Inhibitory Activity of the Drosophila melanogaster Serpin Necrotic Is Dependent on Lysine Residues in the D-helix*

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Necrotic is a member of the serine protease inhibitor or serpin superfamily. It is a potent inhibitor of elastase and chymotrypsin type proteases and is responsible for regulating the antifungal response in Drosophila melanogaster. Necrotic contains three basic lysine residues within the D-helix that are homologous to those found in the heparin-binding domain of antithrombin and heparin co-factor II. We show here that substitution of all three lysine residues for glutamines caused cellular necrosis and premature death in Drosophila in keeping with a loss of function phenotype. The lysine to glutamine substitutions had no effect on the overall structure of recombinant Necrotic protein but abolished the formation of stable complexes with target proteases. Individual substitutions with either glutamine or alanine demonstrated that lysine 68 was the most critical residue for inhibitory activity. Despite the homology to other serpins, Necrotic did not bind, nor was it activated by sulfated glycans. These data demonstrate a critical role for basic residues within the D-helix (and lysine 68 in particular) in the inhibitory mechanism of the serpin Necrotic.

The serine protease inhibitors or serpins are found in most branches of life including mammals, plants, viruses, and prokaryotes, where they inhibit a wide range of proteolytic cas-

cades (1). The superfamily is defined by more than 30% sequence homology with the archetypal member α1-antitrypsin and conservation of tertiary structure. This structure is composed of three β-sheets (A–C) and an exposed mobile reactive loop that presents a peptide sequence as a pseudosubstrate for the target protease (2). After docking, the enzyme cleaves the P1–P1’ peptide bond of the serpin (3), and the protease is inactivated by a dramatic conformational transition that swings it 70 Å from the upper to the lower pole of the protein in association with insertion of the reactive loop as an extra strand (s4A) in β-sheet A (4, 5) (Fig. 1A). The serpin-enzyme complex is then removed from the circulation and targeted for destruction via members of the low density lipoprotein receptor family (6, 7).

Necrotic (Nec)3 is a serpin that regulates the extracellular activation of the Drosophila Toll-mediated antifungal response (8). Fungal infection triggers an extracellular protease cascade that results in the cleavage and activation of the Toll ligand, Spaetzle (Fig. 2). Removal of functional Nec results in an abundance of cleaved Spaetzle and constitutive activation of the Toll pathway. Drosophila strains containing nec null alleles display obvious black patches of cellular necrosis localized on the cuticular joints and die within 48 h of eclosion (9, 10). It is this phenotype that gives the protein its name. We have shown previously that Nec is a broad range protease inhibitor with preference for elastase- and chymotrypsin-like proteases (11). However, Nec has two major differences when compared with the archetypal serpin α1-antitrypsin. First, it has a 100 residue N-terminal extension of unknown function, and second, it contains three lysine residues at the N-terminal portion of the D-helix. Other serpins, such as antithrombin and heparin co-factor II, that have homologous D-helix lysine residues bind sulfated glycans such as heparin (12–14). This results in a conformational transition within the serpin and a 100-fold increase in the association rate constant with the target protease (13–17).

We have used a biochemical and genetic approach to evaluate the role of the D-helix lysine residues in Nec. We show here that these residues are required for serpin inhibitory activity both in vivo and in vitro. The most important residue was lysine 68 because the K68Q and K68A substitutions increased the stoichiometry of inhibition (SI) to such an extent that it prevented complex formation. Interestingly, these residues were not required for binding sulfated glycans. These results demonstrate a novel role for positively charged D-helix residues in serpin inhibitory function.

**EXPERIMENTAL PROCEDURES**

*Materials*—Restriction enzymes were purchased from Roche Applied Sciences; T4 DNA-ligase and the IMPACT-NT pro-

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3 The abbreviations used are: Nec, Necrotic; SI, stoichiometry of inhibition; HNE, human neutrophil elastase.
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protein purification system were from New England Biolabs (Hitchin, Hertfordshire, UK); and oligonucleotides, porcine pancreatic trypsin, bovine pancreatic α-chymotrypsin, porcine pancreatic elastase, bovine serum thrombin, and the substrate MeO-Suc-Ala-Ala-Pro-Val-p-NA were from Sigma. Human neutrophil elastase (HNE) was purchased from Athens Research and Technology (Athens, GA). The rabbit anti-Nec antibody was a kind gift from Dr. J.-M. Reichhart (Université Louis Pasteur, Strasbourg, France).

Protein Purification—Expression and purification of wild-type and mutant Nec was achieved by fusion with an N-terminal tag consisting of a chitin-binding domain and a protein-splicing element (Intein). The Intein sequence undergoes specific self-cleavage in the presence of thiols, such as cysteine or dithiothreitol, allowing separation of the target protein from the chitin-bound affinity tag. Previous studies revealed that full-length Nec failed to express in Escherichia coli (11). Recombinant Nec without the N-terminal extension (Nec-ΔN) was amplified using PCR from Nec cDNA (10) and cloned into the Intein pTYB12 expression vector using SpeI and NotI restriction sites. E. coli BL21(DE3) cells containing the pTYB12-Nec-ΔN construct were induced at an optical density of 0.5–0.6 with 0.1 mM isopropyl β-D-thiogalactopyranoside and grown overnight at 23 °C. The cells were harvested by centrifugation, resuspended in 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, and lysed by sonication in the presence of a mixture of protease inhibitors. The lysis extracts were isolated by high speed centrifugation and then applied to a column containing the IMPACT-NT chitin-resin. On-column self-cleavage of the Intein sequence was carried out by overnight incubation with 50 mM dithiothreitol at 4 °C. The protein was further purified through a Superdex 200 gel filtration column (Amersham Biosciences) and stored in 50 mM Tris, pH 8.0. The resulting protein was assessed by SDS, non-denaturing, and 8 M urea PAGE as detailed previously (18). In each case 3 μg of protein was loaded in each lane. CD experiments were performed using a JASCO J-810 spectropolarimeter.

Site-directed Mutagenesis—pTYB12-Nec-ΔN mutant constructs were prepared by site-directed mutagenesis using the QuikChange™ XL kit (Stratagene). Oligonucleotide primers consisting of the point mutation flanked on both sides by 10–12 bases of template sequence were used. DpnI restriction enzyme was added following the reaction and incubated for 37 °C for 1 h to digest the parental supercoiled template DNA. DpnI-treated DNA from each reaction was transformed into XL10-Gold ultracompetent cells.

Complex Formation Assays—Recombinant Nec protein was incubated with varying quantities of serine proteases for 15 min at room temperature in 50 mM Hepes, 150 mM NaCl, 0.01% (w/v) dodecyl-maltoside, pH 7.4 (buffer A). The reactions were stopped by incubation with 1% (w/v) SDS-PAGE loading buffer and boiling for 3 min. The samples were pulse centrifuged and separated by 10% (w/v) SDS-PAGE, and the protein was visualized by staining with Coomassie Blue.

Determination of Reaction Parameters Describing Protease Inhibition—The inhibition rate constant (kᵢ) describing the inhibition of HNE by Nec-ΔN and Nec-ΔN mutants was determined under pseudo-first order conditions (i.e. [I] ≈ 10[E]₀) using the progress curve method (19, 20). Rate constants of inhibition were measured at 25 °C in 50 mM Hepes, 150 mM NaCl, 0.01% (w/v) dodecyl-maltoside, pH 7.4 by adding the
enzyme (final concentration, 20 nM) to a mixture of Nec protein (from 200 nM to 1.6 nM) and the substrate (1.5 mM, \(K_m = 0.23\) mM) for HNE and recording the release of product as a function of time. The progress curves were then analyzed according to Equation 1 (19, 20),

\[
[P] = v_s t + \frac{v_s - v_0}{k_{obs}} (1 - e^{-k_{obs}t})
\]  
(Eq. 1)

where \(v_s\) is the initial velocity, \(v_0\) is the steady state velocity at completion of the reaction, and \(k_{obs}\) is the pseudo-first rate order rate constant for the approach toward steady state. The values of each variable were obtained by fitting the progress curve to Equation 1 using nonlinear regression analysis and were then used to calculate \(k_{obs}\) according to Equation 2 under the assumption that the inhibition takes place through a simple bi-molecular reaction.

\[
k_{obs} = \frac{k_d [I]}{1 + [S]/K_m} + k_d
\]  
(Eq. 2)

**Role of the D-helix in the Inhibitory Activity of Necrotic**

**RESULTS**

**Charged Residues in the D-helix Are Required for Nec Function in Vivo**—Homology models show a concentration of basic residues (Lys68, Lys70, and Lys71) at the N-terminal domain of the D-helix of Nec (Fig. 1B). These residues are in positions similar to those of residues within the positively charged heparin-binding domains of antithrombin (Arg129, Arg132, and Lys133) and heparin co-factor II (Arg189, Arg192, and Arg193) (Fig. 1C). To determine whether these residues are important for inhibitor function in vivo, we created UAS-nec transgenic Drosophila lines in which the three lysine residues were replaced by glutamines (K68Q, K70Q, and K71Q). The transgene (UAS-nec K68Q/K70Q/K71Q) was then expressed ubiquitously in Drosophila using the GAL4 daughterless driver. Unlike wild-type UAS-nec transgenic flies, expression of the UAS-nec containing K68Q/K70Q/K71Q in the D-helix (Œ) fail to rescue nec null mutant flies.

**Biochemical Analysis of K68Q/K70Q/K71Q Nec-N**—To define the contributions that lysines 68, 70, and 71 make to protein function, we expressed and purified recombinant wild-type and K68Q/K70Q/K71Q Nec-DN using the IMPACT-NT system as detailed under “Experimental Procedures.” The residues Ala-Gly-His-Met-Thr were added to the N terminus of the assumption that the inhibition takes place through a simple bi-molecular reaction.

\[
k_{obs} = \frac{k_d [I]}{1 + [S]/K_m} + k_d
\]  
(Eq. 2)
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Lys₆⁸, Lys₇₀, and Lys₇¹ in the D-helix of Nec Are Required for Protease Inhibition—To test for inhibitory activity, wild-type and K68Q/K70Q/K71Q Nec-ΔN were incubated at a 1:1 molar ratio with enzymes that are known to be a target for Necrotic: bovine α-chymotrypsin, HNE, porcine pancreatic elastase, and human neutrophil cathepsin G (11). In each case wild-type Nec-ΔN formed an SDS-stable complex with the protease, but K68Q/K70Q/K71Q Nec-ΔN was cleaved to generate a smaller species with a molecular mass of ~38 kDa (Fig. 5). The kinetics of inhibition of Nec was further assessed by the determination of both the association rate constant ($k_a$) and the SI against HNE. Wild-type Nec-ΔN inhibited human neutrophil elastase with a $k_a$ of $1.5 \times 10^5$ M⁻¹ s⁻¹ and a stoichiometry of inhibition of 1.2 (Table 1). However, no value was measurable for either of these parameters when K68Q/K70Q/K71Q Nec-ΔN was incubated with HNE. These data show that Lys₆⁸, Lys₇₀, and Lys₇¹ are required for Nec to form a covalently linked serpin-protease complex.

Dissection of the Contribution of Each D-helix Lysine Residue to the Inhibitory Activity of Nec—To determine the contribution made by each D-helix lysine residue, we prepared recombinant Nec-ΔN protein containing either the K68Q, K70Q, or K71Q substitution. Because glutamine has polar properties, we also prepared recombinant Nec-ΔN protein containing either the K68A, K70A, or K71A substitution. Each recombinant protein migrated as a single species on 10% (w/v) SDS and nondenaturing PAGE (Fig. 6a and data not shown). All of the mutants

wild-type and K68Q/K70Q/K71Q Nec-ΔN as a result of the cloning strategy so that the N terminus of the recombinant protein became AGHMTVF-. Both wild-type and K68Q/K70Q/K71Q Nec-ΔN migrated as a single band on 10% (w/v) SDS and nondenaturing PAGE (Fig. 4a) and CD spectra showed the characteristic serpin α/β fold with a flat-bottomed negative minimum extending between 208 and 222 nm for both wild-type and K68Q/K70Q/K71Q Nec-ΔN (Fig. 4b). An overlay showed a nearly identical profile between 205 and 225 nm for the wild-type and mutant proteins. To test whether cleavage of the reactive center loop resulted in a stable, relaxed species, wild-type and K68Q/K70Q/K71Q Nec-ΔN were incubated with porcine pancreatic trypsin at an enzyme to serpin molar ratio of 1:500 in 50 mM Hepes, 150 mM NaCl, 0.01% (w/v) dodecyl-maltoside, pH 7.4, for 15 min at 22 °C. Both the wild-type and mutant Nec-ΔN migrated more rapidly on an 8 M urea gradient gel following incubation with enzyme (data not shown), in keeping with the formation of a hyperstable serpin with the reactive loop inserted into β-sheet A. These results indicate that both wild-type and K68Q/K70Q/K71Q Nec-ΔN were in a native, monomeric conformation capable of undergoing the stressed to relaxed transition that is typical of an inhibitory serpin.

FIGURE 4. Characterization of K68Q/K70Q/K71Q Nec-ΔN. a, 10% (w/v) SDS-PAGE (left) and nondenaturing PAGE (right) of recombinant K68Q/K70Q/K71Q Nec-ΔN following chitin column purification and gel filtration using a Superdex 200 column. The protein migrates as a single band on both gels, indicating that it is present in its monomeric conformation. b, circular dichroism spectrum of recombinant K68Q/K70Q/K71Q Nec-ΔN at 22 °C (solid line) shows a flat bottom profile typical of a native serpin. The CD profile of wild-type Nec-ΔN (dotted line) is included for comparison.

FIGURE 5. Inhibitory activity of K68Q/K70Q/K71Q Nec-ΔN. 10% (w/v) SDS-PAGE comparing the interaction of wild-type and K68Q/K70Q/K71Q Nec-ΔN with cathepsin G (CATG), HNE, porcine pancreatic elastase (PPE), and bovine α-chymotrypsin (CHYM) serine proteases. Recombinant protein was incubated at a 1:1 serpin/protease ratio for 15 min. The inhibitory complex (small arrow) and cleaved Nec are indicated.

TABLE 1

|        | $k_a$  | SI    | $k_a \times SI$ |
|--------|--------|-------|-----------------|
| Nec-ΔN | $1.5 \times 10^5$ | 1.2   | $1.8 \times 10^5$ |
| Nec-ΔNK₆⁸Q | NR     | NR    | NR              |
| Nec-ΔNK₇₀Q | NR     | NR    | NR              |
| Nec-ΔNK₇₁Q | NR     | NR    | NR              |
| Nec-ΔNK₆⁸A | $2.5 \times 10^4$ | 3.5   | $8.7 \times 10^3$ |
| Nec-ΔNK₇₀A | $2.1 \times 10^4$ | 3.5   | $6.7 \times 10^3$ |
| Nec-ΔNK₇₁A | $2.6 \times 10^4$ | 3.4   | $9.1 \times 10^3$ |
| Nec-ΔNK₆⁸Q₇₀A | $0.7 \times 10^4$ | 3.1   | $2.2 \times 10^3$ |
| Nec-ΔNK₆⁸Q₇₁A | $8.7 \times 10^4$ | 1.7   | $1.5 \times 10^5$ |
| Nec-ΔNK₇₀Q₇₁A | $1.5 \times 10^5$ | 1.2   | $1.8 \times 10^5$ |

The errors for the analysis of the kinetic parameters are <10%. The results represent the means of at least three independent experiments. NR, not possible to determine result. Nec-ΔN is K68Q/K70Q/K71Q Nec-ΔN.
with HNE that were 60-fold slower than that of wild-type Nec-ΔN (Table 1). The SI values for these mutants were 3-fold higher when compared with wild-type Nec-ΔN. There was no observable inhibition of HNE by K68Q Nec-ΔN. Similar results were obtained for Nec-Δ when the lysine residues at 68, 70, and 71 were substituted for alanines (Table 1). Both K70A and K71A Nec-Δ were effective inhibitors of HNE despite an increase in the SI. However, there was no observable inhibition of HNE by K68A Nec-ΔN. Taken together, these data show that all three lysines contribute to the inhibitory activity of Nec-ΔN, but that the largest contribution is from Lys68.

The Loss of Function of E64Q Mutation Nec Is Not Mediated by Breaking the Salt Bridge with Glu64—Wild-type Nec protein contains a Glu residue at position 64, four residues, and one complete helical turn prior to Lys68. The distance between the side chains of Lys68 and Glu64 (6.28 Å) is compatible with the formation of a salt bridge (Fig. 1D). To test the hypothesis that this salt bridge may be required for serpin activity, we prepared, expressed, and purified recombinant Nec-ΔN containing the E64Q or E64A mutation. SDS-PAGE, nondenaturing PAGE, 8 M urea PAGE, and circular dichroism studies confirmed that E64Q and E64A Nec-ΔN folded as a monomeric serpin (data not shown). Both E64Q and E64A Nec-ΔN formed an SDS-stable complex with HNE with $k_a$ and SI values that were comparable with those of wild-type Nec-ΔN (Table 1). These results suggest that the deleterious effects of the K68Q and E64A mutations are not due to a stabilizing interaction between Lys68 and Glu64.

Lys68, Lys70, and Lys71 in the D-helix of Nec Have No Role in Heparin Binding—In view of the homology between Lys68, Lys70, and Lys71 in the D-helix of Nec and the heparin-binding domain of antithrombin and heparin co-factor II, we assessed whether the D-helix lysines-mediated binding to glycans. Wild-type Nec-ΔN eluted from a heparin affinity column containing both high and low molecular weight oligosaccharides as a single peak at $\sim$0.2 M NaCl. By comparison, K68Q/K70Q/K71Q Nec-ΔN eluted primarily at a peak of 0.3 M NaCl. These results suggest that Nec-ΔN has a modest affinity for sulfated glycans and that this effect is not mediated by the Lys68/Lys70/Lys71 residues in the D-helix. Furthermore, neither high nor low molecular weight oligosaccharides changed the intrinsic tryptophan fluorescence profile of Nec-ΔN, demonstrating that there is no conformational transition upon heparin binding.

**DISCUSSION**

Nec is a member of the serine protease inhibitor (serpin) superfamily that regulates the extracellular activation of the *Drosophila* Toll-mediated antifungal response (8). It functions as a broad range protease inhibitor (11) and contains three lysine residues in the N-terminal portion of the D-helix. These residues are likely to be of particular importance as other serpins that have homologous D-helix lysine residues bind and are activated by sulfated glycans such as heparin (21). We have therefore assessed the role of these D-helix lysine residues on the structure and function of Nec. To assess the role of these residues in vivo, Lys68, Lys70, and Lys71 were mutated to structurally similar but uncharged glutamine residues (K68Q, K70Q, and K71Q). This triple mutant failed to rescue flies lacking

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**FIGURE 6.** Characterization and assessment of the inhibitory activity of recombinant K68Q, K70Q, and K71Q Nec-ΔN. a, 10% (w/v) SDS-PAGE (left panel) and 7.5% (w/v) nondenaturing-PAGE (right panel). b, 10% (w/v) SDS-PAGE comparing the interaction of wild-type, K68Q/K70Q/K71Q, K68Q, K70Q, and K71Q Nec-ΔN with HNE. In each case the recombinant protein was incubated with HNE at a 1:1 serpin:enzyme ratio for 15 min. The bands corresponding to complexed and cleaved serpin are indicated.
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functional Nec (Fig. 3) and demonstrated that Lys68, Lys70, and Lys71 are important for the inhibitory activity of the protein.

The K68Q/K70Q/K71Q Nec mutant was then expressed as a recombinant protein to determine the mechanism by which the triple mutant inactivates Nec. Our previous studies have shown that full-length Nec cannot be expressed in E. coli (11), and so the mutants were prepared in Nec in which the first 99 residues of the N terminus had been deleted (Nec-ΔN). This truncated protein adopts a typical serpin fold and displays inhibitory activity against HNE and chymotrypsin-like proteases (11). Recombinant K68Q/K70Q/K71Q Nec-ΔN was purified as a monomeric protein that had a CD profile almost identical to that of wild-type Nec-ΔN (Fig. 4). Thus the mutants do not prevent protein folding. However K68Q/K70Q/K71Q Nec-ΔN had no inhibitory activity against HNE, cathepsin G, porcine pancreatic elastase, or bovine α-chymotrypsin (Fig. 5). Indeed progress curve kinetics confirmed a complete lack of inhibition against the target HNE (Fig. 5 and Table 1). This is surprising because, despite being important in heparin binding, no mutants have been described in these residues that cause a loss of inhibitory function in other serpins.

The effect of the K68Q/K70Q/K71Q mutant on Nec-ΔN was further characterized by preparing and expressing each of the point mutations individually (Fig. 6a). All three residues contribute to inhibitory activity. Both K70Q and K71Q reduced the association rate constant with HNE by 20-fold if the kₐ × SI is used to calculate a second order rate constant (22) (Table 1). However, in both cases the Nec-ΔN mutant was still able to form an SDS-stable complex (Fig. 6b). The result was most striking for K68Q Nec-ΔN. This mutant was a substrate for HNE and was unable to form an SDS-stable complex. These data demonstrate that Lys68 is the most important of the three residues in mediating inhibitory activity. To confirm these data we also prepared and expressed each of the point mutations with an alanine replacing the lysine residue. Once again only the replacement of Lys68 had a significant effect on the inhibitory activity of Nec-ΔN.

Analysis of Nec-ΔN modeled on antithrombin (13) showed that residue Glu64 was within sufficient proximity to form a salt bridge with Lys68. We assessed whether this linkage was necessary for the inhibitory activity of Nec-ΔN by replacing Glu64 with a glutamine or an alanine. Recombinant E64Q Nec-ΔN had only a modest reduction in association rate constant and increase in SI with HNE. Nec-ΔN containing the E64A mutation had no effect on the association rate constant or SI with HNE. Thus the salt bridge between residues Glu64 and Lys68 is not a requirement for Nec-ΔN to be a competent protease inhibitor.

The cluster of lysine residues (Lys68, Lys70, and Lys71) within the D-helix is in a similar spatial location to those of antithrombin and heparin co-factor II (Fig. 1). Thus the lack of activity of the K68Q/K70Q/K71Q mutant of Nec-ΔN may result in part from a failure to bind and be activated by sulfated glycans. This was assessed by analyzing the binding of wild-type and K68Q/K70Q/K71Q Nec-ΔN on a heparin affinity column. Recombinant wild-type Nec-ΔN eluted from the column in 0.3 M NaCl. This is modest when compared with antithrombin, which elutes from heparin-Sepharose heparin at ~1.0 M NaCl but is similar to heparin co-factor II and plasminogen activator inhibitor-1, which elute at 0.4 M NaCl (23). Surprisingly, substitution of Lys68, Lys70, and Lys71 Nec-ΔN for uncharged glutamines showed a slight increase in binding affinity for heparin. The cause for this is unclear, but it is possible that the K68Q/K70Q/K71Q mutant exposes other positively charged residues that can bind to heparin. Finally, the addition of heparin to Nec did not cause any conformational transition when assessed by intrinsic tryptophan fluorescence. Taken together these data show that the K68Q/K70Q/K71Q mutant of Nec-ΔN does not mediate its effect by blocking the heparin activation of Nec.

The serpin superfamily of inhibitors utilizes a dynamic mechanism to regulate a wide range of biological pathways (1). The “suicide” mechanism of inhibition allows tight regulation of enzyme activity through the distortion of the active site of the protease (5). In heparin-binding serpins the conformation of the C terminus of the D-helix is coupled to the state of β-sheet A (24–26). It is clear from the data presented here that residues in the D-helix also play a critical role in inhibitory activity of the non-heparin-activated serpin Nec. It is likely that mutations in the D-helix perturb the top of β-sheet A, which in turn will affect the degree of loop insertion in the native state as well as the rate of loop insertion once the protease has bound to the serpin. Either of these mechanisms would explain the abolition of inhibitory activity caused by mutations in the D-helix.

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