The myxoma and malignant rabbit fibroma poxviruses are lethal tumorigenic viruses of rabbits whose virulence is modulated by the production of a virus-encoded secreted serine proteinase inhibitor, SERP-1. This viral protein was detected in medium harvested from myxoma and malignant rabbit fibroma virus-infected cells, and its inhibitory profile has been characterized by gel and kinetic analysis. SERP-1 forms complexes with and inhibits the human fibrinolytic enzymes plasmin, urokinase, and two-chain tissue-type plasminogen activator (association rate constants 3.4 \times 10^6, 4.3 \times 10^5, and 3.6 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \text{ respectively.}) It is also able to inhibit \( C_{1s} \), the first enzyme in the complement cascade with an association rate constant which was unaffected by the addition of heparin (1.3 \times 10^5 \text{ M}^{-1} \text{s}^{-1}). SERP-1 acts as a substrate for and is cleaved by thrombin, porcine trypsin, human neutrophil elastase, porcine pancreatic elastase, thermolysin, subtilisin, bovine \( \alpha \)-chymotrypsin, and factor Xa. Incubation with kallikrein and cathepsin G had no effect.

The structure of SERP-1 has been modeled on other members of the serpin family which revealed the characteristic serpin architecture apart from the absence of the D-helix. Structural analysis and kinetic assays demonstrate that the absence of this region does not prevent inhibitory activity and furthermore allow the identification of cysteine residues involved in internal and intermolecular disulfide bonding.

The superfamily of serine proteinase inhibitors or serpins includes a number of well-characterized regulatory proteins such as \( \alpha_1 \)-antitrypsin, \( \alpha_1 \)-antichymotrypsin, antithrombin III, and heparin cofactor II (1). The inhibitory specificity of these proteins is in the main defined by the residues at the P₁-P₄' positions of the reactive center (2). These amino acids act as a pseudosubstrate for the cognate proteinase which then binds in a 1:1 ratio with the serpin and is inactivated by complex formation (3).

There are other members of the serpin family which are not proteinase inhibitors such as ovalbumin (4), thymoxine binding globulin (5), and angiotensinogen (6), and some serpins whose function has yet to be determined. Important examples of the last group are the serpins expressed by the poxviruses. These proteins have gained membership to the family by virtue of amino acid sequence homology and have been identified in the vaccinia virus genome (7-9), the cowpox virus (10), the fowlpox virus (11), and myxoma and malignant rabbit fibroma viruses (12).

The viral serpins are important in determining virulence as their deletion from the viral genome significantly reduces host mortality (10, 12). For example, rabbits infected with the MYX \(^{1}\) or MRV develop primary tumors at the site of inoculation. The viruses then spread through the host lymphatic channels to induce a rapidly lethal systemic infection characterized by severe immunosuppression, the appearance of secondary tumors distal to the inoculation site, and death within 2 weeks caused by opportunistic Gram-negative infection (13). Deletion of the region from the viral genome that encodes the SERP-1 member of the serpin superfamily results in a reduction in the severity of bacterial infections and improved rabbit survival (12).

Although it is apparent that the viral serpins are important for biological virulence, their mode of action remains unclear. The vaccinia viral serpin is able to block cell fusion (14, 15), and there is evidence that the cowpox viral serpin may interfere with the host inflammatory response (16, 17). The effect of this serpin on the host immune response has recently been clarified by Ray et al. (18), who showed that the cowpox viral serpin is able to inhibit interleukin-1β-converting enzyme.

In this paper we report the characterization of the inhibitory specificity of the viral serpin SERP-1. Our data suggest that targets for the SERP-1 protein of MYX and MRV include members of the fibrinolytic and complement pathways. Furthermore we have modeled the structure of this protein on other members of the serpin family which allows the identification of cysteine residues involved in internal and intermolecular disulfide bonding.

**MATERIALS AND METHODS**

Human neutrophil elastase and plasma \( \alpha_1 \)-antichymotrypsin were from Drs. C. Ward and S. Afford, Lung Immunobiological Research Laboratory, General Hospital, Birmingham, U.K. Recombinant \( \alpha_1 \)-antitrypsin was from Dr. H. P. Schnelli, Ciba-Geigy, Basel Switzerland recombinant arginine (P₁) \( \alpha_1 \)-antitrypsin was from Delta Bio-

\(^{1}\) The abbreviations used are: MYX, myxoma virus; MRV, malignant rabbit fibroma virus; SERP-1, serpin coded for by myxoma virus and MRV; MRV-S₁, SERP-1-deleted mutant of MRV; tPA, two-chain tissue-type plasminogen activator; Suc, succinyl; MeO, methoxy; pNA, p-nitroanilide; Bz, benzoyl; Chromozym TRY, carboxbenzoxyl-valyl-glycyl-arginine-4-nitroanilide acetate; Chromozym X, N-methoxycarbonyl-d-norleucyl-glycyl-L-arginine-4-nitroanilide acetate.
technology, Nottingham, U. K.; human α-thrombin was from Dr. J. M. Freyssinet, Institut d'Hématologie et d'Immunologie, Faculté de Médecine, Strasbourg, France; and antithrombin III was purified as described previously (19). C13 was from Dr. P. A. Pemberton, Immunology Division, Children's Hospital, Boston; and C1 inhibitor was purified by a modification of the method of Harrison (20) with the elution step on DEAE-Sepharose being replaced with a dextran sulfate-Sepharose column (5 x 30 cm) equilibrated with TCE (50 mM Tris, 20 mM sodium citrate, 5 mM EDTA, pH 7.4) containing 50 mM NaCl. After washing with TCE containing 0.1 mM NaCl, C1 inhibitor was eluted with a 2-liter linear gradient from TCE, 0.1 M NaCl to TCE, 0.5 M NaCl. Fractions containing C1 inhibitor were pooled, dialyzed against 20 mM KH2PO4, 150 mM KCl, pH 7.0, before loading onto hydroxylapatite as described previously (20). Human plasmin, kallikrein, Bz-Pro-Arg-pNA, Chromozym TRY, and Chromozym X were obtained from Boehringer Mannheim, East Sussex, U. K.; and Val-Leu-Lys-pNA and Isoelu-Pro-Arg-pNA were from Kabivitrum Middlesex, U. K. Two-chain tPA, urokinase, and all other enzymes and substrates were from Sigma Chemical Co., Dorset, U. K.

Cells and Viruses—The source and culture of MRV and MYXV were as described previously (21); and the serum-depleted mutant of MRV, MRV-S1, was prepared as described by Upton et al. (12). SERP-1 is only expressed during proteinase-activated phase of viral replication; therefore the secreted protein was concentrated in a Centriprep-10 cell (Amicon) and sterilized by filtration through a 0.2-μm membrane. The glycosylated viral serpin was detected in the media from infected cells by SDS-polyacrylamide gel electrophoresis and Western blots; MYXV has two copies of the SERP-1 gene and MRV (a recombinant between MYXV and the sheep fibroma viruses) one copy (22).

Assessment of Serpin-Enzyme Complex Formation—The crude late phase viral supernatant from MYX- or MRV-infected cells containing SERP-1 (or else from MRV-S1-infected cells) was incubated with a range of serine proteinases (approximately 12 pmol of serpin/6 pmol of enzyme) at 37 °C for 10 min or 1 h in 50% (v/v) 0.03 mM sodium phosphate, 0.16 mM NaCl, 0.1% polyethylene glycol 4000, pH 7.4, reaction buffer. The reaction was stopped by the addition of 3% SDS loading buffer and heating at 95 °C for 2 min. The proteins were then separated by 7.5-15% (w/v) SDS-polyacrylamide gel electrophoresis (22) and electroblotted onto nitrocellulose paper at 400 mA for 2 h in 0.0125 M Tris, 0.48 M glycine, 20% (v/v) methanol, pH 8.8 (23). Adequate transfer was determined by staining with Ponceau S, which also allowed the position of the molecular weight markers to be determined. The nitrocellulose paper was blocked by shaking with 0.05 M Tris, 0.002 M CaCl2, 0.05 M NaCl, pH 8.0 with 5% (w/v) skimmed milk powder, 0.02% Nonidet P-40 for 30 min. SERP-1 was visualized by shaking with 0.5% (v/v) polyclonal rabbit anti-SERP-1 antiserum2 in blocking buffer for 1 h and then after washing, with a second antibody for a further h. The second antibody was either 0.4% (v/v) horseradish peroxidase-labeled swine anti-rabbit antibody or, 0.1% (v/v) mouse anti-rabbit antibody. The nitrocellulose paper was then washed with 0.05 M Tris, 0.002 M CaCl2, 0.05 M NaCl, pH 8.0, and SERP-1 visualized with aminothio carbazole or by autoradiography.

Active-site Titration of Serine Proteinases—The active-site titrations of bovine α-chymotrypsin, porcine trypsin, and human α-thrombin were performed according to the method of Chase and Shaw (24) using the suicide substrates p-nitrophenyl acetate for bovine α-chymotrypsin and p-nitrophenyl-p'-guanidino benzoate for porcine trypsin and human α-thrombin. Bovine α-chymotrypsin was then used to determine the activity of recombinant α1-antitrypsin, recombinant arginine (P1) α1-antitrypsin, and α1-antitrypsin, whereas porcine trypsin was used to determine the activity of plasma α1-antitrypsin, plasma antithrombin III, α2-macroglobulin, and C1 inhibitor. Active-site titration was performed by incubating 50 mM enzyme with increasing concentrations of inhibitor and 0.03 mM sodium phosphate, 0.16 M NaCl, 0.1% polyethylene glycol 4000, pH 7.4, reaction buffer in a volume of 100 μl. The residual proteolytic activity was determined by adding reaction buffer containing the appropriate substrate (final concentration, 0.16 mM) to a final volume of 1 ml and observing the change in A405 for 3 min. Active-site values are calculated by plotting residual proteolytic activity against the amount of inhibitor and extrapolating to the 100% line as described by Chase et al. (5). These inhibitors were then used to calculate the active-site values of 10 serine proteinases using the substrates shown in Table 1. In all cases the enzyme and inhibitor were incubated at 37 °C for at least 5 times the predicted half-life for complex formation as determined from the published association rate constants. The active-site values for tPA and urokinase were later verified to be correct by titration against SERP-1.

Active-site Titration of SERP-1—The active-site of SERP-1 was determined in a similar manner using a 50 nM concentration of an enzyme of known activity which had previously been determined as described by Upton et al. (12). The enzyme and inhibitor were incubated in 100 μl at 37 °C for 30 min before adding the substrate. In all of the above active-site titrations 1:1 enzyme/inhibitor interactions have been assumed. The metallocproteinase thermolysin was included as a negative control as equimolar active-site concentrations of enzyme and serpin should result in complete cleavage at the reactive center.

Determination of Association Rate Constants—These were determined under second order conditions as described by Beatty et al. (3). Active-site equimolar concentrations of enzyme and latitude phase viral supernatant were incubated at 20 °C with reaction buffer for varying time intervals. The reaction was stopped and the quantity of free enzyme determined by adding reaction buffer containing the substrate to a final volume of 1 ml. The value of the association rate constant (k on) was calculated from the half-life (t 1/2) of enzyme inhibition using Equation 1

\[
\ln(1 - \frac{E_t}{E_0}) = \frac{k_{\text{on}} [I]}{E_0}
\]

where E0 is the initial enzyme concentration.

Modeling of SERP-1—All model building, energy minimization, and molecular dynamics studies were conducted using Quanta/CHARMM version 3.0 (Polygen Corporation, Waltham, MA). The simulations were performed on a Silicon Graphics Personal Iris 4D2, equipped with stereo graphics facility.

Homology Modeling—A three-dimensional model of residues 8-556 of SERP-1 (34) was constructed using the atomic coordinates from the crystal structure of ovomucin (25). Sequence alignments based on those of Huber and Carrel1 (26) were computed using the Needleman-Wunsch algorithm with modification to penalize for the introduction of gaps in regions of defined secondary structure (27). This confirmed the 50% amino acid homology between SERP-1 and ovomucin in all major areas of secondary structure. The final model was constructed using amino acid substitution by residue replacement on the ovomucin backbone with tools and default atom geometries present in the protein modeling package of Quanta/CHARMM. No crystal data were available for modeling the amino-terminal 13 amino acids and carboxyl-terminal 7 amino acids of SERP-1, and these were therefore excluded.

Molecular Dynamics—For all simulations and calculations the default values for CHARMM molecular mechanics and dynamics parameters were used. The polar hydrogen atom descriptions (hydrogen bound to carbon not explicitly represented) were selected from the AMBER 95.PRT file. Models were minimized using the adopted basis Newton Raphson algorithm, which was continued until the root mean square gradient was less than 0.08 kcal/mol/Å.

After minimization, the structure was equilibrated to 300 K for 2 ps, followed by relaxation at 300 K for 10 ps. The conformation exhibiting the lowest energy during molecular dynamics was chosen, minimized, and solvated with a 10 Å shell of (TIP3P) water molecules. All water molecules that came too close to the structure were removed. The system was composed of 8065 atoms, being made up of 3403 protein atoms and 1554 water molecules. Finally a 20 ps molecular dynamics run at 300 K was performed and the final structure energy minimized and analyzed.

RESULTS

To determine whether SERP-1 acts as a substrate or an inhibitor for a panel of serine proteinases, the proteins were selected from MYXV- or MRV-infected cells or from cells infected with MRV-S1 were mixed with the appropriate proteinases and the status of SERP-1 monitored by Western blots. SERP-1 was identified on the Western blot as having a molecular mass of 55 kDa (Fig. 1, lanes 1 and 13). There was a higher molecular mass band at 110 kDa, which resolved after incubation with 5% (v/v) β-mercaptoethanol, suggesting that it represented SERP-1 dimer (data not shown). There was also a minor lower molecular weight band detected at 40 kDa.
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**Table I**

| Enzyme                      | Inhibitor (Ref.) | $k_{\text{max}}$ (mM) | Substrate (Final concentration) (mM) |
|-----------------------------|------------------|------------------------|-------------------------------------|
| Human neutrophil elastase   | rAT              | $1.9 \times 10^5$      | MeO-Suc-Ala-Ala-Pro-Val-pNA 0.29     |
| Cathepsin G                 | ACh              | $5.1 \times 10^7$      | Suc-Ala-Ala-Pro-Phe-pNA 0.16         |
| Human plasmin               | ArgAT            | $2.5 \times 10^6$      | Val-Leu-Lys-pNA 0.18                |
| Kallikrein                  | ArgAT            | $8.9 \times 10^4$      | Bz-Pro-Phe-Arg-pNA 0.22             |
| tPA                         | ArgAT            | $1.4 \times 10^2$      | Isoleu-Pro-Arg-pNA 0.17             |
| Urokinase                   | ArgAT            | $8.9 \times 10^3$      | Isoleu-Pro-Arg-pNA 0.17             |
| Subtilisin                  | $\alpha_2$-M     |                        | Chromozym TRY 0.37                  |
| Porcine pancreatic elastase | AT               | $1.0 \times 10^4$      | Succ-Ala-Ala-Ala-pNA 0.19           |
| Factor Xa                   | ATIII            | $6.3 \times 10^4$      | Chromozym X 0.28                    |
| C$_{1\beta}$                | Cl-inhibitor     | $4.3 \times 10^4$      | Chromozym TRY 0.15                  |

**Fig. 1.** Analysis of SERP-1 interactions with a variety of serine proteinases by Western blotting. Lane 1, late MYX; lane 2, MYX with tPA incubated for 10 min; lane 3, MYX with tPA incubated for 60 min; lane 4, MYX with urokinase incubated for 10 min; lane 5, MYX with urokinase incubated for 60 min; lane 6, MYX with plasmin incubated for 10 min; lane 7, MYX with plasmin incubated for 60 min; lane 8, MYX with thrombin incubated for 10 min; lane 9, MYX with thrombin incubated for 60 min; lane 10, MYX with porcine trypsin incubated for 10 min; lane 11, MYX with porcine trypsin incubated for 60 min; lane 12, early MYX; lane 13, late MYX. Which could represent a proteolytic digestion product of SERP-1 or possibly nonspecific binding of the antibody to another protein as it was present in the early media (Fig. 1, lane 12), and SERP-1 is expressed from a late promoter.

MYX SERP-1 formed complexes of 130, 90, and 106 kDa with human plasmin, urokinase, and tPA, respectively (Fig. 1; lanes 2-7). It is plausible that the 106-kDa complex with tPA may represent cleavage of the dimer, although this is unlikely as subsequent studies have demonstrated tPA-SERP-1 complex in batches of late supernatant which have no detectable dimer. A 50-kDa cleavage product of SERP-1 but no higher molecular weight complexes were detected with thrombin, porcine trypsin (Fig. 1, lanes 8-11), human neutrophil elastase, porcine pancreatic elastase, thromolysin, subtilisin, bovine $\alpha_2$-chymotrypsin, and factor Xa (data not shown). Incubation with kallikrein and cathepsin G had no effect. The 110-kDa dimer of SERP-1 was also cleaved in each case and did not form any detectable complexes.

Having confirmed complex formation the active-site concentration of MYX SERP-1 was determined against human plasmin assuming 1:1 complex formation. This value varied between SERP-1 batches, but a typical value was 3.4 $\mu$g/ml (Fig. 2). Similar inhibitory profiles were obtained for urokinase and tPA. Interestingly, this deduced active-site concentration is significantly less than the value of 18 $\mu$g/ml for the protein mass of SERP-1 estimated from the Coomassie-stained SDS-polyacrylamide gel electrophoresis. This would suggest either a comigrating protein on the SDS-polyacrylamide gel electrophoresis or inactive SERP-1. The latter hypothesis was supported by the autoradiograph which showed cleaved SERP-1 as well as enzyme-inhibitor complexes (Fig. 1). This cleavage of SERP-1 is likely to be at the active site as preincubation of late MYX supernatant with thrombin resulted in a similarly cleaved species which was inactive as an inhibitor of human plasmin.

The SERP-1-containing MYX sample did not turn over the substrate in the absence of plasmin, urokinase, or tPA. Furthermore, the crude early phase MYX supernatant (which lacks SERP-1) did not inhibit plasmin, urokinase, or tPA after incubation at 37°C for 30 min (data not shown). Interestingly, early samples from MYX-infected cells (before SERP-1 was expressed) increased the hydrolysis of substrate in the presence of tPA but not significantly with plasmin or urokinase. Although the mechanism of tPA enhancement by early supernatants was not investigated substrate hydrolysis was enhanced 2.5-fold by early MYX samples in the presence of tPA at concentrations at which the late phase extract fully inhibited tPA activity.

**Fig. 2.** Inhibition of 5 pmol of human plasmin by crude late myxoma virus extract.
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the serpin. This modified virus failed to secrete proteins capable of inhibiting plasmin, urokinase, or tPA. Furthermore, MRV extract containing SERP-1 also inhibited C1S, but that lacking the serpin had no inhibitory activity.

Association Rate Constants—The association rate constants of SERP-1 with human plasmin, urokinase tPA, and C1S were determined as $3.4 \pm 0.3 \times 10^4$, $4.3 \pm 0.5 \times 10^4$, $3.6 \pm 0.6 \times 10^4$, and $1.3 \pm 0.1 \times 10^4$ M$^{-1}$ s$^{-1}$, respectively. The association rate constant of SERP-1 with C1S was unaffected by heparin ($1.2 \pm 0.6 \times 10^4$ M$^{-1}$ s$^{-1}$). All values are the weighted mean of two readings with standard error, and the final concentration of each enzyme was 50, 30, 40, and 80 nM, respectively. The substrates and their final concentrations are as shown in Table 1.

Modeling of SERP-1—Fig. 3 shows the alignment of SERP-1 with ovalbumin and four other serpins modified from Huber and Carrel (26). The interesting features of this alignment are the extended carboxyl-terminal tail of SERP-1 and the complete absence of a sequence corresponding to the D-helix.

The serpin was modeled on the structure of uncleaved ovalbumin by standard homology modeling techniques (28, 29) with the resulting structure being energy minimized before manual repositioning of poor torsion angles and close atom contacts. The final structure is shown in Fig. 4, projected onto the ovalbumin backbone. In the final model, root mean square deviation between the ovalbumin and SERP-1 main chain carbon atoms was 1.7 Å. The alignment of SERP-1 with ovalbumin clearly shows that the loss of the D-helix in SERP-1 (arrowed) produces very little perturbation of the structure, with the ordering and orientation of the major secondary structural elements remaining unchanged.

The SERP-1 structure was also compared with the structures of cleaved α1-antitrypsin (30) and α1-antichymotrypsin (31) and gave root mean square deviations for main chain carbon atoms of 2.3 and 2.7 Å, respectively.

Of the 4 cysteine residues in SERP-1, Cys13, Cys16, and Cys15 are sufficiently close to potentially form a disulfide bridge (Fig. 5), whereas Cys14 is too distant to be able to form a disulfide bond.

**DISCUSSION**

The poxviruses MRV and MYX are lethal tumorigenic viruses of rabbits (13) whose virulence is dependent upon the synthesis and secretion of a variety of proteins which permit virus propagation in rabbits but which have little effect on

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**Fig. 3.** Alignment of SERP-1 with other serpins. Amino acid alignment of SERP-1 (12) with ovalbumin, α1-antitrypsin, α1-antichymotrypsin, C1 inhibitor, and barley Z protein (26). Sequence numbering and secondary structure are based on ovalbumin.

**Fig. 4.** Comparison of SERP-1 and ovalbumin. Stereo view of SERP-1 (gray) superimposed in the structure of ovalbumin (white) showing the high degree of homology between the actual ovalbumin structure and the computed SERP-1 structure. The position of the ovalbumin D-helix is arrowed. As an active inhibitor the reactive center loop of SERP-1 is likely adopt a canonical structure rather than the three-turn helix (shown here).

**Fig. 5.** The 4 cysteines in SERP-1. Ribbon diagram of SERP-1 showing the positions of the 4 cysteine residues in the molecule. Cys13, Cys14, and Cys15 are in or close to the A-helix with Cys14 lying 34 Å away on strand s2C of the C-sheet close to the reactive center. The shaded area shows the position of the absent D-helix.
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replication in cultured cells. SERP-1 is an example of one such virulence factor and is notable because it is encoded in two gene copies in MYX, a single copy in MRV but is absent from a closely related virus, Shope fibroma virus, that causes only benign tumors in infected rabbits. The role of SERP-1 in viral pathogenesis is further underscored by the fact that the SERP-1 deletion in MRV (12) and a comparable double deletion in MYX (15) significantly reduce virulence in infected rabbits. The SERP-1 protein has approximately 30% amino acid homology with members of the serpin superfamily (12), the 34% identity with ovalbumin allowing homology modeling based on the well-refined structure of this protein (25). As SERP-1 acts as a functional inhibitor it is likely that the reactive center loop adopts a partially reinserted canonical conformation (32, 33) rather than the helical form shown in Fig. 4. An unusual feature of SERP-1 is the lack of a D-helix which is normally highly conserved among the serpins (26). Modeling work presented here indicates that the absence of this helix is still compatible with correct folding and maintenance of the general serpin architecture. Indeed, C1 inhibitor lacks some 60% of the D-helix, whereas barzul Z protein lacks the structural features corresponding to helices A –F1, the first three strands of the C β-sheet of ovalbumin (1.7 Å) as the structures of α1-antitrypsin and α1-antichymotrypsin are those of cleaved serpins, with a six- rather than a five-membered A-sheet (32). Despite this SERP-1 still retains a high degree of homology with these cleaved proteins.

Superposition of the predicted structure of SERP-1 on other serpins showed that the root mean square deviation for main chain carbon atoms was 2.3 and 2.7 Å when compared with α1-antitrypsin and α1-antichymotrypsin respectively. These values are higher than that based on ovalbumin (1.7 Å) as the structures of α1-antitrypsin and α1-antichymotrypsin are those of cleaved serpins, with a six- rather than a five-membered A-sheet (32). Despite this SERP-1 still retains a high degree of homology with these cleaved proteins.

Fig. 1 demonstrated a 110-kDa form of the SERP-1 monomer which disappeared on treatment with β-mercaptoethanol. This is compatible with dimer formation with other proteins through disulfide bridges, suggesting the presence of at least 1 freely available cysteine residue. Modeling has allowed the prediction of the positions of all 4 cysteine residues. Cysβ is in the extended amino-terminal tail, Cys3 is both in the A-helix, and Cysαβ and Cysαβ are both in the A-helix, and Cysαβ is in the second strand of the C β-sheet. For a disulfide bridge to form, the S-S distance must be 2.05 Å (36); and as Cysαβ and Cysαβ are constrained in the A-helix, only Cysβ could come into close enough contact with another cysteine residue to form a disulfide bond. It is plausible that Cysβ could disulfide bond internally with either Cysβ or Cysαβ, leaving the other cysteine residue free. Thus there are at least 2 free cysteines in SERP-1, Cysαβ and either Cysβ or Cysαβ, which could potentially disulfide bond with a second SERP-1 molecule.

The Western blot (Fig. 1) shows that the 110-kDa SERP-1 dimer is noninhibitory in that it is unable to form higher molecular weight complexes with the fibrinolytic enzymes plasmin, tPA, or urokinase. Other serpins which form Cys-linked dimers, protease nexin I, α1-antitrypsin, and α1-antichymotrypsin, retain their inhibitory function despite dimer formation.3 This suggests that dimerization of SERP-1 compromises the conformation of the reactive center of the molecule. Fig. 5 illustrates that Cysαβ lies very close to the reactive center and thus dimerization involving this residue would result in perturbation and obfuscation of the reactive center, rendering the serpin inactive. As the dimer is inactive both SERP-1 molecules must be in noninhibitory conformations so that Cysαβ–Cysαβ is the most likely linkage with the addition of the proteinase leading to cleavage and degradation of the dimer and not complex formation.

Despite good structural and sequence homology to the other serpins, SERP-1 has a unique combination of arginine-asparagine residues at the P1-P′′ positions (12) which is atypical and makes it difficult to predict the inhibitory specificity. Our results show that SERP-1 inhibits three enzymes which play an important role in the fibrinolytic pathway: plasmin, urokinase, and tPA, but not a fourth enzyme, kallikrein. Furthermore it also inhibits the first enzyme in the complement pathway C1s. It is apparent that not all of SERP-1 in a particular preparation is active and therefore available to form complexes. The inactive material acts as a substrate for the enzyme and is degraded with a consequent downward band shift of 4–5 kDa. This is compatible with specific cleavage within the reactive center loop as is seen with other serpins (37, 38). A similar band shift is seen following incubation of SERP-1 with enzymes such as thrombin and porcine trypsin (Fig. 1). Preincubation of the late MYX supernatant with thrombin resulted in a loss of inhibitory activity supporting the conclusion that the downward band shift of SERP-1 represents reactive center loop cleavage.

The assays of complex formation allowed the functional active-site titration of SERP-1 with plasmin. This showed the value to be very low and therefore the kinetic experiments were performed directly on unfractionated supernatants. Such assays are valid as the late MYX supernatant does not itself turn over the enzyme substrates and the early MYX supernatant, which lacks SERP-1, failed to inhibit plasmin, urokinase, or tPA. As a further control MRV, which is >90% identical to MYX (21), also expressed SERP-1 which inhibited plasmin, urokinase, tPA, and C1s. The MRV-S1 mutant, with a deletion in the SERP-1 gene (12), failed to secrete proteins which inhibited any of these enzymes. Thus the findings must reflect the inhibitory profile of SERP-1 and not other "contaminating" protease inhibitors.

The association rate constants between the fibrinolytic enzymes and SERP-1 were approximately 4 × 108 M⁻¹ s⁻¹, and that with C1s was 1.3 × 109 M⁻¹ s⁻¹. These values, which are only moderately fast, are as high as others such as C1s with C1 inhibitor (34, 39) and protein C with protein C inhibitor (40), which are thought to be physiologically relevant. In the context of the inflammatory microenvironment of infected rabbit tissues the serpin and enzyme concentrations are likely to be high, and therefore complex formation and inhibition are more likely to result (41). Furthermore, MYX and MRV are rabbit viruses and all of the enzymes tested are human, which may serve to reduce the association rates from their proper physiological values. An alternative explanation is that the moderately fast association rate constants indicate that the effect of SERP-1 on the fibrinolytic and complement enzymes is of secondary importance and that the enzyme inhibited by SERP-1 in vivo has yet to be determined. It is of interest, however, that despite the low association rate constant, SERP-1 is only the second naturally occurring inhibitor of C1s to be reported.

SERP-1 clearly has an important role in determining virulence and has now been shown to be a specific inhibitor of enzymes within the fibrinolytic and complement pathways. The method by which the inhibition of this pathway leads to the demise of the rabbit is unclear but may be linked to the profound immunosuppression produced by the virus. Such immunosuppression may be mediated by inhibition of the complement cascade or by inhibition of cell surface urokinase.

3 D. L. Evans, unpublished observations.
which in turn may serve to reduce neutrophil migration (42).
The inhibition of these components of the inflammatory
response may impair host response and so allow viral replication and secondary Gram-negative infection. This modifi-
cation of the host immune response by viral serpins has also
been demonstrated in the cowpox virus which encodes a serpin
that is a specific inhibitor of interleukin-1β-converting en-
zyme (18). Interestingly this serpin, which has the amino acid
residues aspartic acid-cysteine at the P1′,P′ positions, has no
effect on the fibrinolytic enzymes plasmin or tPA but medi-
ates its effect by modifying host cytokine response. It is
plausible that SERP-1 may also act on the host cytokine
response in addition to its effect on the fibrinolytic and
complement pathways.

Acknowledgment—The atomic coordinates from the crystal
structure of ovalbumin were kindly supplied by Dr. P. E. Stein, Department
of Haematology, University of Cambridge, U. K.

REFERENCES
1. Carrell, R. W., and Boswell, D. R. (1986) in Proteinase Inhibitors (Barrett,
A., and Salvesen, G., eds) pp. 403–420, Elsevier Science Publishing Co.,
Amsterdam
2. Schechter, I., and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27,
157–162
3. Beatty, K., Bieth, J., and Travis, J. (1980) J. Biol. Chem. 255, 3931–3934
4. Hunt, L. T., and Dayhoff, M. O. (1980) Biochem. Biophys. Res. Commun. 96,
864–871
5. Flink, I. L., Bailey, T. J., Gustafson, T. A., Markham, B. E., and Morkin,
E. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7708–7712
6. Doolittle, R. F. (1983) Science 222, 417–419
7. Boursnell, M. E. G., Fouaia, J. J., Campbell, J. L., and Binns, M. M. (1988)
J. Gen. Virol. 69, 2995–3005
8. Kotwal, G. J., and Moss, B. (1988) Virology 167, 524–537
9. Smith, G. L., Howard, S. T., and Chan, Y. S. (1989) J. Gen. Virol. 70,
2333–2343
10. Pickup, D. J., Ink, B. S., Hu, W., Ray, C. A., and Joklik, W. K. (1986) Proc.
Natl. Acad. Sci. U. S. A. 83, 7896–7897
11. Tomsley, F., Binns, M., Campbell, J., and Boursnell, M. (1988) J. Gen. Virol.
69, 1025–1040
12. Upton, C., Macen, J. L., Wishart, D. S., and McFadden, G. (1990) Virology
170, 619–631
13. Strayer, D. S., Skeates, E., Cabriac, G. F., Sharp, P. A., Corbell, L. B.,
Sell, S., and Leibowitz, J. L. (1983) J. Immunol. 130, 399–404
14. Law, K. M., and Smith, G. L. (1992) J. Gen. Virol. 73, 549–557
15. Turner, P. C., and Moyer, R. W. (1992) J. Virol. 66, 2076–2081
16. Panzner, G. J., Pickup, D. J., Fredrickson, T. N., McIntyre, L. J., and
Buller, R. M. L. (1989) Virology 172, 262–273
17. Chua, T. P., Smith, C. E., Reith, R. W., and Williamson, J. D. (1990)
Immunology 89, 202–208
18. Ray, C. A., Black, R. A., Kronheim, S. R., Greenstreet, T. A., Sleath, P. R.,
Salvesen, G. S., and Pickup, D. J. (1992) Cell 69, 597–604
19. Evans, D. L., McGregan, M., Scott, R. W., and Carrell, R. W. (1991) J. Biol.
Chem. 266, 22307–22312
20. Harrison, R. A. (1983) Biochemistry 22, 5001–5007
21. Block, W., Upton, C., and McFadden, G. (1985) Virology 140, 113–124
22. Lassenmi, U. K. (1970) Nature 227, 680–685
23. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci.
U. S. A. 76, 4350–4354
24. Chase, T. Jr., and Shaw, E. (1970) Methods Enzymol. 19, 20–27
25. Stein, P. E., Leslie, A. G. W., Finch, J. T., and Carrell, R. W. (1991) J. Biol.
Chem. 221, 941–950
26. Huber, R., and Carrell, R. W. (1988) Biochemistry 27, 8551–8566
27. Leek, A. M., Levitt, M., and Chothia, C. (1986) Proteins 1, 77–78
28. Blundell, T. L., Sibanda, B. L., Sternberg, M. J. E., and Thornton, J. M.
(1987) Nature 326, 547–552
29. Greer, J. (1990) Proteins 7, 317–334
30. Loebmann, H., Tokuoka, R., Deisenhofer, J., and Huber, R. (1984) J.
Mol. Biol. 177, 531–556
31. Baumann, U., Huber, R., Bode, W., Groase, D., and Lesjak, M. (1991) J.
Mol. Biol. 218, 595–606
32. Carrell, R. W., Evans, D. L., and Stein, P. E. (1991) Nature 353, 578–578
33. Mast, A. E., Enghild, J. J., and Salvesen, G. (1992) Biochemistry 31, 2720–
2725
34. Lennick, M., Brew, S. A., and Ingham, K. C. (1986) Biochemistry 25, 3890–
3898
35. Larrard, R., and Svensson, B. (1989) Carlsberg Res. Commun. 54, 173–
190
36. Creighton, T. E. (1988) BioEssays 6, 57–63
37. Potempa, J., Watovska, Z., and Travis, J. (1986) J. Biol. Chem. 261, 14330–
14334
38. Potempa, J., Fedak, D., Dubin, A., Mast, A., and Travis, J. (1991) J. Biol.
Chem. 266, 21425–21437
39. Pemberton, P. A. (1989) The serpins: structure/function relationships, Ph.D.
dissertation, University of Cambridge, U. K.
40. Pratt, C. W., Macik, B. G., and Church, F. C. (1989) Thrombosis Res. 53,
585–592
41. Hooper, M. J., and Griffin, J. H. (1988) J. Biol. Chem. 263, 11613–11616
42. Boyle, M. D. P., Chiolo, V. A., Lawman, M. J. P., Gee, A. P., and Young,
M. (1987) J. Immunol. 139, 167–174
43. Travis, J., Owen, M. G., Carrell, R. W., Rosenberg, S., Hallewell, R. A., and
Barr, P. J. (1986) J. Biol. Chem. 261, 4334–4339
44. Paton, P. A., Rodis, N., Schifferli, J. A., Biocff, R., Courlas, M., and
Schafran, P. (1970) J. Biol. Chem. 265, 10796–10791
45. Scott, C. F., Carrell, R. W., Glasser, C. B., Kueppers, F., Lewis, J. H., and
Colman, R. W. (1980) J. Clin. Invest. 67, 631–634
46. Ellis, V., Scully, M. V., and Kakkar, V. V. (1986) Biochem. J. 238, 329–
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