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Ligand Binding Properties of the Siderocalins NGAL and
Q83

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1. ABBREVIATIONS

AA	Arachidonic acid
AS	Ammonium sulphate
acac	Acetylacetonate
Amp	Ampicillin
Cat	Catechol
Cmp	Chloramphenicol
CPMG	Carr, Purcell, Meiboom and Gill
CV	Column volumes
DAUDA	11-[5-(Dimethylamino)-1-naphtalenesulfonylamino]-undecanoic acid
DHBA	Dihydroxybenzoic acid
DHBS	Dihydroxybenzoyl-serine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
EDTA	Ethylene-diamine-tetra-acetic acid
Ent	Enterobactin
FABP	Fatty Acid Binding Protein
FID	Free Induction Decay
GdmCl	Guanidinium chloride
HSQC	Heteronuclear Single Quantum Coherence
INEPT	Insensitive Nuclei Enhanced by Polarization Transfer
IPTG	Isopropyl- β -D-thiogalactopyranosid
Kan	Kanomycin
K _D	Dissociation constant
kDa	Kilodalton

K _{sp}	Solubility product
LB	Lysogeny broth
MMP-9	Matrix Metalloproteinase-9
NaAc	Sodium acetate
NGAL	Neutrophil Gelatinase-Associated Lipocalin
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
OD ₆₀₀	absorbance at 600 nm (“optical density”)
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDB	RCSB Protein Data Bank
Pi	Phosphate
ppm	Parts per million
TRIS	Trishydroxymethylaminomethane

Amino acids are abbreviated in one- or three-letter code.

2. INTRODUCTION

2.1. Preview

In this thesis, the human protein NGAL and its quail homologue Q83 were investigated. Basically they are secreted transport proteins, however, associated with a wide variety of functions. NGAL and Q83 belong to the class of siderocalins, a subclass of lipocalins. This class is characterized by forming a β -barrel, capable of inclosing mainly hydrophobic ligands. The functions of NGAL and Q83 are directly connected to their ligand binding properties and therefore this is the main subject of discussion.

The introduction is structured as follows. First an overview of the class of lipocalins and siderocalins is presented. The importance of siderocalins in iron trafficking is outlined and in this context the most important ligand of NGAL and Q83 will be delineated. Then the two proteins are characterized more closely, including a description of the newfound secondary binding site of Q83. Subsequently the main methods exploited, biomolecular NMR spectroscopy and fluorescence quenching analysis are presented. The last paragraph of the introduction will give a brief overview of the work done in this thesis.

2.2. Lipocalins

Lipocalins are a class of secreted proteins that exist in a wide range of organisms, namely vertebrates, invertebrates, plants and gram negative bacteria. They are essential in various physiological processes. The two basic characteristics defining the class of lipocalins are already evident by the name derived from the Greek “lipos” (fat) and “kályks” (calyx). The latter term refers to the structural similarity among the group. All lipocalins build a calyx of an eight-stranded antiparallel β -barrel with a highly hydrophobic interior. Enclosed in this barrel is an internal binding site for small hydrophobic ligands, which directly leads to their second

commonality, related to their function. Lipocalins are able to bind and transport small hydrophobic molecules such as lipids, steroids, retinoids and bilins (3).

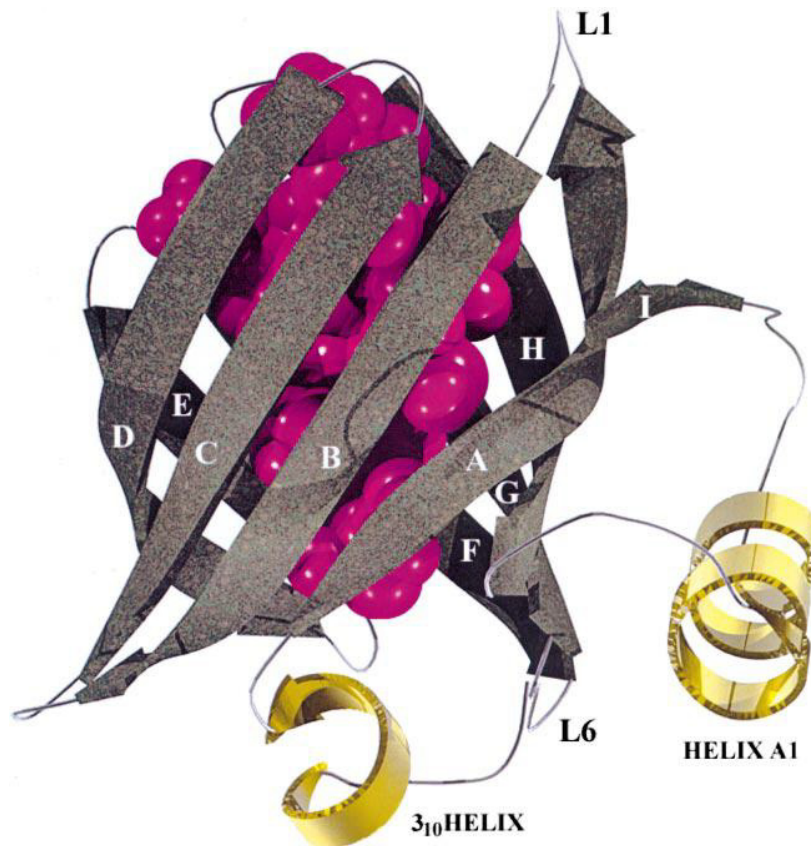


Fig. 1. Prototypical structure of a lipocalin. The collection of purple balls represents an overlay of a variety of different ligands. The usually narrower bottom of the calyx might be closed by a 3₁₀-helix at the N-terminus. The C-terminus is comprised by an α -helix, followed by a short B-strand (I). The eight anti-parallel B-strands that form the barrel are named A - H. Figure from Flower et al. (1).

In many cases the ligands are released again after binding to specific cell surface receptors. So lipocalins are important in ligand trafficking, transporting various molecules to their site of action. As there is such a wide range of ligands involved, lipocalins play essential roles in diverse processes like prostaglandin synthesis, pheromone transport, olfaction and retinoid binding (2). Their function certainly is intimately connected to their ligand binding properties. However, some special lipocalins were found to be associated with fundamentally different biological processes, for instance mediation of apoptosis (22), which does not easily fit into the picture of simple transport proteins. Considering these seemingly unrelated processes it can be guessed already that lipocalins' functions might be more

sophisticated than could be expected from a shallow view. In addition to ligand binding, some lipocalins have secondary functions, either as enzymes, e.g. prostaglandin D synthase (34) or by formation of protein-protein complexes, such as the later described NGAL-MMP-9 complex (11). Some mechanisms still remain unexplained to a great extent.

The structural similarities in lipocalins occur despite the surprisingly low level of sequence homology across the family. Fig. 2 shows an unwound view of the lipocalin fold. The main structurally conserved regions are marked as boxes. Below are shown the amino acid sequences of some lipocalins corresponding to those motifs, indicating the low sequence homology. Only a few residues, marked in pink, occupy fixed positions with respect to the fold.

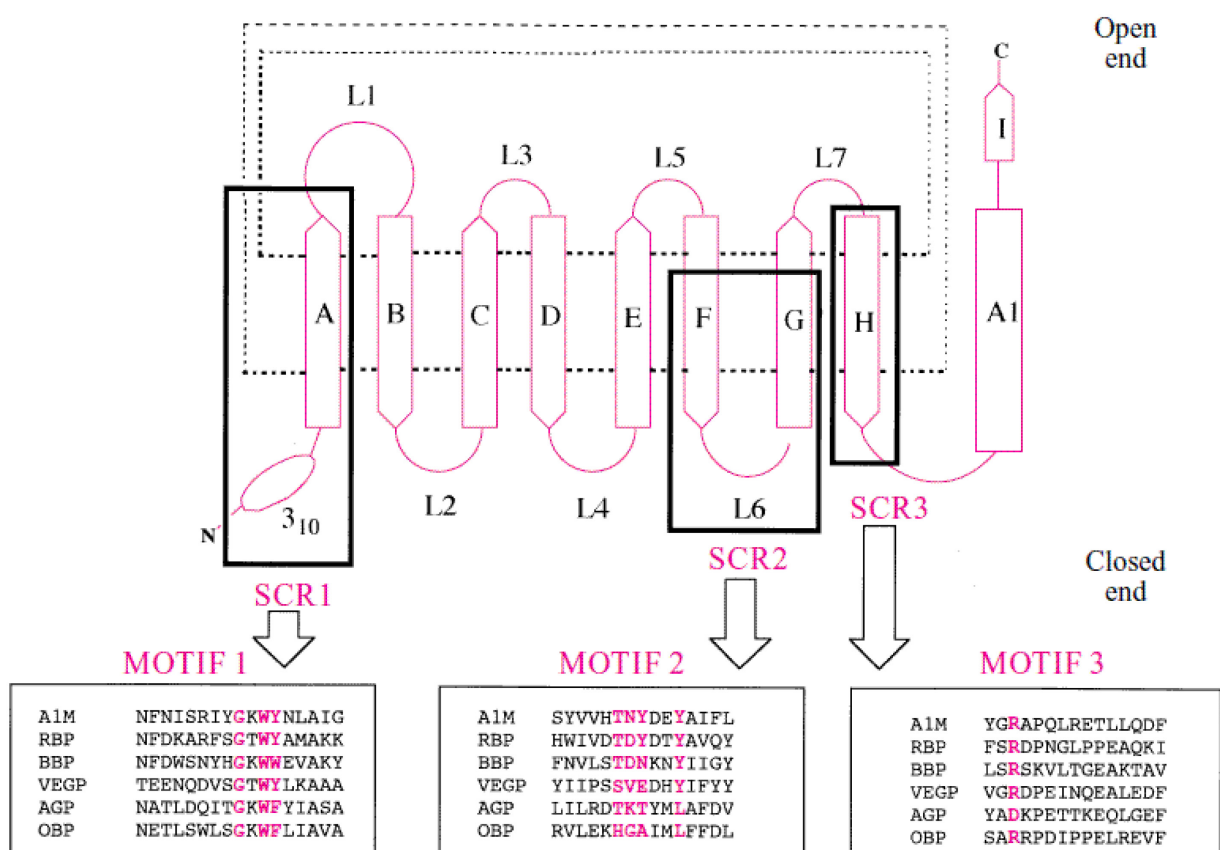


Fig. 2. Unwound view of the lipocalin fold. The pair of dotted lines indicates hydrogen-bond connection. The opening of the ligand-binding site is at the top side. This end has four β -hairpin loops, in contrast to the closed end, that contains only three. Figure from Flower et al. (3).

2.3. Siderocalins

Siderocalins are those proteins among the lipocalin family that are able to bind siderophores inside their calyx. Siderophores in turn are chelating molecules secreted by microorganisms, which are able to bind iron with extremely high affinity. The physiological relevance of siderocalins in this context is quite obvious. During infection Bacteria excrete siderophores in order to “steal” iron from the host. Siderocalins produced by the body in response therefore prevent iron from being delivered to the bacteria by capturing the iron-bound siderophores. Indeed, it has been shown that 24p3¹-deficient mice are more sensitive to bacterial infections (27).

Before dealing with other processes siderocalins might be associated with, this process of sequestering iron will be covered in more detail. The crucial role iron plays in organisms makes this function a key host defense mechanism against bacterial infection (37).

2.4. Biological Relevance of Iron

Iron is needed in organisms in relatively large amounts. The reasons are the numerous enzymes and proteins containing iron as an essential cofactor.

An adult human of 70 kg holds about 5 g of iron, which is about 1 mM for body volume. A bacterial cell in comparison requires an internal cell concentration of approximately 1 μ M (39).

Although iron is abundant in earth’s crust, its availability in aqueous environment is limited by the extreme insolubility of ferric hydroxide ($K_{sp} \approx 10^{-38}$) (38). So at pH 7 the maximum concentration of the free aqueous ion $[Fe^{3+}]$ is about 10^{-18} M (23). However, even lower concentrations of free iron are toxic to the human body. So there have to be mechanisms in order to tightly regulate its free concentration and transport. This is achieved through a variety of iron-binding proteins including transferrin and ferritin². The serum iron transport protein, transferrin, is known to maintain the ferric ion concentration at 10^{-24} M (15). Hence pathogenic bacteria

¹ 24p3 is a murine siderocalin and the homologue of NGAL.

must compete against this thermodynamic limit in order to obtain iron from the serum or tissue of its human host. As a limiting nutrient, iron is extremely important in microbial growth. That's why bacteria have consequently evolved aggressive iron acquisition processes like the above stated siderophores, selective iron chelators, produced and secreted specifically in response to iron deficiency. In the following paragraph a closer look into the structure and binding mechanism of a well understood siderophore, enterobactin, will be provided.

2.5. Enterobactin

Enterobactin, also named enterochelin in some sources, is a derivative of a cyclic tri-serine lactone and tri-catechol. Catechol in turn is the ortho isomer of benzenediol.

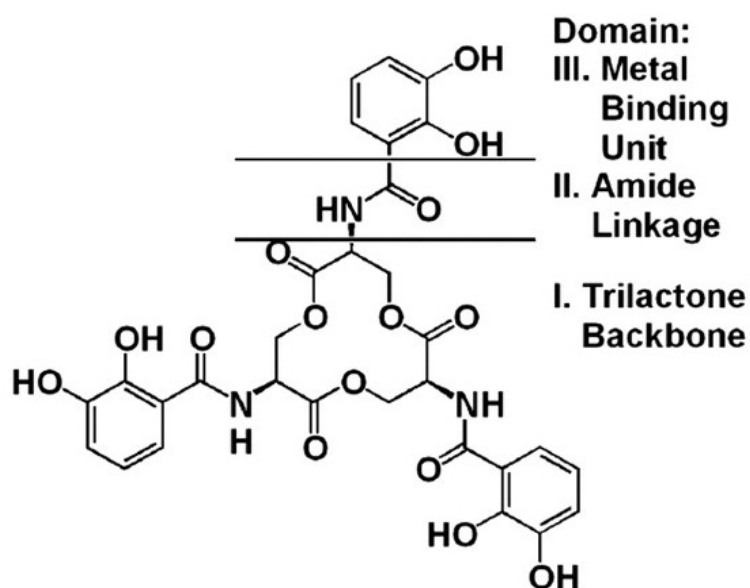


Fig. 3. Structure of enterobactin. The metal binding units are the catechols, that are linked to the trilactone by amide groups. Figure from Raymond et al. (15).

Enterobactin binds Fe^{III} with an extremely high affinity (23) to form the complex $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$. Enterobactin is a hexadentate ligand, which means it is chelated to the central ferric ion at six positions, namely through the oxygen atoms of the three catecholates.

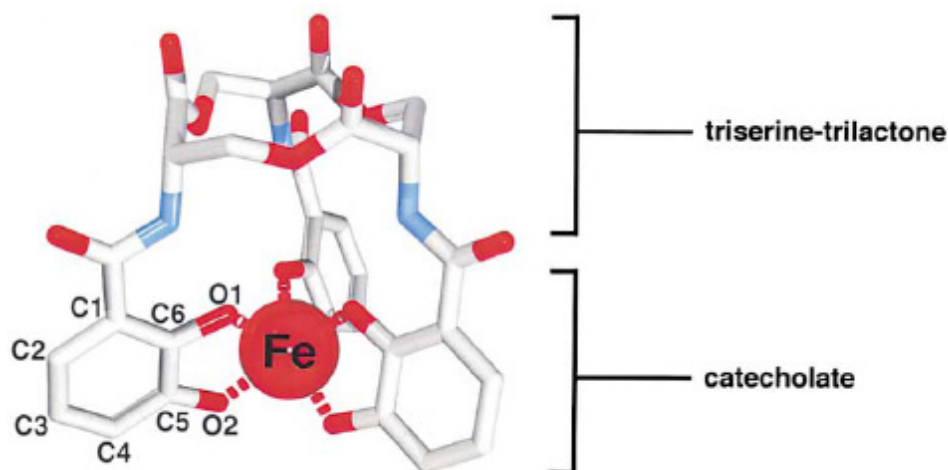


Fig. 4. Ribbon structure of enterobactin in complex with iron. The metal is bound by six deprotonated hydroxyls. Figure from Goetz et al. (6).

The Fe-free enterobactin at physiological pH exists in two forms in quite equal fractions. In one configuration the ortho-oxygen of the catechol is protonated and forms a hydrogen bond to the amide oxygen. Therefore the oxygen atoms of the catechol are oriented in the same direction as the amide oxygen. Upon deprotonation, the catecholate ring rotates around the amide bond and the ortho-oxygen proton gets hydrogen-bonded to the amide proton (15).

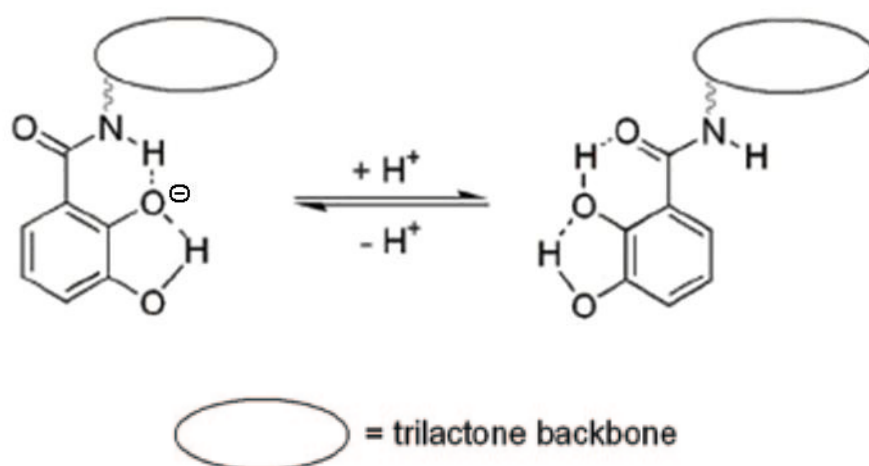


Fig. 5. Two existing configurations of Fe-free enterobactin. Upon protonation the catechol groups are rotating by 180 ° towards the outside of the molecule. Figure adapted from Abergel et al. (7).

The same transition coming along with deprotonation is happening upon coordination of Fe^{III} . This configuration is named catecholate binding mode and is found at neutral pH. However at lower pH the conformation can change to the so called salicylate binding mode (7).

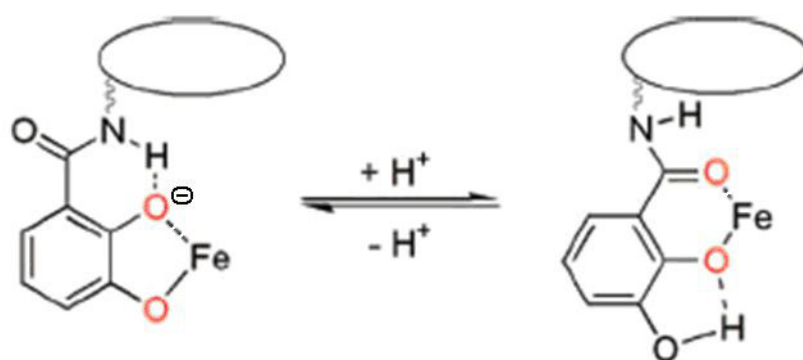


Fig. 6. Catecholate and salicylate binding mode of enterobactin with iron. The catecholate configuration, shown left, can change to salicylate mode at low pH. Figure adapted from Abergel et al. (7).

The salicylate form $[\text{Fe}^{\text{III}}(\text{EntH}_3)]^0$, as will be discussed later, is predicted to be sterically incompatible with binding to the siderocalin, because of clashes with the walls of the inflexible calyx. This appears to be important for iron release in acidic endosomes, which will be discussed in chapter 2.6.1.

The whole process upon binding of Fe^{III} is nicely seen in the 3D model of enterobactin shown in Fig. 7. In the free molecule on the left, the catechol groups are locked into one rigid conformation by hydrogen bonds. Triggered by metal complexation, accompanied by deprotonation, the oxygens are turned towards the inside of the molecule by rotating the catechol rings around the amide bonds. This dynamic conformation seems optimal for enterobactin in order to perform its function as a siderophore. The “open” conformation favors rapid initial binding of iron and hence is suitable for prospecting the metal, whereas the conformation change to the “closed” geometry forms a stable complex after sequestering the metal (15).

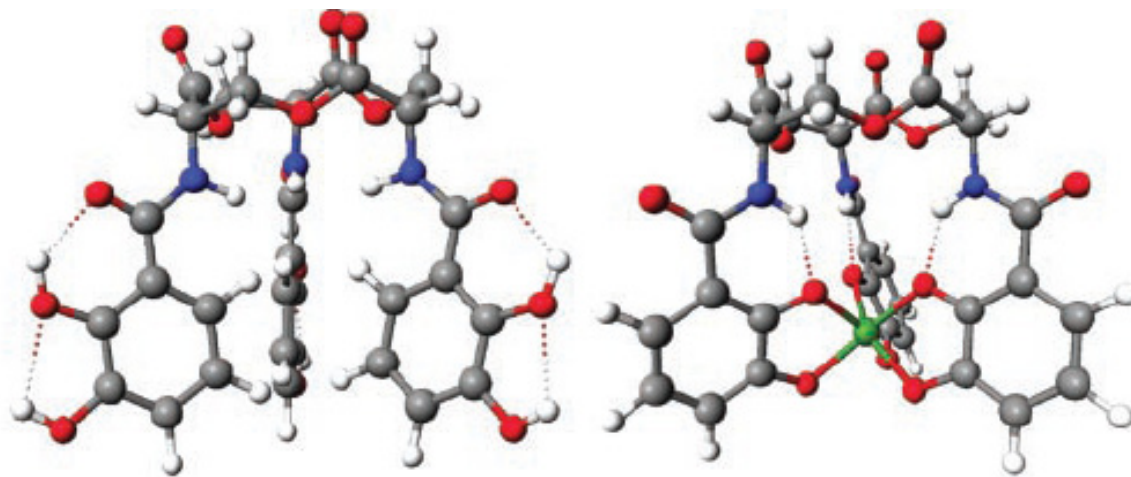


Fig. 7. Ribbon structure of free and Fe-bound enterobactin. The structure on the left can be regarded as an iron prospector. The closed configuration on the right forms a compact complex. Figure from Raymond et al. (15).

2.6. NGAL

NGAL (neutrophil gelatinase-associated lipocalin, also called 24p3, siderocalin or lipocalin2) is a human siderocalin. It is a 178 amino acids protein of 21 kDa, found in specific granules, secretory vesicles of blood granulocytes (47). NGAL forms a protein-protein complex with the proenzyme form of matrix metalloproteinase-9 (pro-MMP-9, or progelatinase B) via an intermolecular disulphide bridge (6). The solution structure of NGAL (20) and its crystal structure (35) reveal the typical eight-stranded antiparallel β -barrel with a “funnel-like” shape, made up by two almost orthogonal β -sheets (Fig. 8). The access to the calyx, the binding site within the barrel cavity, is provided by the one open end of the barrel. The opposite site, where the barrel gets narrower, seems to be closed by a short 3_{10} -helix. Also at this end of the barrel lies the free cysteine residue required for association with pro-MMP-9.

After activation, MMP-9 acts as a protease, degrading components of the extracellular matrix, such as collagens and gelatins (20). In this context NGAL has a regulatory influence on the proteolytic properties of MMP-9 (36). Matrix metalloproteinases are associated to destructive tissue remodeling, e. g. in invasive processes or metastasis of malignant cells (48). NGAL might play a role in

regulating these processes, as up-regulation of NGAL mRNA has been reported in a number of cancer tissues (49).

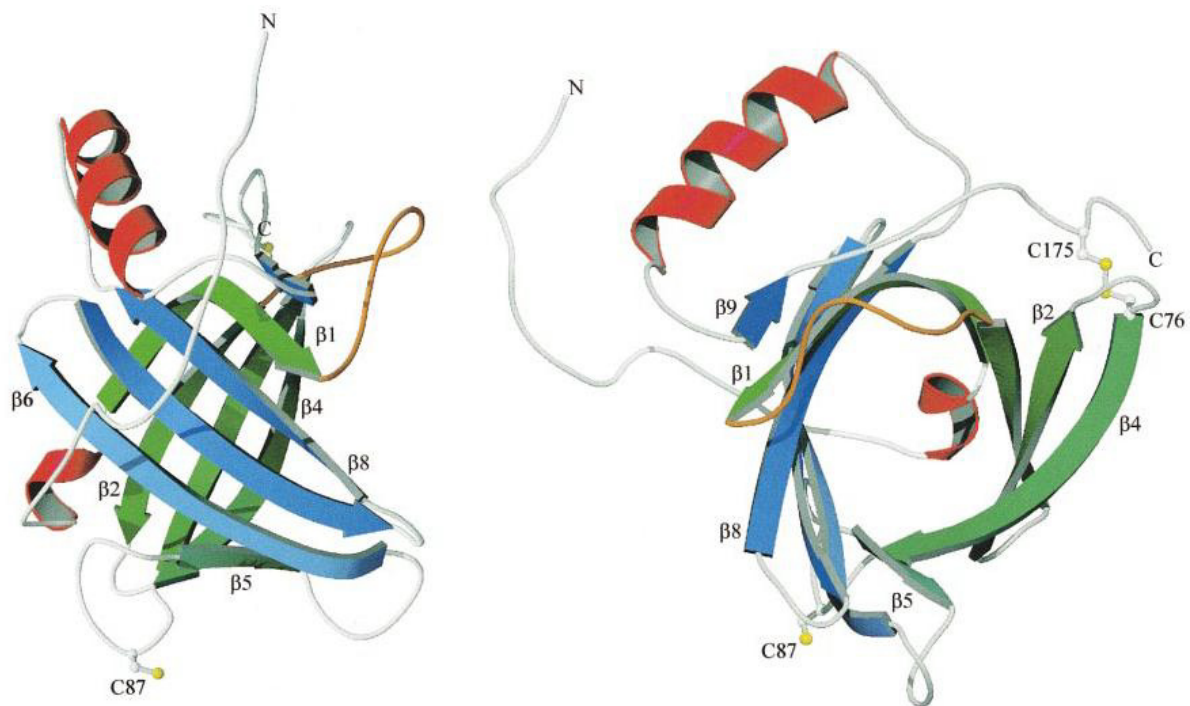


Fig. 8. NMR-derived solution structure of NGAL. The left picture reveals the typical lipocalin fold. The right view looks into the open end of the barrel. Cysteine side chains are shown by ball-and-stick representations, indicating the disulphide bridge between C₁₇₅ and C₇₆ and the unpaired C₈₇ for association with MMP-9. Figure from Coles et al. (20).

So far two distinct physiological functions of NGAL have been discussed. On the one hand its role in innate immune response, sequestering bacterial siderophores, and then its regulatory influence on MMP-9. However, NGAL is associated with even more versatile processes. For instance it is supposed to function as growth factor in multiple cell types, acting as an iron donor for the cytoplasm (24). Iron uptake into endosomes might happen after binding of NGAL to a specific membrane receptor, such as reported for megalin (21). Another cell surface receptor that promotes endocytosis of NGAL, named 24p3R, has been found (9) and others are likely to exist. NGAL may thus be an alternative to transferrin in iron trafficking. At least it seems evident that there are different iron transport systems, as for instance deletion of the transferrin pathway does not block organogenesis (10). It might be that the importance of NGAL varies between diverse developmental stages. Especially the iron delivery pathway via the NGAL receptor 24p3R is supposed to be

primarily active during cell differentiation and organogenesis. NGAL was shown to induce formation of kidney epithelia from metanephric mesenchymal cells (41). NGAL mediated iron trafficking via the 24p3R seems to be an important complement to iron delivery by ferritin during development. The role of 24p3R in the adult human, where highest expression levels are found in the brain, remains elusive. Several findings propose importance of NGAL and its receptor in apoptosis by delivering or withdrawing iron from the cell (9). Self-evidently, these attributes are dependent on the presence or absence of a ferric siderophore bound to the calyx. Recent studies provide indirect evidence for the existence of mammalian siderophores mediating iron delivery (4, 40). Although several compounds synthesized by mammals, such as catechol or dihydroxybenzoic acid, revealed effective binding to NGAL (5), exact physiological processes remain elusive. A putative endogenous ligand is likely to show close structural similarities to enterobactin and therefore many studies were using this effectively binding molecule as a surrogate (7).

2.6.1. Enterobactin Binding Site - The Calyx

NGAL binds enterobactin at physiological conditions with a dissociation constant of 0.4 nM (7). Furthermore it binds other catecholate-type molecules like parabactin, bacillibactin or 2,3-dihydrobenzoic acid (17, 18). The crystal structure of the enterobactin-bound NGAL ($\text{NGAL}/[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$) shows that the protein forms three sub-pockets in the calyx where each is occupied by one catecholate ring of the ligand (6). However, ferric enterobactin is not highly stable and therefore a mixture of its decay products dihydrobenzoyl-serine and dihydrobenzoic acid appears in the crystal structure of the co-purified protein-ligand-complex.

The calyx appears flatter and broader than is typical for most lipocalins. Moreover it is comparatively hydrophilic, containing several positively charged residues (35). Particularly the side chains of R₈₁, K₁₂₅ and K₁₃₄ are placed between the catechol rings. The interacting force seems to be a mixture of electrostatic and cation- π interactions. Then there is a hydrogen bond formed between Y₁₀₆ and the meta-oxygen of one catecholate ring and a herringbone-type π - π -stacking interaction between catechols and W₇₉ (7).

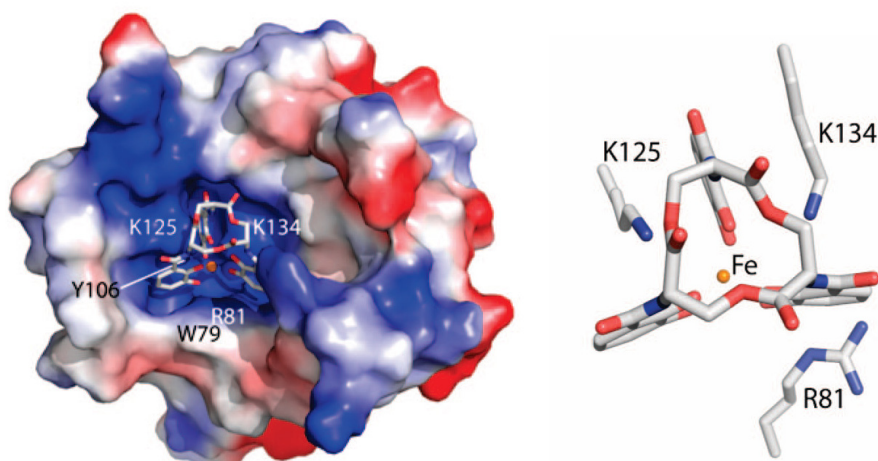


Fig. 9. The calyx of NGAL with bound $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$. The graphic to the left shows electrostatic potential by blue (positive) and red (negative) coloring, indicating the very positively charged binding site, unusual for lipocalins. Residues crucial for binding are highlighted in stick view. The aromatic rings of the ligand placed between the positively charged side chains are visualized in the right picture. Figure from Abergel et al. (7).

The calyx seems to be quite rigid as there are no major conformational changes upon binding. Only side chains of W₇₉ and R₈₁ show significant variation in response to ligand binding (6).

Iron release from NGAL occurs in acidic endosomes (10) and therefore the effects of pH variation on the protein might be interesting. The structure of NGAL was shown to be stable over the range of pH 7.4 to 2.0 (6, 35). However, as already stated in the part about enterobactin, in the low-pH salicylate form of this ligand the catechol rings are turned outwards (15). This conformation would lead to steric clashes with the walls of the inflexible calyx and hence does not bind. Fluorescence quenching analyses show that there is a dramatic decrease in affinity at pH 3 compared to pH 4, represented by a 300-fold increase in K_D . In contrast the change in K_D is very low between pH 7.4 and 4 (7). The pK_a values of $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$ are 5.2, 3.2 and 2.7, corresponding to the stepwise protonation of the meta-oxygens of the catechol rings, resulting in the neutral salicylate complex $[\text{Fe}^{\text{III}}(\text{EntH}_3)]^0$. Hence, the major decrease in affinity corresponds to the second protonation step of $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$. It should be noted, that the dramatic decrease in affinity is primarily caused by steric effects and not due to loss of ligand charge, although electrostatic forces definitely contribute to the general affinity. A similar effect can be observed upon addition of iron-free enterobactin, which in general

has a lower affinity than the ferric enterobactin but the same overall charge. At pH 7.4 the K_D for $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$ is 0.4 nM and for $[\text{H}_3(\text{Ent})]^{3-}$ 3.6 nM (7).

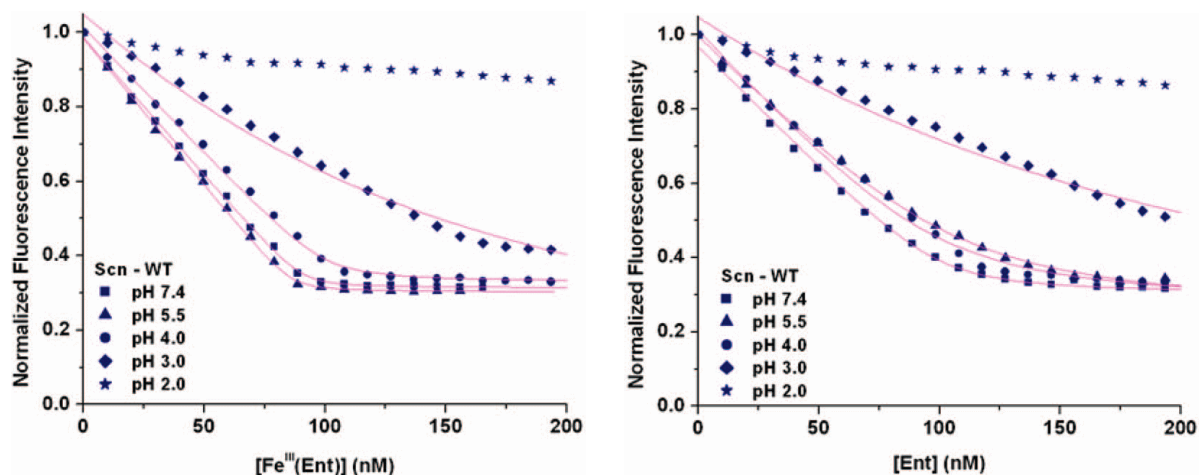


Fig. 10. NGAL titration with ferric- (left) and Fe-free- (right) enterobactin at different pH, monitored by fluorescence quenching analysis. Protein fluorescence at 340 nm is quenched upon ligand binding. The free enterobactin shows slightly less overall affinity but reveals the same drastic decrease from pH 4 to 3. Figure from Abergel et al. (7).

Another experiment shows the increase in fluorescence upon acidification of the enterobactin-saturated and therefore quenched $\text{NGAL}/[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$ complex. The data show that significant release of the ligand starts at pH 3.5 (7).

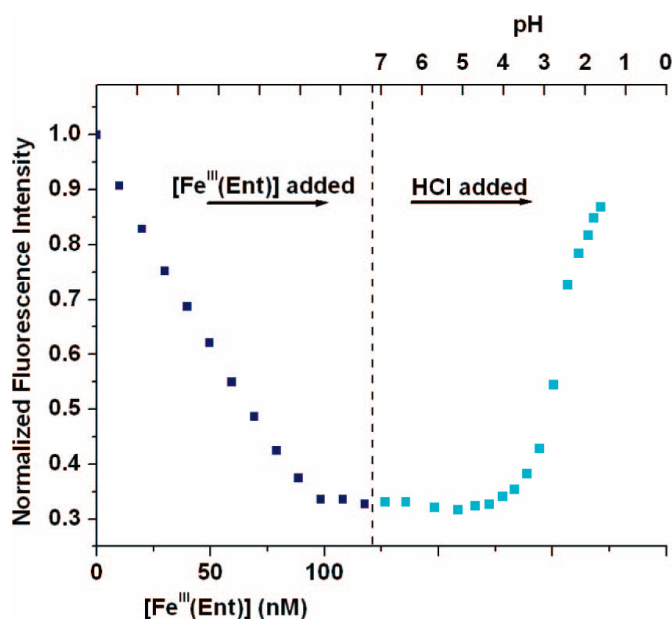


Fig. 11. Ligand release from $\text{NGAL}/[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$ upon HCl-titration monitored by fluorescence quenching.

Significant release of enterobactin starts below pH 4. Figure from Abergel et al. (7).

NGAL releases iron in acidic endosomes where the pH is around 5. At that pH the catecholate form of the Fe-Ent complex is still predominant but the slightly lower affinity at that pH might trigger enterobactin release, whereas the ligand is successively removed from chemical equilibrium. However, effective release of iron possibly requires preceding hydrolysis of the Ent-triserine lactone, as is found in the NGAL/FeDHBx crystal structure.

2.7. Q83

Q83 is the quail homologue of NGAL and shows 23% identity and 64% sequence similarity to the human protein (27). The solution structure (14) reveals the typical lipocalin fold, the β -barrel formed by eight antiparallel β -strands. Also the 3_{10} helix, flanking the β -barrel at the N-terminus, is present. An evident structural difference to NGAL is the existence of a 10-residue α -helix at the back of the calyx, where a large Ω -loop is located in the human homologue.

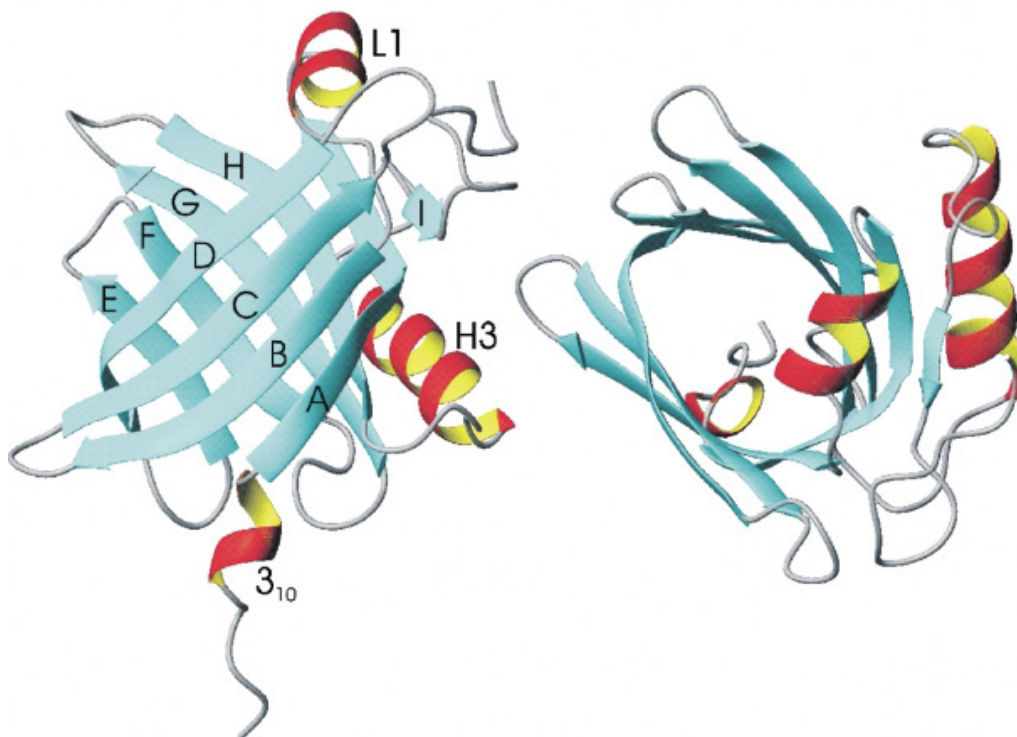


Fig. 12. Solution structure of Q83. Directly visible is the highly conserved lipocalin fold. In the loop region (L1) Q83 contains a short α -helix. The right view looks into the open end of the protein. Figure from Hartl et al. (14).

In line with its specific ligand enterobactin, Q83 just like NGAL features a wider calyx than typical for lipocalins. The conserved amino acids that form the basic triad of NGAL (Arg₈₁, Lys₁₂₅ and Lys₁₃₄), essential for siderophore binding, have their analogues in Lys₈₃, Arg₁₀₂ and Arg₁₁₃ in Q83. These positively charged residues, located at the binding site inside the calyx, can be seen as a characteristic structural feature for siderocalins. Indeed, Q83 binds enterobactin in a similar mode than NGAL and also with nearly identical dissociation constant of 0.54 nM (27). As the solution structure of the [Ga^{III}(Ent)]³⁻-bound form presents, each of the three catechols of enterobactin occupies one distinct binding pocket, just like shown for NGAL. The catechol ring that shows cation- π interaction with Lys₈₃ seems to be additionally stabilized by π - π stacking with Trp₆₂, equivalent to NGAL's Trp₇₉ (27).

So Q83 is a member of the siderocalin family, however, little is known about its physiological functions. Q83 was first identified after transformation with the *v-myc* oncogene in quail embryo fibroblasts. In these cell lines Q83 is highly overexpressed, as *v-myc* abrogates the suppression of the Q83 gene (14).

2.7.1. Fatty Acid Binding Site

Q83 has been shown to bind unsaturated fatty acids, like arachidonic acid, with nanomolar dissociation constant in a binding site distinct from the calyx (46).

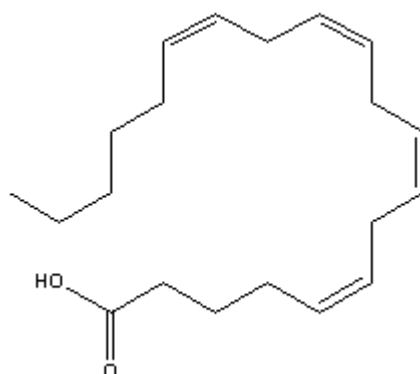


Fig. 13. Structure of arachidonic acid.

Fatty acids are binding towards the closed end of the calyx, thus both ligands, siderophore and fatty acid, can bind simultaneously. This was shown by methods of biomolecular NMR spectroscopy, which is a valuable tool not only for solving solution structures, but also for characterizing interaction sites, checking protein fold or monitoring conformational dynamics. An introduction to biomolecular NMR will be given in chapter 2.7.

2.7.2. Dual Binding Mode

Fig. 14 shows the HSQC spectra of Q83 in the free form and after addition of $[\text{Ga}^{\text{III}}(\text{Ent})]^{3-}$ and arachidonic acid. Gallium was used as a surrogate for iron in the enterobactin complex, as the paramagnetic properties of iron would interfere in NMR experiments. The significant change in the chemical shifts of several amino acids upon addition of the ligands can easily be observed. The binding site for arachidonic acid could be located by comparing the chemical shifts of the free and bound form. Definitely other resonances are shifting than for enterobactin, indicating different localization of binding. Furthermore spectrum D shows that both, AA and Ent, can bind simultaneously, as it differs from the two spectra B and C. The same spectrum for $\text{Q83}/[\text{Ga}^{\text{III}}(\text{Ent})]^{3-}/\text{AA}$ was obtained, independent of which ligand was added first. Additional evidence that the two binding sites are distinct is given by the fact that binding of Ent leads to quenching of intrinsic fluorescence, whereas addition of AA does not.

2.7.3. Solution Structure of the Q83/GaEnt/AA complex

The solution structure of the $\text{Q83}/[\text{Ga}^{\text{III}}(\text{Ent})]^{3-}/\text{AA}$ complex, which is shown in Fig. 15, provides further insight into the dual ligand binding mode of Q83. It confirms that the typical lipocalin fold is conserved and that AA binds at the closed end of the barrel in hydrophobic environment. AA is positioned in a U-shape, the polar head and the methyl end pointing towards the closed end of the calyx. The carboxylate group might build hydrogen bonds to Thr₉₀ and Tyr₉₂. Also Arg₆ is found to be located near the polar head of AA, however, its position is not well defined within the structural ensemble (46).

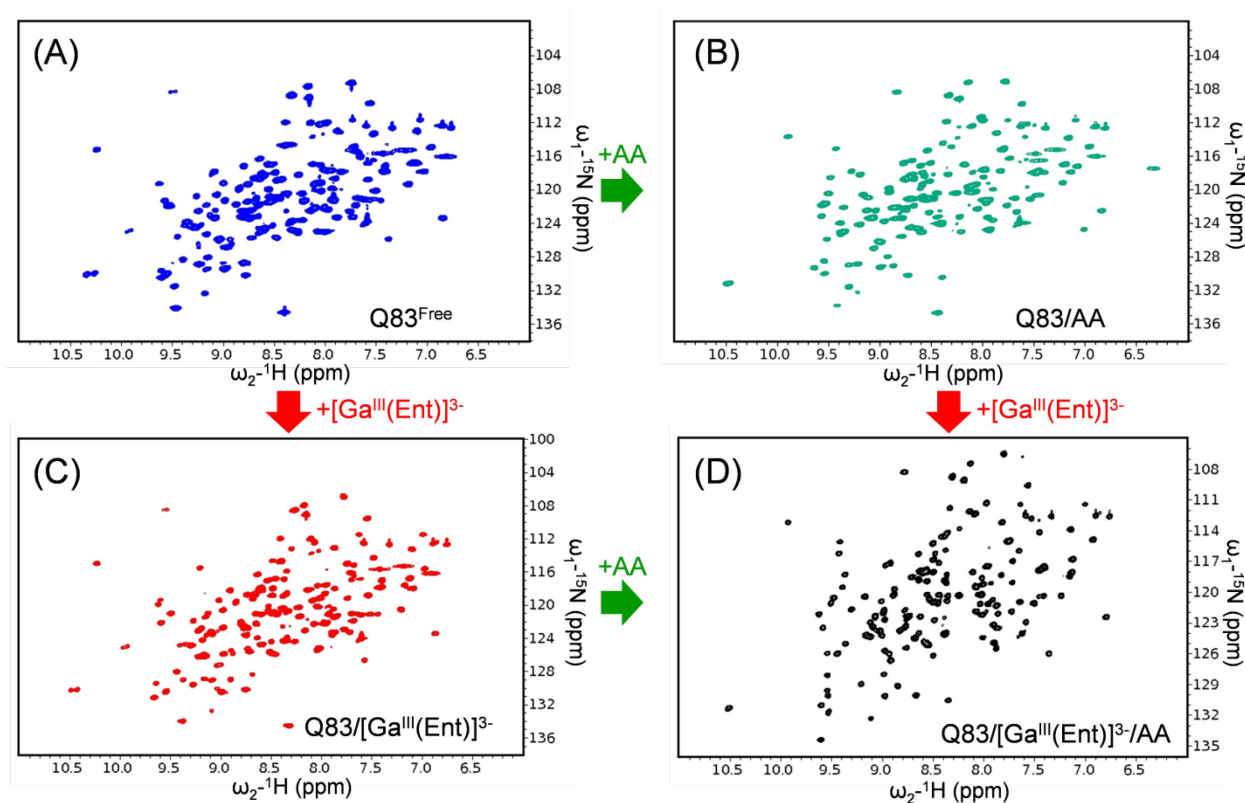


Fig. 14. HSQC spectra of Q83 before and after addition of either one or both ligands, $[\text{Ga}^{\text{III}}(\text{Ent})]^{3-}$ and AA. The spectra show that both compounds can bind simultaneously and at distinct binding sites. The same spectrum D is obtained regardless of which ligand is added first. Figure from Coudeville et al. (46).

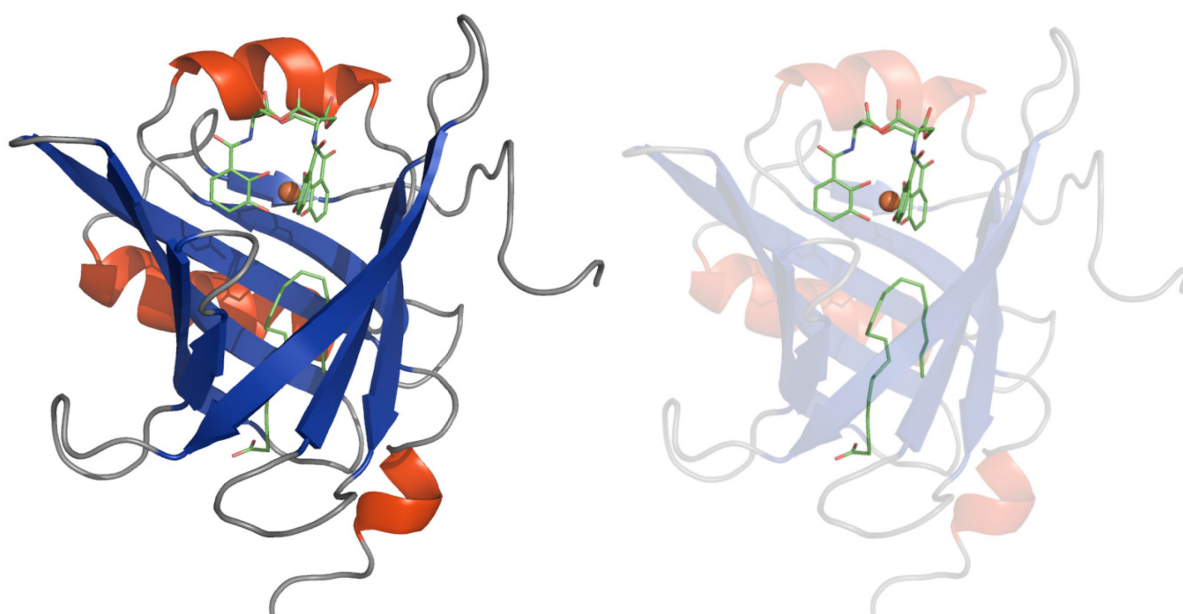


Fig. 15. Solution structure of the $\text{Q83}/[\text{Ga}^{\text{III}}(\text{Ent})]^{3-}/\text{AA}$ complex. Enterobactin is bound in its usual way. The fatty acid is located towards the narrower end of the barrel and might eventually enter the protein from this end. Figure from Coudeville et al. (46).

2.8. Objective

There are two main issues addressed. First, does NGAL share the binding properties of the newfound second binding site with its quail homologue? If so, this might play a key role in deciphering the diverse biological functions of the protein. AA for instance acts as a precursor in prostaglandin synthesis and consequently is involved in the regulation of inflammation, one of the unexplained functions associated with NGAL.

The second quest is to shed more light onto the molecular ligand binding mode of NGAL and Q83. This might on the one hand be helpful for defining the siderocalin protein family and on the other hand provide a molecular basis for explaining their pleiotropic functions.

Two methods emerged to be particularly favorable for examining these questions. Fluorescence quenching analysis can effectively be used to monitor binding and extract dissociation constants. For providing detailed insight into molecular processes, biomolecular NMR spectroscopy serves as a particularly valuable tool.

2.9. Biomolecular NMR

For investigation of macromolecules, 2D or 3D experiments are used. Here chemical shifts of two or three nuclei of different kind, usually ^1H , ^{15}N and ^{13}C , are tracked in one experiment. Therefore the protein has to be labeled with the NMR active isotopes of nitrogen or carbon during expression. The frequencies for every probed nucleus are plotted on an axis. So overlapping chemical shifts can be distinguished by correlating them with nuclear spins of neighboring atoms. Elementary for these experiments is the possibility of magnetization transfer between vicinal nuclei, which can be described by quantum mechanics. In most experiments magnetization transfer via chemical bonds is employed, but there are also methods that employ correlation of nuclei adjacent in space.

Of course only the evolution of one atom can be probed directly when recording the FID, however, in every experiment the pulse sequence is repeated many times. Each of these repetitions contains a different time interval during which the

magnetization is evolving on an element that is not directly observed. This evolution affects each FID recorded and therefore incrementing the time interval opens up a new time dimension for the other NMR active nucleus. Fourier transformation in this time domain gives the additional frequency dimension in the spectrum.

There is a wide variety of established NMR experiments that can be used to gain sophisticated structural and dynamical information.

2.9.1. ^1H - ^{15}N HSQC

A particularly often used experiment is the HSQC. The HSQC is a 2D experiment in which the correlation between ^1H and ^{15}N is exploited. The resulting spectrum is usually plotted with the offsets of the different ^{15}N nuclei in the ω_1 dimension and the offsets of the directly attached protons in the ω_2 dimension. It is normally represented in a contour plot, where points of equal intensity are joined by lines.

Each peak in such a spectrum corresponds to one amide group and therefore to one amino acid in the protein. In an unfolded protein, peaks might be poorly separated due to the absence of secondary structure. In a structured protein, where all amino acids have a defined environment, the spectral dispersion is larger.

The peaks have to be assigned via various triple resonance experiments, probing the spins of ^1H , ^{13}C and ^{15}N .

HSQC Pulse Sequence

The pulse sequence works by transferring the magnetization from the proton to the nitrogen by INEPT in the preparation phase. The INEPT enhances the signal for the ^{15}N nuclei as it transfers magnetization from the protons to the nitrogens. At a given magnetic field strength the equilibrium magnetization of protons is ten times higher than that of ^{15}N nuclei due to their higher gyromagnetic ratio.

The INEPT starts with a 90° x-pulse on ^1H , rotating the magnetization from z to -y. The following magnetization transfer is done in three periods, A, B and C,

indicated in Fig. 16. In period A, an anti-phase state on the ^1H spin is generated by a spin echo. The spin echo refocuses the offsets of the ^1H and ^{15}N spins but lets their coupling evolve for the whole time period. In period B coherence transfer is achieved by a 90° y-pulse on spin ^1H and a simultaneous 90° x-pulse on ^{15}N . The anti-phase magnetization evolves into in-phase by a second spin echo in period C, which now could be recorded on ^{15}N .

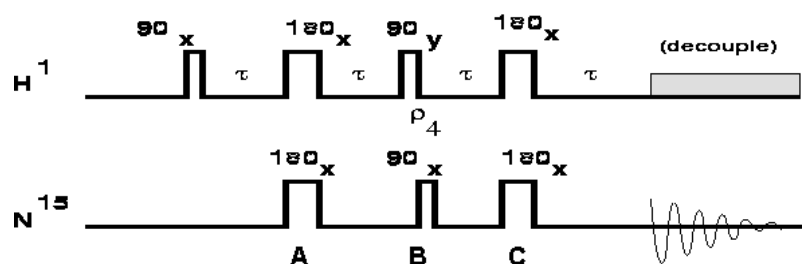


Fig. 16. INEPT pulse sequence. Transverse magnetization is generated on ^1H and transferred to ^{15}N .

In the HSQC the time interval for the second spin echo, t_1 , is incremented in the pulse train. The magnetization of the nitrogen spin evolves for the whole time period t_1 . The t_1 time corresponds to the ^{15}N chemical shifts in the final frequency spectrum.

At last the magnetization of ^{15}N is transferred back to the ^1H , where it is observed.

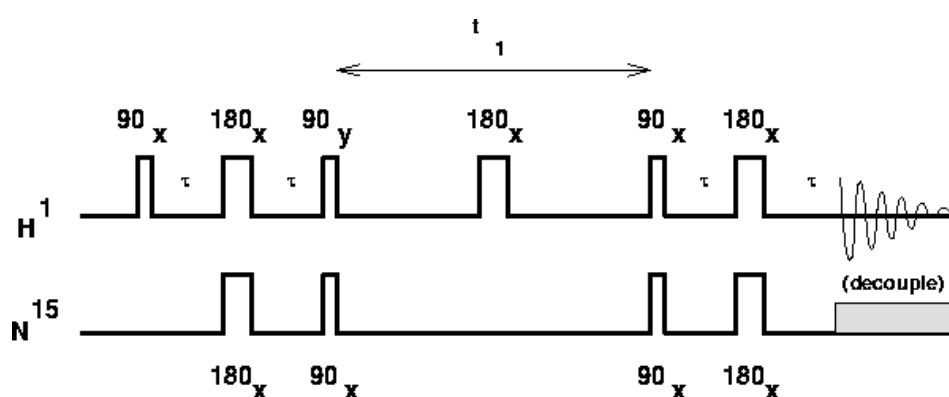


Fig. 17. HSQC pulse sequence. Magnetization is transferred from ^1H to ^{15}N and back again to ^1H , where it is observed. The time interval t_1 , during which magnetization is evolving on ^{15}N , is incremented in the pulse train, opening up the second frequency dimension.

2.9.2. Peak Assignment

The peaks in the ^1H - ^{15}N HSQC spectrum have to be assigned to the corresponding amino acid residues. There are several 3D NMR experiments to achieve that. Those described further down were used in this thesis. In the spectra of these experiments, the ^1H and ^{15}N chemical shifts for the different amino acids are visible, like in the ^1H - ^{15}N HSQC, but additionally there is a frequency dimension for ^{13}C that reveals chemical shifts for adjacent carbons.

HNCA

In this experiment magnetization is transferred from proton through nitrogen to the C_α and back again to the proton. So at the peak for a given backbone amide group in the ^1H - and ^{15}N -dimension, the spectrum shows the C_α chemical shift of the same amino acid. Although the pulse sequence is optimized to transfer magnetization to that ^{13}C , due to comparable coupling constants transfer also occurs to the C_α of the preceding residue. Therefore also the chemical shift of this nucleus is visible. The intensity of the peaks depends on the coupling constants between the nitrogen and the C_α . In α -helices the peak at the C_α chemical shift of the preceding residue is usually observed with lower intensity, whereas this is often not the case in β -sheets. Complemented with other triple resonance experiments, the HNCA can be consulted to track the amino acid network in the protein.

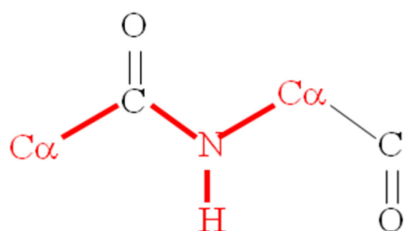


Fig. 18. HNCA magnetization transfer. The nuclei at which magnetization is located during the experiment are colored in red. The transfer is carried out via the bonds indicated by red lines.

HNCO

Here magnetization is transferred from the proton via the nitrogen to the carbonyl carbon of the preceding residue in the amino acid sequence. This experiment usually gives very intense peaks and therefore enables efficient allocation of carbonyl carbons to the peaks belonging to the amide group of the successor amino acid in the ^1H - ^{15}N spectrum.

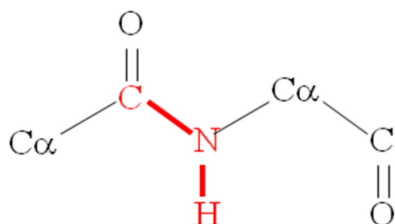


Fig. 19. HNCO magnetization transfer.

HN(CA)CO

In the HN(CA)CO magnetization is transferred from the proton via two nuclei, nitrogen and C_α , to the carbonyl carbon and back to the proton again. However, in the same experiment magnetization is also partly transferred to the carbonyl carbon of the preceding residue, via the C_α of that residue. Therefore the spectrum usually shows a peak for the carbonyl carbon at the ^1H and ^{15}N chemical shifts of the same amino acid and, at the same ^1H - ^{15}N shifts, a peak of the preceding carbonyl carbon. The peak intensities again depend on the coupling constants between the nuclei of magnetization transfer. Often it cannot be determined clearly which of the two carbonyl peaks corresponds to the actual or the preceding amino acid, respectively. However, in combination with the HNCO, the HN(CA)CO is an effective method to track the amino acid network, in addition to the HNCA.

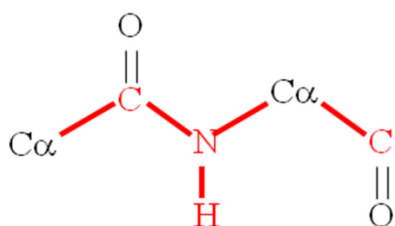


Fig. 20. HN(CA)CO magnetization transfer.

2.9.3. Protein Dynamics - CPMG

Biomolecular NMR spectroscopy provides tools to study dynamics in a wide time regime. Many events relevant for protein function occur in the range of microseconds to milliseconds, a time scale that can be investigated by the CPMG experiment.

In solution NMR the proteins are flexible to some extent and therefore not all molecules will be found in the same conformational state during measurement. States that are less populated or maintained for a shorter time range, respectively, are not distinctly visible in traditional experiments. If one considers a protein site to be in either one of two possible conformations A and B with an exchange rate, $k_{ex} = k_{AB} + k_{BA}$, then separate peaks are only observed if k_{ex} is much smaller than the chemical shift difference ($\Delta\omega$) between the states (slow exchange). The peak intensities are defined by the populations of the different conformations (p_A, p_B). If the exchange rate gets higher, the peaks are broadening and shifting towards each other. A k_{ex} similar to $\Delta\omega$ refers to intermediate exchange. The timescale of fast exchange is characterized by $k_{ex} \gg \Delta\omega$.

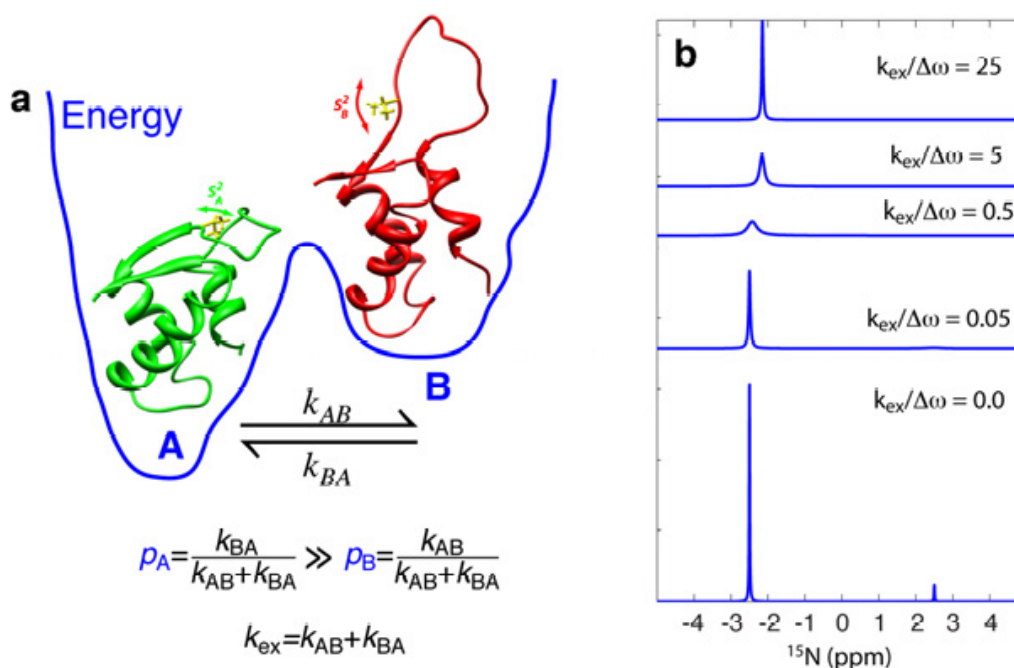


Fig. 21. Exchange between two differently populated states in a protein. (a) Graphical representation of ground and excited state. The populations of the two states and the exchange rate are determined by the rate constants. (b) NMR spectrum for different exchange rates. Figure from Vallurupalli et al. (52).

Peak broadening is a direct consequence of dephasing magnetization due to interconversions between the conformations. In the CPMG experiment a variable number of 180° or π pulses, so called spin echoes, are applied during a fixed time interval in order to refocus the signal. The higher the frequency of applied spin echoes, the narrower the signal gets. This frequency, ν_{CPMG} , is plotted against the effective transverse relaxation rate $R_{2,\text{eff}}$ to give the so called dispersion curve.

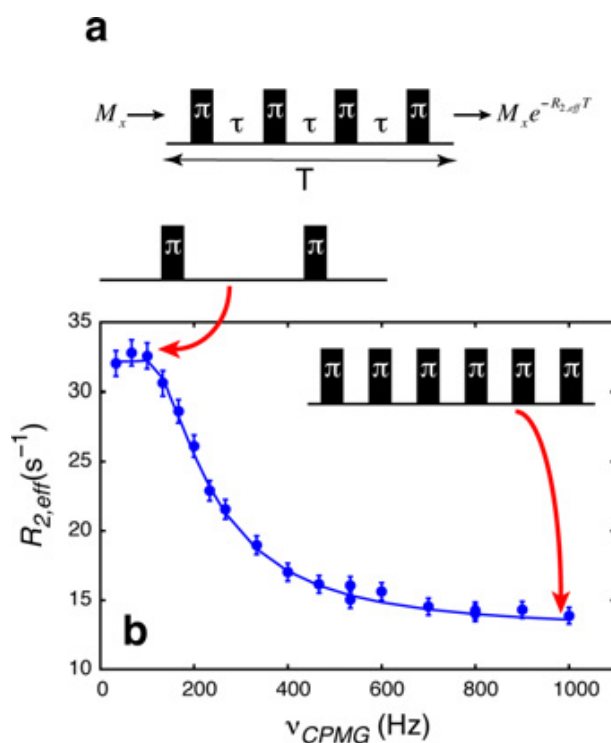


Fig. 22. (a) Schematic representation of the constant time delay in the CPMG experiment. A various number of refocusing pulses is applied during that time, which gives the CPMG frequency ν_{CPMG} . (b) CPMG dispersion curve. The decrease of $R_{2,\text{eff}}$ with ν_{CPMG} indicates chemical exchange. Figure from Vallurupalli et al. (52).

$R_{2,\text{eff}}$ is calculated from the intensity (I) of the peak of interest, the duration of the constant time delay (T) and the intensity of a reference spectrum (I_0) that lacks the constant time delay, $R_{2,\text{eff}} = -1/T \ln(I/I_0)$. Depending on the timescale of the exchange (fast, intermediate and slow) the curve can be fit with an appropriate function to extract the exchange parameters, p_A , p_B , k_{ex} and $\Delta\omega$. However, there are too many parameters to give a unique solution. p_A , p_B and k_{ex} are independent of the field strength, whereas $\Delta\omega$ is not. Hence, by measuring the CPMG at two different field strengths, ambiguity in dispersion curves can be resolved.

2.10. Fluorescence Quenching

Fluorescence quenching is a common approach to monitor binding of ligands to proteins. This method makes use of the intrinsic fluorescence of the amino acids phenylalanine, tyrosine and tryptophan, whereas the latter shows most intense fluorescence. Usually tryptophan is rather rare in proteins. NGAL and Q83 contain two residues of this kind.

2.10.1. Calyx Binding Site

As a tryptophan residue is located directly at the siderophore binding site of NGAL and Q83, binding of enterobactin in the calyx can be tracked by fluorescence quenching experiments. The tryptophans of the protein in its unbound form can be excited at 280 nm, leading to a peak in the emission spectrum with a maximum around 345 nm. When $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$ is added stepwise to the protein solution, whereas an emission spectrum is recorded after each step, fluorescence intensity decreases continually.

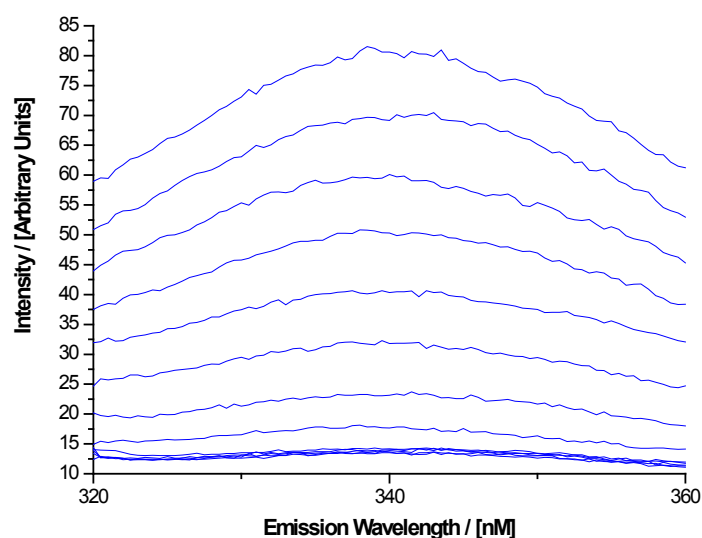


Fig. 23. Emission spectra of Q83 at different $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$ concentrations, excited at 280 nm. Lower intensity curves correspond to higher ligand concentrations. When saturation is reached, further addition of ligand does not decrease fluorescence significantly, indicated by the close by curves at the bottom.

Every intensity value at a given ligand concentration is obtained by integration of the signal in the spectral range and not just from a single wavelength. This reduces the error due to fluctuations of the spectrometer and peak drift as a result of ligand addition.

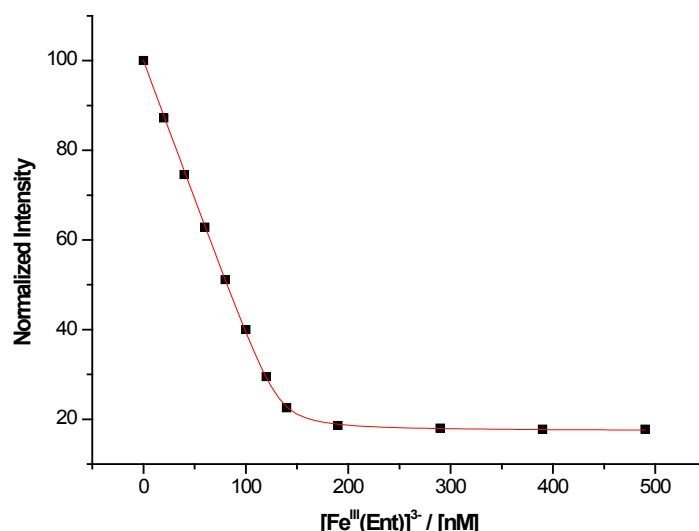


Fig. 24. The integrals of the measured intensities of each curve in Fig. 23 plotted against $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$ concentration. The red curve shows the fit with the equation shown below.

The plot of fluorescence intensity versus ligand concentration can be fit with the following equation, assuming a single binding site model. The fit reveals the dissociation constant of ligand and protein.

$$I = I_{\text{max}} - I_{\text{sat}} \frac{[P] + [L] + K_D \sqrt{([P] + [L] + K_D)^2 - 4[P][L]}}{2[P]}$$

I_{max} ... maximum intensity

I_{sat} ... maximum intensity minus intensity at saturating concentration of the ligand

$[P]$... protein concentration

$[L]$... ligand concentration

K_D ... dissociation constant

2.10.2. Fatty Acid Binding Site

For the fatty acid binding site dissociation constants cannot be measured directly by fluorescence quenching, as fluorescent amino acids are not involved in binding here. Fluorescence quenching works efficiently only across distances of about 10 nm, moreover, fatty acids are not effective quenchers like Ent with its aromatic rings. Still, ligand affinities can be investigated by an indirect method of fluorescence quenching. The binding site was first saturated with the fluorescent molecule DAUDA (11-[5-(Dimethylamino)-1-naphtalenesulfonylamino]-undecanoic acid or 11-(Dansylamino)undecanoic acid).

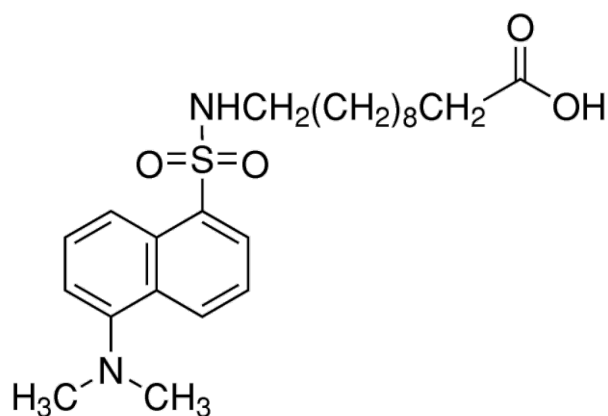


Fig. 25. Structure of DAUDA.

When DAUDA is bound to Q83, it shows intensive fluorescence with a maximum around 490 nm when excited at 345 nm, whereas its fluorescence intensity in buffer is comparatively very low in that range. Hence, binding again can be followed when the molecule is added to the protein solution in steps.

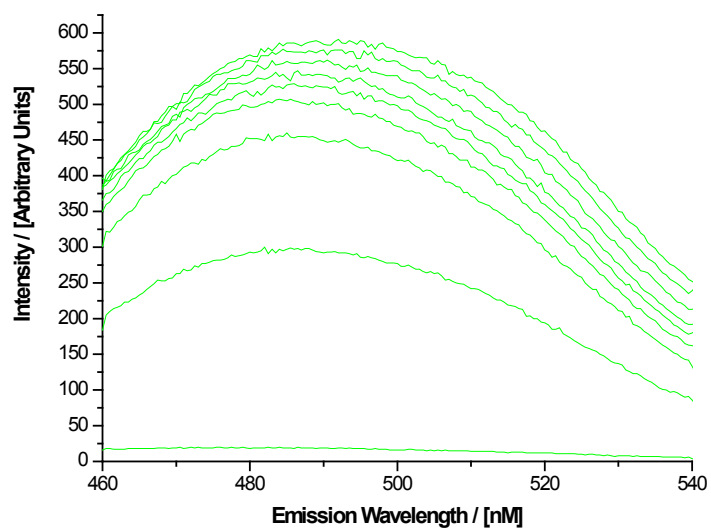


Fig. 26. Emission spectra of Q83 at different DAUDA concentrations, excited at 345 nm. Higher intensity curves correspond to higher DAUDA concentrations.

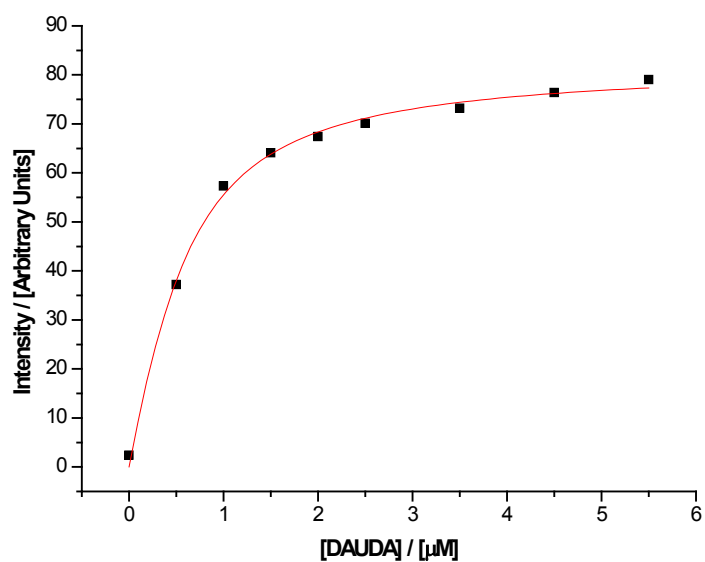


Fig. 27. The integrals of the measured intensities of each curve in Fig. 26 plotted against DAUDA concentration. The red curve shows the fit with the equation shown below.

With the following equation, similar to the one used in the preceding passage, only this time increasing fluorescence intensity with ligand concentration is observed, the K_D of the protein for DAUDA can be defined.

$$I = I_{\max} \frac{[P] + [L] + K_D \sqrt{([P] + [L] + K_D)^2 - 4[P][L]}}{2[P]}$$

Addition of AA to the DAUDA-saturated protein leads to competitive binding, whereas fluorescence intensity decreases again. The K_D values can be constructed from the plot of fluorescence intensity versus the natural logarithm of the ligand concentration, at constant DAUDA concentrations. This graph can be fit with the function for a sigmoidal curve.

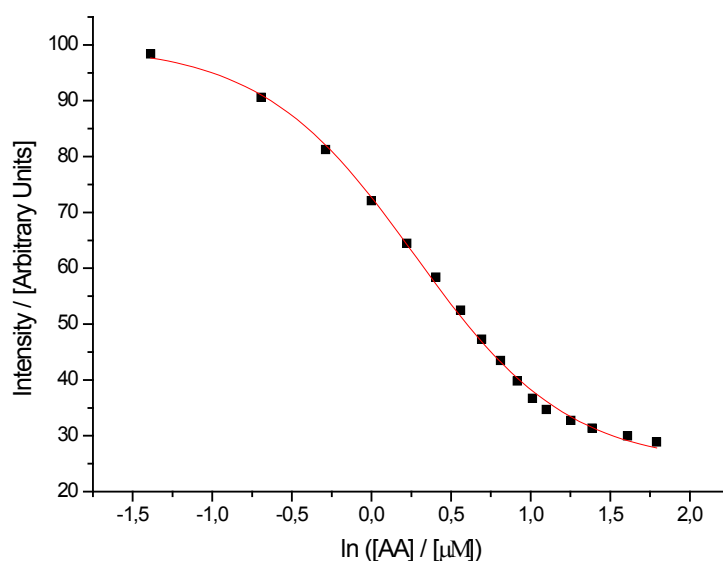


Fig. 28. The integrals of the measured intensities at different AA concentrations plotted against the natural logarithm of these concentrations. The red curve shows the sigmoidal fit. At the inflection point the AA concentration equals the DAUDA concentration.

At the ligand concentration of the inflection point the same amount of ligand and DAUDA is bound. Consequently the ratio of DAUDA and the ligand at that point equals the ratio of their K_D s.

$$K_{D1} / K_{D2} = [\text{DAUDA}] / [\text{AA}]$$

As the DAUDA concentration and its K_D is known and the concentration of ligand at the inflection point can simply be calculated by taking the exponential of the logarithmic value, the K_D of the ligand can be obtained.

2.11. Process Overview

The following paragraph should provide a rough overview about the work done.

In order to delineate the siderophore binding mechanism, mutants of the basic triad in the Q83 calyx were designed and examined towards binding to Ent. For further specifications of interacting forces the ligand was applied at different charge by substituting the trivalent iron with the tetravalent vanadium. Furthermore Ent affinity was tested at increasing salt concentrations for Q83 and NGAL.

Catechol and DHBA, suggested mammalian siderophores, were investigated at varying iron concentrations and pH values. Results were compared to those for Ent in order to trace possible differences in the binding mechanism. Crystallization of NGAL in combination with these ligands should extend structural information on binding of these compounds.

For characterizing binding in the secondary binding site, various mutants of Q83 were designed. Mutated amino acids were chosen in reference to the solution structure of the Q83/AA complex. By determining dissociation constants for the different mutants, crucial residues for binding were detected. Besides AA, several other fatty acids were examined towards binding to Q83.

Considering the two binding sites, an upcoming question is to which extent these are coupled with each other. This was dealt with by fluorescence assays, probing affinities of one ligand in presence of another, bound at the other binding site. Moreover CPMG experiments of ligand-free and Ent-bound NGAL should elucidate protein dynamics and its changes upon Ent binding.

3. MATERIALS AND METHODS

3.1. Buffers and Solutions

Autoclaving was done at 121 °C for 15 min.

Antibiotics were applied at following final concentrations:

Ampicillin 100 µg/ml

Kanamycin 50 µg/ml

Chloramphenicol 50 µg/ml

LB

20 g LB-Broth stock powder

→ add dH₂O to 1 l

→ autoclave

LB Agar (for ≈ 20 plates)

500 ml LB

7.5 g agar

→ autoclave

→ cool to 55°C,

→ add antibiotics

→ pour into plates

→ store at 4°C

8M Urea

480 g Urea

→ add H₂O to 1 l

M9 Medium

858 ml dH₂O

100 ml of 10x M9 salts

→ autoclave

→ cool to room temp.

1 g NH₄Cl (or ¹⁵NH₄Cl if needed)

4 g glucose (or 3 g ¹³C-glucose if needed)

10 ml trace elements

100 µl 1M CaCl₂

2 ml 1M MgSO₄

→ add antibiotics

10x M9 Salts

65 g Na₂HPO₄

30 g KH₂PO₄

5 g NaCl

→ add dH₂O to 1 l

→ adjust pH to 7.4

→ autoclave

Trace Elements

EDTA 5 g/l

FeCl₃·2H₂O 0.83 g/l

ZnCl₂ 84 mg/l

CuCl₂·2H₂O 13 mg/l

CoCl₂·2H₂O 10 mg/l

H₃BO₃ 10 mg/l

MnCl₂·4H₂O 1.6 mg/l

NaPi Buffer

50 mM NaCl (2,92 g)

10 mM NaH₂PO₄ (1,38 g)

10 mM Na₂HPO₄ (1,78 g)

→ add dH₂O to 1 l

→ adjust pH to 6.5

→ filtrate

→ degas, if used in chromatography

PBS Buffer

150 mM NaCl (8.76 g)

5 mM NaH₂PO₄ (0.69 g)

5 mM Na₂HPO₄ (0.89 g)

→ add dH₂O to 1 l

→ adjust pH to 7.4

→ autoclave

6M GdmCl

573 g GdmCl

→ add dH₂O to 1 l

dNTP Mix

2 mM dATP

2 mM dCTP

2 mM dGTP

2 mM dTTP

→ in dH₂O

→ store at -20°C

3.2. Q83

3.2.1. Template

The template for the recombinant Q83 was available in a pET3d expression vector. The construct encodes a 157 amino acid residue protein, lacking an N-terminal signal sequence of the precursor protein. In vivo this 22 amino acid sequence is removed by post-translational cleavage.

The calculated molecular weight of the recombinant protein is 18 079 Da and its calculated pI 6.97.

The extinction coefficient was calculated to 24 535 M⁻¹ cm⁻¹ at 280 nm.

MTVPDRSEIA GKWYVVALAS NTEFFFLREKD KMKMAMARIS FLGEDELKVS YAVPKPNGCR
KWETTFKTS DDGEVYSEE AEKKVEVLDT DYKSYAVIYA TVVKDGRTLH MMFLYSSE
VSPAATAIFR KLAGERNYTD EMVAMLPRQE ECTVDEV

Fig. 29. Amino acid sequence encoded by the Q83 template. The different residues that were mutated in the following are highlighted.

3.2.2. Site-Directed Mutagenesis

Cloning of the different mutants was done using the “QuickChange[®] Site-Directed Mutagenesis Kit” from Stratagene.

The mutants were designed using the following primers, ordered from Sigma-Aldrich[®].

R6A

5'- GACTGTGCCGGACGCGAGCGAGATTGCAGGG -3'

5'- CCCTGCAATCTCGCTCGCGTCCGGCACAGTC -3'

R6Q

5'- GACTGTGCCGGACCAGAGCGAGATTGCAGGG -3'

5'- CCCTGCAATCTCGCTCTGGTCCGGCACAGTC -3'

Y51F

5'- GGACGAGCTGAAGGTCTCCTTCGCAGTCCCCAAACC -3'

5'- GGTTTGGGGACTGCGAAGGAGACCTTCAGCTCGTCC -3'

K68A

5'- GGGAGACAACCTTCAAGGCGACCAGTGATG -3'

5'- CATCACTGGTCGCCTTGAAGGTTGTCTCCC -3'

Y76F

5'- GGTGAAGTCTTCTACTCAGAGGAAGCC -3'

5'- GGCTTCCTCTGAGTAGAAGACTTCACC -3'

K83A

5'- CTCAGAGGAAGCCAAGGCAAAGGTGGAGGTGCTGGACACTGAC -3'

5'- GTCAGTGTCCAGCACCTCCACCTTTGCCTTGGCTTCCTCTGAG -3'

Y92F

5'- GCTGGACACTGACTTCAAGAGCTATGC -3'

5'- GCATAGCTCTTGAAGTCAGTGTCCAGC -3'

R102A

5'- CAGTAATCTATGCAACTGCGGTGAAGGACGGAAGGACCC -3'

5'- GGGTCCTTCCGTCCTTACCCGCAGTTGCATAGATTACTG -3'

R113A

5'- GGACCCTGCACATGATGGCGCTCTACAGC -3'

5'- GCTGTAGAGCGCCATCATGTGCAGGGTCC -3'

Y115F

5'- GCACATGATGAGGCTCTTCAGCAGAAGCCC -3'

5'- GGGCTTCTGCTGAAGAGCCTCATCATGTGC -3'

R117A

5'- GCACATGATGAGGCTCTACAGCGCAAGCCCTGAGG -3'

5'- CCTCAGGGCTTGCGCTGTAGAGCCTCATCATGTGC -3'

3.2.3. PCR

PCR reactions were done on an “Eppendorf Mastercycler® gradient”.

Reaction Mix

10x Reaction Buffer	5 µl
Forward Primer (100 ng/µl)	2 µl
Reverse Primer (100 ng /µl)	2 µl
Template (123 ng/µl)	0.5 µl
dNTP Mix	1 µl
<u>dH₂O</u>	<u>39.5 µl</u>
PfuTurbo® DNA Polymerase	1 µl

Cycling Conditions

95° C 2 min

95° C 30 sec

55° C 1 min > 20 cycles

68° C 14 min

4° C hold

For R113A the PCR had to be repeated as the subsequent sequencing of the transformed and minipreped DNA gave the plasmid without insert. The primer for this mutation shows a relatively low melting temperature, so 53° C was chosen for annealing. Furthermore the elongation step was set down to 7 minutes.

All PCR products were digested for 5 h with the restriction enzyme DpnI in order to get rid of the methylated, non-mutated DNA template.

3.2.4. Transformation

The PCR products were transformed into *Escherichia Coli DH5a* cells, derived from Novagen, using the following protocol.

- Thaw DH5α aliquot on ice (15 min)
- Add 5 µl of mutant PCR-mix
- Keep on ice for 5 - 30 min
- Heat shock: 1 min at 42 °C
- Keep on ice for 2 min

- Add 300 µl of LB medium (no antibiotics added)
- Grow for 45 min in shaking incubator at 37° C and 180 rpm
- Plate 50 - 100 µl on an LB_{Amp} Ager Petri dish
- Incubate over night at 37° C

Ampicillin could be used to select for the cells containing the plasmid, as the pET3d vector contains an ampicillin resistance cassette.

3.2.5. Miniprep

The plasmid DNA was obtained from the transformation products using the following protocol.

Small scale centrifugations were done on a tabletop centrifuge at maximum speed.

- Add one colony from transformation plate to 10 ml LB_{Amp} in a 50 ml Falcon tube
- Grow over night in shaking incubator at 37° C and 180 rpm
- Centrifuge at 3220 g for 10 min at 4° C
- Extract DNA using the “QIAprep[®] Spin Miniprepr Kit” from Qiagen
- Eluted DNA with 30 µl H₂O

The DNA concentration was detected with a nanodrop UV/VIS photometer.

The plasmids were checked for the right mutation by sequencing at VBC-Biotech.

3.2.6. Expression

The different plasmids with the mutations and the original plasmid were transformed into the expression strain *Escherichia Coli BL21(DE3)pLysS* derived from Novagen, using the following protocol.

- Thaw BL21 aliquot on ice (15 min)
- Add 0.2 µl of Miniprep product (100 - 400 ng/µl)
- Keep on ice for 5 - 30 min
- Heat shock: 45 sec at 42 °C
- Keep on ice for 2 min
- Add 300 µl of LB medium (no antibiotics added)
- Grow for 45 min in shaking incubator at 37°C and 180 rpm
- Plate 50 - 100 µl on an LB_{Amp,Cmp} Ager Petri dish
- Incubate over night at 37°C

Chloramphenicol could be used in addition to ampicillin to select for the right colonies, as the expression strains contain the pLys plasmid with chloramphenicol resistance.

The expression procedure is described in the following protocol.

- Add one colony from transformation plate to 2 ml LB_{Amp,Chl} in a 15 ml unsealed tube
- Grow “day culture” for 7 h in a shaking incubator at 37°C and 180 rpm

- Add 50 µl of day culture to 20 ml of M9_{Amp,Chl} in a 50 ml Erlenmeyer flask
- Grow over night in shaking incubator at 37° C and 180 rpm
- Add the 20 ml of overnight cultures to 1 l of M9_{Amp,Chl} (¹⁵N- or ¹⁵N-¹³C-labeled if needed) in a 2 l Erlenmeyer flask (with indentations, assuring sufficient oxygen supply)
- Grow in shaking incubator at 37° C and 180 rpm until OD₆₀₀ reaches 0.5 (≈ 3h)
- Induce with IPTG to a final volume of 1mM
- Express for 5 h in the shaking incubator at 37° C and 180 rpm
- Harvest cells at 5000 g for 15 min
- Discarded the supernatant
- Resuspend pellet in 15 ml PBS buffer
- Freeze at -80° C, if cell suspension is not used directly

The slightly red discolouring of the mixture produced by the Q83/Enterobactin-Fe^{III} complex served as a good indicator for effective expression.

3.2.7. Purification

During the whole purification process the protein was kept at 4° C if possible.

The red colour of the Q83/Enterobactin-Fe^{III} complex, allowed localizing the protein visually during the whole procedure.

Centrifugation steps for cell debris, streptomycin and ammonium sulphate precipitation were carried out at 35 000 g.

Centrifugation for concentrating the protein in “Amicon Centrifugal Filter Units” was done at 3220 g.

Cell Lysis

The cells were broken open by 15 min of sonication, pulsing 30 % of time. The cell debris was separated from the solution, containing the overexpressed protein, by centrifugation.

Nucleic acids were precipitated by bringing the supernatant to 1 % streptomycin, followed by centrifugation.

Ammonium Sulphate Precipitation

Q83 is a good soluble protein which precipitates at ammonium sulphate (NH_4SO_4) concentrations higher than 60 %. The percentage refers to ammonium sulphate concentration in saturated water. Hence, in order to get rid of a lot of less soluble protein, an ammonium sulphate precipitation at 60 % was done. The amount of NH_4SO_4 in g that had to be used to reach the desired concentrations was calculated with the online tool of ©Encor Biotechnology Inc. (<http://www.encorbio.com/protocols/AM-SO4.htm>). Precipitate was spinned down and the supernatant was brought to 95 % ammonium sulphate concentration, again followed by centrifugation. The red pellet was resuspended in 5 ml NaPi buffer.

Chromatography

All chromatographic methods were done with “Äkta™ Explorer” and “Äkta™ Purifier” systems.

Anion-Exchange

The calculated pI of the used Q83 protein is 6.97, which means in the pH 6.5 NaPi buffer it is rather positively charged. In contrast DNA that is still present is negatively charged because of its phosphate backbone and therefore can be effectively separated from the protein by anion-exchange-chromatography. For that purpose a 6 ml “Resource Q” prepacked column was used, equilibrated with 20 ml NaPi buffer. The sample was loaded onto the column with a flow of 1 ml/min,

so the pressure did not exceed 0.4 MPa. DNA was highly retarded, whereas the flow through containing Q83 was directly collected into a 15 ml “Amicon Centrifugal Filter Unit” with a 10 kDa cut-off. Q83, with its molar weight of 18 kD, was safe not to pass the membrane and was concentrated to less than 1 ml by centrifugation for about 30 min. The Resource Q column was washed in the given order with 5 CV 1 M NaCl, 5 CV 1 M NaOH, 5 CV 1 M NaCl, 5 CV H₂O and 5 CV 20 % ethanol, in which it was stored.

Size-Exclusion

Size-Exclusion-Chromatography was done in order to separate Q83 from proteins that could not be cleared by the former methods. A “HiLoad 16/60 Superdex 75 prep grade” prepacked column was equilibrated with 160 ml of NaPi buffer, whereas the bed volume is about 120 ml. The sample was loaded onto the column through a 1 ml loop. The pressure was kept below 0.4 MPa by a flow of 1 ml/min. Q83 eluted after about 70 ml and was collected in fractions of 0.8 ml into a 96 well plate. The Q83 containing fractions were spotted by their red discolouring and pipetted into a centricon. Concentrating was done as described before by centrifugation for about 40 min until a concentration of less than 500 µl was reached. The HiLoad column was washed in the given order with 0.5 CV 0.5 M NaOH, 3 CV H₂O and 3 CV 20 % ethanol, in which it was stored.

Getting Rid of the Co-Purified Ligand

For subsequent studies on Q83, the protein had to be present in its free form, without enterobactin bound. Therefore the first step was to unfold the protein so it had no specific affinity to the ligand anymore. This was done by bringing the sample to 6M GdmCl and incubating it at 70°C for 30 min. Separation of the now unbound ligand from the protein was done with a “PD-10 Desalting column”. This is a prepacked, disposable column for separation of high ($M_r > 5000$) from low molecular weight substances ($M_r < 1000$). After equilibration with 25 ml of 6 M GdmCl buffer, the sample had to be applied in a volume of 2.5 ml ideally. The flow through was discarded and then elution was started with 3.5 ml buffer, whereas

the flow through was collected immediately. Compared to the protein, the enterobactin-iron complex was highly retarded, indicated by the remaining red band on the column. The manufacturer quotes a protein yield typically greater than 95% with less than 4% low molecular weight contamination when following the described procedure. After washing with 50 ml dH₂O, the columns were reused several times.

Proper refolding of the protein was ensured by dialysis. Therefore the 3.5 ml of elute from the desalting column were diluted to 30 ml with NaPi buffer and transferred to a dialysis bag with a 10 kDa cut-off. The solution was dialysed 3 times against 500 ml of NaPi buffer.

The sample was concentrated in a 15 ml “Amicon Centrifugal Filter Unit” with a 10 kDa cut-off. After washing three times with buffer it was typically concentrated to a volume of about 1 ml.

Verifying the Protein

The protein concentration was determined by absorption measurement on a nanodrop UV/VIS photometer. The concentration was calculated from absorbance by Lambert Beer’s law ($A = \epsilon cd$). The extinction coefficient ϵ for Q83 at 280 nm is $24\,535\text{ M}^{-1}\text{ cm}^{-1}$.

The final protein yield usually was about 10 - 15 mg per liter of expression culture.

For verifying the protein, ¹H-¹⁵N HSQC spectra of the ¹⁵N-labelled samples were recorded and compared to the spectrum for the pure and properly folded Q83.

3.3. NGAL

3.3.1. Template

The recombinant NGAL construct was available in the pET38b expression vector. The template encodes a 176 amino acid residue protein. In contrast to the precursor protein, residue Cys₈₅ is mutated to a serine, in order to prevent dimerisation of the protein due to disulfide bridge formation.

The calculated molecular weight of the recombinant protein is 20 408 Da and its theoretical pI 9.21.

The extinction coefficient was calculated to 27 515 M⁻¹ cm⁻¹ at 280 nm.

```
      10      20      30      40      50      60
MTSDLIPAPP LSKVPLQQNF QDNQFQGKQY VVGLAGNAIL REDKDPQKMY ATIYELKEDK
      70      80      90     100     110     120
SYNVTSVLFR KKKCDYWIRT FVPGSQPGEF TLGNIKSYPG LTSYLVRVVS TNYNQHAMVF
      130     140     150     160     170
FKKVSQNREY FKITLYGRTK ELTSELKENF IRFSKYLGLP ENHIVFPVPI DQCIDG
```

Fig. 30. Amino acid sequence encoded by the NGAL template. The serine, replacing the wild-type-cysteine, is highlighted.

3.3.2. Expression

Expression was done similar to Q83, except for the following differences.

The pET38b vector exhibits kanamycin resistance. Hence, kanamycin was used instead of ampicillin to select for positive bacteria.

After induction with IPTG, the protein was expressed over night at 24 ° C.

3.3.3. Purification

Purification was done similar to Q83, except that different concentrations for the ammonium sulphate precipitation were used. NGAL is less soluble and therefore the first step was done at 40 % NH_4SO_4 and the second step, at which NGAL should precipitate, at 65 % NH_4SO_4 .

3.4. Ligand Preparation

An aqueous solution of metal-free enterobactin was provided from cultures of an E. Coli strain deregulated in enterobactin biosynthesis. The iron-bound form $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$ was obtained after adding FeCl_3 to the $(\text{Ent})^{6-}$ -solution.

In NMR experiments, gallium, which is non-paramagnetic, was used as a substitute for iron, in order to avoid paramagnetic effects. The $[\text{Ga}^{\text{III}}(\text{Ent})]^{3-}$ -complex was prepared by adding $\text{Ga}(\text{acac})_3$ to the metal-free solution of $(\text{Ent})^{6-}$.

Catechol, 2,3-DHBA, arachidonic acid, lauric acid, palmitic acid, oleic acid and linoleic acid were received as powder from Sigma-Aldrich®. Stock solutions of 10 mM were obtained by solubilization in DMSO. Further dilutions were done in NaPi buffer.

DAUDA was also derived from Sigma-Aldrich® as powder. A 10 mM stock solution was produced in ethanol. Subsequent dilutions were done in NaPi buffer.

3.5. NMR

All NMR experiments were carried out in the three in-house Varian™ spectrometers with magnetic field strength resulting in ^1H Larmor frequencies of 500, 600 and 800 MHz.

All samples were brought to protein concentrations between 400 μM and 2 mM in NaPi buffer.

D₂O was added to a final concentration of 10 % in order to supply the sample with a deuterium reference. This “lock-solvent” can be used to detect changes in the magnetic field strength. By shim coils the field can consequently be locked to a constant value.

All experiments were carried out at 25 °C.

Data processing was done by NMRPipe and NMRDraw (42). The obtained spectra were analyzed with CARA and Sparky (43).

Backbone assignment of the ¹H-¹⁵N HSQC spectrum of the ligand-free NGAL and the NGAL/[Ga^{III}(Ent)]³⁻-complex was done using HNCA, HNCB and HN(CA)CO.

The assigned HSQC spectrum of NGAL is available on the “RCSB Protein Data Bank” and was overlaid to the measured spectrum, so it aided as a model for the assignment of the enterobactin-free form of NGAL.

CPMG data was processed with Sparky and MS Excel. The obtained matrices, including the peak heights for the various amino acid residues at the different CPMG cycles, were analyzed with MATLAB and rdnmr.

3.6. Fluorescence Quenching

All fluorescence experiments were carried out on a Perkin Elmer LS 50B fluorimeter. Measurements were done in NaPi buffer pH 6.5 or 7.4 at room temperature.

Ligands were added to a 2 ml volume of protein solution in a quartz cuvette. Proper mixing was ensured by pipetting up and down with a 100 µl pipette.

The dilution factor in the cuvette due to ligand addition was disregarded as its effect was evanescent compared to the error of the experiment.

Data processing was done with MS Excel and Origin.

Ideally around 15 spectra at regular intervals were recorded until saturation was reached and 10 more thereafter.

3.6.1. Calyx

A 10 μM $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$ or $[\text{V}^{\text{IV}}(\text{Ent})]^{2-}$ solution was added to 0.1 μM protein in 2 μl steps. At an excitation wavelength of 280 nm, the emission spectra were recorded between 320 and 360 nm.

3.6.2. Fatty Acid Binding Site

Q83 mutants

The K_D values for DAUDA were directly determined in every experiment. Therefore, first a 200 μM DAUDA solution was added to 2 ml of 2 μM protein in steps of 2 μl or more. The emission spectrum between 460 and 540 nm measured at an excitation wavelength of 345 nm. At the point where saturation was reached and emission did not change significantly anymore, arachidonic acid, also at 2 μl steps of 200 μM stock solution was added, following the same procedure as described before.

Q83 wild type

The K_D of DAUDA for the wild type Q83 was determined to 100 nm. Therefore it was not necessary to do the DAUDA-titration at every experiment. A solution saturated with DAUDA, namely 5 μM for a protein concentration of 2 μM , was provided in the standard volume of 2 ml in the cuvette. The different ligands were again added in 2 μl steps or more in concentrations dependent on their affinity.

Q83 in the presence of $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$

A more complicated method had to be applied in order to probe K_D s for AA in the presence of bound $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$, because the enterobactin bound in the calyx quenches DAUDA fluorescence. Hence, only the binding of the fatty acid to the Ent-free Q83 can directly be monitored. However, in the presence of both, Ent-free and Ent-bound Q83, conclusions can be drawn on the affinity to Ent-bound protein by measuring DAUDA fluorescence quenching.

The setups shown in Table 1 were applied in the cuvette for AA titrations. The $[Q83^{free}]/[Q83^{Ent}]$ ratios are given in assumption that Ent is all bound to the protein. Titration with AA was done as usual in 2 μ l steps with 250 μ M and 500 μ M stock solutions. The K_D calculation is carried out and explained in the results part.

	$[Q83^{free}] / [Q83^{Ent}]$
3 μ M Q83 1 μ M $[Fe^{III}(Ent)]^{3-}$ 5 μ M DAUDA	2 μ M / 1 μ M
4 μ M Q83 2 μ M $[Fe^{III}(Ent)]^{3-}$ 5 μ M DAUDA	2 μ M / 2 μ M
6 μ M Q83 4 μ M $[Fe^{III}(Ent)]^{3-}$ 5 μ M DAUDA	2 μ M / 4 μ M

Table 1. The left column shows the mixtures applied in the cuvette for AA titrations. The right column specifies the concentrations of protein in the free and Ent-bound form.

3.7. Crystallography

X-Ray Crystallography should give information about the orientation of ligands bound to the calyx of NGAL.

Primarily a small scale crystallisation screen for NGAL was set up, trying 96 different conditions. All crystallisation conditions were composed of sodium acetate, ammonium sulphate, sodium chloride and dH_2O at different concentrations and pH values. The screen was arranged by a pipetting robot. The plate was stored in a hotel at 22 °C and 4 °C where it was monitored by cameras.

For those conditions that worked best, NGAL was crystallized by the hanging drop vapour diffusion method. With this method, crystals big enough for x-ray investigation can be obtained. Two different methods, co-crystallisation and soaking, were applied for producing protein crystals with 2,3-DHBA bound to the calyx.

On a glass cover slip 1.3 μ l of 500 μ M protein were mixed with 0.5 μ l solution of precipitant and 0.2 μ l of 100 mM ligand. For crystallisation of NGAL without ligand, 0.5 μ l precipitant solution was added to 1.5 μ l protein. Eight different conditions were selected, specified in the following table.

Condition no.	1	2	3	4	5	6	7	8
[NaAc]/[M]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
[AS]/[M]	1.2	1.2	1.2	1.34	1.34	1.34	1.34	1.91
[NaCl]/[mM]	40	67	93	120	40	67	93	67
pH	4	4	4	4	4.5	4.5	4.5	5

Table 2. Crystallisation conditions for which NGAL crystals were obtained.

The wells of a 48-well plate were filled with 150 μ l of the accordant precipitant solution and sealed with a cover slip, the drop facing downwards inside the well. The well consequently forms a closed thermodynamic system. The drop is separated by air from the solution at the bottom, higher in concentration of precipitant. Consequently water evaporates from the drop, increasing its concentration towards equilibrium. So protein and salt inside the drop is concentrating slowly and gradually, reducing the chance of precipitation before crystals are formed. The plate was stored in the hotel at 22 °C.

Obtained crystals were examined in the in-house x-ray machine to check if their diffraction pattern showed the expected space group and if diffraction was good enough to allow proper resolution. For those that diffracted well, a selection of eight crystals was sent to the synchrotron in Grenoble for collecting high resolution data.

4. RESULTS

4.1. Enterobactin Binding Mode of Q83 and NGAL

4.1.1. Cation- π Interactions - The Basic Triad

Q83 was shown to bind $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$ with a dissociation constant of 0.54 nM (27) in its classical lipocalin binding site, the calyx. As for NGAL, also for Q83 especially basic residues like Lysine and Arginine are supposed to be directly involved in siderophore binding by forming cation- π interactions or hydrogen bonds. The basic triad of NGAL (Arg₈₂, Lys₁₂₅ and Lys₁₃₄) has its analogy in Q83's Lys₈₃, Arg₁₀₂ and Arg₁₁₃ (27). In order to probe the relevance of Q83's basic residues, the mutants K83A, R102A and R113A were tested for their affinities to $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$.

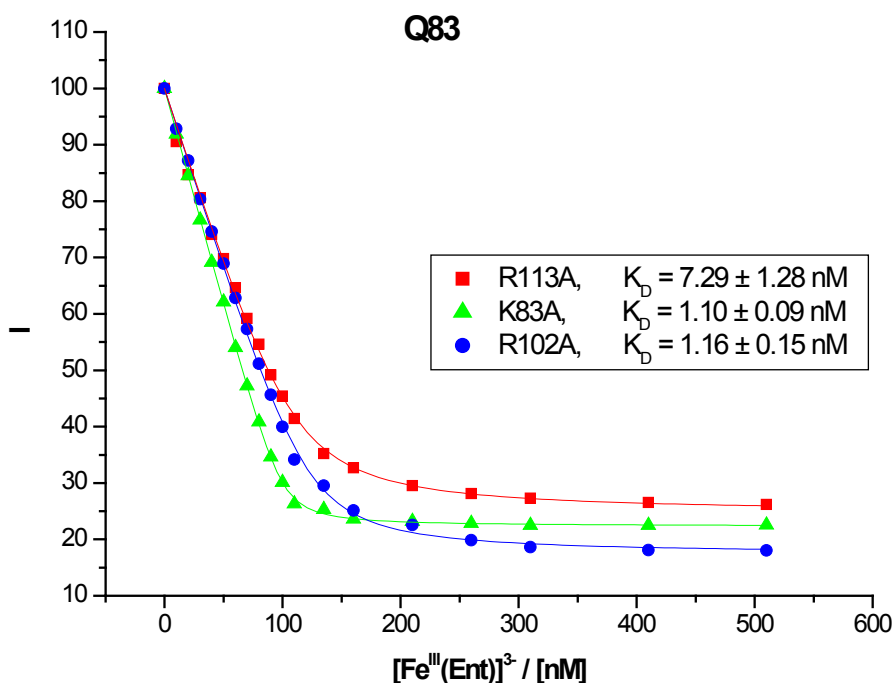


Fig. 31. Fluorescence quenching of Q83 mutants upon addition of enterobactin. The curves are fit with the equation presented in the introduction.

The results show only a slight decrease in affinity for K83A and R102A. Arg₁₁₃ emerges to be the most important, with more than ten times decrease in affinity when mutated to alanine. However, change in affinity is definitely not dramatic and therefore it seems that the loss of one cation- π interaction only has a limited effect on binding. Comparable results were reported for NGAL when tested with enterobactin analogues, especially designed to disrupt one cation- π interaction (18). Therefore it can be inferred that binding to enterobactin is essentially analogous for Q83 and NGAL.

4.1.2. Coulombic Interactions

As suggested by the solution structure of the Q83/[Ga^{III}(Ent)]³⁻ complex and partly shown by the results above, cation- π interactions of the basic triad and π - π -stacking with Trp₆₂ play important roles in enterobactin binding. Still other electrostatic forces, particularly coulombic interactions, might also contribute significantly.

Higher ionic strength of the buffer might weaken coulombic interactions to the ligand in the calyx dramatically compared to its effect on cation- π interactions. Hence, determining the K_D s at different salt concentrations should reveal the predominant electrostatic forces for siderophore binding. The K_D s for both, NGAL and Q83 for [Fe^{III}(Ent)]³⁻ were measured at different NaCl-concentrations by fluorescence quenching (Fig. 20).

A decrease in affinity from 0.76 nM at 50 mM NaCl to 2.2 nM at 2 M could be observed. This decrease with ionic strength is not dramatic and so coulombic interactions are not considered to be the major forces for the complex formation.

Similar conclusions can be drawn from the results for NGAL (Fig. 33). The K_D values also showed the tendency to increase with higher salt concentrations. The reported K_D of 0.41 nM at low salt concentration (7) increased to 1 nM at 1 M NaCl and 2.8 nM at 2 M NaCl.

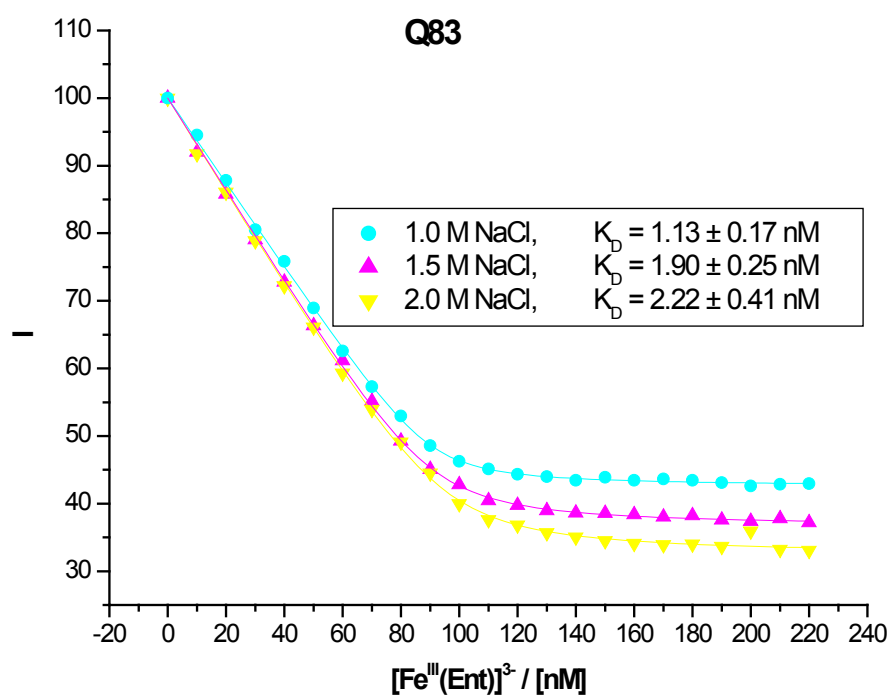


Fig. 32. Fluorescence quenching analysis of Q83 at different salt concentrations.

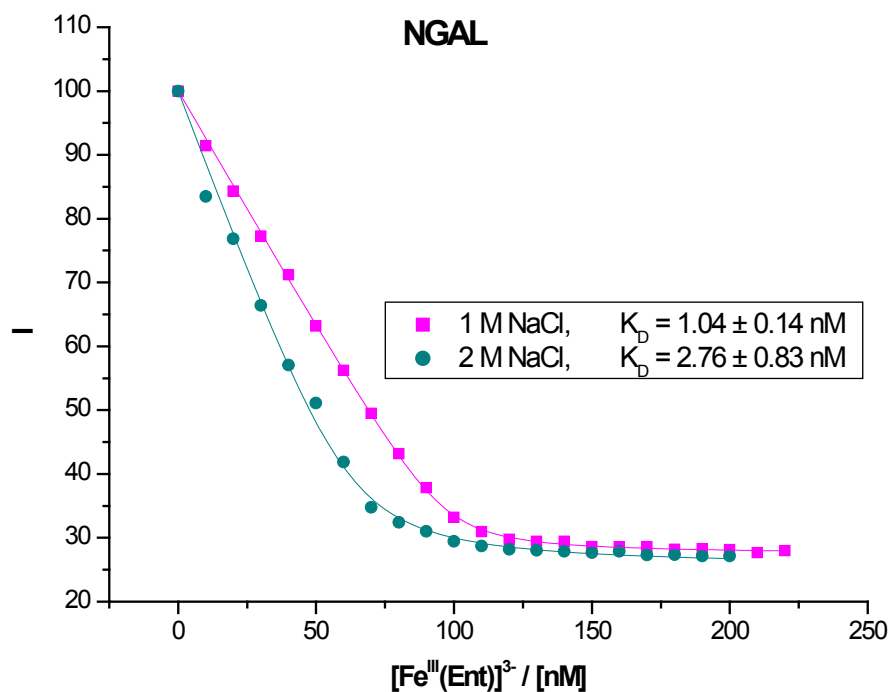


Fig. 33. Fluorescence quenching analysis of NGAL at different salt concentrations.

To confirm these results, the affinity for Q83 to a differently charged ligand was investigated. Therefore enterobactin was coordinated to vanadium instead of iron, resulting in an overall charge of the complex $[\text{Va}^{\text{IV}}(\text{Ent})]^{2-}$ of -2 instead of -3 for the iron-complex $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$.

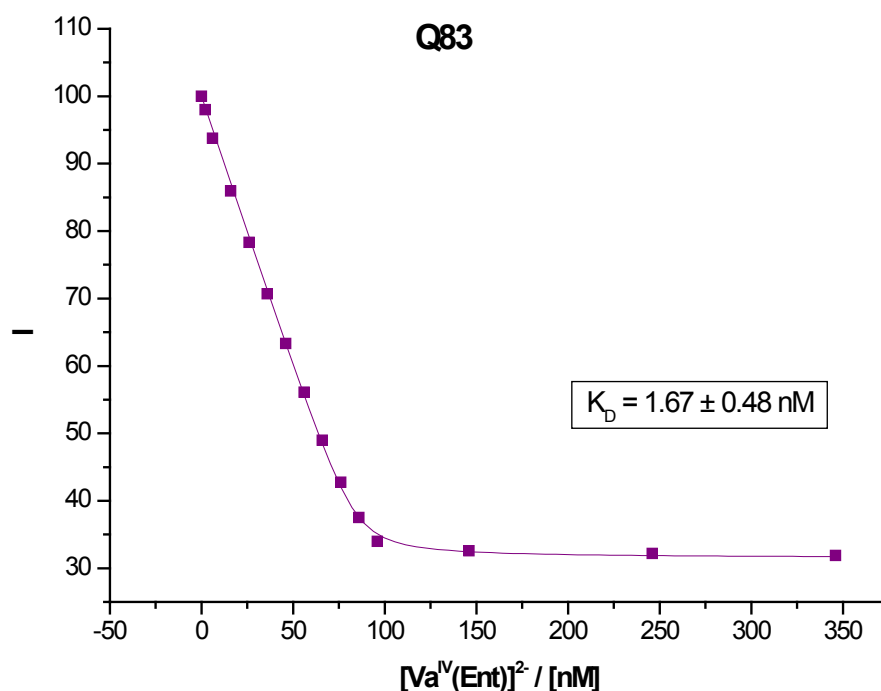


Fig. 34. Fluorescence quenching of Q83 upon addition of $[\text{Va}^{\text{IV}}(\text{Ent})]^{2-}$.

The measured K_D of 1.67 nM again did not reveal a great change in affinity, supporting the assumption that coulombic interactions are of limited importance for the Q83-Ent interaction. A comparable result of 2.26 nM was reported for NGAL (32).

Generally the results confirm a very similar binding mode for Q83 and NGAL towards siderophores.

4.2. Dynamics of NGAL

4.2.1. ^1H - ^{15}N HSQC Peak Assignment

The HSQCs of the ligand-free and Ent-bound form of NGAL were assigned by HNCA, HNCO and HN(CA)CO. The HSQC of ligand-free NGAL, which is available on the PDB, was used as a template. The following residues could not be assigned for the free form: V32, M49, Y50, S66, V67, L71, I78, L122, I170, D171 and I174.

In the bound form several peaks were considerably shifted and therefore assignment was more ambiguous. The following amino acids could not be assigned: S3, V32, A35, N37, D43, V67, L68, F81, V82, L122, F131, Q145, G158, V168 and I174.

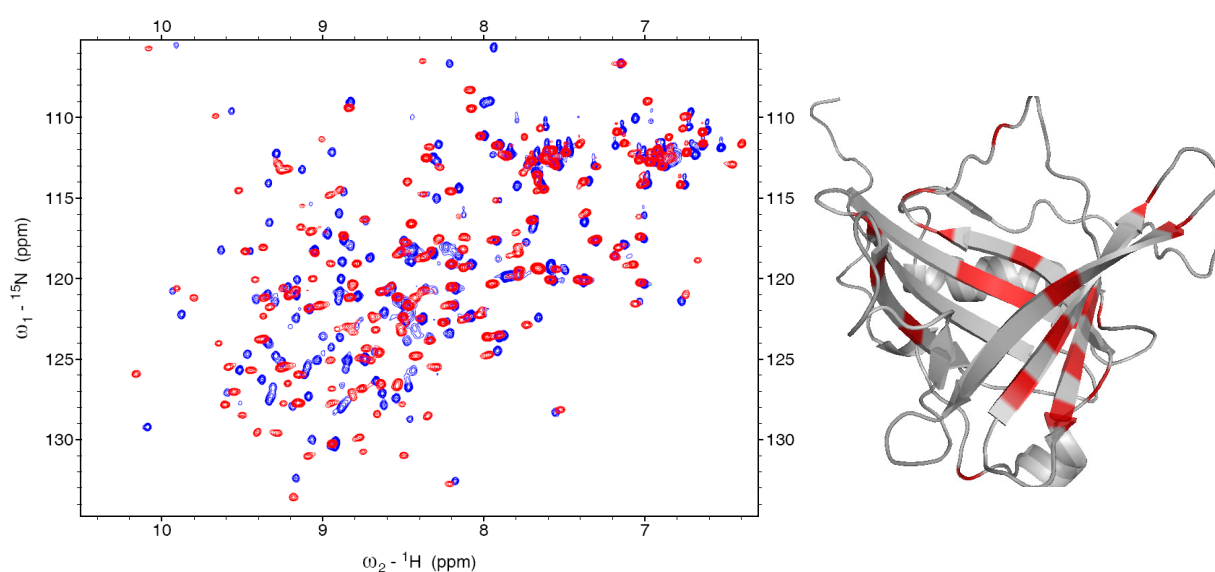


Fig. 35. Overlaid ^1H - ^{15}N HSQC spectra of free (blue) and Ent-bound (red) NGAL. In the structure on the right, peaks that are significantly shifted in the HSQC are highlighted in red.

4.2.2. CPMG

For free NGAL the CPMG experiment revealed pronounced backbone dynamics in the B-barrel and in various loop regions. The dispersion plots of the residues Q17, K28, V31, E42, D45, L68, F69, R70, C74, D75, W77, T80, F81, V82, F90, G93, T102, M118, Q126, K132, T134, L135, E141, L142, T143, E148, H163, D171, Q172 and

D175 show significant exchange in the intermediate exchange regime. Intermediate exchange means that the chemical shift difference between the conformations and the exchange rate are of similar magnitude.

Dynamics is almost entirely quenched upon Ent binding. Only slight intermediate exchange was observed in the dispersion plots of the residues Q26, G33, K60, S61, G93 and S103.

Fig. 36 shows the dispersion plots of residue T134, which is located in the β -barrel, for the free and Ent-bound NGAL. The absence of dynamics in the bound form is clearly visible. It should be noted that R_2^0 , the value $R_{2,eff}$ is approximating at infinite ν_{CPMG} , is bigger for the free form. This indicates additional exchange that is not refocused by the Π pulses.

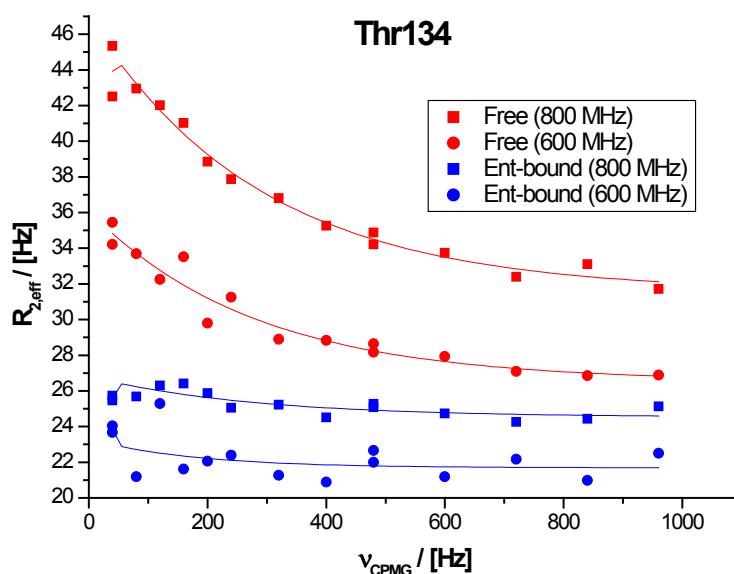


Fig. 36. Dispersion plots for Thr134 of the free and Ent-bound NGAL. Exchange is revealed for the free but not for the Ent-bound form. The CPMG was measured at an 800 MHz and 600 MHz spectrometer.

Fig. 37 shows the dispersion plots for residue S103, which revealed most significant exchange in the Ent-bound protein. Dynamics is much less pronounced than for T134 in the free form as can be seen by the lower $\Delta R_{2,eff}$. Again the plots reveal additional exchange in the free form, indicated by the higher R_2^0 values.

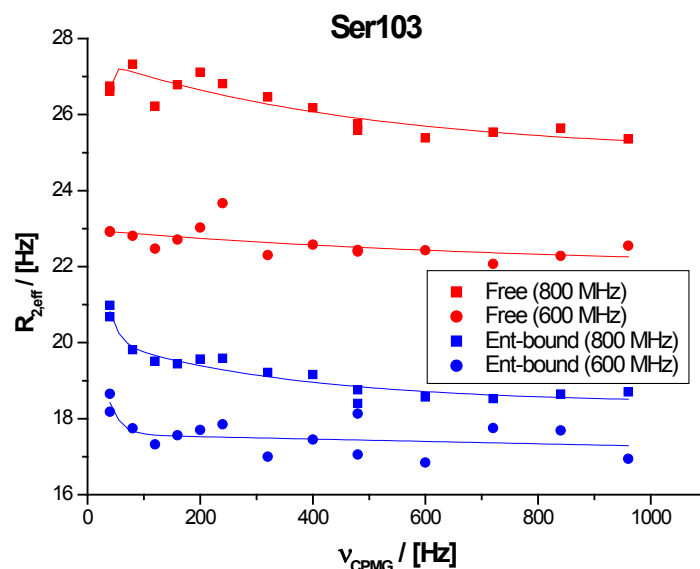


Fig. 37. Dispersion plots for Ser103 of the free and Ent-bound NGAL. Slight exchange is revealed for the bound form. The free form displays a higher R_2^0 value indicating additional exchange that is not refocused by the Π pulses.

In Fig. 38 residues that showed exchange in the free form are highlighted in the left and those that revealed slight exchange in the bound form are highlighted in the right structure. The two residues for which the dispersion plots are displayed above are marked in a different color.

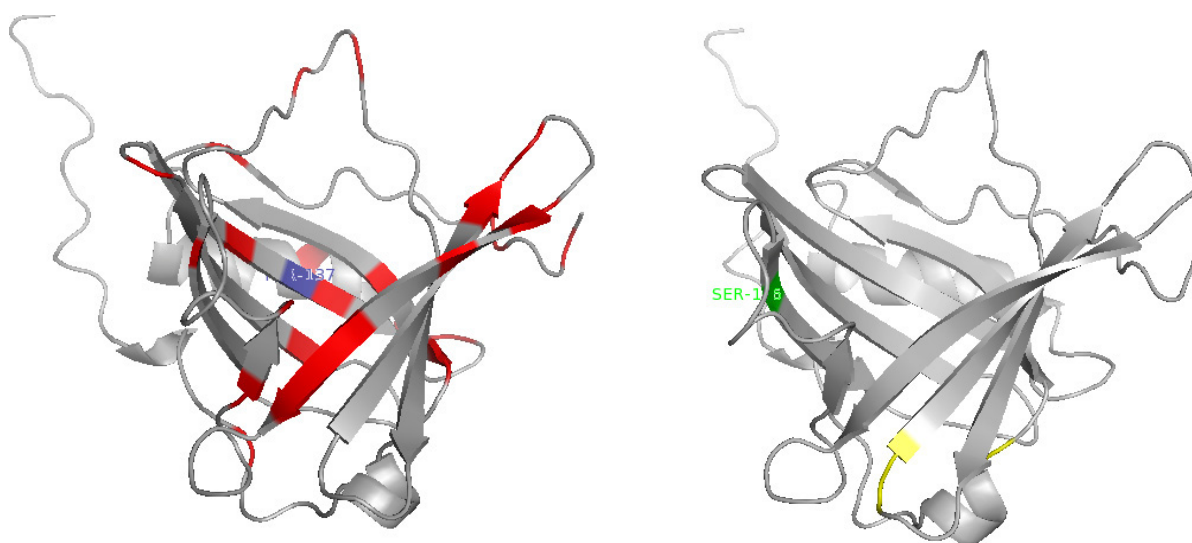


Fig. 38. Structure of NGAL with residues that revealed exchange in the free (left) and Ent-bound (right) form highlighted. T134 is marked in blue (left) and S103 in green (right).

4.3. Mammalian Siderophores - Catechol Binding Mode of Q83 and NGAL

As pointed out in the introduction, NGAL is not only capable of binding bacterial siderophores in its calyx but most likely also plays a role in iron transport mediated by mammalian siderophores. The compounds found so far are structurally tightly related to enterobactin. Catechol basically is the iron-binding moiety of enterobactin and is derived in mammalian as well as in bacterial metabolism as a dietary compound. Recent studies revealed its ability in NGAL-mediated iron transport and the supposable link to NGAL's partly high expression levels in aseptic diseases (4).

The catechol derivative 2,5-dihydroxybenzoic acid was shown to be the iron-binding component of the mammalian siderophore associated to the murine homologue of NGAL, 24p3 (5). In contrast, the iron binding moiety of enterobactin is 2,3-dihydroxybenzoic acid.

In order to gain further insight into the binding mode of these compounds to NGAL, the interactions were monitored by fluorescence and NMR titrations. The results might also be informative for iron delivery mechanisms of NGAL.

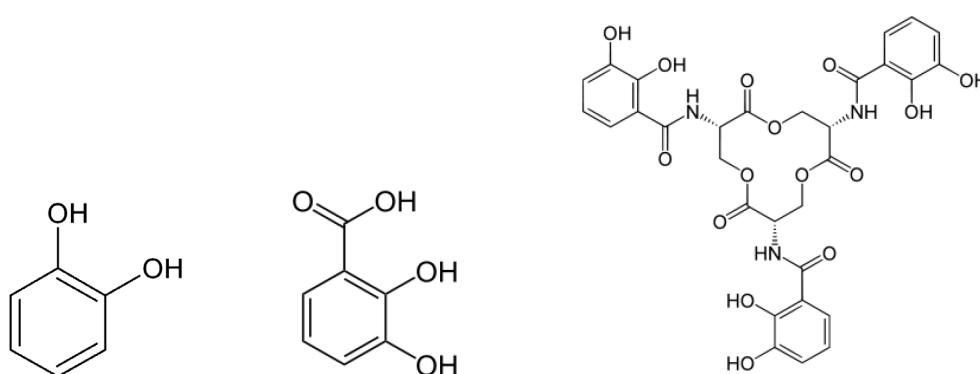


Fig. 39. Structures of catechol (left), 2,3-Dihydroxybenzoic acid (middle) and enterobactin (right).

The measurements were done at pH 7.4, as especially for catechol the affinity significantly decreases below pH 7 (4).

4.3.1. Fluorescence Quenching

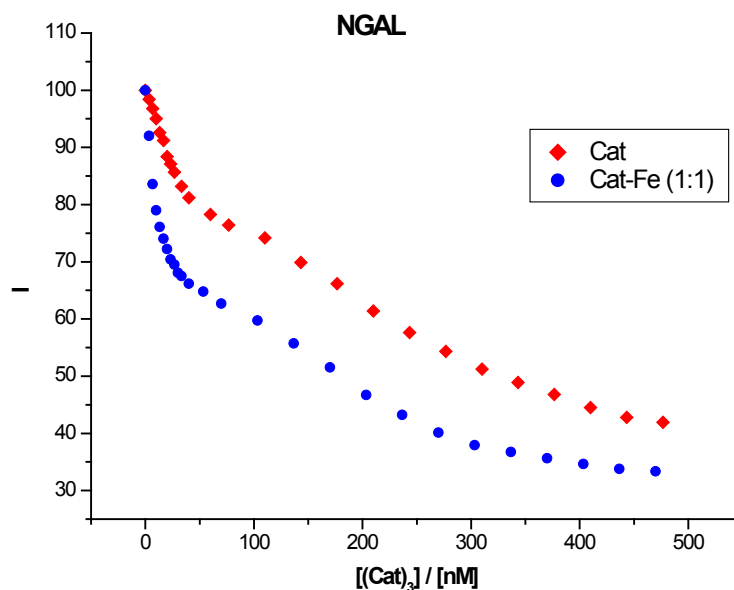


Fig. 40. Fluorescence quenching of NGAL upon addition of Cat and Cat-Fe^{III}. The concentration on the x-axis is plotted for three Cat moieties to allow better comparison with Ent. The observable “shoulder” in the titration curve and the fact that it cannot be fit with the single binding site model suggests sequential binding.

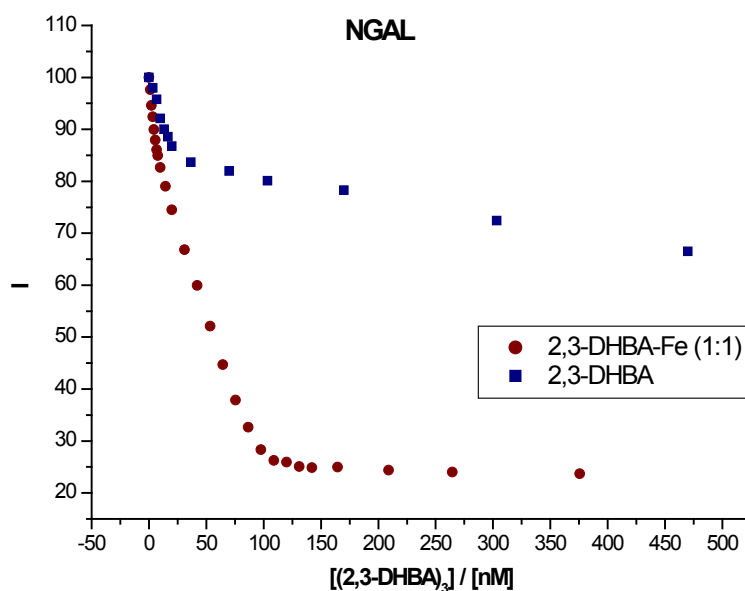


Fig. 41. Fluorescence quenching of NGAL upon addition of 2,3-DHBA and 2,3-DHBA-Fe^{III}. The curve cannot be fit with the single binding site model. The presence of iron increases affinity more extensively than is the case for Cat.

The fluorescence quenching plots for catechol display a “shoulder”, which makes it impossible to extract K_D values with a single binding site model. The results indicate the successive binding of the ligand to the distinct binding pockets in the calyx. In the presence of one equivalent iron and at pH 7 the predominant form of catechol is supposed to be $\text{Fe}(\text{Cat})_2$ (4). Therefore it is suggested that in the beginning of the titration primarily $\text{Fe}(\text{Cat})_2$ is occupying two binding pockets, corresponding to the first distinct decay in the plot.

The plots for 2,3-DHBA also cannot be fit with a single binding site model. A sequential binding mode can be inferred, although it is not as explicitly visible than for catechol. The presence of iron is more important for binding of 2,3-DHBA than catechol.

Basically similar behavior upon addition of catechol and 2,3-DHBA was observed for Q83. The plots are not shown as they only provide qualitative information and are therefore redundant with respect to the plots for NGAL.

4.3.2. ^1H - ^{15}N HSQC Titrations

In order to gain further information on the binding mechanism, HSQC titrations with $\text{Cat-Ga}^{\text{III}}$ and 2,3-DHBA- Ga^{III} for NGAL and Q83 were carried out. The information that can be obtained from the spectra is essentially similar for both proteins. Therefore again only plots for NGAL are shown.

Fig. 42 shows an overlay of spectra of NGAL without ligand, at 1 equivalent (eq) $\text{Cat-Ga}^{\text{III}}$ and saturated with $\text{Cat-Ga}^{\text{III}}$ (5 eq). After addition of 1 eq, several peaks of the free form are vanished and occur at different chemical shifts. Upon saturation with ligand, another subset of peaks appears, whereas the first subset vanishes.

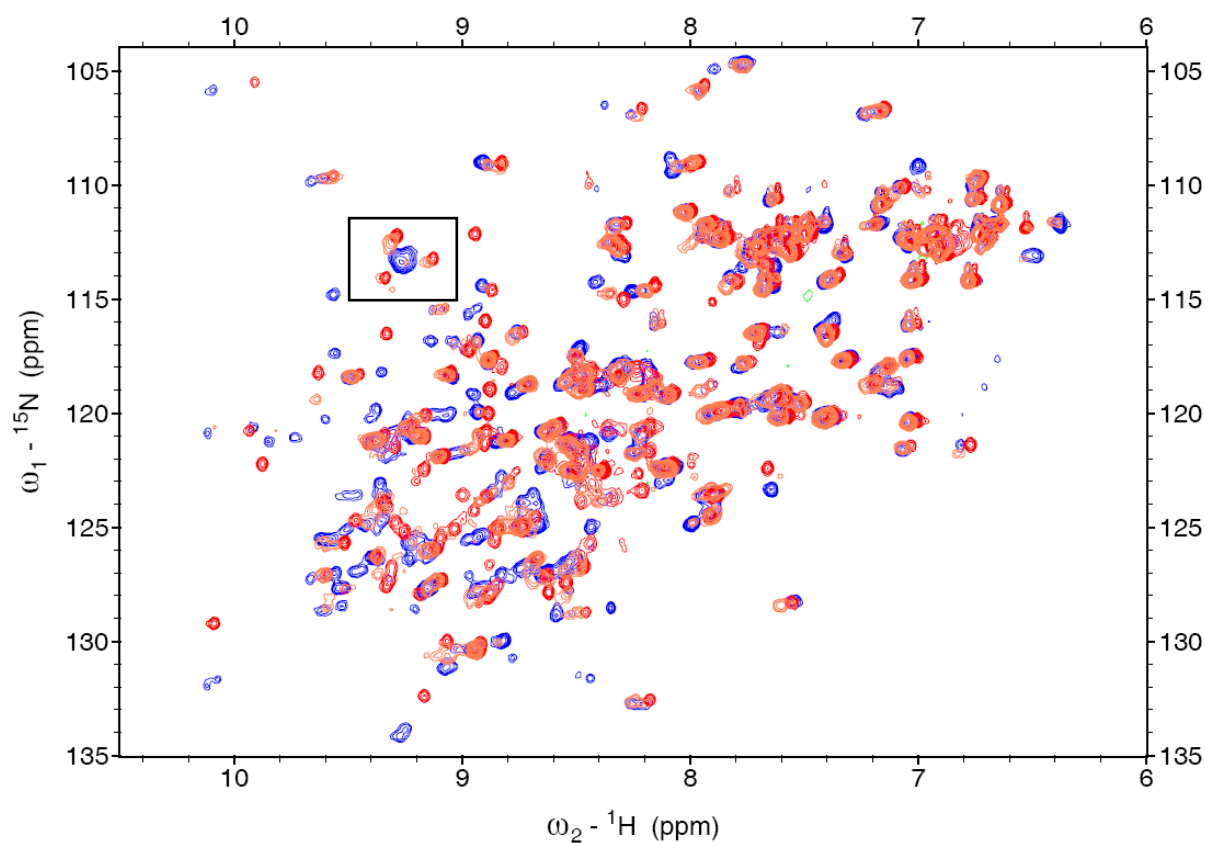


Fig. 42. HSQC titration of NGAL with Cat-Ga^{III}. Red - free form, orange - 1 eq Cat, blue - 5 eq Cat. The framed section is shown in the close-up below.

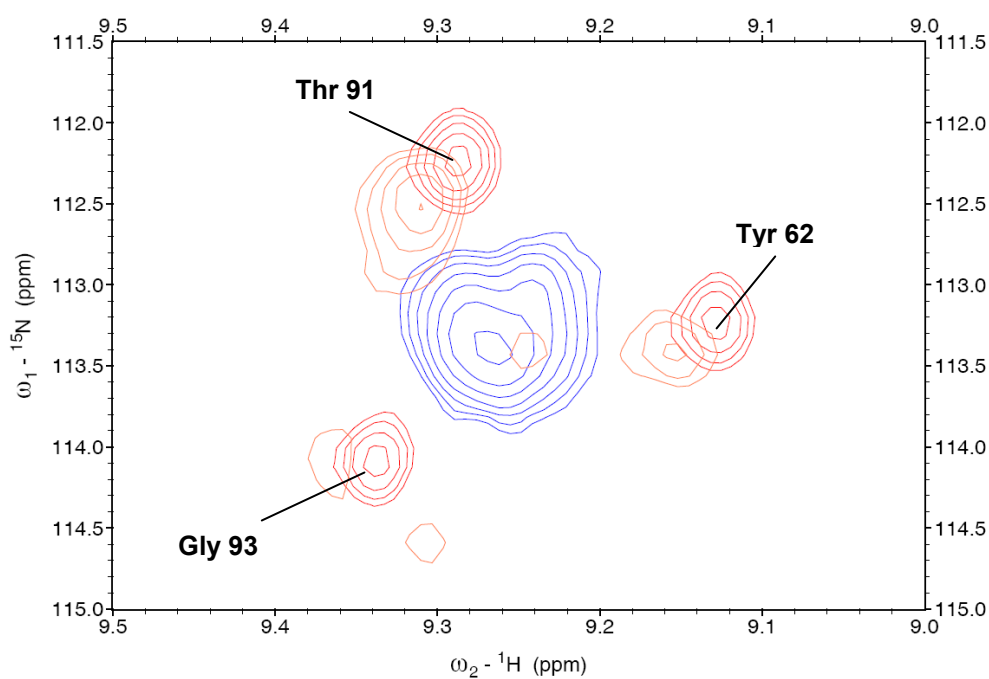


Fig. 43. Close-up of the peak shifts of Gly93, Thr91 and Tyr62 upon titration with Cat-Ga^{III}.

The close-up in Fig. 43 displays the peaks of Gly93, Thr91 and Tyr62 that appear at distinct chemical shifts in the free form and show similar chemical shifts at saturation with catechol. For Thr91 it is clearly visible that the peak at 1 eq is not directly located in between the peaks for the free and saturated protein. This means that there are more than the two forms, free and saturated, present at that ligand concentration and thus confirms the sequential binding mode. There are several possible states of the binding site present at intermediate ligand concentrations (e.g. 1 eq) with distinctly occupied binding pockets.

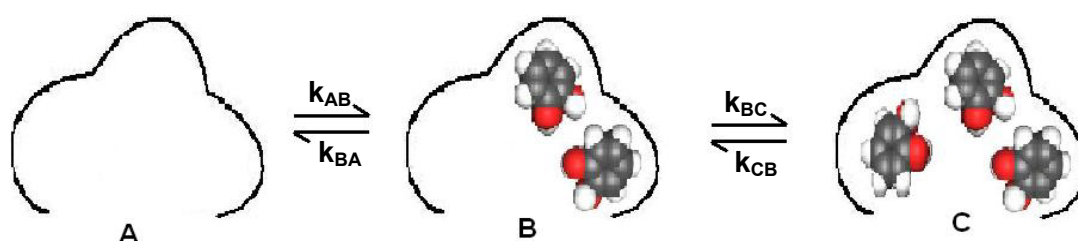


Fig. 44. Exchange between different loading states. The scheme represents the three binding pockets in the calyx of NGAL, loaded with a different number of catechol moieties (A, B, and C). The state of only one binding pocket occupied is not shown. Furthermore more states are possible if the three binding pockets are distinguished. Participation of Ga^{III} is also not included in that scheme.

The fact that only one subset of peaks is observable in the spectrum at 1 eq catechol suggests fast exchange between different loading states in the calyx. Considering for instance exchange between state A and B this means that $k_{AB} + k_{BA} \gg \Delta\omega_{AB}$. The peak appears at the average of the chemical shifts of exchanging states weighted with their populations, $\omega = p_A \cdot \omega_A + p_B \cdot \omega_B + p_C \cdot \omega_C$.

In Fig. 45 the NGAL spectrum at saturation with Ent is compared to the spectrum at saturating conditions of catechol. The spectra are almost identical which suggests a similar binding mode. Residues corresponding to the peaks that are shifted are highlighted in the protein structure below.

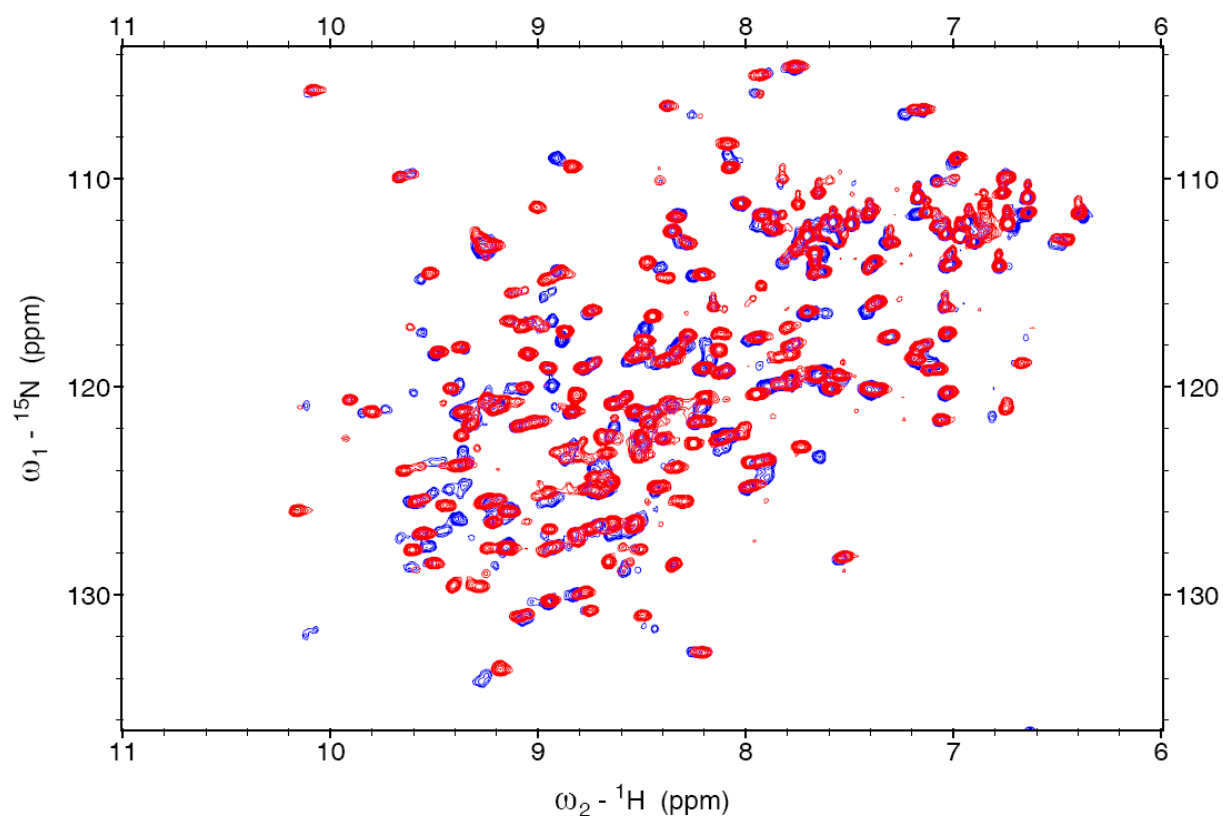


Fig. 45. HSQC spectra of NGAL saturated with Ent-Ga^{III} and Cat-Ga^{III}. Red - Ent-bound, blue - Cat-bound.

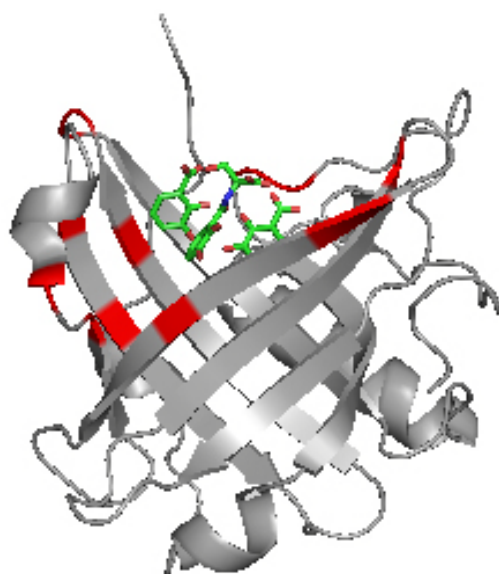


Fig. 46. Structure of NGAL complexed with apo enterobactin. Residues that show significantly different chemical shifts in Fig. 45 are highlighted in red.

The spectra for the titrations with 2,3-DHBA-Ga^{III} are shown below. The same conclusions than for binding of catechol can be drawn for 2,3-DHBA.

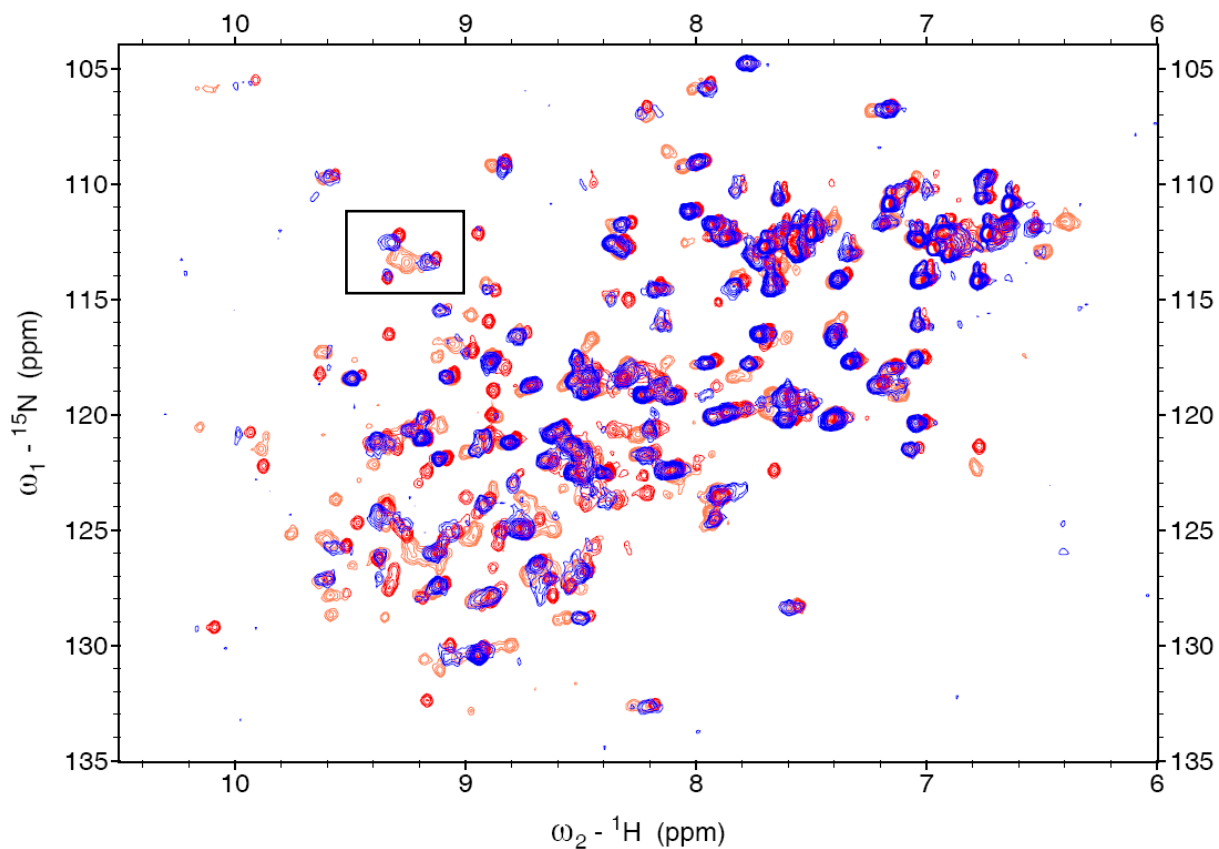


Fig. 47. HSQC titration of NGAL with 2,3-DHBA-Ga^{III}. Red - free form, blue - 1 eq DHBA, orange - 5 eq DHBA. The framed section is shown in the close-up below.

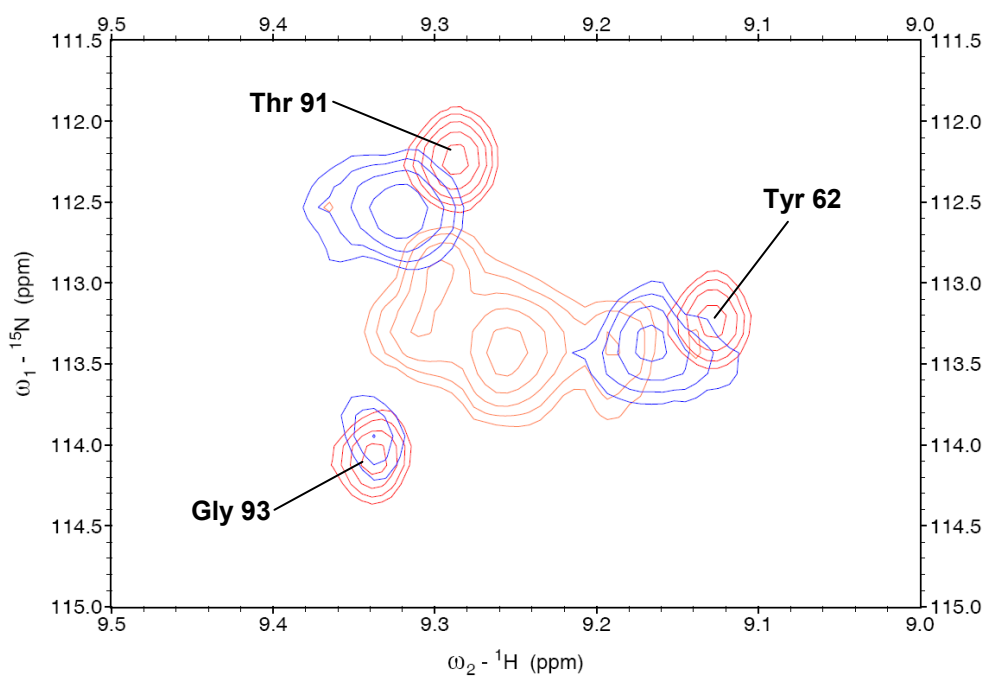


Fig. 48. Close-up of the peak shifts of Gly93, Thr91 and Tyr62 upon titration with 2,3-DHBA-Ga^{III}.

4.3.3. X-Ray Crystallography

X-ray crystallography was done in order to gain further insight into the orientation of ligands inside the calyx of NGAL. After doing a small scale crystallization screen, effective conditions were found to obtain crystals of NGAL usable for x-ray diffraction. Best yield was obtained at crystallization conditions of 1.2 M ammonium sulphate, 0.1 M sodium acetate and 93 mM NaCl at pH 4. 2,3-DHBA was tried to be bound to the calyx by co-crystallization and soaking of the crystals that were grown in ligand-free conditions. However, for all trials the electron density map revealed the sulfate-ion in the calyx but no ligand. Apparently the calyx is easily accessible for small, soluble molecules. The results of x-ray crystallography could not be employed for investigation of ligand binding.

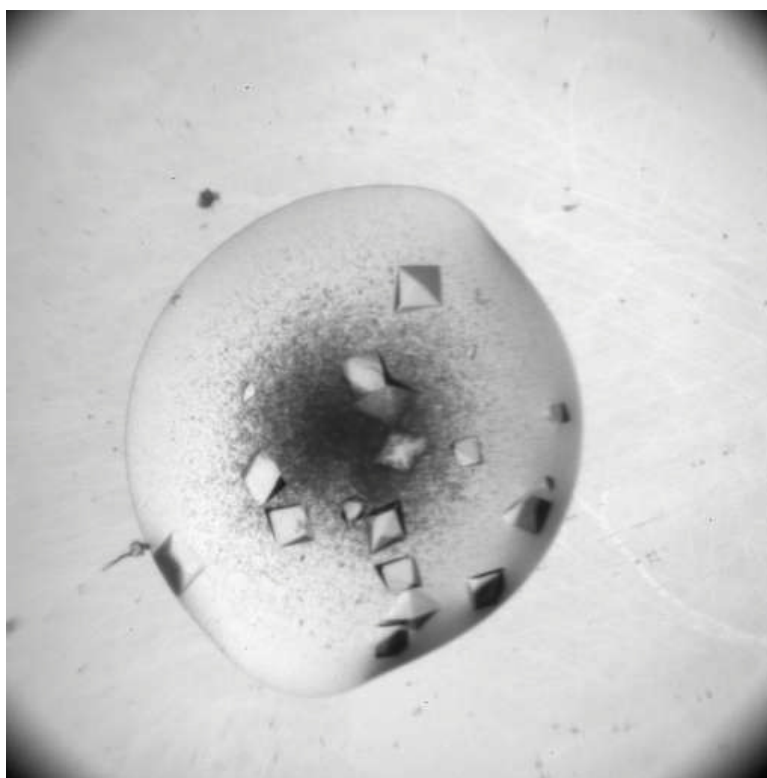


Fig. 49. Crystals of NGAL in a hanging drop. Crystallisation conditions in the drop were 1.2 M AS, 0.1 M NaAc and 93 mM NaCl at pH 4.

4.4. Fatty Acid Binding Mode of Q83

4.4.1. Delineating Significant Residues

The binding site for fatty acids is located towards the closed end of the calyx. In consideration of the solution structure (Q83/AA) binding seems to be mainly stabilized by hydrophobic contacts between aromatic residues of the protein (Tyr₅₁ and Tyr₇₆) and the aliphatic chain of AA. Tyr₉₂ seems to be important for hydrogen bonding to the polar head of the fatty acid. Also the side chains of Thr₉₀ and Arg₆ are located near the AA carboxylate. The mutants R6A, R6Q, Y51F, Y76F, T90A and Y92F were tested for their affinities to AA.

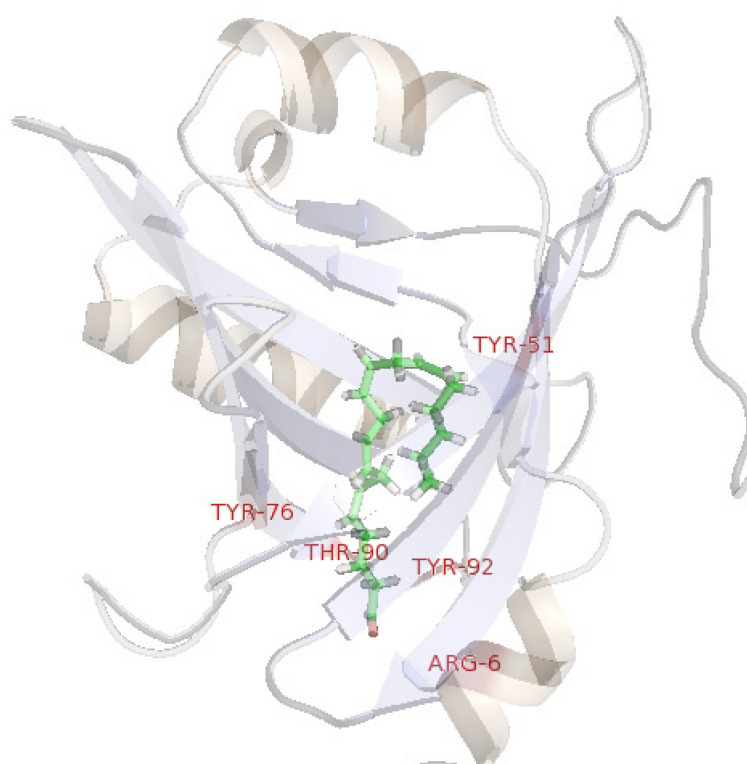


Fig. 50. Structure of Q83 with mutated residues marked in red. Arachidonic acid is shown in stick representation.

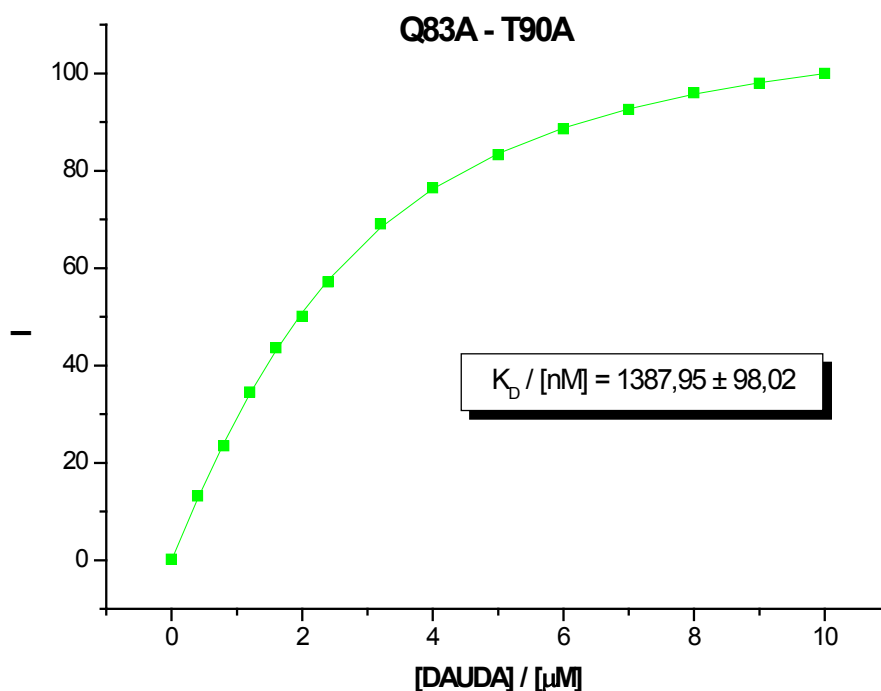


Fig. 51. DAUDA titration of the mutant T90A, monitored by fluorescence analysis. The K_D for DAUDA was determined for every mutant. The saturated protein was subsequently titrated with AA.

Most significant reduction in affinity was observed for deprivation of the hydroxyl group of Tyr₉₂ or Thr₉₀, respectively. The Y92F mutant showed a K_D of 1864 nM, compared to 26 nM for the wild type. A dissociation constant of 1078 nM was revealed for the mutant T90A. The hydroxyl groups of Tyr₇₆ and Tyr₅₁ seem to be less crucial. A K_D of 174 nM for Y76F and 108 nM for Y51F was measured. Mutation of Arg₆ into glutamine slightly reduced the affinity to 42 nM. A stronger effect was shown for mutation of the same residue to alanine, resulting in a K_D of 120 nM.

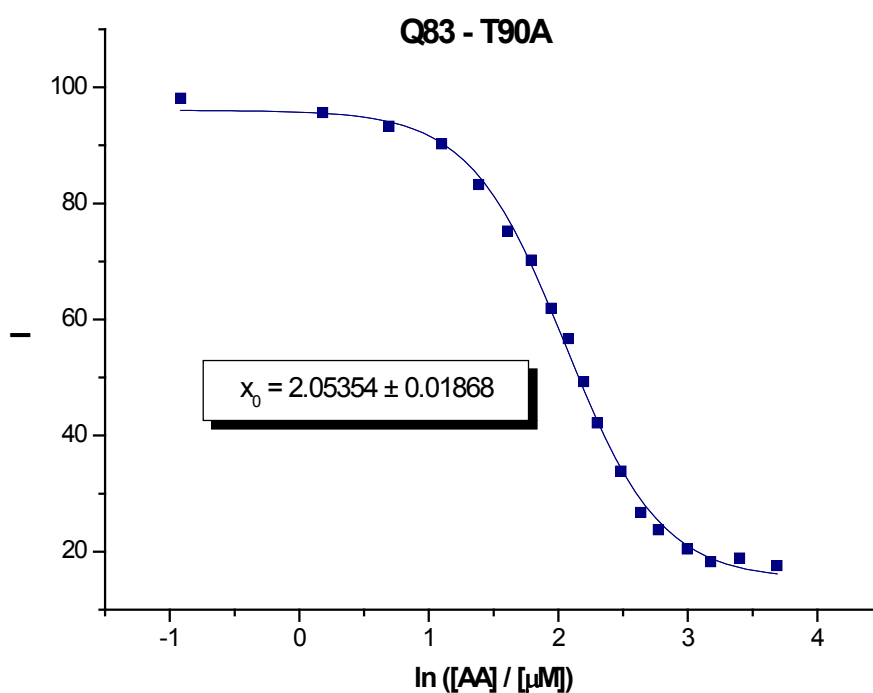


Fig. 52. AA titration of the mutant T90A after saturation with DAUDA. x_0 gives $\ln([AA])$ at the inflection point. Its exponential reveals the AA concentration at which DAUDA and AA are bound to the protein in equal amounts.

Q83 mutant	Ligand	K_D / [nM]
R6A	DAUDA	115
	AA	42
R6Q	DAUDA	120
	AA	98
Y51F	DAUDA	296
	AA	108
Y76F	DAUDA	160
	AA	174
T90A	DAUDA	1388
	AA	1078
Y92F	DAUDA	265
	AA	1864

Table 3. Affinities of different Q83 mutants for DAUDA and AA.

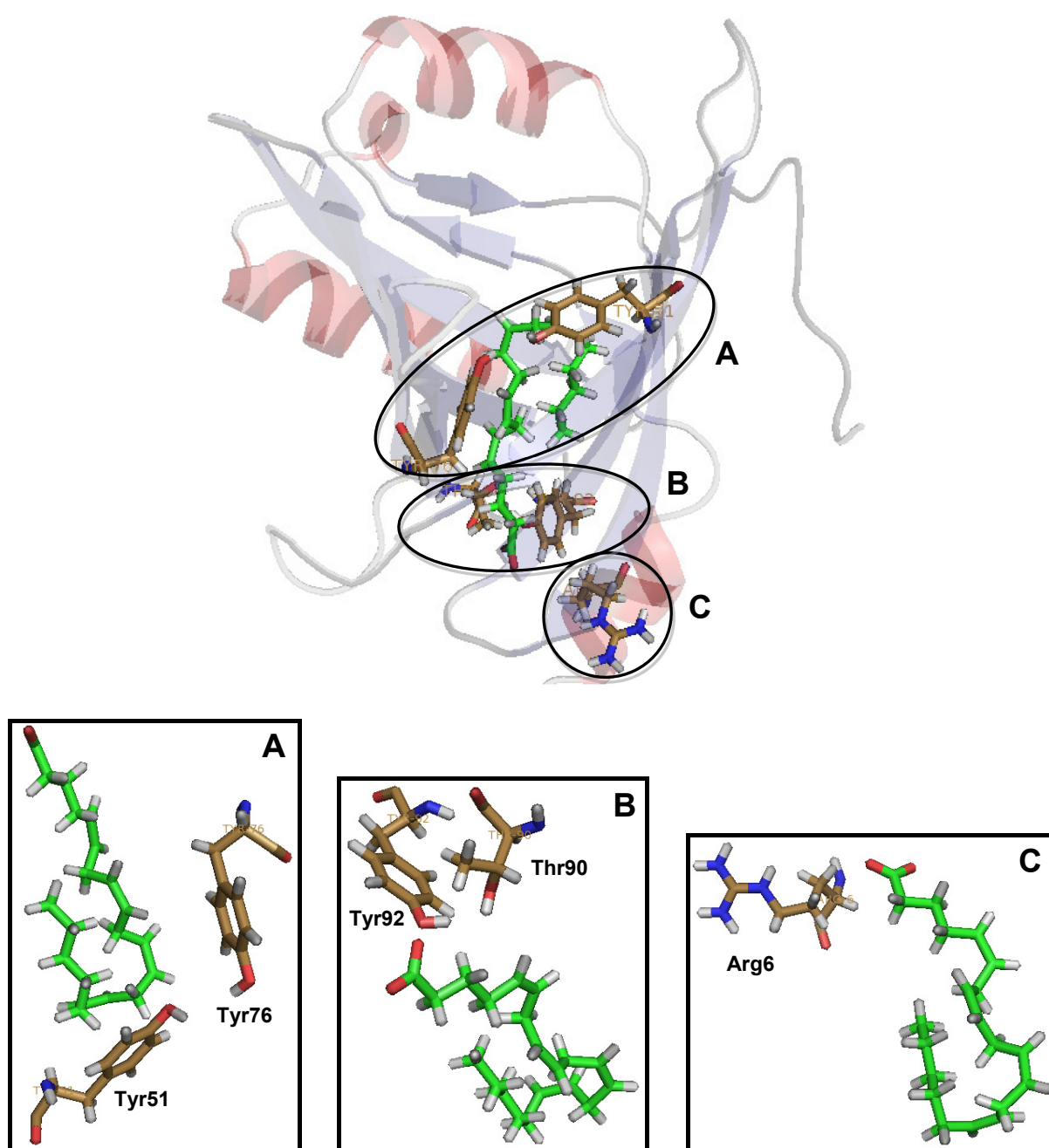


Fig. 53. Positions of the mutated residues with respect to bound arachidonic acid. The orientation of the close-ups is different to the protein structure above.

4.4.2.Chain Length Dependence

Various fatty acids with different chain length and number of double bonds were investigated upon binding to Q83 by fluorescence quenching.

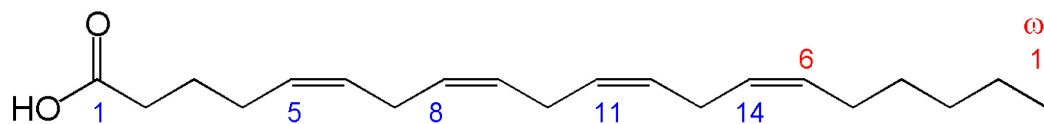


Fig. 54. Arachidonic acid (AA). The configuration of all four double bonds is cis. The actual structure is not linear but bent around C-10.

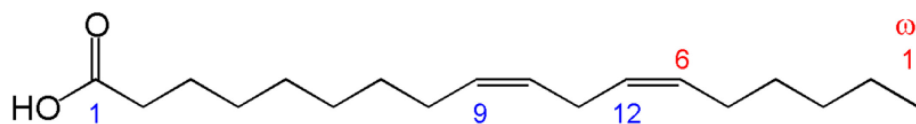


Fig. 55. Linoleic acid (LA). The actual structure is curved due to the rigid cis double bonds.

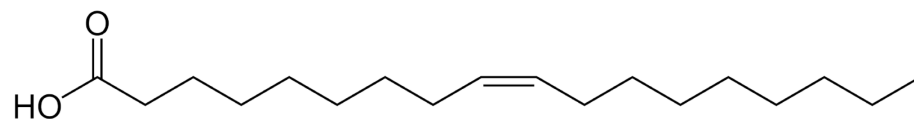


Fig. 56. Oleic acid (OA). The real structure reveals a kink due to the cis bond.

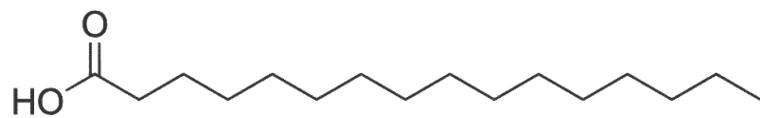


Fig. 57. Palmitic acid (PA).

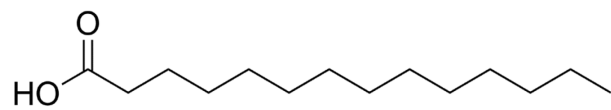


Fig. 58. Myristic acid (MA).

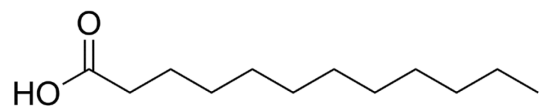


Fig. 59. Lauric acid (LAUA).

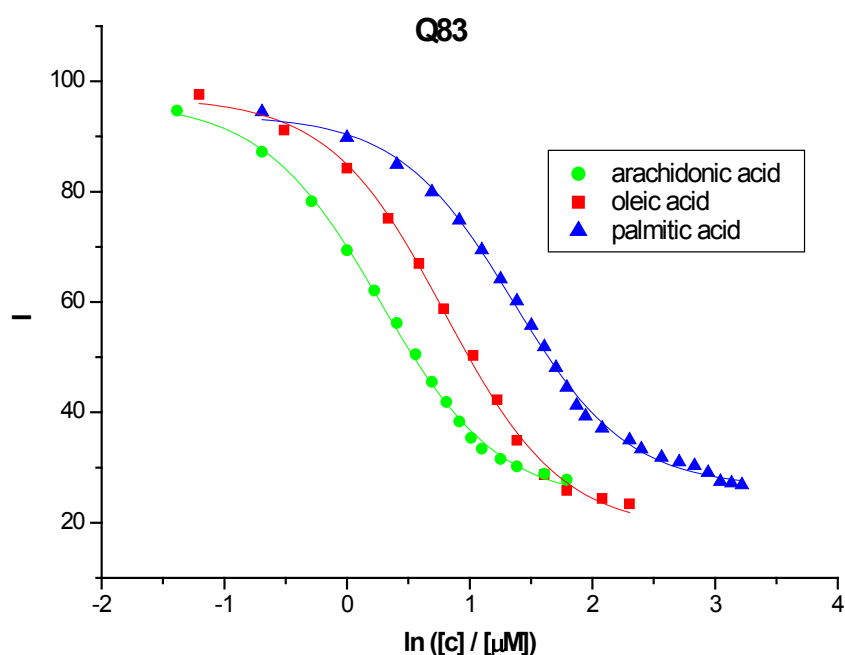


Fig. 60. Replacement of DAUDA bound to Q83 upon addition of fatty acid. Fluorescence intensity is plotted against the natural logarithm of fatty acid concentration. The inflection points are revealed by a sigmoidal fit.

The affinity for fatty acids decreased quite linearly with chain length. The K_D of 26 nM for arachidonic acid (C20:4 all-cis-5,8,11,14) approximately doubled to 40 nM for linoleic (C18:2 cis,cis-9,12) and oleic acid (C18:1 cis-9). Reduction of double bonds doesn't seem to affect the affinity. For the other tested fatty acids affinity further decreased with reduction of chain length from 77 nM for palmitic acid (C16:0) to 100 nM for myristic acid (C14:0) and 200 nM for lauric acid (C12:0).

Ligand	K_D / [nM]
DAUDA	104
AA	26
OA	44
LA	43
PA	77
MA	103
LAUA	200

Table 4. Dissociation constants of Q83 for different fatty acids.

If the K_D values are plotted against the number of C-atoms of the corresponding fatty acids, the curve can be fit with a first order exponential decay.

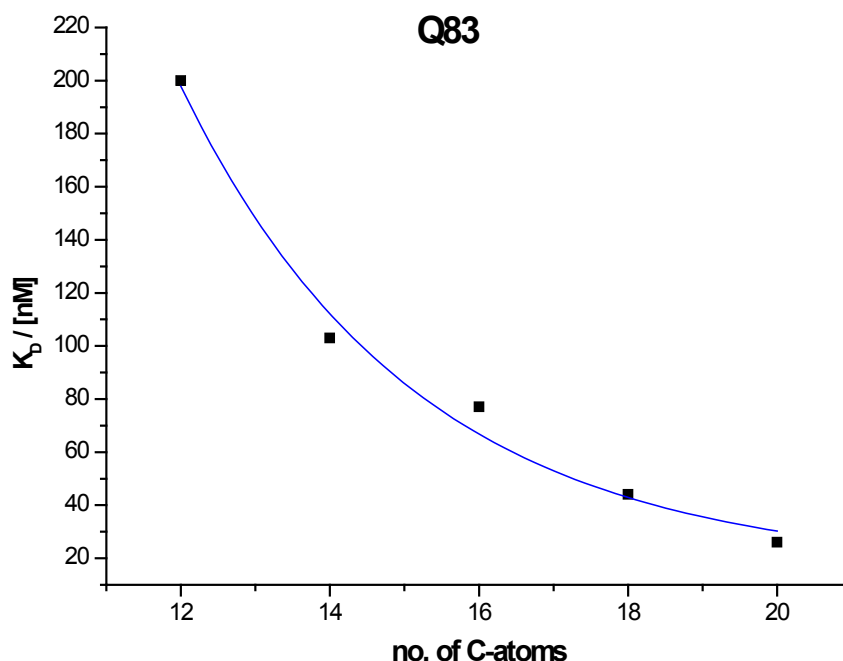


Fig. 61. Dissociation constants plotted against the number of carbons of the corresponding fatty acids.

Considering that $K = -e^{-\Delta G/RT}$, the exponential decay indicates that, beginning from lauric acid (C12), every additional CH_2 group reduces the ΔG by a constant factor. Rewriting the equation gives $\Delta G = -RT \ln K$. The calculated ΔG values for the different fatty acids and the corresponding $\Delta\Delta G$ per CH_2 group are given in the following table. Except for the outlier of palmitic acid the results indeed show a relatively constant contribution of every additional CH_2 group to the binding affinity. The conclusion is that increasing affinity with chain length can be attributed to the hydrophobic effect. The nonpolar fatty acid chain minimizes the contact with the solvent by burying inside the protein, thereby increasing the entropy of the solvent. The longer the hydrocarbon chain, the higher is the gain of entropy and therefore the absolute value of ΔG . It should be noted that ΔG values are given for the dissociation reaction, which means that a positive ΔG favours the protein-ligand complex.

Fatty acid	Lipid numbers	ΔG /[J/mol]	$\Delta\Delta G$ /[J/mol] per CH ₂
LAUA	C12:0	37837	
MA	C14:0	39465	814
PA	C16:0	40179	357
LA	C18:2	41608	715
OA	C18:1	41552	686
AA	C20:4	42842	617

Table 5. Calculated ΔG values for binding of the different fatty acids and contributions per CH₂ group.

4.5. Dual Ligand Binding Mode of Q83

The solution structure of the Q83-Ent-AA-complex and HSQC peak shifts revealed that the protein can bind both ligands simultaneously (46). By fluorescence quenching analysis it should be investigated how the binding of one ligand affects the affinity for the second ligand at the other binding site of Q83. Possible allosteric mechanisms upon binding of specific unsaturated fatty acids or enterobactin binding should be revealed.

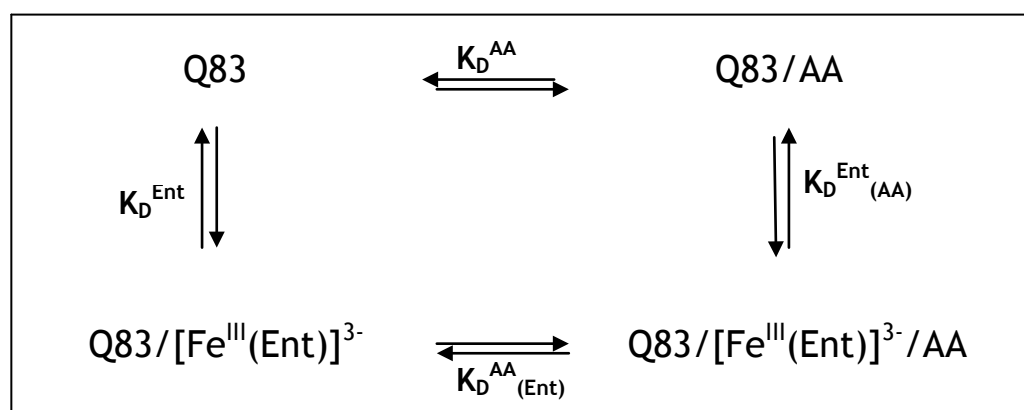


Fig. 62. Schematic representation of the dual binding mode of Q83. The K_D superscript denotes the ligand which is binding. The subscript in brackets indicates the corresponding Q83 form.

4.5.1. Affinity for Ent to AA-bound Form

The K_D for enterobactin in the presence of bound arachidonic acid ($K_D^{\text{Ent}}_{(\text{AA})}$) could be determined directly by ordinary tryptophan fluorescence quenching.

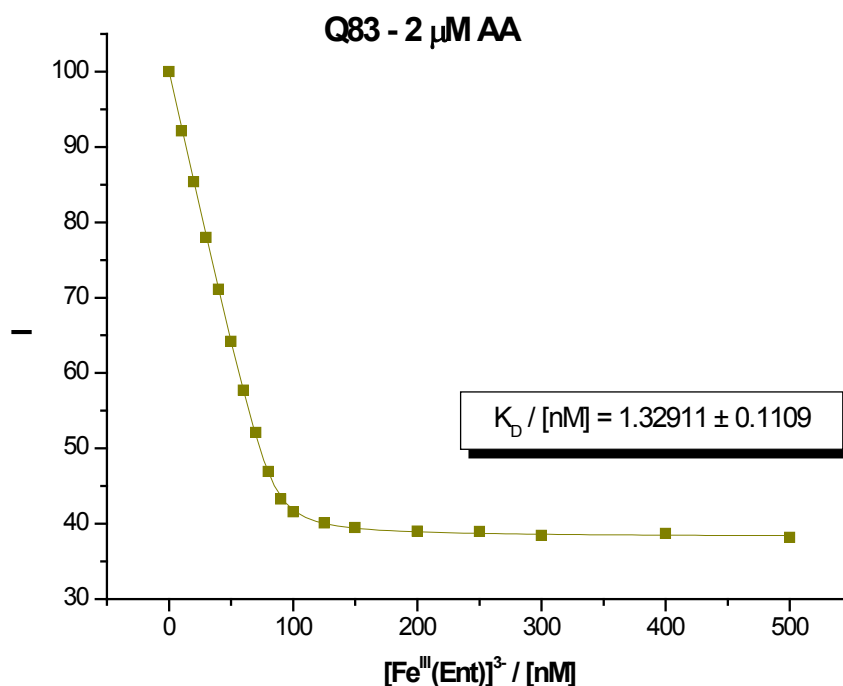


Fig. 63. Fluorescence quenching upon addition of Ent at saturating AA concentration.

A slight decrease in affinity of Q83 for $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$ in the AA-bound form ($K_D^{\text{Ent}}_{(\text{AA})} = 1.33 \text{ nM}$) was observed, compared to the free form ($K_D^{\text{Ent}} = 0.54 \text{ nM}$). The raise in K_D is not dramatic but indicates that the change in the potential energy surface upon AA binding is transmitted to the Ent binding site.

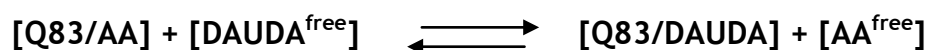
4.5.2. Affinity for AA to Ent-bound Form

The affinity for arachidonic acid when enterobactin is bound ($K_D^{\text{Ent}}_{(\text{AA})}$) is more complicated to investigate, as the enterobactin bound in the calyx quenches DAUDA fluorescence. Hence, only the binding of the fatty acid to the Ent-free

Q83 can be monitored directly. However, in the presence of both, Ent-free and Ent-bound Q83, conclusions can be drawn on the affinity to Ent-bound protein by measuring DAUDA fluorescence quenching.

Some approximations have to be made for those calculations and therefore the obtained dissociation constants should rather be considered as qualitative bench marks than exact quantitative values.

First an apparent dissociation constant ($K_{D(app)}$) of AA and DAUDA-bound Q83 is determined. For this purpose a fluorescence quenching experiment is consulted, where DAUDA was applied at saturating concentration (5 μ M DAUDA and 2 μ M Q83) and the mixture was titrated with AA. This is exactly the experiment that was carried out for measuring the K_D of AA and Q83 but this time the K_D for the following dissociation reaction is calculated.



This is a coupled equilibrium between two dissociation reactions but for determining the $K_{D(app)}$ it is treated as if $[DAUDA^{free}]$ would be constant. $K_{D(app)}$ can be expressed in the following way.

$$K_{D(app)} = [AA^{free}] * [Q83/DAUDA] / [Q83/AA]$$

$K_{D(app)}$ is obtained by fitting the titration curve the same way as done for direct quenching in the calyx (Fig. 63).

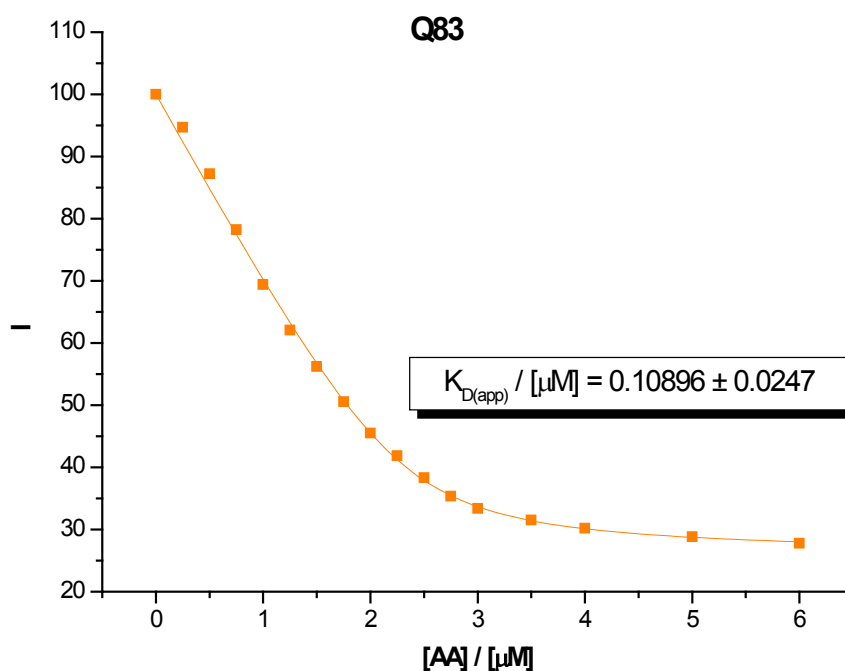


Fig. 64. Fluorescence decrease due to DAUDA replacement by AA in the Q83 secondary binding site. The curve is fit by the one-ligand equation which provides an apparent K_D , $K_{D(app)}$.

In other experiments, Ent-bound and Ent-free Q83 was provided in the cuvette in certain ratios, whereas Ent was assumed to be all bound to the protein. The following setups were tried.

	$[Q83^{free}] / [Q83^{Ent}]$
3 μM Q83 1 μM $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$ 5 μM DAUDA	2 μM / 1 μM
4 μM Q83 2 μM $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$ 5 μM DAUDA	2 μM / 2 μM
6 μM Q83 4 μM $[\text{Fe}^{\text{III}}(\text{Ent})]^{3-}$ 5 μM DAUDA	2 μM / 4 μM

The calculation is shown for a $[Q83^{free}] / [Q83^{Ent}]$ ratio of $2 \mu\text{M} / 2 \mu\text{M}$. Fluorescence decrease was monitored as usual upon addition of AA and plotted against $\ln[AA]$.

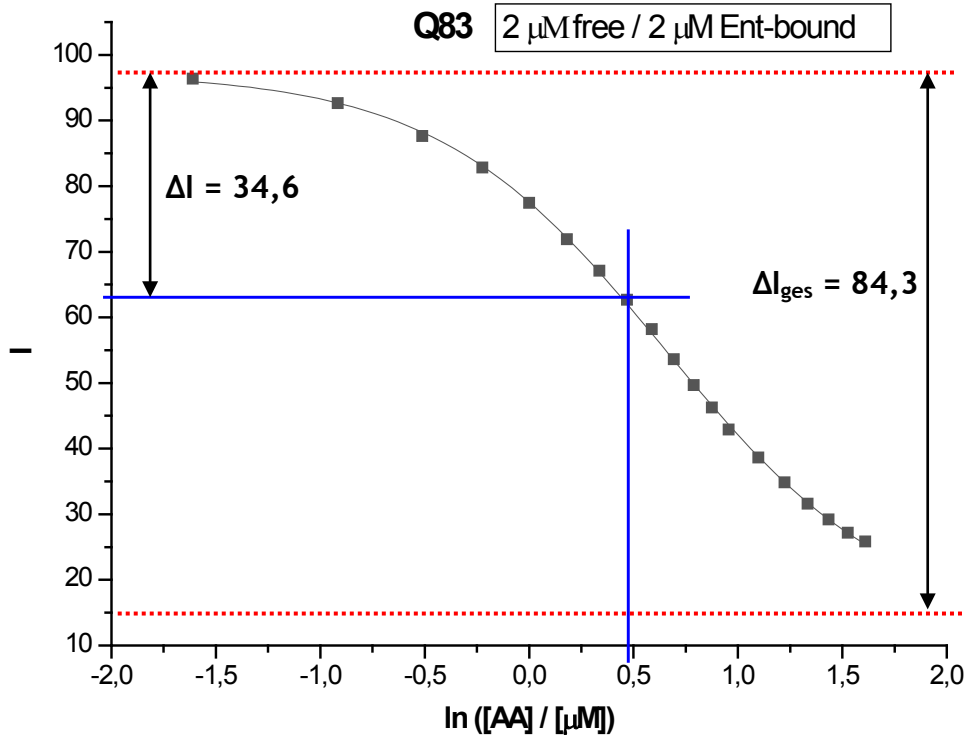


Fig. 65. Fluorescence decrease upon addition of AA. Only the DAUDA replacement in the Ent-free Q83 is monitored as the fluorescence of DAUDA bound to Ent-bound Q83 is quenched. The calculation explained in the text was done at $1.6 \mu\text{M}$ AA, indicated by the blue lines, corresponding to 41% of total intensity decline.

The minimal intensity at complete saturation with AA is determined. The ratio of Ent-free protein bound to AA ($[Q83^{free}/AA]$) to total Ent-free protein ($[Q83^{free}]$) can be calculated at a certain AA concentration by dividing the decrease of fluorescence intensity at that concentration through the total decrease in intensity.

$$[Q83^{free}/AA] / [Q83^{free}] = \Delta I / \Delta I_{ges}$$

In Fig. 64 this is shown schematically for $[AA] = 1.6 \mu\text{M}$ or $\ln([AA]/[\mu\text{M}]) = 0.47$, respectively. The following calculation is carried out at that AA concentration.

$$[Q83^{\text{free}}/AA] / [Q83^{\text{free}}] = 0.41$$

As the Ent-free Q83 concentration ($[Q83^{\text{free}}]$) is known (2 μM), the Ent-free Q83 concentration, which is bound to AA ($[Q83^{\text{free}}/AA]$) can be calculated.

$$[Q83^{\text{free}}/AA] = 2 \mu\text{M} * 0.41 = 0.82 \mu\text{M}$$

Assuming that all protein is either in DAUDA-bound or AA-bound form, the DAUDA-bound protein concentration ($[Q83/DAUDA]$) is obtained.

$$[Q83/DAUDA] = [Q83^{\text{free}}] - [Q83^{\text{free}}/AA] = 1.18 \mu\text{M}$$

The values for $[Q83/DAUDA]$ and $[Q83^{\text{free}}/AA]$ are then substituted into the equation for $K_{D(\text{app})}$. Now the free AA concentration ($[AA^{\text{free}}]$) can be calculated.

$$K_{D(\text{app})} = [AA^{\text{free}}] * [Q83/DAUDA] / [Q83/AA] \Rightarrow [AA^{\text{free}}] = 0.076 \mu\text{M}$$

A second equation is expressed, describing the K_D of Ent-bound Q83 for AA.

$$K_D^{\text{Ent}}_{(AA)} = [AA^{\text{free}}] * [Q83^{\text{Ent}}_{\text{free}}] / [Q83^{\text{Ent}}/AA]$$

$K_D^{\text{Ent}}_{AA}$... K_D of Ent-bound Q83 for AA

$[AA^{\text{free}}]$... concentration of free AA

$[Q83^{\text{Ent}}_{\text{free}}]$... concentration of Ent-bound protein without AA bound

$[Q83^{\text{Ent}}/AA]$... concentration of Ent- and AA-bound protein

$[AA^{free}]$ is already known. $[Q83^{Ent}/AA]$ is obtained by subtracting the AA concentration that is bound to Ent-free Q83 and the free AA concentration from the total AA concentration at the investigated titration point.

$$[Q83^{Ent}/AA] = [AA] - [Q83^{free}/AA] - [AA^{free}] = 0.704 \mu M$$

Consequently $[Q83_{free}^{Ent}]$ can be calculated as follows.

$$[Q83_{free}^{Ent}] = [Q83^{Ent}] - [Q83^{Ent}/AA] = 1.296 \mu M$$

Finally $K_D^{Ent}_{AA}$ is obtained.

$$K_D^{Ent}_{(AA)} = [AA^{free}] * [Q83_{free}^{Ent}] / [Q83^{Ent}/AA] = 0.139 \mu M = \underline{139 \text{ nM}}$$

With a second calculation at the point of 3 μM $[AA]$ a K_D^{Ent} of 123 nM was obtained.

For the ratio 2 μM free / 1 μM Ent-bound Q83, a dissociation constant of 160 nM was determined. A lower affinity was obtained for the setup 2 μM free / 4 μM Ent-bound Q83 with a K_D of 247 nM.

In summary the measurements revealed a decrease of AA affinity by a factor of five to ten for Ent-bound Q83 compared to free protein. Thus the effect of bound Ent on AA binding is bigger than for the reverse case.

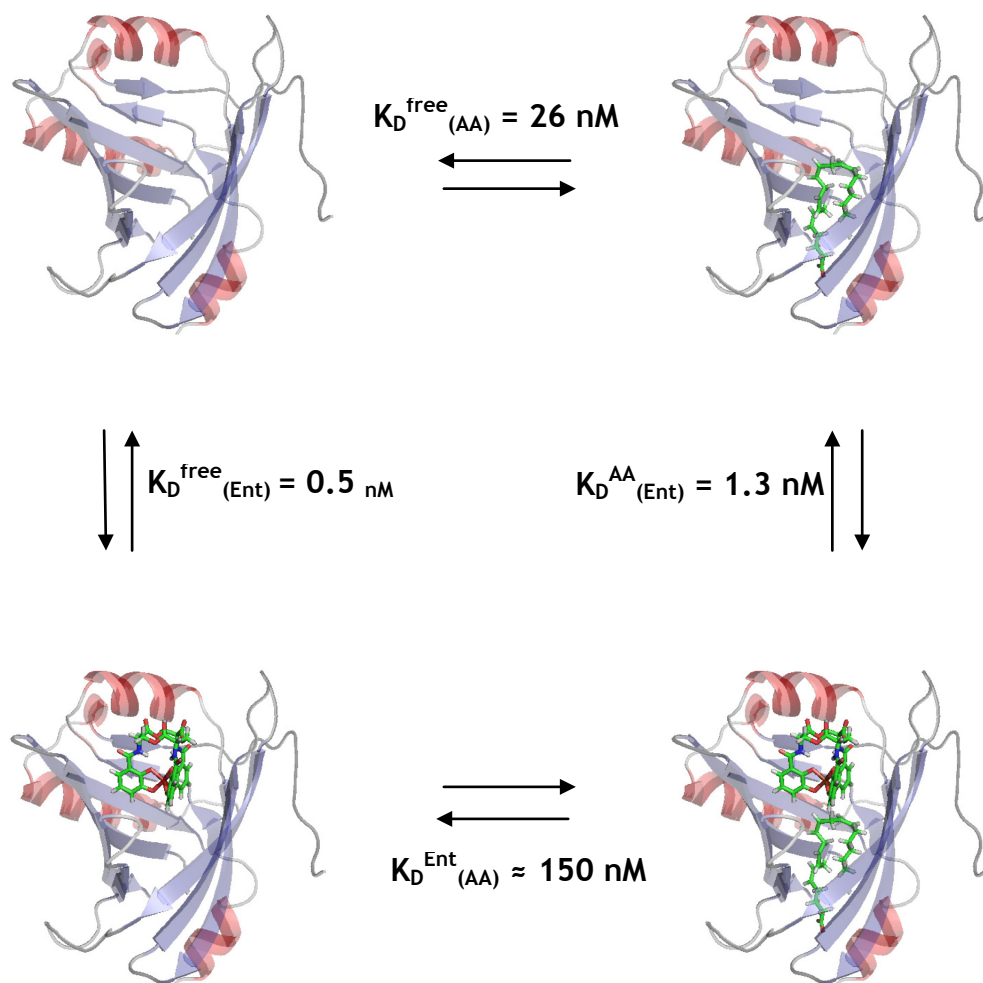


Fig. 66. Dual binding mode of Q83. The scheme is analogous to Fig. 62. Binding of one ligand reduces the affinity for the other ligand in the second binding site. Bound enterobactin seems to have a significant effect on the affinity for arachidonic acid, whereas minor influence was found for the reverse case.

5. DISCUSSION

5.1. Siderophore Binding

So far NGAL has been the only eukaryotic protein shown to bind siderophores. The data provided here completes evidence for Q83 to be a member of the siderocalin family. In fact it binds siderophores with a very similar binding mode and affinity to NGAL, in spite of a substantially different calyx construction.

Structural features for the Ent-siderocalin interaction could be characterized by mutant studies, complementing the picture from the already available Ent/Q83[Ga^{III}(Ent)]³⁻ solution structure. Residues essential for interaction are Trp₆₂, Lys₈₃ and Arg₁₁₃, analogous to Trp₇₉, Lys₁₂₅ and Lys₁₃₄ in NGAL. This set of structurally conserved residues seems to be representative for the siderocalin protein family.

Evidence that Q83 and NGAL are homologs is completed by the fact that both proteins are involved in the same biological processes, e.g. immune response (8, 37), inflammation (13), tissue differentiation (10) and cancer progression (14). The roles of NGAL and Q83 in innate immune response and iron delivery pathways can directly be attributed to their abilities to bind siderophores. Other functions are most likely also intimately connected to the ligand binding properties of the proteins but eventually related to a secondary binding site.

5.2. Fatty Acid Binding

A very exceptional feature of Q83 is the existence of two distinct binding sites (46). Even if biological mechanisms are not yet understood, the fatty acid binding site might play a key role for deciphering the various functions of the protein. AA, which showed the highest affinity among the tested fatty acids, is a key inflammatory intermediate and acts in regulation of several signalling enzymes.

Fatty acid binding to NGAL could not be demonstrated so far but still it seems to be evident that the human protein also comprises the secondary binding site. Besides analogous functions, NGAL exhibits a very similar structure to Q83. Residues which were shown to be crucial for binding in Q83 are present at comparable regions in NGAL. Thr₉₀ and Tyr₉₂ of Q83, which turned out to be of major importance, might have their analogues in Thr₁₁₄ and Tyr₁₁₆ in the human protein.

A possible reason why binding could not be observed might be that fatty acids, in contrast to siderophores, possibly access their binding site via the narrower end of the β -barrel. In this region an N-terminal extension of the amino acid sequence is found in NGAL, which is not present in Q83. This loop sequence is responsible for the 3_{10} -helix to be located directly in front of the opening at that end of the protein and consequently fatty acid binding might be prevented in NGAL (Fig. 67). Modification of the helix in vivo, e.g. proteolytic cleavage would then be necessary to make the secondary binding site accessible.

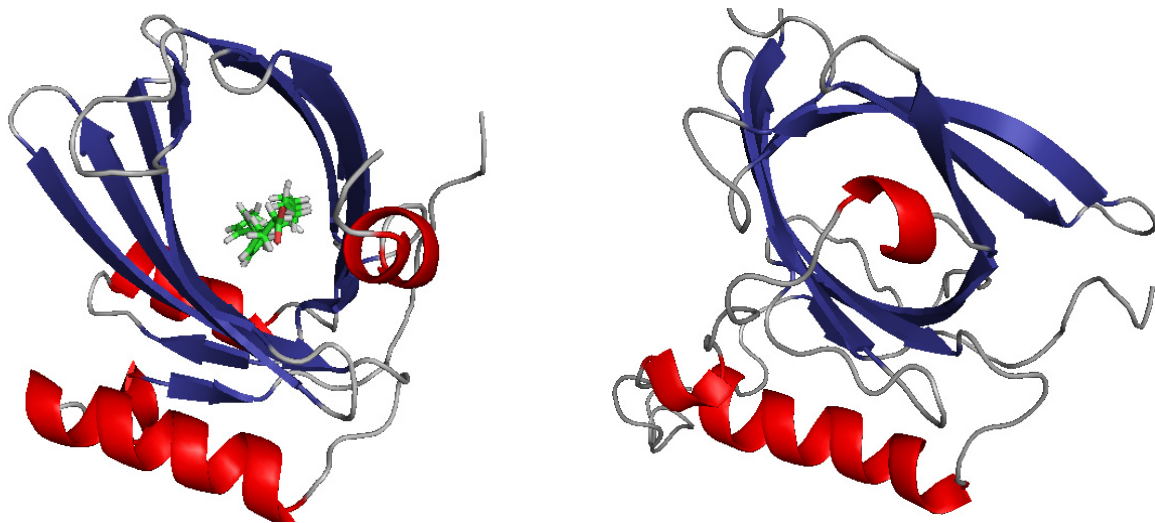


Fig. 67. Bottom view of Q83 (left) and NGAL (right). The 3_{10} -helix in NGAL inhibits access to the interior of the protein.

In an attempt to demonstrate ligand binding in the fatty acid binding site, a mutant of NGAL that lacks the N-terminal extension was cloned but could not be expressed successfully.

From studies on Q83 it can be inferred that binding of Ent is not beneficial for the affinity to AA. The Ent bound protein revealed an approximately six times higher dissociation constant for AA than the free form of Q83. This reduction in affinity is possibly due to a decrease in protein dynamics upon ligand binding. The CPMG for NGAL revealed that pronounced dynamics in the free form of the protein is almost entirely quenched in the Ent-bound protein.

5.3. Outlook

Further research for Q83 could include a more detailed investigation of its dual binding mode in consideration of possible allosteric mechanisms. As two binding sites are present in a single protein, biologically relevant correlation is suggested, however, could not be demonstrated so far. Saturation with AA reduced the affinity for Ent by a factor of two. Other fatty acid ligands could be tried for a possibly more significant effect on Ent-binding. For the reverse case, probing affinities for fatty acids in the presence of bound Ent, a more effective method would have to be established, providing reliable dissociation constants for various fatty acids.

If the findings of Q83 could be applied on its human homologue NGAL, this would be a major step towards elucidation of its pleiotropic functions.

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7. APPENDICES

Zusammenfassung

NGAL ist ein 178 Aminosäuren großes, menschliches Protein und gehört zur Familie der Siderocaline. Es kommt in sekretorischen Vesikeln von Granulozyten vor. Siderocaline sind eine Unterklasse der Lipocaline. Charakteristisch für diese Proteinfamilie sind zum Einen die Struktur eines acht-strängigen, antiparallelen β -Fasses und zum Anderen die Bindung von kleinen, hydrophoben Liganden. Siderocaline im Speziellen binden als Liganden sogenannte Siderophore, eisen-komplexierende Moleküle. Siderophore werden beispielsweise von Bakterien ausgeschieden, um Eisen ihres Wirtes zu komplexieren und daraufhin wieder in die Zelle transportiert zu werden. Eisen wirkt auf Grund seiner geringen Bioverfügbarkeit oft als limitierender Faktor bakteriellen Wachstums. Siderocaline können in diesem Zusammenhang als Mechanismus des Wirtsorganismus verstanden werden, um dieser Eisendepletion entgegen zu wirken. NGAL spielt neben der eben beschriebenen Funktion bei bakteriellen Infektionen aber eine Rolle bei einer Vielzahl anderer Prozesse, wie z. B. Entzündungen, Eisentransport, Gewebsdifferenzierung und Krebsentwicklung. Es scheint sehr wahrscheinlich, dass diese Funktionen direkt mit den Ligandenbindungseigenschaften zusammenhängen.

Q83 ist das Wachtel-Homologe von NGAL und ist als solches in der Forschung wesentlich weniger behandelt. Trotz sehr geringer Sequenzidentität mit NGAL weist es vergleichbare Funktionen und sehr ähnliche Struktureigenschaften auf. Die bei Q83 kürzlich entdeckte, zweite Bindungsstelle für Fettsäuren (46), könnte sich als wesentlicher Faktor zur Aufklärung seiner pleiotropen Funktionen erweisen.

In der vorliegenden Diplomarbeit wurden die Bindungseigenschaften von NGAL und Q83 zu verschiedenen Liganden untersucht. Fluoreszenzquenching Experimente und makromolekulare NMR Spektroskopie an bestimmten Mutanten von Q83 lieferten detaillierte Informationen des Siderophor-Bindungsmechanismus. Außerdem konnte die Fettsäure-Bindungsstelle von Q83 charakterisiert werden. Ein Einblick in die

Dynamik von freiem NGAL und des Protein-Ligand-Komplexes, mit dem Siderophor Enterobactin gebunden, wurde ebenfalls mittels NMR spektroskopischer Untersuchungen gegeben.

Abstract

NGAL is a 178 amino acids human protein, belonging to the siderocalin family. It is found in secretory vesicles of granulocytes. Siderocalins are a subclass of lipocalins. This protein family is characterized structurally by the formation of an eight-stranded, antiparallel β -barrel and functionally by binding small, hydrophobic ligands. The ligands of siderocalins are so called siderophores, iron-chelating molecules. Siderophores are for instance excreted by bacteria, in order to capture iron from its host and transport it into the cell. Due to its low bioavailability, iron often accounts as limiting factor for bacterial growth. In this context siderocalins can be seen as the host's mechanism to counteract iron-depletion. Besides this function in bacterial infection, NGAL plays a role in various other processes, e.g. inflammation, iron delivery, tissue differentiation and cancer progression. These functions are most likely intimately connected to the ligand binding properties.

Q83 is the quail homologue of NGAL and is essentially less examined. In spite of low sequence identity with NGAL, it shows very similar functions and an analog structural fold. Q83's newfound secondary binding site for fatty acids (46) might be a key feature for deciphering its pleiotropic functions.

In this thesis the binding properties of NGAL and Q83 to different ligands were investigated. Fluorescence quenching experiments and macromolecular NMR spectroscopy on various Q83 mutants provided detailed information on the siderophore binding mechanism. Furthermore the fatty acid binding site of Q83 was characterized. An insight into the dynamics of free NGAL and the protein-ligand complex, with the bound siderophore enterobactin, was given using NMR spectroscopic methods.

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