Preorganization of the catalytic Zn^{2+}-binding site in the HNH nuclease motif – a solution study

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Abstract

The structure of the active site in a metalloenzyme can be a key determinant of its metal ion binding affinity and catalytic activity. In this study, the conformational features of the Zn^{2+}-binding HNH motif were investigated by CD-spectroscopy in combination with isothermal microcalorimetric titrations. Various point mutations, including T454A, K458A and W464A, were introduced into the N-terminal loop of the nuclease domain of colicin E7 (NColE7). We show that the folding of the proteins was severely disturbed by the mutation of the tryptophan residue. This points to the importance of W464, being a part of the hydrophobic core located close to the HNH-motif. ITC demonstrated that the Zn^{2+}-binding of the mutants including the W464 site became weak, and according to CD-spectroscopic measurements the addition of the metal ion itself can not fully recover the functional structure. Titrations with Zn^{2+}-ion in the presence and absence of the Im7 protein proved that the structural changes in the unfolded mutant included the HNH-motif itself. The metal-binding of the NColE7 mutants could be, however, fully rescued by the complexation of Im7. This suggests that the formation of a preorganized metal-binding site – existing in the wild-type enzyme but not in the W464 mutants – was induced by Im7. The low nuclease activity of all W464A mutants, however, implies that the interactions of this tryptophan residue are required for precise location of the catalytic residues, i.e. for stabilization of the fine-structure and of the tertiary structure. Our results contribute to the understanding of the metal binding site preorganization.

Keywords: HNH-motif; metallonuclease; Zn^{2+}-binding; protein folding; ITC

1. Introduction

Metal-binding sites in proteins may have structural or catalytic role. In the former case the metal ion induces the formation of a specific structure, allowing for the protein function. The catalytic sites are often preorganized to form the optimal conformation for metal ion binding even in the absence of metal ions. The coordination site of catalytic Zn^{2+}-ions frequently contains two amino acid side-chains close in the sequence, separated only by one or a couple of residues, providing an anchor for the metal binding. The third ligand further in the sequence (within 200 residues) is responsible for the structural properties of the active site. This arrangement can provide a high affinity Zn^{2+}-binding site with some flexibility [1]. It is commonly observed, that the catalytic Zn^{2+}-binding site consists of mainly hydrophilic residues, but this is surrounded by a hydrophobic core. As an example, the mutation of residues F93, F95 and W97 in the carbonic anhydrase II enzyme resulted in decrease of selectivity towards Zn^{2+} over other metal ions, and decreased the stability of the structure [2]. Based on theoretical calculations compared with crystal structures it was observed, that the donor atom – Zn^{2+} distances are longer in the catalytic, than in the structural Zn^{2+}-sites [3]. The longer distances may allow for the binding of an additional ligand, the change of the coordination number during reaction, and affect the Lewis acidity of Zn^{2+}. This can explain the role of protein environment in effective catalysis in contrast to the metal-ligand model complexes. Catalytic Zn^{2+}-sites rarely contain Cys residues, because the S-donoratoms increase the electron density on the metal ion and thus, it is less probable to act as a Lewis acid. The water molecule in the catalytic site can be ionized to a hydroxide ion (e.g. carbonic anhydrase), polarized by a general base to generate nucleophile for catalysis (e.g. carboxypeptidase *A*) or exchanged to the substrate (e.g. alkaline phosphatase) [1]. In the hydrolytic enzymes the Zn^{2+}-ion is responsible not only for activating a molecule for nucleophilic

attack, but also for polarizing the scissile bond, and stabilizing the negatively charged transition state. The Zn^{2+}-polarized water molecule can also provide a proton for the leaving group [4].

An interesting ββα Zn^{2+}-binding site is represented by the HNH-motif of colicin E7, a bacterial toxin, which is produced by *E. coli* cells under stress conditions [5,6]. The C-terminal nuclease domain of colicin E7 (NColE7, residues 446-576) kills the attacked cell by nonspecific digestion of RNA and/or chromosomal DNA molecules [6-9]. The host cell protects itself by coexpression of the Im7 immunity protein forming a stable complex with NColE7 at the nucleic acid-binding site [8]. For reviews on colicins see e.g. refs. [10,11]. The HNH motif formed by the C-terminal 45 residues of NColE7 coordinates a single Zn^{2+}-ion by H544, H569 and H573 in a distorted tetrahedral coordination sphere. This arrangement allows for the binding of a water molecule that can be exchanged to the scissile phosphodiester group of DNA.

It is known that the nuclease activity of NColE7 is completely lost upon deletion of the N-terminal KRNK sequence (residues 446-449) [12], while the structure [13], metal-ion and DNA-binding affinity [12] of the truncated mutant is unchanged. Studies on N-terminal point mutants yielded similar results [14]. It is intriguing why and how the seemingly disordered N-terminus – lying outside the active site and DNA-binding helices – influences the catalytic reaction. Previously we examined the 25 residues long N-terminal loop [15]. By computational modelling three important residues (T454, K458 and W464) were selected to mutate them to alanines (Fig. 1). The triple mutation had a dramatic effect on nuclease activity, protein structure and metal binding.

In this study, we reveal which of the mutated residues has the highest impact and how the N-terminal loop influences the structure and function of NColE7. To study the metal ion binding of the T454A/K458A-NColE7 (TK), T454A/W464A-NColE7 (TW) double, and W464A-

NColE7 (W) single mutants of NColE7 we have applied isothermal titration calorimetry and circular dichroism spectroscopy. The conformational changes were monitored by the latter method in solution phase. The catalytic activity in DNA hydrolysis experiments was followed by agarose gel electrophoresis.

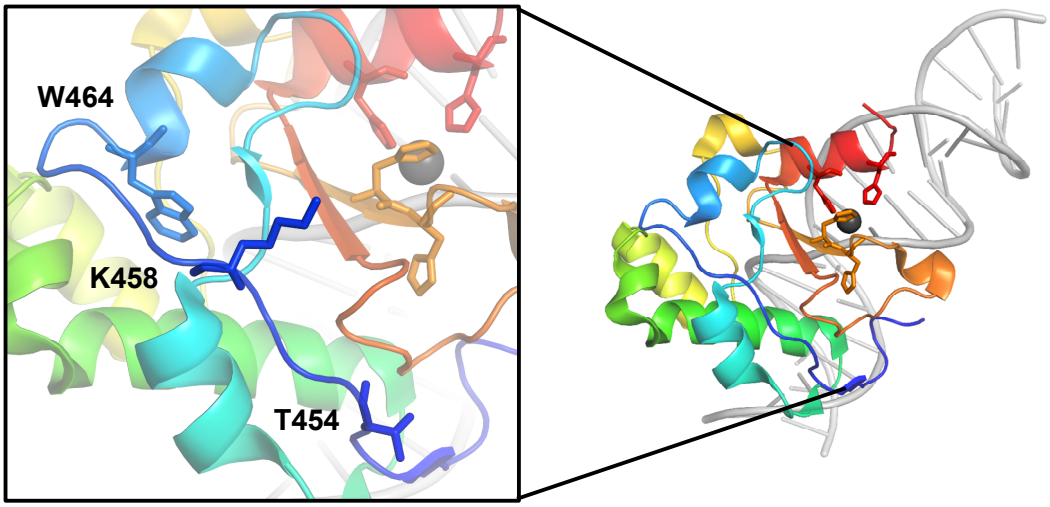


Fig. 1. Crystal structure of NColE7 in its complex with DNA, merged from the structures with Protein Data Bank codes 3FBD [16] (in the absence of metal ion) and 1M08 [17] (in the absence of DNA). The location of the three mutation sites is magnified.

2. Experimental

2.1. Expression and purification of mutant NColE7 proteins

The genes of the NColE7 and Im7 proteins were amplified by PCR from the pQE70 plasmid (a generous gift of Prof. K.-F. Chak, Institute of Biochemistry and Molecular Biology, National Yang Ming University, Taipei, Taiwan [17]). The PCR fragment was cloned into a pGEX-6P-1 vector that provides an N-terminal GST fusion. The mutations have been introduced

by applying the primers described previously for the triple mutant [15]. The constructed plasmids containing the target genes were transformed into *E. coli* DH10B cells and *E. coli* BL21 (DE3) cells for cloning and protein expression, respectively. The same procedure as described earlier [14] was applied for protein purification. All the proteins were stored in 20 mM HEPES, at pH = 7.7.

2.2. Nuclease activity assay

The cleavage of 15 μM (base pairs) pUC19 plasmid by proteins (1.4 μM) in the presence of 4.6 μM zinc(II)-acetate was followed by agarose gel electrophoresis. Incubation time before electrophoretic run was 0-65 min at 37 °C. 10 μl aliquots were loaded onto an ethidium bromide containing 1% agarose gel with 2 μl of 6× DNA Loading Dye (Thermo Sci.). The electrophoresis was performed in a Bio-Rad Wide Mini Sub Cell® GT system at 6.7 V/cm in TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH = 8.0). For comparison 1 μl of the GeneRuler™ (Fermentas) 1 kbp DNA ladder was also loaded to the gel.

2.3. CD-spectroscopic measurements

Synchrotron radiation circular dichroism (SRCD) spectra were recorded at the CD1 beamline of the storage ring ASTRID at the Institute for Storage Ring Facilities (ISA), University of Aarhus, Denmark [18,19], as described before [14]. In parallel CD-measurements were performed on a Jasco-815 spectropolarimeter in the wavelength range of 180-260 nm, in a 0.2 mm pathlength quartz cell. The concentration of the protein solutions was adjusted to 1.6×10^{-5} M in 10 mM HEPES, pH = 7.7. From each raw spectra the water baseline was subtracted.

2.4. ITC measurements

Isothermal calorimetric titrations were performed with the MicroCal Auto-ITC_{200} (GE Healthcare Life Sciences) instrument similarly as described in [15]. The protein samples (~50 μM) were dialyzed for 12 hours in 7000 MWCO Thermo Scientific Slide-A-Lyzer casettes against 20 mM cacodylate buffer, pH = 7.0. The concentration of the protein after dialysis was determined by HPLC amino acid analysis. $ZnCl_2$ was dissolved in the same buffer (c = 400 μM). The dilution heat of $ZnCl_2$ with the buffer was determined for each experiment and the integrated data of dilution heats were subtracted from the corresponding data of protein titrations. 200 μl of protein solutions were titrated with 2 μl aliquots of 400 μM $ZnCl_2$ up to 40 μl and a spacing of 240 s. For titrations carried out in the presence of the Im7 protein, the protein samples (mixture of ~ 40 μM nuclease and 50 μM Im7 protein) were dialyzed and titrated with 400 μM $ZnCl_2$. Analysis of the titration curves was performed with the MicroCal Origin 7.0 software, applying the one-site binding model.

3. Results

3.1. Point mutations reveal that W464 is critical for structural integrity and strong Zn^{2+}-binding of NColE7

In a previous study we performed a computational screen within the 25 residues long N-terminal loop of NColE7 to identify the impact of these residues on the stability of the protein structure [15]. Three important amino acids were selected for further investigation T454, K458, W464. The corresponding triple alanine mutant of NColE7 (TKW) was expressed and purified. It showed low level of catalytic activity in the *in vitro* DNA cleavage assays. In addition a distorted solution structure was detected by CD spectroscopy, and its metal-ion binding affinity proved to

be ~ 2000 times weaker as compared to the wild-type enzyme. Since the intrinsically disordered N-terminal loop is far from the C-terminal catalytic site, this result was surprising. To identify the mutation with most significant contribution to the special features of the TKW mutant we expressed and purified the following double and single point mutants: T454A/K458A-NColE7 (TK), T454A/W464A-NColE7 (TW) and W464A-NColE7 (W).

According to CD spectroscopic measurements (Fig. 2), it is evident that the W464A mutation is responsible for the severe changes in the structure of the proteins. The spectra of TW and W mutants are similar to that of TKW, while the spectrum of the TK mutant is identical to NColE7.

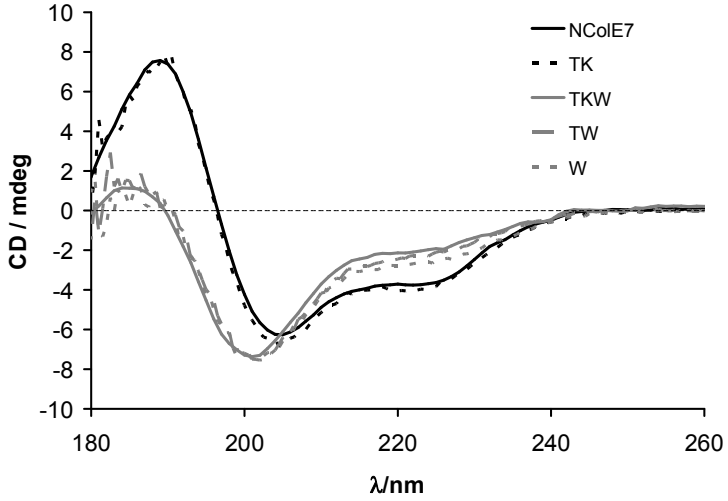


Fig. 2. CD-spectra of the NColE7 mutants, without metal-ion addition.

The metal ion is essential for the catalytic activity of NColE7 [20]. Therefore, to elucidate the effect of the structural changes on the metal ion binding, ITC measurements were carried out (Fig 3). As shown in Table 1 (vide infra), all mutants with the W464A mutation have a decreased Zn^{2+}-binding affinity, while the TK mutant retained the strong metal binding property. This

proves that the mutants possessing a partially unfolded structure bind Zn^{2+}-ions weakly. Our observations confirmed that the structural changes also affect the metal binding site.

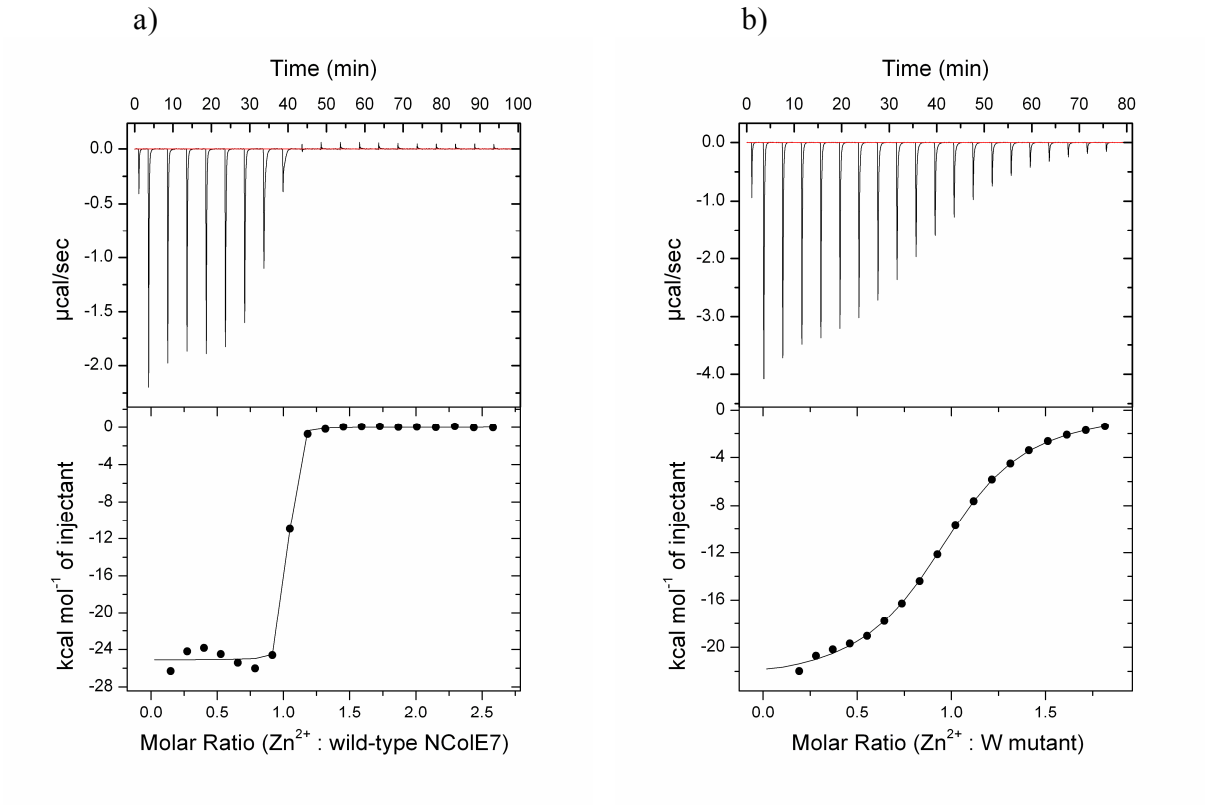


Fig. 3. Representative examples of microcalorimetric titrations of the investigated proteins with Zn^{2+}-ions: a) wild-type NColE7 and b) W mutant.

Supporting the above observations, the CD spectral pattern of the W mutant approached that of NColE7 on increasing Zn^{2+} concentration, but the same spectrum could not be obtained even at 7 fold metal ion excess (See Fig S1 in ESI). At high excess of Zn^{2+}-ions precipitation and denaturation of the proteins occurred similarly to the TKW mutant [15]. In contrast, there was no further change in the CD spectra of the wild-type NColE7 upon addition of more than one equivalent of metal ion [14]. This is in agreement with the weak Zn^{2+}-ion binding established by

the ITC experiments and suggests that the presence of the metal ion itself may not allow for the rescue of the protein structure.

Based on the above results one would expect an impaired nuclease activity in the case of proteins with W464A mutation. Plasmid DNA cleavage experiments were performed to test the catalytic activity of the mutant proteins. Agarose gel electrophoresis detection of the products is shown in Fig. 4. Indeed, the nuclease activity correlates well with the above observations on differences in the structure and metal ion binding affinity. Notably, the TK mutant cleaved almost all large size DNA within one hour, but its nuclease activity was slightly lower, than that of NColE7. As expected, the TW and W mutants had low catalytic activity, shown by the slightly increased amounts of the relaxed form of the plasmid during the first one hour of the incubation.

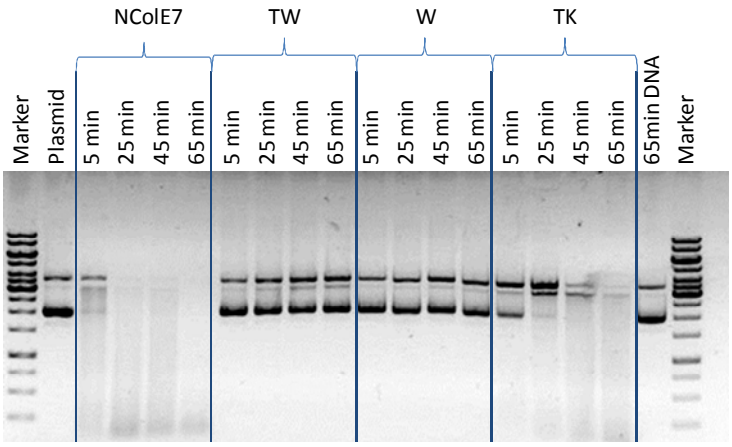


Fig. 4. Agarose gel electrophoresis monitoring of the changes in pUC19 plasmid DNA in the DNA cleavage assay as a function of the time during the incubation with NColE7, TW, W or TK mutants.

It is worthwhile mentioning that even though much less efficiently, the TW and W mutants were able to cleave DNA on longer timescale (data not shown). This residual activity was also enough to kill the bacterial cells when the genes of the proteins were cloned without the gene of the Im7 protein [21].

3.2. Activation of the Zn^{2+}-binding in a preorganized site

Considering the severe changes in the protein conformation and Zn^{2+}-binding as the consequences of the W464A mutation, it is difficult to explain the low, but detectable catalytic activity of these mutants. On the other hand, the CD spectrum of the TKW triple mutant was nearly identical to that of the NColE7/DNA complex in the presence of DNA. Its DNA binding also proved to be as strong as that of NColE7 [15]. This suggests that the substrate binding induces a similar protein structure to that of NColE7, and that all the W464A mutants behave in a similar manner. Recently we have shown that the Im7 protein also binds to the TKW mutant strongly, and the CD spectra revealed similar structural stabilization to the above described phenomenon [22]. The CD spectrum of the W mutant in the presence of Im7 is also identical to that of NColE7 (Fig. S2). The fact, that macromolecular interactions improve the structure of the W464A mutants, while the addition of Zn^{2+}-ions did not, indicates that the preorganization of the metal binding residues is critical for metal binding of the HNH-motif. To approve this hypothesis, i.e. to check whether the metal ion binding residues are properly positioned within the active site of the NColE7 mutants upon the interaction with Im7, we performed further ITC experiments. Titrations of the nucleases by Zn^{2+}-ions were carried out in the presence of the Im7 protein (Fig. 5).

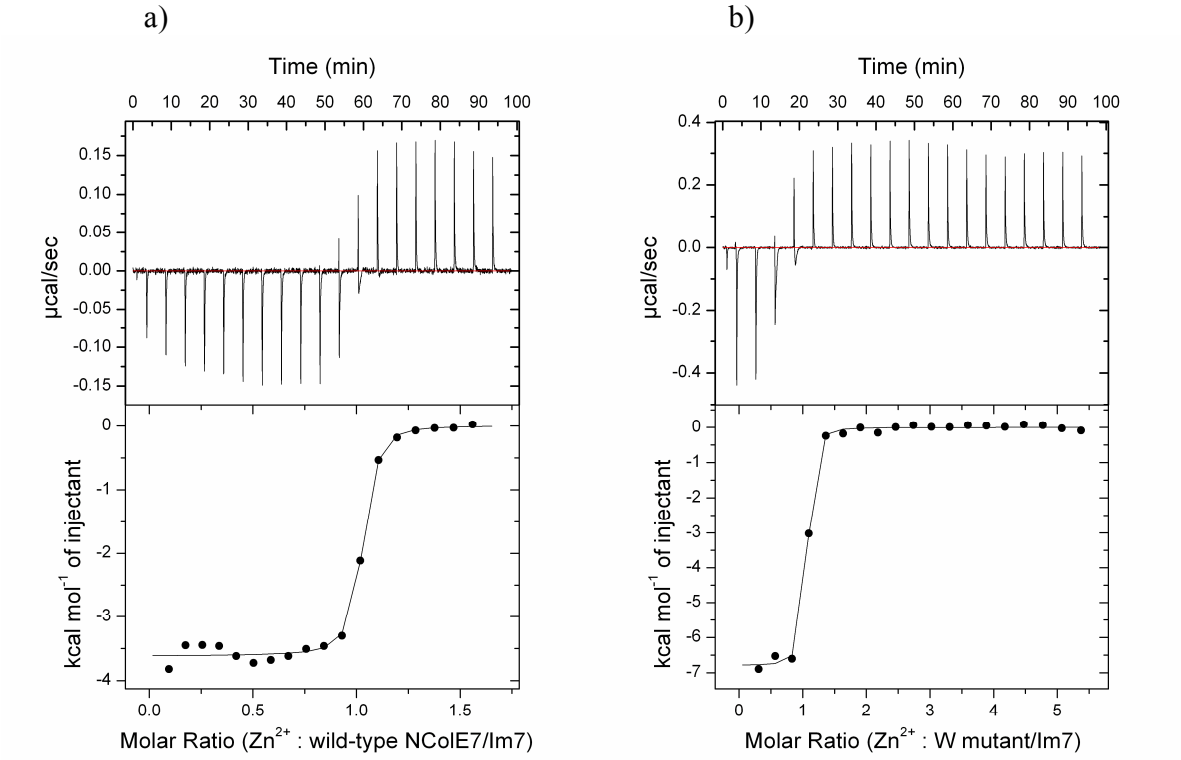


Fig. 5. ITC measurements in the presence of the Im7 protein. Titration of a) NColE7 and b) W mutant with Zn^{2+}-ions under the conditions described in the experimental section.

Table 1. Apparent dissociation constants of the Zn^{2+}-protein complexes calculated from the ITC titration curves in the absence and presence of Im7. The errors indicate the uncertainty of curve fitting. Control titration experiments of Im7 with Zn^{2+} confirmed, that Im7 does not bind metal ion under the same conditions (data not shown).

mutant	**K_d (protein)**	**K_d (protein/Im7)**
NColE7	10 ± 3 nM	61 ± 18 nM
TKW	11 ± 1 µM	33 ± 23 nM
TK	230 ± 40 nM	n.d.
TW	51 ± 3 µM	n.d.
W	5.6 ± 0.3 µM	55 ± 25 nM

As it is demonstrated by the titration curves and the derived data collected in Table 1, the K_d values characterizing the Zn^{2+}-binding of the NColE7, as well as, the TKW or W mutants are the same within the experimental error in the presence of Im7. This means that the metal-binding was completely rescued by the interactions between the nuclease and Im7 proteins.

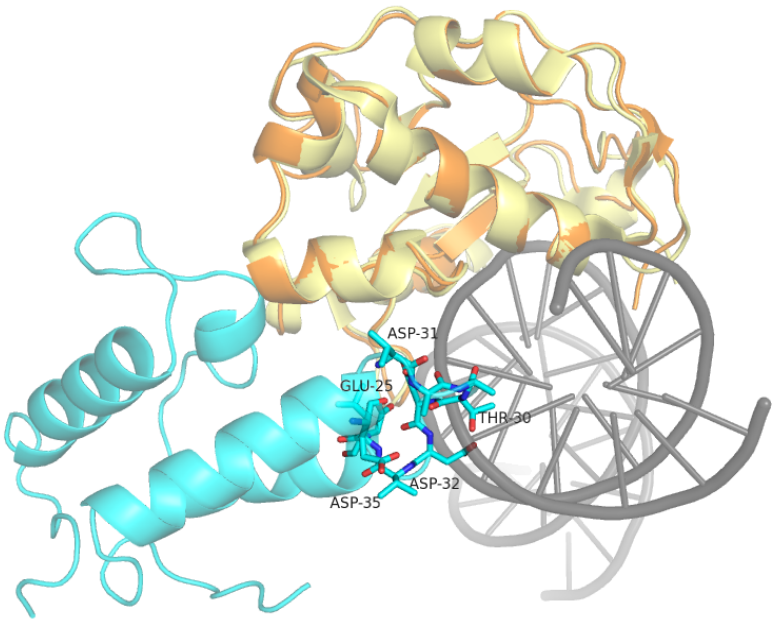
4. Discussion

The T454A/K458A/W464A mutations are located within the N-terminal loop of NColE7, a part of the protein that does not form any stable secondary structure elements and is supposed not to be directly involved in DNA-binding or cleavage. The dramatic effect of the W464A modification on the nuclease activity and the severe structural changes were therefore, surprising. As shown by CD- and ITC-measurements, the absence of the tryptophan side-chain also influenced the Zn^{2+}-binding affinity within the active site. The results suggest that the metal binding site in NColE7 is preorganized, while it is destroyed in the investigated proteins containing the W464A mutation. The structure of the active centre can only partially be rescued by the addition of Zn^{2+}-ions, in agreement with our previous investigation on the HNH motif itself [23]. This indicates that in contrast to the Cys_2His_2 zinc finger motifs with similar Zn^{2+}-binding $\beta\beta\alpha$ fold [24], the His_3 coordination site has a weak ability to form a structural metal binding site. The important fact that the binding of the inhibitory protein Im7 can induce the reformation of the metal ion binding site emphasizes the crucial role of the protein environment around the HNH-motif. As it was already shown for the TKW triple mutant, the binding to the substrate DNA also induces the proper folding [15] explaining the catalytic activity of the mutants, although the hydrolysis reaction is much less efficient as compared to that in the presence of WT NColE7. Remarkably, the Im7 protein and substrate DNA have an overlapping, albeit different binding site on the NColE7 protein (Fig. 6a) suggesting that the structure of

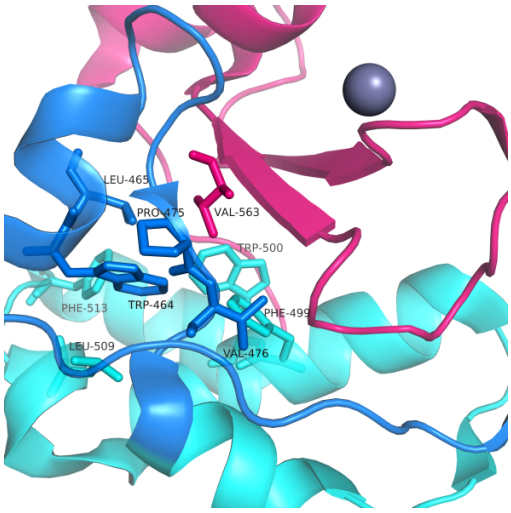
NColE7 can be stabilized in several ways. However, the induced structure may not be stable enough to provide the optimal conditions for the reaction, accounting for the low catalytic activity. This is in agreement with the observation that crystallization experiments failed with the TKW/DNA complex, while the other point mutants of NColE7 could readily cocrystallize with DNA in paralell experiments (Fig. S3). It is difficult to answer the question, why the rescued Zn^{2+}-binding and secondary structure is not enough for the effective nuclease activity? The reason may be in the tertiary structure of the protein and/or the precise local conformations of residues. The example of NColE7 shows that the understanding of enzymatic mechanism may not be simplified by assigning a role for the most crucial catalytic residues only. However, we can learn how to influence the structure of enzymes by introducing targeted point mutations.

Based on the results of this work the above observed phenomena were unequivocally attributed to the W464A mutation out of the three mutation sites in the TKW protein. The role of the tryptophan residues in the structure stabilization has already been demonstrated for a number of peptides [25-27] and proteins [28,29]. Examples include e.g. the conformational stabilization of Porphyromonas gingivalis HmuY hemophore [30], integrin $\alpha_5\beta_1$ [31] and the staphylococcal nuclease [32]. The CD spectral changes of the W140A mutant of the latter protein were reported emphasizing the importance of the Trp residues in its folding. The deletion of 13 residues (including W140) from the C-terminus of this nuclease resulted an unfolded protein, however its folding could be induced under certain conditions, and it is active in presence of Ca^{2+}-ions [33]. In the NColE7 protein the W464 residue is located in a hydrophobic pocket close to the HNH-motif (Fig. 6b and c).

a)



b)



c)

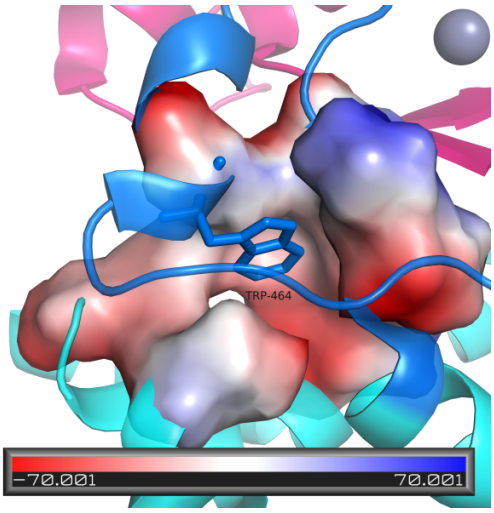


Fig. 6. a) Superposition of the nuclease domain from the NColE7/Im7 crystal structure (PDB code: 7CEI [34], Im7 in blue and NColE7 in orange) and from the NColE7/DNA crystal structure (PDB code: 3FBD [16], DNA in grey and NColE7 in yellow). A negatively charged loop of the Im7 protein would overlap with the DNA therefore, the DNA binding of the nuclease domain is

only possible in the absence of the Im7 protein. b) The location of W464 in a hydrophobic pocket formed by L465, P475, F499, W500, L509 and F513 shown in the structure with PDB code: 1M08 [17]. c) The vacuum electrostatics surface of the same residues around W464 was generated by PyMOL [35]. In both b) and c) panels the N-terminal loop residues are dark blue, while the amino acids from the HNH motif are in violet.

The alignment of the protein sequences of bacterial nuclease toxins, containing their active centre at the C-terminus show that the Trp residue is highly conserved (Scheme S1). The multiple interactions in which W464 is involved, can explain the high impact of this residue on the function of the protein and on the stabilization of the ββα-structure of the HNH-motif. The structure of HNH-motif has multiple importance. It not only provides the binding site for the metal ion, but has direct role in the reaction mechanism. One of the name-giving conserved HNH residues, N560 is located in the flexible HNH-loop between the two β-sheets [36,37]. Its extended hydrogen bond interactions assure conformational stability essential for the tight placement of DNA in the active site and for the position of H545 that polarizes a water molecule to perform the nucleophile attack. The intrachain hydrogen bonding network in a loop is a special structural feature of HNH-motif, combining flexibility and function.

Our results revealed a complex case study on how to influence, destroy and rescue a preorganized catalytic Zn^{2+}-site. The solution studies in the case of metalloenzymes are particularly important, since the crystal packing in the solid state influences the stability of protein. We have proved that the metal-binding site of NColE7 is preorganized in solution phase, and the proper folding of the active site in NColE7 mutants may be induced by the substrate or the Im7 protein. There are several crystal structures of NColE7 and its mutants published in the

literature but most of them are either in complex with the Im7 protein [34,36-39], or with DNA [8,16,39,40] while only two were obtained in the absence of Im7 and/or DNA [13, 17] The same distribution is valid for the NColE9 crystal structures (see Table S1). As we observed, Im7 or DNA-binding may stabilize the structure. Therefore, the conclusions drawn from these structures must be carefully interpreted and augmented with solution studies.

The understanding of the protein structure vs. function relationships is a key point in the design of artificial enzymes. Based on the results shown here we will be able to influence the folding and thus the function of the NColE7 metallonuclease. The above results will advance our knowledge in the design of controlled artificial nucleases for gene therapeutic purposes [21].

Acknowledgements

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