Genetic engineering, production and characterisation of monomeric variants of the dimeric *Serratia marcescens* endonuclease

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Abstract The *Serratia* nuclease is a non-specific endonuclease which cleaves single- and double-stranded RNA and DNA. It is a member of a large family of related endonucleases, most of which are dimers of identical subunits, with the notable exception of the *Anabaena* nuclease which is a monomer. In order to find out whether the dimer state of the *Serratia* nuclease is essential for its function we have produced variants of this nuclease which based on the crystal structure (Miller, M.D. and Krause, K.L. (1996), Protein Science 5, 24–33) were expected to be unable to dimerise. We demonstrate here that these variants, H184A, H184N, H184T and H184R, are monomers and have the same secondary structure, stability towards chemical denaturation and activity as the wild-type enzyme. This allows to conclude that the dimeric state is not essential for the catalytic function of the *Serratia* nuclease. In contrast, the S179C variant which is also a monomer shows little activity, presumably because this amino acid substitution changes the structure of the enzyme.

Key words: Circular dichroism; Folding; Quaternary structure; Site-directed mutagenesis; Stability

1. Introduction

The extracellular *Serratia marcescens* nuclease is a non-specific endonuclease, capable of cleaving single- and double-stranded RNA and DNA with very high specific activity [2–6]. Like many other non-specific nucleases, it exhibits certain sequence preferences [3,6,7], suggesting that local structural features of the substrate influence the rate of cleavage. It requires Mg^{2+} for phosphodiester bond cleavage and produces 5′-phosphorylated oligonucleotides [8–10]. Mutational analyses which were based on the crystal structure [11] and a comparison with the amino acid sequence of several related enzymes [12–14] have allowed to identify amino acid residues essential for catalysis and to suggest a mechanism of action for this enzyme [15,16] which was corroborated by kinetic analyses using various natural and chemically modified substrates [5,17].

The *Serratia* nuclease is a member of a family of non-specific nucleases found in both prokaryotic and eukaryotic organisms, some of which have been characterised in detail: in addition to the *Serratia* nuclease, NucA from *Anabaena* sp. [13,18,19], Nuc1 from *Saccharomyces cerevisiae* [12,20], endonuclease G from *Bos taurus* [14,21], and a RNA/DNA non-specific nuclease from the mold *Syncephalostrum racemosum* [22]. Others have been identified on the basis of their sequence homology to the *Serratia* nuclease, for example a homologous nuclease from *Borrelia burgdorferi* whose sequence was obtained in a genome sequencing project [23].

While the *Serratia* nuclease [24,25,1], Nuc1 [12] and endonuclease G [26] were reported to be homodimers, NucA is a monomer [19], raising the question whether the dimeric state of the *Serratia* nuclease, Nuc1 and endonuclease G is essential for the activity of these enzymes and/or whether these enzymes have to dissociate into monomers for catalysis. This is not only a question of academic interest but has a technological aspect to it, as the *Serratia* nuclease (Benzonase) is an enzyme of commercial importance, which is used to degrade nucleic acids in biochemical and pharmaceutical preparations. For this purpose efforts are being undertaken to immobilise the enzyme on solid support. This would be much easier to accomplish with an active monomeric variant.

Based on the detailed crystallographic analyses of the *Serratia* nuclease [1,27] which have clearly identified the physiological dimer interface, we have designed variants of the *Serratia* nuclease which should not be able to dimerise. We report here the genetic engineering, production and characterisation of such variants. Our results demonstrate that the dimer state of the *Serratia* nuclease is not necessary for the catalytic function of this enzyme, as monomeric variants with the same specific activity have been identified.

2. Materials and methods

2.1. In vitro mutagenesis and sequencing

In vitro mutagenesis to obtain the S179C, H184N, H184T and H184R variants was carried out using the 2-PCR method [15,28]. The H184A variant was generated applying an inverse PCR strategy [19,29]. The mutations were verified by sequencing using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS, on an ABI 373A DNA sequencer (Applied Biosystems) according to the supplier's protocol.

2.2. Overexpression and purification of Serratia nuclease and variants

The wild-type *Serratia* nuclease and the variant enzymes S179C, H184A, H184N, H184T and H184R were produced as His_6GlySer-tagged proteins in *E. coli* and purified as described before [25,5].

2.3. Gel filtration analysis

Preparative and analytical gel filtration experiments were carried out at 25°C on a Beckman Biosys 2000 HPLC system using a Merck Superformance 600-16 Fractogel EMD BioSEC (S) and a Pharmacia Superdex 75 HR 10/30 column. The protein concentration was 40 μM.

2.4. Analytical ultracentrifugation analysis

Analytical ultracentrifugation experiments were carried out at 20°C and 45000 min^{-1} in a Beckman Optima XL-A centrifuge equipped with absorption optics and an An-50 8-place rotor. The protein con-

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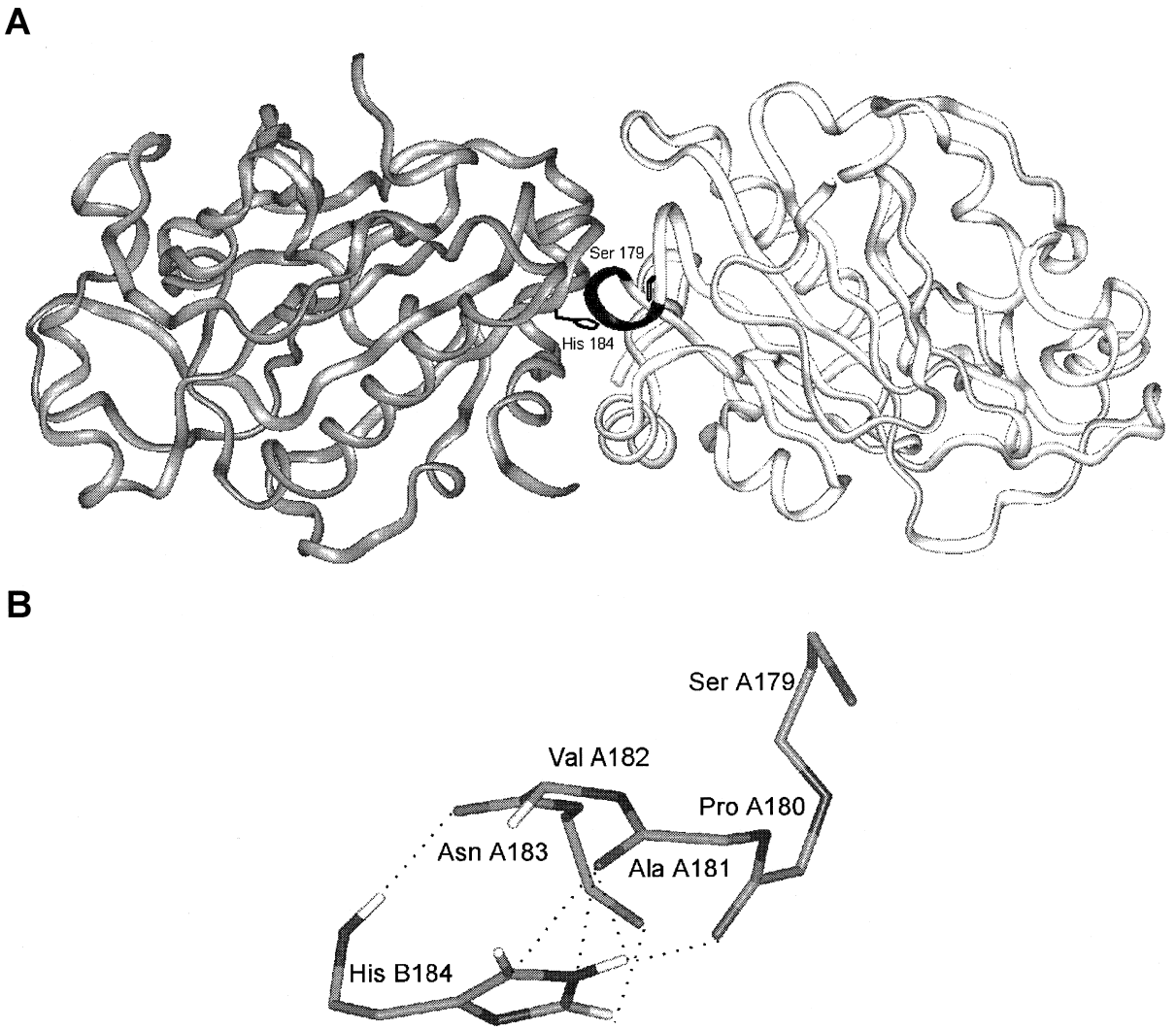

Fig. 1. A: Dimer of the *Serratia* nuclease (based on the coordinates of the *Serratia* nuclease, Brookhaven Protein Data Bank, accession number 1SMN). The positions of His-184 and Ser-179 are shown only in subunit A and B, respectively. The loop containing residues 180–183 in subunit A which make contacts to His-184 in subunit B is highlighted in black. B: Part of the His B184 environment and protein-protein contacts to residues in subunit A of the *Serratia* nuclease dimer, as identified in [1].

centration was 6 μM. Sedimentation velocity data were evaluated with the program package AKKUPROG [30].

2.5. Activity measurement with DNA as substrate (hyperchromicity assay)

The DNase activity of the wild-type *Serratia* nuclease and the different variants was determined using a hyperchromicity assay with high molecular weight DNA from herring sperm as described previously [16].

2.6. Protein engineering

The selection of amino acid substitutions to produce monomeric *Serratia* nuclease variants was based on the coordinates of the crystal structure ([11]; PDB entry 1SMN).

2.7. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For protein analysis 10% SDS-PAGE was used [31].

2.8. Circular dichroism (CD spectra)

CD spectra were recorded with a Jasco J-710 circular dichrograph in 0.05 cm cells at protein concentrations of 5 μM as described previously [15]. Chemical denaturation experiments were carried out us-

Table 1
Hydrogen bonds and non-bonded contacts of His-184 in the dimer interface of the *Serratia* nuclease

Subunit 1		Subunit 2	
Residue	Atom	Residue	Atom
Intermolecular hydrogen bonds (≤ 3.5 Å)			
Pro-180	O	His-184	NE2
Ala-181	O	His-184	NE2
Val-182	O	His-184	N
Water-mediated hydrogen bonds (≤ 3.5 Å)			
H_2O-29	ND1	His-184	OH2
Non-bonded protein-protein contacts (≤ 3.5 Å)			
Pro-180	O	His-184	CE1
Ala-181	C	His-184	NE2
Ala-181	O	His-184	CD2
Asn-183	O	His-184	CE1

As the interactions originating from subunit A and subunit B are essentially the same, interactions are given only for one subunit (modified from [1]).

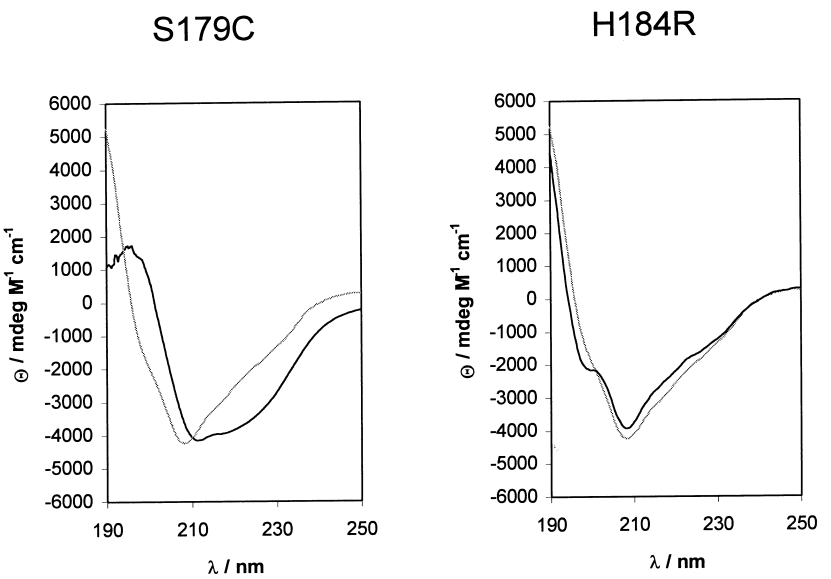

Fig. 2. Far-UV CD spectra of the S179C and the H184R variants. The spectrum of the wild-type protein is shown in grey for reference.

ing guanidinium chloride in a concentration range from 0–6 M. For thermal denaturation the protein samples were heated at a rate of $\Delta T = 20°C\ h^{-1}$. Both chemical and thermal denaturation curves were recorded by measuring the CD effect at $\lambda = 220$ nm. In addition, in the thermal denaturation experiments CD spectra were recorded at 10°C intervals in a wavelength range from 190 to 250 nm.

3. Results

The dimer interface of the *Serratia* nuclease contains a large surface area of complementary charge and shape. Together the two subunits form a single globular entity. His-184 is in a suitable distance to Pro-180, Ala-181, Val-182 and Asn-183 of the second subunit to form protein-protein contacts [1] (Fig. 1, Table 1). We have chosen His-184 as a prime candidate to disrupt the steric and electrostatic complementarity in the interface region as substitution of this residue promised to have a large impact on the integrity of the dimer interface. We introduced residues that either cannot form the specific contacts to the second subunit or in addition are large and bulky and introduce additional charge. The replacement of His-184 with Ala, Asn, Thr and Arg resulted in proteins of good solubility.

In addition, we have produced the S179C variant that previously had been described as a monomeric variant of the nuclease [1]. The Ser-179 to Cys exchange is interesting because it would not be expected to directly disrupt the dimer interface although this residue is located near the interface (Fig. 1). In contrast to the His-184 variants S179C was only poorly expressed.

The quaternary structure of the wild-type enzyme, the S179C and the presumptive monomeric H184 variants was analysed by gel filtration and analytical ultracentrifugation. Previously produced crosslinked dimers of the *Serratia* nuclease (Franke and Pingoud, in preparation) and the S179C monomer [1] were used to calibrate a gel filtration column. The gel filtration experiments clearly showed that the H184 variants are monomers (Table 2). The monomeric state of the S179C and the H184R variants was confirmed by sedimentation velocity experiments in the analytical ultracentrifuge (Table 3).

CD spectra recorded in a wavelength range from 195–250 nm demonstrate that the His-184 variants (Fig. 2, shown is only the spectrum of H184R) have the same secondary structure composition and, thus, presumably the same tertiary structure as the wild-type enzyme. In the case of S179C a significantly different far-UV CD spectrum was obtained which indicates changes in the secondary structure composition and, therefore, also in the overall structure of this variant (Fig. 2).

The activity data (Table 4) are in agreement with the structural data. The monomeric His-184 variants have within the limit of error the same nucleolytic activity (10^6 Kunits units mg^{-1}) and similar k_{cat}- and K_M-values as the wild-type enzyme whereas the S179C variant shows only residual nucleolytic activity (10^3 Kunits units mg^{-1}) probably due to changes in the structure.

As monomers of naturally occurring dimers tend to be less stable when compared to their dimeric form [32] we have subjected the wild-type enzyme as well as the monomeric variants H184R and S179C to chemical and thermal unfolding experiments. The stability was determined by measuring the CD effect at 220 nm at guanidinium chloride (GdmCl) con-

Table 2
Elution volumes of monomeric and dimeric variants of the *Serratia* nuclease

Variant	S140C_X	Wt	S179C	H184R	H184T	H184N	H184A
V_E	0.86	0.91	1	1.04	0.97	0.99	1.04

The wild-type protein elutes between a crosslinked dimer (S140C_X) and the monomeric variants S179C, H184A, H184N, H184T and H184R which reflects a monomer-dimer equilibrium.

Table 3
Sedimentation data for dimeric and monomeric variants of the *Serratia* nuclease

Variant	Dimer (theoret.)	Monomer (theoret.)	Wt	S179C	H184R
$S_{20,w}$ [S]	3.49	2.62	3.5	2.5	2.5

The theoretical sedimentation coefficients were calculated for a protein of spherical shape ($a/b = 1$) in the case of the monomer and for a prolate ellipsoid ($a/b = 3/1$) in the case of the dimer.

centrations varying between 0 and 6 M (Fig. 3). Assuming a simple unfolding model, the free energy $\Delta G°(H_2O)$ of the different variants of the nuclease was calculated. This assumption is justified as the GdmCl induced unfolding is reversible and gives no indication of populated intermediates. $\Delta G°(H_2O)$ was found to be -10.0 kJ mol^{-1} (per subunit) for the wild-type enzyme, -10.1 kJ mol^{-1} for the S179C variant and -11.4 kJ mol^{-1} for the H184R variant.

In addition to the GdmCl induced denaturation, the thermal unfolding of the wild-type enzyme and the two monomeric variants S179C and H184R was analysed. Heating samples of the nuclease from 20 to 85°C led only to a slight increase of the CD effect at 220 nm for the wild-type enzyme ($\Delta\Theta \approx -1000$ mdeg M^{-1} cm^{-1}) suggesting a restructuring rather than unfolding, and to no significant change for the S179C variant. The H184R variant is also resistant to heat induced unfolding up to a temperature of 60°C. At higher temperature unfolding begins but is not complete at 85°C as a residual CD effect of $\Theta = -2000$ mdeg M^{-1} cm^{-1} remains. CD spectra that were recorded between 190 and 250 nm at 10°C intervals revealed that in the case of the wild-type enzyme and the H184R variant the CD spectrum in a wavelength range from 210 to 250 nm becomes similar to the 20°C spectrum of the S179C variant, whereas the CD spectrum of the S179C variant remains more or less unchanged upon heating (data not shown). We conclude from this that upon heating the wild-type enzyme and the H184R variant change their structure in a way that it becomes similar to that of the S179C variant. In agreement with this is the finding that the wild-type nuclease and the H184R variant loose their nucleolytic activity at 60°C but regain it when cooled down to ambient temperature (data not shown).

4. Discussion

The *Serratia* endonuclease is an extracellularly secreted enzyme. Cleaving both single- and double-stranded DNA and RNA it presumably has a nutritive function, making available for its host precursors for the synthesis of nucleic acids. Related enzymes continue to be discovered in prokaryotic and eukaryotic organisms, where they may serve also other than nutritive functions. For example, endonuclease G from *B. taurus* is associated with the inner membrane of mitochondria and involved in the replication of mitochondrial DNA [14].

As far as this has been investigated, all members of the *Serratia* nuclease family of enzymes like the *Serratia* nuclease [24,15,1] are homodimers, with the exception of the *Anabaena* nuclease which is a monomer [19]. We have been interested to find out whether the catalytic activity of the *Serratia* nuclease is dependent on its dimeric state or whether it is active both as a dimer or a monomer. To address this question we previously have crosslinked the two subunits of a genetically engineered variant and demonstrated that this obligatory dimer is fully active (Franke and Pingoud, in preparation). While this result demonstrated that the dimer state is compatible with activity, it did not resolve the problem whether a monomeric variant could also be active. For this purpose, we have now produced monomeric variants of the *Serratia* nuclease which carry single amino acid substitutions. Replacement of His-184 which is located in the dimer interface, by Ala, Thr, Asn and Arg, all resulted in monomeric variants as shown by gel filtration and analytical ultracentrifugation experiments. This demonstrates that His-184 is essential for dimerisation. From gel filtration experiments it is clear that the H184R variant does not show any tendency to dimerise at concentrations up to 40 μM. This implies that H184R is a monomer under the conditions where it cleaves its substrate. While the His-184 variants have a different quaternary structure compared to the wild-type enzyme, their secondary structure composition is the same as that of the wild-type enzyme, suggesting that also the tertiary structure is unchanged. This is corroborated by the stability measurements which demonstrate that the His-184 variants are as stable as the wild-type enzyme towards guanidinium chloride induced denaturation. Most importantly, the monomeric His-184 variants have a very similar specific activity in cleaving DNA as the dimeric wild-type enzyme. Taken together these results allow to conclude that the *Serratia* nuclease is not dependent on its dimeric state for catalytic activity. Hence, the catalytic centres of the dimeric enzyme are completely independent of each other. This does not mean that the natural dimeric state of the *Serratia* nuclease needs not be without advantage for *S. marcescens*. It could well be that at low concentrations of enzyme and macromolecular nucleic acids, likely to be present in the natural habitat of this bacterium, processive cleavage and, therefore, complete digestion of a nucleic acid substrate would benefit from the dimeric state.

We have also analysed the S179C mutant which had been

Table 4
Specific activities and kinetic parameters for the cleavage of high molecular weight DNA by the wild-type *Serratia* nuclease and the monomeric variants S179C, H184N, H184T and H184R

Variant	Activity (KU mg^{-1})	k_{cat} (M(nt)/M(E) s^{-1})	K_M (μM(nt))	$k_{cat}\ K_M^{-1}$ (μM^{-1} s^{-1})
wt	3.7×10^6	1126	85	13
S179C	5.7×10^3	1	n.d.	n.d.
H184R	5.8×10^6	1721	85	20
H184N	4.0×10^6	1197	106	11
H184T	5.7×10^6	1667	75	22

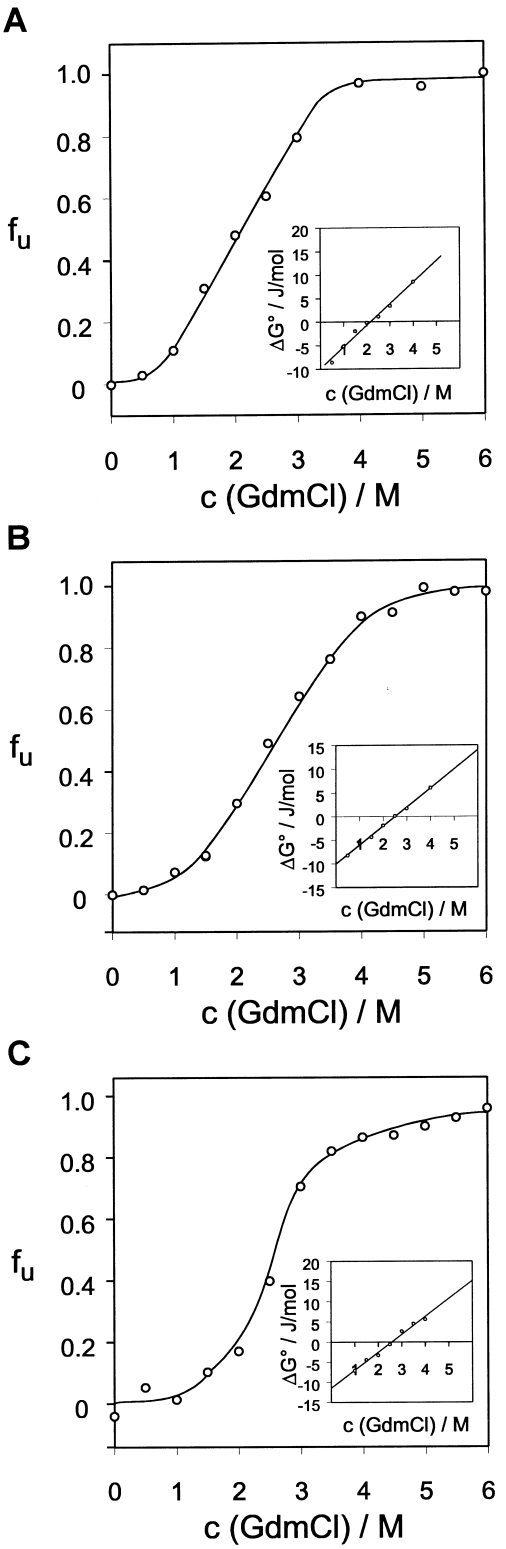

Fig. 3. GdmCl induced unfolding of the wild-type *Serratia* nuclease (A), the S179C variant (B) and the H184R variant (C). The transition curves in which the fractions of unfolded protein versus the GdmCl concentration are plotted were obtained by monitoring the CD at $\lambda = 220$ nm. The data for the plot of unfolding free energy change ΔG were calculated from the transition curves. The straight line was obtained by linear regression. Extrapolation to 0 M GdmCl yields $\Delta G°(H_2O)$ (data given in the text).

reported before to be monomeric [1]. This result could be confirmed, however, S179C displays only 0.1% of the activity of the wild-type enzyme and the His-184 variants described above. Both its monomeric state and its inactivity came as a surprise as Ser-179 is not directly involved in subunit interactions and far from the catalytic centre. Furthermore, the Ser→Cys substitution is conservative. The CD spectroscopic analysis showed that the S179C variant does not have the same secondary structure composition as the wild-type enzyme or the His-184 variants. Chemical denaturation experiments of the S179C variant revealed an enzyme with a stability comparable to that of the wild-type enzyme and the H184 variants. It is noteworthy that the His-184 variants and the wild-type enzyme at 60°C have a similar CD spectrum as the S179C variant at 20°C and loose their activity at this temperature but regain it when cooled to ambient temperature. We conclude from this that the wild-type enzyme dimer dissociates upon heating and then, as the monomeric His-184 variants, undergoes a structural rearrangement that is similar to the one that occurs as a consequence of the Ser→Cys substitution.

The *Serratia* nuclease (tradename: Benzonase) is an enzyme of commercial importance, as it is used to remove nucleic acids from biochemical and pharmaceutical preparations. For this purpose, it would be advantageous if the enzyme could be immobilised on solid support. So far, such efforts were unsuccessful, as the immobilised dimer slowly dissociates into its subunits, leading to 'bleeding' of the *Serratia* nuclease if not both subunits are covalently linked to the support (Dr. K. Holschuh, personal communication). We expect the His-184 variants because of their monomeric state to pose no problems in this respect.

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