α. Also, the conservation of a number of residues which have been shown to specifically affect the trimer structure and binding in human TNF-α suggest the structure is similar as well. The notable exception in the prediction of the secondary structure is a large α-helix predicted to be in TNF-α4 towards the C-terminal end. However, previous alignments have shown that TNF-α4 still has a high sequence similarity with the other isoforms and has a number of conserved residues when aligned with TNF-α from humans and mice. The predicted α-helix may just be a part of the C-terminal extension or an inaccurate prediction.

Based on results obtained in this investigation, it can be deduced that TNF-α from common carp would at least share a very similar secondary structure to TNF-α in humans and mice. There does not seem to be an α-helix present in the structure, despite one being present in

mammalian TNF-α. It is also predicted that the structure can form a trimer much like TNF-α in humans due to the presence of conserved residues which affect formation of the trimer. The next step in further work would be to perform structural studies on the isoforms of TNF-α in common carp. This would be done to determine the structure of TNF-α in common carp and see how this structure does compare to the known structures of TNF-α in humans and mice. This could also answer several questions regarding the cytokine such as what structural differences would each of isoforms have in regards to each other. The structure could also be used as a basis for further research into the function of TNF-α in fish and whether the isoforms have a function specific to them.

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