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Chemical Rescue of Active Site Mutants of *S. pneumoniae* Surface Endonuclease EndA and Other Nucleases of the HNH Family by Imidazole

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The His-Asn-His (HNH) motif characterizes the active sites of a large number of different nucleases such as homing endonucleases, restriction endonucleases, structure-specific nucleases and, in particular, nonspecific nucleases. Several biochemical studies have revealed an essential catalytic function for the first amino acid of this motif in HNH nucleases. This histidine residue was identified as the general base that activates a water molecule for a nucleophilic attack on the sugar phosphate backbone of nucleic acids. Replacement of histidine by an amino acid such as glycine or alanine, which lack the catalytically active imidazole side chain, leads to decreases of several orders of magnitude in the nucleolytic activities of members of this nuclease family. We were able, however, to restore the activity of HNH nuclease variants (i.e., EndA (*Streptococcus pneumoniae*), *Sma*Nuc (*Serratia marcescens*) and NucA (*Anabaena* sp.)) that had been inactivated by His→Gly or His→Ala substitution by adding excess imidazole to the inactive enzymes in vitro. Imidazole clearly replaces the missing histidine side chain and thereby restores nucleolytic activity. Significantly, this chemical rescue could also be observed in vivo (*Escherichia coli*). The in vivo assay might be a promising starting point for the development of a high-throughput screening system for functional EndA inhibitors because, unlike the wild-type enzyme, the H160G and H160A variants of EndA can easily be produced in *E. coli*. A simple viability assay would allow inhibitors of EndA to be identified because these would counteract the toxicities of the chemically rescued EndA variants. Such inhibitors could be used to block the nucleolytic activity of EndA, which as a surface-exposed enzyme in its natural host destroys the DNA scaffolds of neutrophil extracellular traps (NETs) and thereby allows *S. pneumoniae* to escape the innate immune response.

Introduction

The His-Asn-His (HNH) motif is characteristic of the active sites of a large number of specific and nonspecific nucleases.[1] This motif has been found in homing endonucleases (e.g., I-PpoI, I-HmuI) as well as in restriction endonucleases (e.g., KpnI, Hpy99I) and in nonspecific nucleases such as caspase-activated DNase (CAD), Vvn and the colicins E7 and E9.[2] The typical active site architecture of these nucleases consists of two short antiparallel β strands and an α helix arranged around a divalent metal ion, typically Mg^{2+}, which is essential for nucleolytic cleavage (ββα-metal-finger motif). The histidine residue at the first position of the HNH motif was found to be directly involved in catalysis, acting as a general base that activates a water molecule for in-line attack on the phosphodiester bond of its substrate and possibly also responsible for leaving group protonation by means of an OP2-mediated proton shuttle from Nδ(His) to the O3′ leaving group.[3] The last histidine in the conserved motif mediates metal ion binding; in some nucleases it is replaced by asparagine (HNN motif).[4] The active site is stabilized by the central asparagine of the HNH motif.[5]

In a detailed sequence comparison of HNH nucleases a subgroup of proteins was identified. This subgroup is characterized by the DRGH motif located in the first β-strand of the ββα metal finger. The final histidine unit in the DRGH motif corresponds to the first histidine residue of the HNH motif.[6] All characterized members of this subgroup—namely *Serratia marcescens* nuclease (*Sma*Nuc), *Anabaena* sp. nuclease (NucA), *Streptococcus pneumoniae* endonuclease (EndA) and EndoG, a mitochondrial apoptotic nuclease in mammals—are nonspecific nucleases; they have H→N substitution in their active sites (i.e., (DRG)HNN).[7] The nucleolytic activities of these nucleases were abolished by single amino acid substitutions in the active sites involving the first histidine residues in their HNN motifs.[8] For EndA we were able to show that the catalytic activity could be restored by supplementing the reaction buffer with imidazole.[8c,9] This compound is clearly able to occupy the space that would otherwise by occupied by the missing imidazole of the histidine side chain when that histidine is replaced with alanine or glycine; this "chemical rescue" effect is more pronounced for the histidine-to-glycine exchange than for the histidine-to-alanine exchange.[9] These results suggested that the precise positioning of the imidazole ring in the active site is crucial for the activation of the nuclease (Figure 1).

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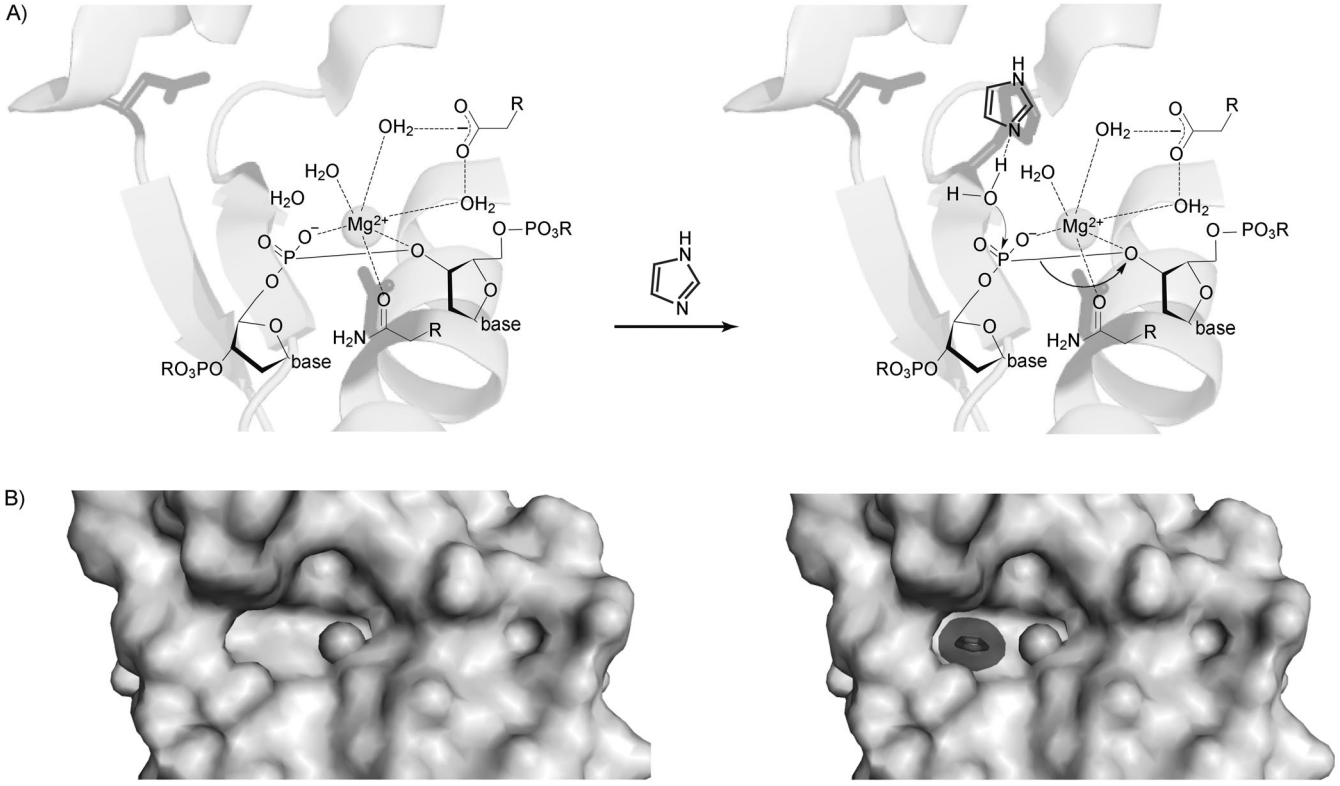

Figure 1. Chemical rescue of the EndA variant H160G. A) In the absence of the histidine side chain, the general base that activates the water molecule is absent. Imidazole can bind to the pocket in EndA to replace the missing histidine side chain. B) Structural model of the EndA variant with bound Mg^{2+} with use of a surface representation for the protein and a sphere for the Mg^{2+} ion, based on the structure of the EndA variant H160A (PDB ID: 3owv). The side chain of Ala160 was removed. Right: the position of the imidazole was derived from the *Sma*Nuc structure (PDB ID: 1ql0) after superpositioning of the two structures.[28] Structures were rendered by use of PyMOL (DeLano Scientific).

The nuclease EndA is a surface-exposed protein from *S. pneumoniae*.[10] This pathogenic bacterium can cause pneumonia and is able to spread from the lung tissue, resulting in severe systemic infections in mammalians.[11] Recent investigations have revealed that EndA is an important virulence factor promoting the invasive behaviour of these bacteria.[11b] Nucleolytic activity on the surface of *S. pneumoniae* leads to the degradation of the DNA scaffolds of neutrophil extracellular traps (NETs). NETs are part of the innate immune response and consist of extracellular DNA fibres and several attached proteins including antibacterial peptides, histones and cell-specific proteases.[12] Bacteria trapped by NETs are disarmed and killed. However, streptococci producing the surface-exposed nuclease can degrade the DNA scaffolds and allow the bacteria to escape NETs, which ultimately leads to invasive spreading of the pathogen.[11b]

The in vitro chemical rescue of EndA H160A or H160G by imidazole that we had previously detected was essential for the characterization of EndA, because the wild-type gene of the nuclease could not be produced in *Escherichia coli*.[8c,9] Only the inactive H160A or H160G variants of EndA could be produced, indicating that the nucleolytic activity during basal expression is toxic for the host even at very low intracellular enzyme concentrations. Toxicity is a general problem for recombinant production of nonspecific nucleases. This problem can be solved by the coexpression of functional inhibitor proteins blocking the nucleolytic activity during the cloning procedures, as has been done for NucA and its inhibitor NuiA, for dEndoG and its inhibitor EndoGI and in other cases.[7a,8b] The nonspecific nucleases *Sma*Nuc and Vvn could be cloned and produced as recombinant proteins because they are not active under the reducing conditions of the intracellular milieu.[13] For correct folding and nucleolytic activity these two nucleases need the formation of intramolecular disulfide bonds, which is promoted by the oxidizing extracellular environment. For EndA, however, no inhibitor protein or other regulatory mechanism has yet been identified. Activation of the nuclease by imidazole thus provided a unique possibility to produce the inactive recombinant protein in sufficient amounts in *E. coli* for a detailed structural and functional investigation of the enzyme.

Chemical rescue based on imidazole supplementation has also been investigated in several previous studies with other enzymes. Deoxyribonuclease II (DNase II) is a ubiquitous enzyme with several histidine residues in the active site.[14] The chemical rescue effect could be demonstrated for two of these histidines. The residues were substituted by alanine, leading to a nucleolytically inactive protein, and exogenously added imidazole partially restored the catalytic activity.[15] Similar results were reported for DNase I: two histidine-deficient DNase I variants were tested and increases in activity on addition of imidazole could be detected.[16] Chemical rescue has also been observed in in vivo studies, both with prokaryotic and with eu-

karyotic cells. For a review of the literature on chemical rescue see ref. [17].

Here we have investigated chemical rescue for three members of the nucleases containing the DRGH motif in vitro and also for one of them in vivo. Although the activation of EndA H160A or H160G by imidazole was reported in previous publications, a more detailed analysis was carried out here, in order to find out whether chemical rescue is a general phenomenon for enzymes of this subgroup of HNH nucleases and whether or not chemical rescue by imidazole is a generally applicable way of controlling their activities.[8c,9] Catalytically inactive variants of *Sma*Nuc from *Serratia marcescens* and NucA from the cyanobacterium *Anabaena* sp. PCC 7120 with His→Gly substitutions in the active site were tested for imidazole activation. Chemical rescue could also be observed for *Sma*Nuc and NucA, and comparison was made with the activity levels of the corresponding wild-type enzymes. From this result we propose that chemical rescue is a general phenomenon for nucleases containing the DRGH motif, and possibly also for the large family of HNH nucleases. The chemical activation of inactive EndA variants was also verified in vivo (*E. coli*) by supplementation of the growth medium with imidazole at nontoxic concentrations. Nucleolytic activation was detectable due to the pronounced reduction in cell viability upon chemical rescue of the EndA activity in vivo. We conclude that the in vivo rescue assay for EndA H160G offers great promise for an in vivo screening system for selection of low-molecular-weight compounds as inhibitors of EndA and possibly as antibiotics directed against *S. pneumoniae*.

Results

Chemical rescue of NucA H124G

The active site variant NucA H124G was successfully cloned and purified in a procedure similar to that established for the wild-type protein. Both nuclease variants were coexpressed with the specific inhibitor NuiA and were separated under denaturing conditions during purification.[8b] In the absence of imidazole, very weak nucleolytic activity of NucA H124G could be detected after prolonged incubation with supercoiled plasmid DNA (0.001% of the wt activity; Figure 2A). Residual activity (0.8% of the wt activity) had also been reported earlier for NucA H124A.[4b] However, catalytic activity could be stimulated by increasing the imidazole concentrations and was directly compared to the activity of the wild type (wt, Figure 2A). The concentration of wt NucA was adjusted to 1 pM to reach a level of activity of wt NucA comparable to that observed for NucA H124G (500 pM) in the presence of 50 mM imidazole. The increases in open circular plasmid were measured and plotted for three individual activity assays (Figure 2B). Initial rates were calculated from the linear phase of the cleavage reaction by linear regression and plotted against the imidazole concentration (Figure 2C). The half-maximum effective concentration for imidazole for the activation of NucA H124G was calculated ($EC_{50\ imidazole} = 35 \pm 8$ mM) by nonlinear regression. The difference in the activities of wt NucA and of the rescued variant at 50 mM imidazole is given by the ratio of the slopes of the fitted curves, which displays a 1000-fold difference (1100 ± 180) once the difference in enzyme concentration is taken into account.

Chemical rescue of *Sma*Nuc H89G

Chemical activation by imidazole was also tested for *Sma*Nuc containing the active site substitution H89G. *Sma*Nuc H89G was cloned successfully and purified as described for the *Serratia* wild-type nuclease.[18] Production of the nuclease in *E. coli* host cells is unproblematic because *Sma*Nuc is inactive under reducing conditions. Activation occurs under oxidizing conditions: upon secretion in vivo and during purification and renaturation through the formation of two intramolecular disulfide bonds in vitro.[13a,18] The nucleolytic activities for wt *Sma*Nuc and the rescued *Sma*Nuc H89G variant were tested by the plasmid DNA cleavage assay. *Sma*Nuc H89G did not display nucleolytic activity in the absence of imidazole but exhibited

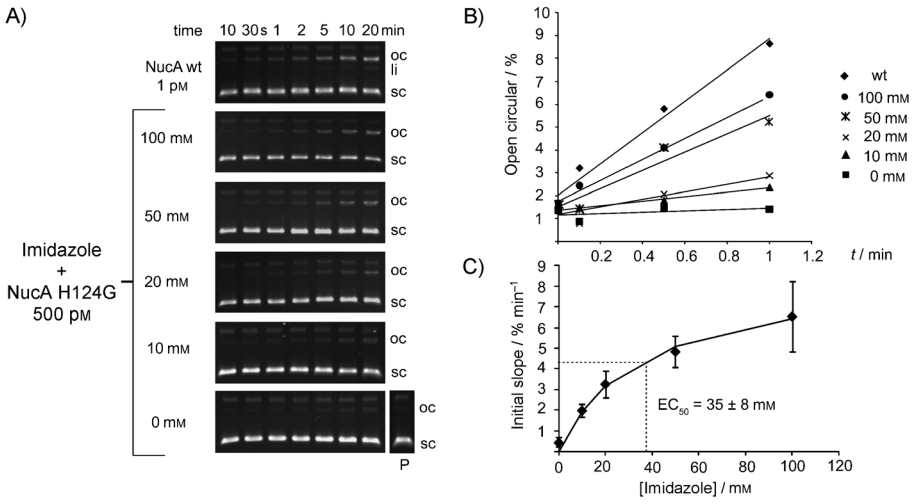

Figure 2. Chemical rescue of NucA H124G. A) Supercoiled plasmid DNA cleavage by NucA H124G (500 pM) in the absence or in the presence of the indicated imidazole concentrations (oc: open circular, li: linear, sc: supercoiled, P: plasmid only). The supercoiled plasmid is converted into the open circular form over time, indicating activation of NucA H124G; for comparison the DNA cleavage kinetics for wt NucA (1 pM) are also shown. B) The percentages of open circular plasmid for the first four time points were plotted and fitted to a linear curve. Individual activity assays were performed and the means of the slopes were calculated ($n = 3$). C) The mean slopes for NucA H124G at different imidazole concentrations were plotted and the half-maximum imidazole concentrations needed for chemical rescue were determined: $EC_{50} = 35 \pm 8$ mM, $n = 3$ (S.E.M.). Note that there is an additional band above the band representing the oc plasmid DNA. This band represents concatemers (see also Figures 3, 4 and 5).[29]

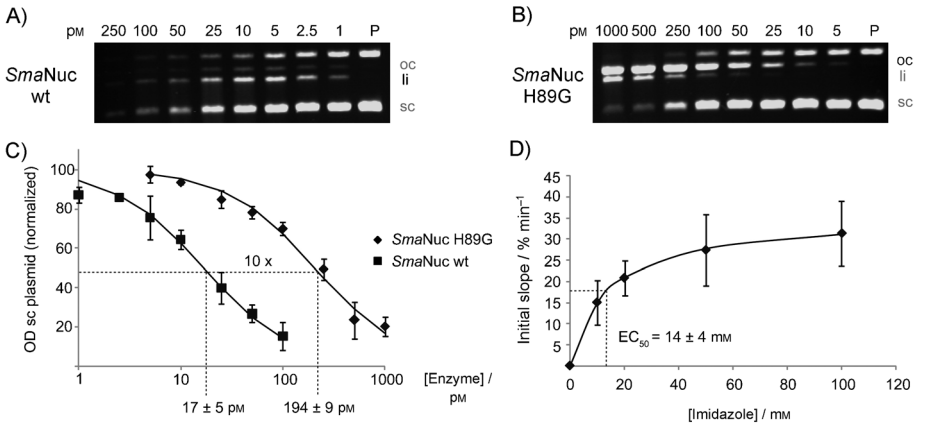

Figure 3. Chemical rescue of *Sma*Nuc H89G. Supercoiled plasmid DNA cleavage by A) wt *Sma*Nuc (1–250 pM), or B) *Sma*Nuc H89G (5 to 1000 pM) in the presence of imidazole (50 mM). The DNA cleavage reaction (oc: open circular, li: linear, sc: supercoiled, P: plasmid only) was stopped after 1 min incubation by addition of loading buffer containing EDTA. C) The intensities of the supercoiled plasmid DNA bands were measured to compare the rates of cleavage of wt *Sma*Nuc and *Sma*Nuc H89G. The half-maximum enzyme concentrations needed for DNA cleavage by wt *Sma*Nuc and by *Sma*Nuc H89G at 50 mM imidazole were determined ($n=3$) and found to correspond to a tenfold difference in activity between wild type und the rescued variant. D) Initial rates of plasmid DNA cleavage by *Sma*Nuc H89G (50 pM) plotted against imidazole concentration. The half-maximum imidazole concentration needed for chemical rescue of *Sma*Nuc H89G was calculated in a similar way as for NucA (Figure 2; $EC_{50}=14\pm4$ mM, $n=3$)

However, increasing the salt concentration to 125 mM, thus weakening the protein–DNA interaction, leads to a switch for wt *Sma*Nuc from the diplotomic to the haplotomic mode of action. Addition of 100 mM imidazole, however, has only a small effect on the diplotomic versus haplotomic reaction mechanism (Figure 4).[19]

The half-maximum effective concentration of imidazole for the activation of *Sma*Nuc H89G was determined in a similar way as for NucA (Figure 3D). The increases in open circular plasmid over time during a plasmid DNA cleavage assay were measured at different imidazole concentrations. Similar time intervals as for the NucA kinetics were chosen and the first four data points were plotted. The curves were linearly fitted and the slope was determined (data not shown). The half effective concentration of imidazole was calculated to be 14 ± 4 mM and was determined essentially as described for NucA (Figure 3D).

increasing catalytic activity with increasing imidazole concentrations (Figure 3).

Notably, wt *Sma*Nuc and the rescued variant displayed different cleavage patterns in degradation of plasmid DNA (Figure 3). DNA degradation by wt *Sma*Nuc follows a diplotomic mechanism.[19] This mode of action leads to the production of linearized plasmid during the reaction without significant accumulation of open circular plasmid as an intermediate (Figure 3A). The prominent linear band is indicative of fast and effective cleavage of plasmid DNA caused by several nicks on both strands in close proximity, leading to fast linearization of the plasmid. In contrast, plasmid degradation by the rescued *Sma*Nuc H89G followed the haplotomic mode (Figures 3B and 4). The open circular plasmid appeared as an intermediate during the cleavage reaction with a cleavage pattern comparable to those displayed by NucA (Figure 2A) and EndA H160G (Figure 5A). This degradation mode is the result of a more random distribution of nicks. Because wt *Sma*Nuc and the rescued *Sma*Nuc H89G display different cleavage patterns their activities could not be compared directly by measurement of the increases in concentration of open circular plasmid. Instead, the rates of decrease of the supercoiled plasmid concentrations for different enzyme concentrations at 50 mM imidazole were quantified (Figure 3C). The half-maximum effective concentrations for scDNA cleavage by wt *Sma*Nuc and by chemically rescued *Sma*Nuc H89G were determined (wt: 17 ± 5 pM, H89G: 194 ± 9 pM) and showed only a tenfold difference in activity. Similar experiments were performed with a monomeric variant of *Sma*Nuc (H184R) and the rescued monomeric enzyme variant (H89G/H185R).[20] The cleavage patterns of the two variant proteins were identical, that is, haplotomic; this indicates that the mode of action was not a consequence of the monomer/dimer state of the nuclease (data not shown).[19]

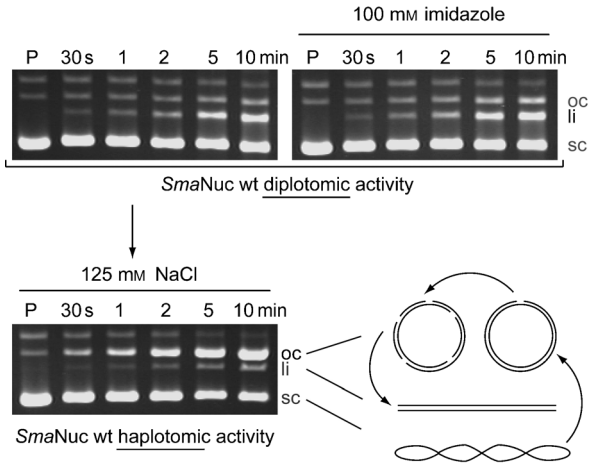

Figure 4. Mechanistic switching of *Sma*Nuc. Depending on the salt concentration, wt *Sma*Nuc (10 pM) shows different cleavage mechanisms for the degradation of plasmid DNA. The diplotomic mechanism results in an accumulation of linearized plasmid DNA, whereas the concentration of open circular plasmid DNA remains low during the reaction. The cleavage mechanism can be shifted to a haplotomic activity by raising the ionic strength, which weakens the DNA–protein interaction. Plasmid DNA cleavage now occurs in two steps consisting of the accumulation of open circular plasmid and its transition to the linear form (see schematic picture of plasmid degradation). A low DNA–protein affinity, as induced by addition of salt, results in a more random accumulation of nicks distributed over the entire plasmid, because the probability of two cleavage events occurring nearby on the same plasmid decreases. The mechanistic switch is only induced to a minor degree by 100 mM imidazole.

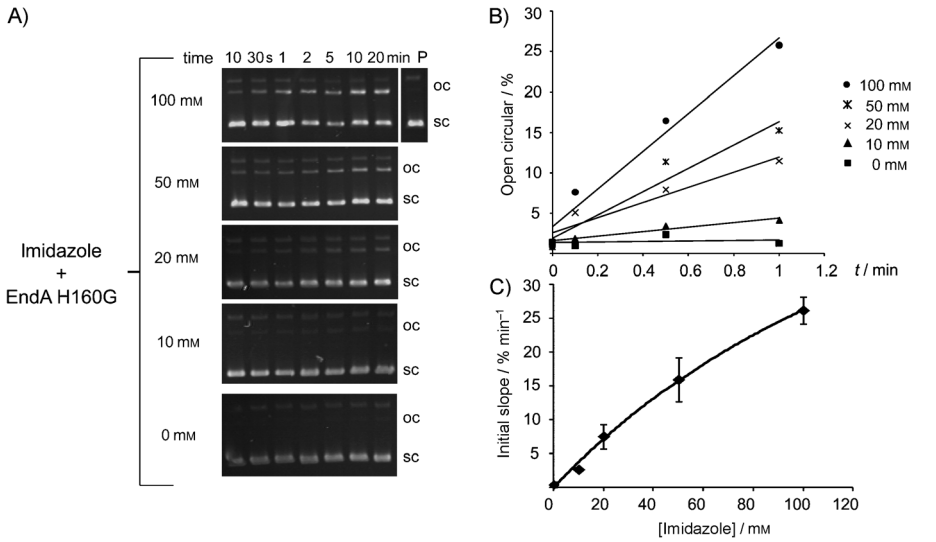

Figure 5. Chemical rescue of EndA H160G. A) Supercoiled plasmid DNA cleavage by EndA H160G (100 pM) in the absence or in the presence of the indicated imidazole concentrations (oc: open circular, li: linear, sc: supercoiled, P: plasmid only). The supercoiled plasmid is converted to the open circular form over time, indicating the activation of EndA H160G. Individual plasmid DNA cleavage assays were performed. B) The percentage values of open circular plasmid for the first four time points were plotted and fitted to a linear curve. C) The mean slopes of three assays ($n=3$) obtained at different imidazole concentrations were plotted. The half-maximum imidazole concentration needed for DNA cleavage by EndA H160G could not be determined accurately ($EC_{50}=200\pm75$ mM, $n=3$) because saturation in the rate versus imidazole concentration plot was not reached.

Chemical rescue of EndA H160G

The chemical rescue of EndA H160A and H160G had been reported by us in 2010.[8c,9] A more detailed analysis, similar to those performed for NucA H124G and *Sma*Nuc H89G, was carried out in this study through investigation of the kinetics of plasmid DNA cleavage at different imidazole concentrations under otherwise identical conditions (Figure 5A and B), in order to compare the three related nucleases. Unlike those for NucA H124G and *Sma*Nuc H89G, the curves obtained for EndA H160G did not reach saturation in the imidazole range investigated, so the half-maximum effective concentration of imidazole could only be estimated with a large error (200±75 mM).

In vivo chemical rescue of EndA H160G

Cloning and production of nonspecific nucleases in *E. coli* can be difficult because of the intrinsic toxicities of these proteins. The nonspecific activity leads to degradation of the host DNA and RNA that cannot be effectively repaired by *E. coli*. Previous work indicated that wt EndA is highly toxic for *E. coli* and that basal expression even during cloning procedures impairs plasmid stability and leads to cell death.[8c] Only the active site variants of EndA (H160A, H160G) were successfully cloned and produced.[8c,9] We tested the ability of imidazole to activate EndA H160A and H160G in living cells (*E. coli*) by supplementing the LB growth medium with different concentrations of imidazole (Figure 6). To test the toxicity of imidazole, the *E. coli* strain BL21 Star DE3 was transformed with pETM-30 for GST production and exposed to high concentrations of imidazole to test the general toxicity of this substance. The cells were grown in a 96-deep-well plate with concentrations of imidazole varying from 0 to 100 mM, without induction of protein production (Figure 6A and B). The optical densities of the 1.5 mL cultures were measured at one-hour intervals. Low imidazole concentrations (10 and 20 mM) did not impair cell growth but a weak toxic effect was detected for 50 and 100 mM imidazole. Similar viability assays were performed for *E. coli* transformed with pETM-30 EndA H160A and pETM-30 EndA H160G, producing GST-EndA H160A and GST-EndA H160G, respectively (Figure 6C and D). Similarly to the case of the in vitro experiments, the in vivo

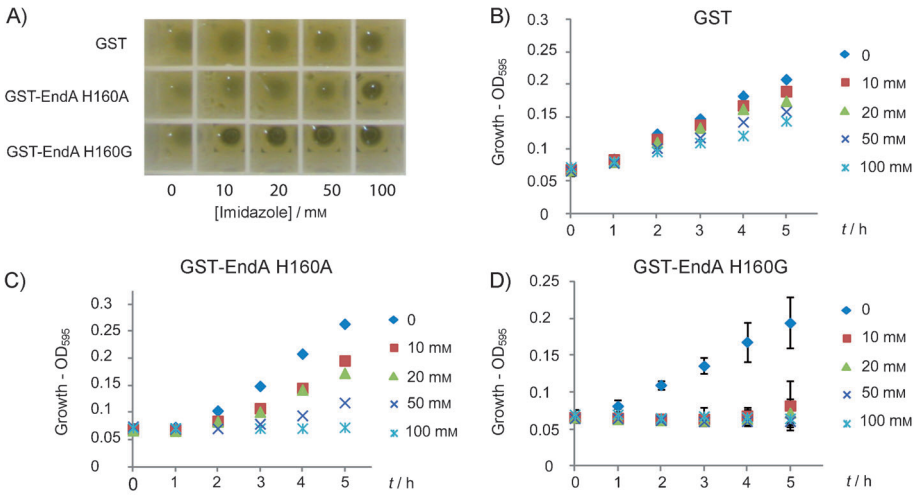

Figure 6. In vivo chemical rescue of EndA H160A and EndA H160G. A) Top view of a 96-deep-well plate filled with LB medium (1.5 mL) inoculated with *E. coli* BL21 Star DE3 transformed with pETM-30 and derivatives producing the proteins indicated. The cultures were grown in the absence or presence of imidazole (0–100 mM). B) to D) Growth was recorded by measuring the OD_{595}: 10 mM imidazole was already sufficient to inhibit the growth of *E. coil* transformed with pETM-30 EndA H160G completely, but had no effect on cells transformed with pETM-30 (GST production). Error bars have been omitted for reasons of clarity in (B) and (C).

rescue was less efficient for EndA H160A than for EndA H160G.[9] For EndA H160A, almost no inhibition of growth was detectable at 10 and 20 mM imidazole, but growth was impaired at 100 mM imidazole. In contrast, the in vivo activation for EndA H160G was detectable in the form of a drastic decrease in cell growth even at 10 mM imidazole, which had no toxic effect for *E. coli* producing only GST (Figure 6D). DNA degradation in *E. coli* cells producing EndA H160G and exposed to 20 mM imidazole could be demonstrated by agarose gel electrophoresis after cell lysis (data not shown).

Discussion

In vitro activation of HNH nuclease variants in which the catalytically essential histidine residue had been substituted by glycine

Chemical rescue of a HNH nuclease was first reported for a variant of the nonspecific nuclease EndA.[8c] In this enzyme variant, the catalytically essential histidine (HNH) at the active site had been replaced by alanine. The missing side chain contains the imidazole residue, and addition of imidazole to the reaction mixture is able to reconstitute the active site of EndA H160A and thereby to restore nucleolytic activity. In order to find out whether this is a more general phenomenon for HNH enzymes, we tested other nonspecific nucleases of the HNH family for chemical rescue. To this end, NucA H124G and *Sma*Nuc H89G containing mutations at positions corresponding to EndA H160 were successfully cloned and purified. Both nuclease variants displayed no or only very weak nucleolytic activity, similarly to what has been reported for the NucA and *Sma*Nuc variants containing an alanine unit instead of the essential histidine.[4b] Chemical rescue by supplementation of exogenous imidazole could be achieved for both enzymes. Nevertheless, the levels of activation by imidazole for both variant enzymes relative to the wild-type enzyme showed a significant difference. The activities of the rescued variants were compared with those of the wild-type *Sma*Nuc and NucA enzymes at 50 mM imidazole, because activation approaches saturation at this imidazole concentration for both enzymes. The differences in activity were calculated on the basis of the increases in open circular plasmid levels during a supercoiled plasmid DNA cleavage assay. The rescued *Sma*Nuc H89G variant displayed an activity ten times lower than that of the wild-type enzyme, whereas the rescued NucA H124G variant was 1000 times less active than wt NucA.

The general differences in activity for the rescued *Sma*Nuc and NucA variants and the corresponding wild-type proteins probably result from decreases in protein–DNA affinity and catalytic efficiency. This is also apparent from the mechanistic switch of *Sma*Nuc H89G, which is shifted from the predominantly diplotomic to the predominantly haplotomic mode of action. It can be assumed that this drop in activity is the result of small rearrangements within the active site due to the amino acid substitution, which lead to suboptimal DNA binding and cleavage. Imidazole is clearly not able to compensate fully for the missing side chain of the active site, because optimal positioning of the imidazole cannot be achieved. The topography of the active site is crucial for the reaction because the correct positioning of imidazole is necessary to activate a water molecule, which attacks the phosphorous atom in-line with the scissile phosphodiester bond.[21]

Imidazole can be considered a second cofactor, together with magnesium, in the chemical rescue experiments and, like magnesium, needs to bind to the active site. Activation of the histidine-deficient variants thus only occurs if both cofactors bind simultaneously to the enzyme and form a quaternary complex with the DNA. The probability of both cofactors being present at the active site in general is therefore lower for the variants than for the wild-type enzyme, which only needs a single cofactor. The requirement for formation of a quaternary complex versus a ternary complex, as in the case of the wt enzyme, reduces the lifetime of the activated state of a rescued variant, resulting in fewer cleavage events over time than in the case of the wild-type enzyme.

From the differences in activity observed for the rescued *Sma*Nuc and NucA variants and the corresponding wild-type enzymes we conclude that activated EndA H160G rescued with imidazole does not represent the fully recovered enzyme. The observed difference in activity between the rescued variant and the wild-type protein seems to be a general consequence of the chemical rescue approach. As seen in all previous publications, only a partial rescue of the enzymes of interest could be achieved.[17] However, a direct comparison of rescued EndA H160G and the wild-type enzyme turned out to be very difficult. The EndA wild-type enzyme was too toxic to establish expression plasmids in *E. coli* and could only be produced in low amounts through the use of a cell-free expression system instead. Nevertheless, the obtained wild-type protein showed a pattern of DNA cleavage comparable with that of the rescued variant, implying a haplotomic mode of action for EndA under these conditions.[8c] Interestingly, activation of EndA H160G with imidazole did not reach saturation at 100 mM imidazole as had been seen for *Sma*Nuc H89G and NucA H124A. This result is rather surprising, because the active sites of the three nucleases studied are structurally similar. However, EndA contains an unusual loop structure protruding from the α-helix of the $\beta\beta\alpha$ metal finger structure. This loop is not found in *Sma*Nuc and NucA. It seems to interfere with DNA binding and/or cleavage as a result of its exposed position, because complete deletion of the loop leads to higher nucleolytic activity.[9]

Intriguingly, close inspection of the active sites of EndA, NucA and *Sma*Nuc shows that the parts of the active sites that could accommodate the imidazole when histidine had been replaced by glycine are quite different in size (Figure 7), which could in part explain why the magnitudes of the chemical rescue effects and their imidazole concentration dependences are different for the three enzymes. *Sma*Nuc, with the tightest binding pocket for the histidine side chain, shows a half-maximum effective concentration of 14 ± 4 mM imidazole for chemical rescue, followed by NucA (35 ± 8 mM) and finally EndA (200 ± 75 mM).

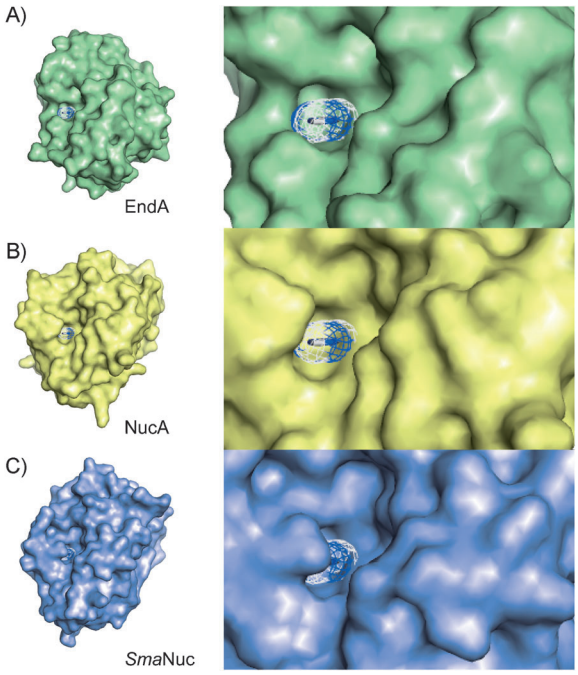

Figure 7. Comparison of the structures of the active sites of A) EndA H160G, B) NucA H124G, and C) *Sma*Nuc H89G, in which imidazole had been modelled to replace the histidine side chain (for details see legend to Figure 1). Structures of the variant proteins were generated by replacement of Ala160 in EndA (PDB ID: 3owv), His124 in NucA (PDB ID: 2o3b) and His89 in *Sma*Nuc (PDB ID: 1ql0) with glycine. Whereas EndA provides a very open binding pocket for imidazole, NucA and, in particular, *Sma*Nuc have considerably tighter binding pockets. All structures were generated by use of PyMOL (DeLano Scientific).

In vivo activation of EndA H160G

In general, cloning and production procedures for nonspecific nucleases are demanding because of their intrinsic toxicities. Nevertheless, stable plasmids for EndA H160A or H160G production in *E. coli* could be established.[8c,9] This result implies that these enzyme variants are catalytically inactive in the cell, as is illustrated by the in vitro activity assays without imidazole. We tested the ability of imidazole to rescue the activities of EndA H160A and EndA H160G in the producing host *E. coli* in a similar way as for the in vitro situation. For the in vivo chemical rescue the growth medium was supplemented with different amounts of imidazole. The rather small chemical compound imidazole is obviously able to enter the cells and can activate EndA H160A and EndA H160G. The in vivo activation leads to nonspecific degradation of the host DNA and RNA, which can be concluded from the massive reduction in cell growth rate and from the direct analysis of DNA degradation in the cells. Interestingly, the efficiency of the chemical activation achieved in *E. coli* differs for EndA H160G and EndA H160A. Cells propagating plasmids encoding EndA H160A are more viable than cells producing EndA H160G, a finding that reflects the in vitro situation. Chemical rescue is less effective for EndA H160A than for EndA H160G, presumably because the methyl side chain of alanine disturbs the optimal positioning of the imidazole ring.[9] In any case, we cannot exclude different protein production levels within the cells. The in vivo assays rely on the fact that basal or "leaky" expression already produces sufficient amounts of protein. We think that basal expression also accounts for the difficulties arising during cloning procedures for nonspecific nuclease genes, especially as seen for the EndA wild-type gene.

Conclusion

This work provides evidence that the chemical rescue effect for HNH/DRGH nuclease variants with substitutions of the catalytically essential histidine seems to be a common feature for members of this family of nucleases. The rescued variants of NucA and *Sma*Nuc displayed lower activities than the corresponding wild-type proteins. Like magnesium, imidazole can be considered to be a cofactor that binds to the active sites of the inactive variants with half-maximum binding in the higher mM range. Imidazole replaces the missing side chain of histidine but the positioning of the imidazole ring seems to be suboptimal, leading to the observed decreases in activity relative to the wt enzymes. From our results we conclude that the rescued EndA variants also are not equivalent to the fully active wild-type enzyme, which could not be produced in sufficient amounts for direct comparison. Nevertheless, the established in vivo activation assay for EndA H160G shows impressive growth reduction even at low imidazole concentrations. The rescued nuclease activity obviously leads to nonspecific degradation of the host DNA/RNA, and this induces toxicity and impairs cell growth. We believe that the in vivo rescue assay for EndA H160G is a promising starting point for a screening of chemical libraries for nuclease inhibitors that might serve as new antibiotics directed against *S. pneumoniae*, which are needed because of the increased emergence of multiple antibiotic resistance of *S. pneumoniae*. Currently, 15 to 30% of cases of *S. pneumoniae* worldwide are multidrug-resistant![22] This screening could be carried out in a high-throughput fashion, similarly to what has been described recently.[23] It is a possibility that some of the inhibitors found in such a screening would inhibit the rescued variant by interfering with imidazole binding to the active site and would not inhibit the wild-type enzyme, but we expect that some of the inhibitors would inhibit wild type EndA as well, and thereby interfere with invasive spreading of the pathogen.

Experimental Section

Plasmid DNA cleavage assay: Several activity tests for nonspecific nucleases have been established during recent years.[24] Nonspecific nucleases that induce single-strand breaks can be monitored by a simple plasmid DNA cleavage assay, which we have used. The compact supercoiled plasmid is transformed by a single nick into an open circular structure that runs significantly more slowly on an agarose gel. A single cleavage event on plasmid substrates leads to an increasing signal in this sensitive assay, whereas the hyperchromicity assay needs the accumulation of several cleavage events.[25] Continuous plasmid degradation leads to further nicking of the supercoiled substrate and of the already open circular form. However, further cleavage of the open circular DNA cannot be visualized by native electrophoresis because a multiple nicked plas-

mid stays in its open circular form. Transformation to the linear form occurs only if two random cleavage events are in close proximity on opposite strands. As a result, during electrophoresis a band representing the linear DNA appears below the band for the open circular plasmid. The ongoing cleavage of circular or linearized plasmid DNA produces oligonucleotides of random sizes, which appear on the agarose gel as a "smear" indicating progressively more extensive degradation. Nucleolytic activity can be quantified by measuring the increase in open circular species normalized to the total amount of DNA. However, the enzyme concentration needs to be adjusted because only the very initial phase of DNA cleavage results in a linear increase in the signal that can be easily fitted. Higher initial velocities are correlated with several nicking events on single plasmids. However, these multiple events do not influence the concentration of open circular plasmid in the reaction mixture. They remain undetected by electrophoresis, resulting in a constant signal although the cleavage reaction actually proceeds. Taking this into consideration, we applied the plasmid DNA cleavage assay to test the chemical rescue effect at different imidazole concentrations for the active site histidine variants NucA H124G, *Sma*Nuc H89G and EndA H160G at rather low enzyme concentrations.

Cloning and purification of wt NucA and NucA H124G: The plasmid pBBInucA H124G was generated by the megaprimer PCR method for site-directed mutagenesis.[26] The megaprimer was amplified with the primer NucA H124G AluI (TTATG ACCGG GGAGG TATAG CTCCT) and R1_NucA (GTTTA GAGGC CCCAA GGGGT TAT) with use of the plasmid pBBInucA as template.[8b] The plasmids pBBInucA and pBBInucA H124G (ampicillin-resistant) were used to transform BL21 Star DE3 (Invitrogen). The cells were plated on LB-agar and incubated overnight at 37 °C. A 25 mL culture was inoculated with a single clone for each plasmid. A 500 mL culture was inoculated with 5 mL of the 25 mL preculture on the next day and incubated at 37 °C. Protein production was induced with isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) at $OD_{600} = 0.4$ for 4 h. The cells were washed with STE buffer. The N-terminal His-tagged protein was purified with Protino Ni-TED 100 packed columns (Macherey–Nagel) by the recommended purification protocol under denaturing conditions. The proteins were renatured during dialysis against Tris·HCl (pH 8.2, 20 mM), Lubrol (0.01%) and glycerol (20%). Aliquots were stored at −20 °C.

Chemical rescue experiments with NucA H124G: All assays were performed at room temperature with the following cleavage buffer: Tris·HCl (pH 8, 20 mM), $MgCl_2$ (5 mM), Triton (0.01%). Wt NucA and NucA H124G were diluted stepwise with cleavage buffer to reach final concentrations of 1 pM and 500 pM, respectively. Plasmid pBluescript SK(+) (pBSK, Stratagene) was used as substrate (15 ng μL^{-1}) to test the nucleolytic activity. NucA H124G activity was chemically rescued with imidazole (stock concentration: 500 mM, pH 8). The reaction was started by addition either of wt NucA or of NucA H124G. Aliquots (10 μL) were taken at indicated time points and the nucleolytic activity was stopped with loading buffer (4 μL) containing EDTA (250 mM). The reaction products were separated by electrophoresis on an agarose-TBE gel (0.8%). The gel was stained with ethidium bromide and documented by use of the BioDocAnalyze System (Biometra). The percentages of open circular plasmid at the time points indicated were determined with the aid of the TINA2.0 software package and plotted. A linear curve was fitted to the first four data points and the slope determined. The slopes were plotted against the corresponding imidazole concentration and the half-maximum effective concentration (EC_{50} value) of imidazole was calculated ($n = 3$). The slopes for wt NucA and the H124G variant (at 50 mM imidazole) were directly compared by calculation of the ratio with allowance for the different enzyme concentrations.

Cloning and purification of *Sma*Nuc wt and *Sma*Nuc H89G: The plasmid pHisNuc H89G was generated by the megaprimer PCR method for site-directed mutagenesis.[26] The megaprimer was amplified with the primer *Sma*Nuc H89G NheI (CGCGG TGGTC AGGCG CCGCT AGCCT) and pL-rev (CGGTT TATCA GCTTG CTTTC GA) with use of pHisNuc (ampicillin-resistant) as template.[8a] The plasmids pHisNuc and pHisNuc H89G were used to transform *E. coli* TGE 900 and plated on LB agar.[27] The cells were incubated overnight at 28 °C and a 25 mL culture was inoculated with a single clone for each plasmid. The cultures were grown at 28 °C and 5 mL of each culture was used to inoculate a 500 mL culture on the following day. The protein production was induced by a temperature shift from 28 °C to 42 °C (4 h) at $OD_{600} = 0.5$. The cells were washed with STE buffer. The N-terminally His-tagged protein was purified under denaturing conditions by use of Protino Ni-TED 100 packed columns (Macherey–Nagel) according to the manufacturer's instructions. The proteins were renatured during dialysis against Tris·HCl (pH 8.2, 20 mM), Lubrol (0.01%) and glycerol (20%) and aliquots were stored at −20 °C.

Chemical rescue with *Sma*Nuc H89G: Activity assays were performed at room temperature with use of the following DNA cleavage buffer: Tris·HCl (pH 8, 20 mM), $MgCl_2$ (5 mM), Triton (0.01%). Wt *Sma*Nuc and the H89G variant were diluted stepwise with DNA cleavage buffer to the concentrations indicated for the titration experiments. Plasmid pBSK was used as substrate (15 ng μL^{-1}) to test the nucleolytic activity. The reaction (10 μL) was started by addition either of wt *Sma*Nuc or of the H89G variant (diluted stepwise with cleavage buffer to 50 pM) to the mixture and stopped after one minute with loading buffer (2 μL) containing EDTA (250 mM). For *Sma*Nuc H89G the reaction mixture was supplemented with imidazole (50 mM). The reaction products were separated by electrophoresis on an agarose-TBE gel (0.8%). The gel was stained with ethidium bromide and documented by use of the BioDocAnalyze System (Biometra). The extents of plasmid DNA cleavage were determined with the aid of the TINA2.0 software package by measurement of the optical densities of the bands representing supercoiled plasmid. The data sets were normalized to the supercoiled plasmid band of the untreated control and the mean of three titration experiments was calculated. The normalized OD values were plotted against the enzyme concentration and the half effective enzyme concentration of wt *Sma*Nuc and the H89G variant was determined.

Mechanistic switching of *Sma*Nuc: The DNA cleavage experiments were performed at room temperature with the same buffer as used for the chemical rescue experiments with *Sma*Nuc H89G supplemented with imidazole (pH 8) or NaCl at the same concentration. Plasmid pBSK was used as substrate (15 ng μL^{-1}) and the reaction was started by addition of wt *Sma*Nuc. Aliquots of 10 μL were taken at the time points indicated and the reaction was stopped with loading buffer (4 μL) containing EDTA (250 mM). The reaction products were separated by electrophoresis on an agarose-TBE gel (0.8%) and analysed as described above.

Cloning and purification of EndA H160G: The cloning, production and purification of EndA H160G was reported previously.[9,26]

Chemical rescue of EndA H160G: The assays were performed in a similar way to the chemical rescue experiments for NucA H124G with use of the following DNA cleavage buffer: Tris·HCl (pH 8,

20 mM), $MgCl_2$ (5 mM). EndA H160G was diluted stepwise with cleavage buffer to a final concentration of 100 pM.

In vivo chemical rescue of EndA H160G: The plasmids pETM-30 (GST expression only) and pETM-30 EndA H160A and pETM-30 EndA H160G (GST-EndA H160A and GST-EndA H160G production) were used to transform *E. coli* BL21 Star DE3. The cells were plated on LB agar containing kanamycin and incubated overnight at 37 °C. An individual clone for each plasmid was used to inoculate a 25 mL culture (with kanamycin). The cultures were incubated at 37 °C and the OD_{600} values were measured the next day. The pre-cultures were diluted in LB medium (100 mL) to a starting OD_{600} of 0.1 for each plasmid. The imidazole concentration was increased stepwise from 10, 20, 50, to 100 mM by addition of appropriate amounts of imidazole (5 M, pH 7.5) to the 100 mL LB culture. Aliquots of 1.5 mL for each imidazole concentration were added to the 96-deep-well plate and incubation was carried out at 37 °C with rotation at 200 rpm. Aliquots (100 μL) of each culture were transferred every hour to a 96-well plate. The OD595 was measured with a microplate absorbance reader (Tecan Sunrise). For the determination of intracellular DNA degradation, cells were pelleted and lysed with SDS (1%); the cell extract was analysed by agarose gel electrophoresis and DNA was stained with ethidium bromide.

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