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The catabolism of intact, reactive centre-cleaved and proteinase-complexed C1 inhibitor in the guinea pig

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SUMMARY

Clearance rates in the guinea pig were determined for intact guinea pig and human C1 inhibitor, the complexes of both inhibitors with human C1s, β factor XIIa and kallikrein, and for each inhibitor cleaved at its reactive centre with trypsin. Intact human and guinea pig C1 inhibitor were cleared from the circulation more slowly (t_{s_2} of 9·7 h and 12·1 h and fractional catabolic rates (FCRs) of 0·09 and 0·117) than any of their cleaved or complexed forms. The reactive centre-cleaved inhibitors were cleared with half-lives of 6·75 h for humans and 10·1 h for the guinea pig. The complexes with target proteases were catabolized much more rapidly, with half-lives ranging from 3·08 h to 4·3 h. The complexes with kallikrein were cleared more slowly than those with C1s and β factor XIIa. Complexes prepared with the guinea pig and human inhibitors were cleared at equivalent rates. The free inactivated proteases were cleared at rates similar to the equivalent complexes, except for kallikrein, which was cleared more rapidly than its complex. The fact that the complexes with different target proteases differed in their catabolism and that protease and complex catabolism were similar suggests that protease may play a direct role in clearance.

Keywords complement serpins protein catabolism

INTRODUCTION

C1 inhibitor (C1INH) is the sole protease inhibitor of C1r and C1s, and is the major inhibitor of kallikrein and Hageman factor (factor XII). It is therefore the major regulator of activation of the classical pathway of complement and of the contact system (reviewed in [1]). Heterozygous deficiency results in hereditary angioneurotic oedema (HAE), which is characterized by intermittent episodes of cutaneous or mucosal oedema [2]. C1INH belongs to the serpin superfamily, members of which are major inhibitors of the inflammatory, coagulation and fibrinolytic serine proteases [3]. The inactivation and clearance of these potentially harmful proteases is a critical issue in the survival of any organism. The inhibitory as well as clearance mechanisms of different members of the serpin superfamily have been shown to be similar in many respects [4–10]. Like other members of the serpin family, C1INH is believed to inhibit target proteases via formation of a stable tetrahedral intermediate [11]. During complex formation or reactive centre cleavage, the inhibitor undergoes specific conformational changes. The catabolism of some protease–serpin complexes has been shown to occur via specific receptors, the serpin enzyme complex receptors (SEC I and SEC II) [12–15]. α_1-antitrypsin (α_1-AT) complexes induced synthesis of α_1-AT in two different cell lines [12]. This synthesis was induced via SEC I. In other studies, serpin–protease complexes were cleared more rapidly from the circulation than were the intact inhibitors [6–10,16]. This increase in complex clearance is facilitated by the recognition of a specific domain on the inhibitors by the SEC receptors [17]. That domain has been identified in the carboxy terminal region of α_1-AT within a region of high homology among the serpins [17]. Presumably, this domain is exposed after the change in conformation that takes place during complex formation. One study analysed the catabolism of intact, cleaved and complexed human C1INH in rats, and showed a much faster clearance of the complexes than of the intact molecule [16]. The catabolic rate of the intact molecule was significantly higher than in humans. In addition, the data suggest the possibility that catabolism may have been influenced by the protease.

In order to minimize the role of species differences in catabolism of serpin–protease complexes, we compared the turnover of human and guinea pig C1INH in its intact, cleaved and complexed forms in guinea pigs. In addition, to clarify further the involvement of the protease, catabolism of the complexes with C1s and kallikrein was compared with that of each PMSF-inactivated protease.

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MATERIALS AND METHODS

Proteins

Human kallikrein and factor XII were purchased from Enzyme Research Labs (South Bend, IN). Activated C1s was a gift from Dr David Bing (Centre for Blood Research, Boston, MA); human and guinea pig C1INH were purified using a modification of the technique of Pilatte *et al.* [18]. C1INH-containing fractions from the phenyl Sepharose column were pooled, concentrated in an Amicon device (Amicon, Danvers, MA) and dialysed against 0·02 M Na/K phosphate, 0·15 M NaCl pH 7·2 (PBS) overnight using a 14 000 MW spectra/Por membrane (Spectrum Medical Industries, Houston, TX). This pool was applied to a Mono-Q column on a fast performance liquid chromatography (FPLC) system (Pharmacia Biotechnology, Piscataway, NJ) equilibrated with PBS, and eluted with a linear NaCl gradient to 500 mM. SDS–PAGE revealed a single band of mol. wt 105 000 for both the human C1INH and the guinea pig C1INH. Factor XII was activated by incubation in the presence of PBS-washed trypsin-coated Sepharose beads (Sigma, St Louis, MO) for 15 min at 37°C at a ratio of 10 U trypsin for 90 μg factor XII. It was then separated from the beads by centrifugation.

Preparation of cleaved and complexed human C1INH and guinea pig C1INH

Human and guinea pig C1INH were cleaved at their reactive centres after iodination, by incubation in the presence of trypsin-coated Sepharose beads for 45 min at 37°C at a ratio of 4 U trypsin per 300 μg inhibitor [19]. The proteins were then separated from the beads by centrifugation. Guinea pig C1INH–C1s complexes were prepared by incubation of radiolabelled protease with unlabelled inhibitor at a molar ratio of 1:1 for 60 min at 37°C. Complex formation was confirmed on SDS–PAGE to insure an appropriate ratio of both proteins. All other complexes were obtained before iodination in the same manner as above. C1INH–kallikrein and C1INH–β factor XIIa complexes were prepared by incubation with an excess of inhibitor (1:3 and 1:10 molar ratios of the protease to inhibitor, respectively). Inhibitor–protease complexes were isolated by chromatography with an FPLC system using a Mono Q column equilibrated in 30 mM Tris–HCl pH 8·0, 150 mM NaCl, and developed with a NaCl concentration gradient from 150 mM to 500 mM. One millilitre fractions were collected at a flow rate of 1 ml/min.

Iodination

Proteins were radiolabelled with ^{125}I using solid-phase iodogen as described previously [19]. The specific activities varied between 0·5 and $2{\cdot}0 \times 10^6$ ct/min per μg. Each animal received the equivalent of 4000–5000 ct/min per g body weight of injected protein.

Clearance studies

Strain 2 guinea pigs ranging in weight from 350 to 800 g were initially sedated with 1·85 mg/100 g body weight of sodium pentobarbital (Burke Co., Columbus, OH) intramuscularly followed by 0·05 ml/100 g body weight of Innovar-Vet (Mallinkrodt, Mundekin, IL) intraperitoneally. A 24 G cannula was then inserted in the cephalic vein on one foreleg. The animals were kept supine on a warmer pad during the first 2 h of the experiments. They were injected with the different radiolabelled proteins via a cephalic vein cannula. Each animal received between 0·5 and 2·0 μg of the designated proteins. Aliquots of blood (300 μl) were obtained in heparinized syringes using 25 G butterfly needles from either jugular vein. At 2 h post-injection, the animals were transferred to an incubator for recovery from anaesthesia. Subsequent timed blood samples were obtained from the animals as previously described during short sedation with 2–3% inhaled Isofluorane gas (Anaquest Inc., Liberty Corner, NJ). Each experiment was terminated after radioactivity levels reached 10–20% of the initial count. The animals were then killed by a 1-ml intracardiac injection of Socumb (Butler Co., Columbus, OH).

Collection and treatment of samples

Aliquots of blood (300 μl) were transferred to a polypropylene tube, plasma was separated by centrifugation and the plasma proteins were precipitated with 10% trichloroacetic acid. The samples were then centrifuged, the supernatants discarded and the precipitates counted for radioactivity for 1 min each in a gamma scintillation counter (ICN Micromedic Systems, Huntsville, AL).

Data analysis

The radioactivity remaining in every sample collected was calculated as a percentage of the initial radioactivity obtained at zero time, and was plotted against time. Each of the proteins and the inhibitor–protease complexes was cleared from plasma in two phases: a rapid distribution phase and a slower clearance phase. Each curve was manually solved following a two-compartment model and analysed according to the method of Matthews to obtain a biological half-life and fractional catabolic rate for each protein injected [21]. The individual values obtained from different animals injected with the same proteins were averaged. We then used the one-way ANOVA non-paired Student's *t*-test to compare the average $t_{\frac{1}{2}}$ and fractional catabolic rates (FCRs) for the intact, cleaved and complexed inhibitors for both guinea pig and human proteins.

Protein sequence analysis

The amino terminal sequence of the isolated guinea pig C1INH was performed using an Applied Biosystems sequencer by Dr Richard A. Harrison (MRC Centre, Cambridge, UK).

RESULTS

Isolation of C1INH and its complexes with proteases

The purification of guinea pig C1INH was similar to the modified method used to purify the human protein. Human and guinea pig C1INH each was functional as determined by reactivity with C1s to form SDS-stable complexes (Fig. 1). The amino terminal sequence of the guinea pig protein was similar to that of the human protein (Fig. 2). Both proteins appeared to be the same size on SDS–PAGE (Fig. 1). The radiolabelled proteins remained active. Both proteins were cleaved with trypsin to yield a single mol. wt 83 000 band on SDS–PAGE, as described previously [20] (Fig. 3). Complexes were prepared as described in Materials and Methods. Before each experiment, the proteases were serially diluted with an equal volume of PBS and protein. Each fraction was incubated with a constant amount of the inhibitor, then analysed on SDS–PAGE and stained with coomassie brilliant blue R250. After the most suitable ratio of protease to inhibitor was determined, larger quantities of the

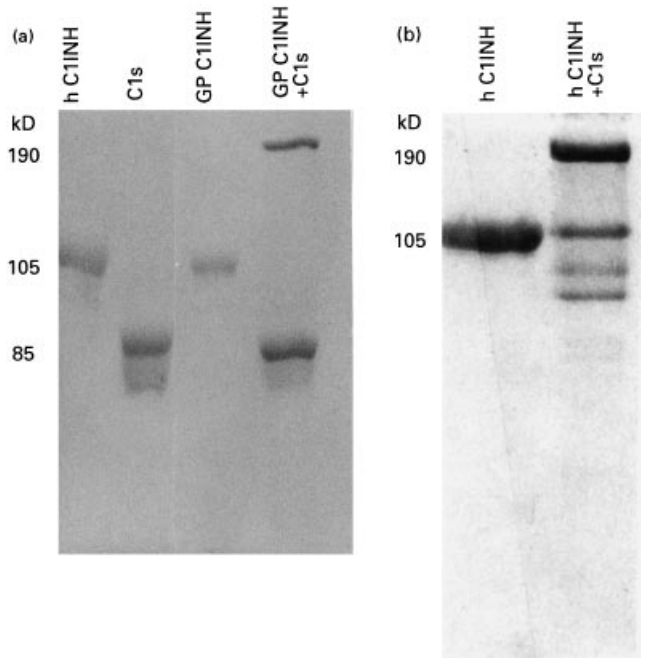

Fig. 1. SDS–PAGE of isolated human and guinea pig C1 inhibitor (C1INH) proteins. (a) Lane 1, human C1INH; lane 2, human C1s; lane 3, guinea pig C1INH; lane 4, guinea pig C1INH incubated with human C1s for 60 min at 37°C. (b) Lane 1, human C1INH; lane 2, human C1INH incubated with human C1s for 60 min at 37°C. Each gel was stained for total protein with coomassie brilliant blue R250.

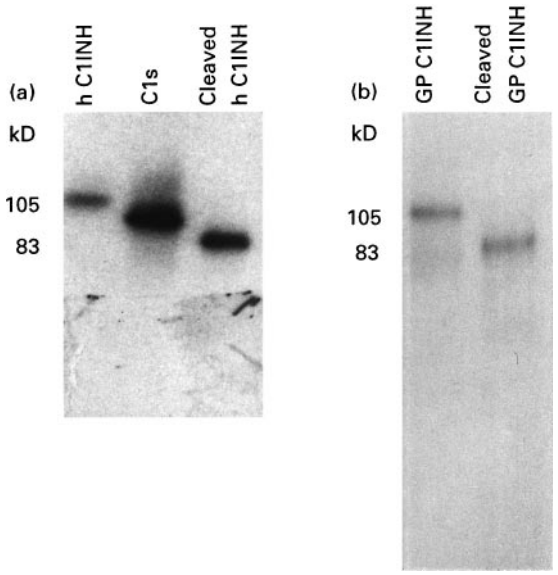

Fig. 3. SDS–PAGE of ^{125}I-radiolabelled human and guinea pig C1 inhibitor (C1INH) proteins following cleavage with trypsin. (a) Lane 1, human C1INH; lane 2, human C1s; lane 3, human C1INH following incubation with trypsin (1% w/w) for 45 min at 37°C. (b) Lane 1, guinea pig C1INH; lane 2, guinea pig C1INH following incubation with trypsin (1% w/w) for 45 min at 37°C. Autoradiograms were performed by exposure of the dried SDS–polyacrylamide gel to Kodak XAR-5 film.

complexes were made at that ratio. In all cases, a slight excess of the inhibitor as well as some degradation products were noted on coomassie brilliant blue-stained SDS polyacrylamide gels. The complexes were separated from contaminants by purification on FPLC using a Mono Q column with a NaCl gradient up to 500 mM as described above. The isolated complexes then were radiolabelled and analysed by SDS–PAGE and autoradiography to check purity (Fig. 4a,b). All cleaved and complexed forms of the inhibitors were used within 48 h of purification and labelling. Intact forms were used within 24 h after labelling. Both C1s and kallikrein complexes revealed a single band of similar size on SDS–PAGE and autoradiography (mol. wt ≈190 000). The complexes with β factor XIIa were smaller (mol. wt ≈130 000) (Fig. 5).

The clearance of intact human and guinea pig C1INH

Intact human and guinea pig C1INHs were slower to clear from the circulation than any of their respective cleaved or complexed forms (Table 1, Fig. 6a,b). When compared with one another, the half-life of the intact human C1INH was shorter than that of the guinea pig (Table 1). However, this difference was not statistically significant ($P > 0{\cdot}05$). There was no statistically significant difference between the FCRs of the intact human and guinea pig inhibitors ($P > 0{\cdot}20$).

Clearance of the reactive centre-cleaved C1INHs

Both human and guinea pig reactive centre-cleaved forms of C1INH were cleared more rapidly than their intact counterparts (Fig. 6a,b). The $t_{½}$of the cleaved human inhibitor was significantly shorter than its intact form ($P < 0{\cdot}001$) (Table 1). Although the half-life of the cleaved human C1INH was shorter than the half-life of its intact form, the difference between them was not as wide as the difference between the two forms of the guinea pig proteins, but was statistically significant ($P < 0{\cdot}05$). The FCRs of intact human and guinea pig inhibitors were also significantly slower than the FCRs of the respective cleaved proteins. Comparison of the clearances of the two cleaved inhibitors revealed that the human inhibitor had a significantly shorter $t_{½}$than the guinea pig protein, but no difference was observed between their FCRs.

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1                   10                  20                  30
N P N A T S S S S Q D P E S L Q D R G E G K V A T T V I S K M L F ..

A T F S S P L K P E S P Q K A X D G - - I T A V T X R E
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Fig. 2. Amino terminal sequence comparison of guinea pig and human C1 inhibitor.

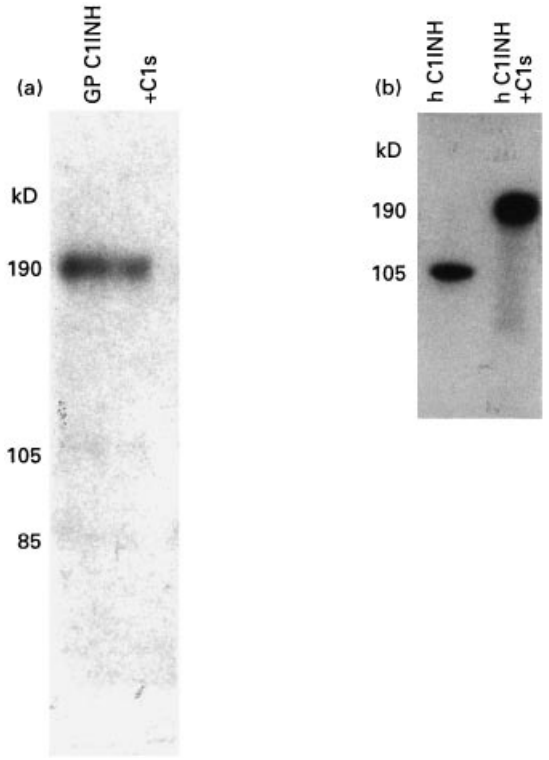

Fig. 4. SDS–PAGE of isolated C1 inhibitor (C1INH)–C1s complexes. Fractions from the Mono Q column chromatogram were pooled, concentrated, radiolabelled and analysed on SDS–PAGE. Autoradiograms were performed by exposure of the dried SDS–polyacrylamide gel to Kodak XAR-5 film. (a) Guinea pig C1INH–human C1 complex. (b) Human C1INH and human C1INH–human C1 complex.

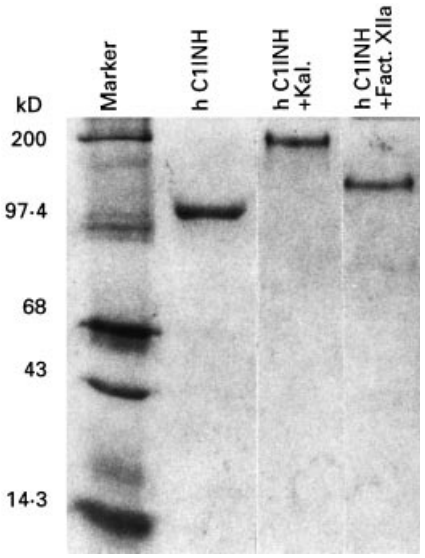

Fig. 5. SDS–PAGE of ^{125}I-radiolabelled complexes of human C1 inhibitor (C1INH) with human plasma kallikrein and β factor XIIa. Lane 1, markers; lane 2, human C1INH; lane 3, isolated human C1INH–human kallikrein complex; lane 4, isolated human C1INH–human β factor XIIa complex. Autoradiograms were performed by exposure of the dried SDS–polyacrylamide gel to Kodak XAR-5 film.

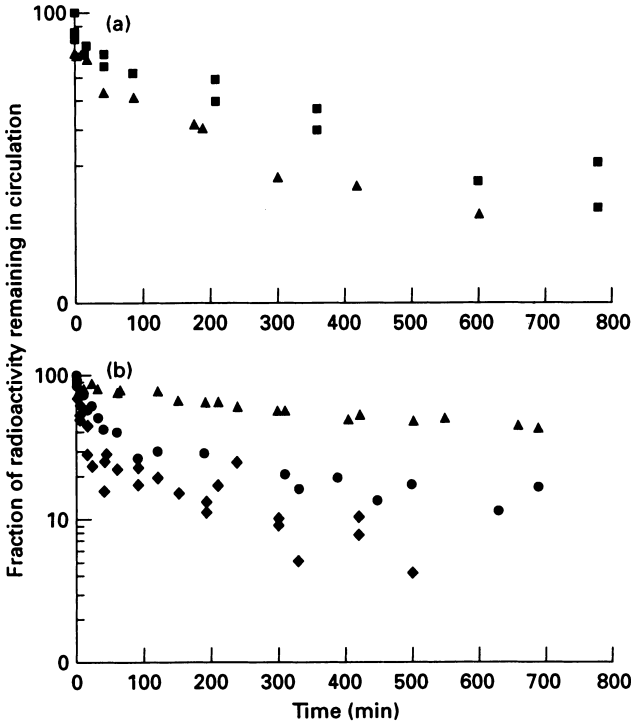

Fig. 6. Clearance of guinea pig C1 inhibitor (C1INH). Isolated proteins were radiolabelled and clearance studies were performed as described in Materials and Methods. (a) Intact (■) and reactive centre-cleaved (▲) human C1INH. (b) Intact guinea pig C1INH (▲), reactive centre-cleaved guinea pig C1INH (●), complex of guinea pig C1INH with C1s (◆).

Clearance of C1INH complexed with C1s, kallikrein or β factor XIIa

The clearances of human and guinea pig C1INH complexed with human C1s, kallikrein and β factor XIIa were evaluated. The complexed human and guinea pig inhibitors each revealed much more rapid turnover rates than their intact or cleaved forms (Figs 6b and 7). The complexed molecules had shorter $t_{\frac{1}{2}}$ and more rapid FCRs than all other forms of the inhibitor (Table 1).

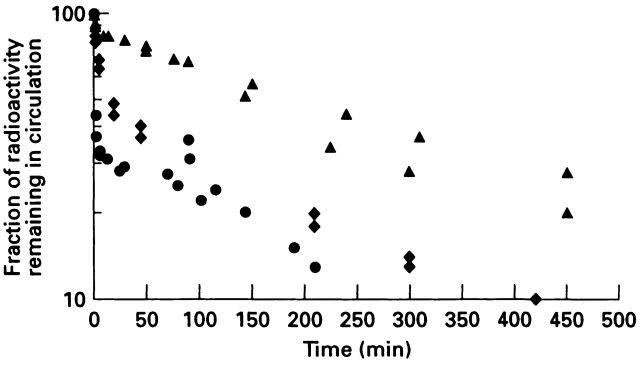

Fig. 7. Clearance of human C1 inhibitor (C1INH)–protease complexes. The complexes were radiolabelled and the clearance studies were performed as described in Materials and Methods. ▲, Human C1INH–human kallikrein complex; ◆, human C1INH–human C1s complex; ●, human PMSF-inactivated kallikrein.

Table 1. Half-lives and fractional catabolic rates of C1 inhibitor (C1INH), proteases and C1INH–protease complexes

	HC1INH	GP C1INH	Cleaved HC1INH	Cleaved GP C1INH	H C1s–H C1INH	H C1s–GP C1INH	HK–hC1INH	HK–GP C1INH	HX11a–h C1INH	HX11a–GP C1INH	HC1s	HK
Half-life												
1	09·0	13·2	6·2	10	2·8	3·4	4·5	4·8	3·5	3·8	2·6	3·0
2	09·8	10·4	6·2	10	3·3	4·4	3·8	3·8	3·2	3·6	3·5	2·7
3	10·4	12·6	7·3	10·3	3·4	3·0	4·0	4·4	3·0	—	—	—
4	—	—	7·3	09·8	2·8	2·9	—	—	—	—	—	—
5	—	—	—	—	—	4·3	—	—	—	—	—	—
Mean	9·7 ± 0·7	12·1 ± 1·47	6·75 ± 0·63	10 ± 0·21	3·08 ± 0·32	3·6 ± 0·7	4·1 ± 0·36	4·3 ± 0·5	3·2 ±	3·7	3·1 ± 0·6	2·85 ± 0·21
FCR												
1	0·079	0·15	0·208	0·21	0·609	0·508	0·18	0·258	0·421	0·366	0·8	0·697
2	0·119	0·107	0·139	0·151	0·634	0·712	0·237	0·342	0·444	0·344	0·7	0·773
3	0·072	0·095	0·172	0·251	0·480	0·722	0·255	0·278	0·379	—	—	—
4	—	—	0·192	0·195	0·536	0·724	—	—	—	—	—	—
5	—	—	—	—	—	0·652	—	—	—	—	—	—
Mean ± s.d.	0·09 ± 0·025	0·117 ± 0·024	0·178 ± 0·03	0·202 ± 0·041	0·565 ± 0·07	0·665 ± 0·092	0·224 ± 0·039	0·293 ± 0·043	0·415 ± 0·033	0·355 ± 0·016	0·75 ± 0·1	0·735 ± 0·054

FCR, Fractional catabolic rate.

The complex with kallikrein cleared significantly more slowly than did the complexes with C1s or β factor XIIa, each of which had very similar half-lives and FCRs. The difference was somewhat greater in the experiments using complexes prepared with the human inhibitor. For each individual protease, there was no difference in the clearance of the complexes prepared with the guinea pig and human inhibitors.

Comparison of the clearances of inactivated C1s and kallikrein with their respective C1INH complexes

Both PMSF-inactivated human C1s and human kallikrein cleared from the plasma rapidly with no significant difference between either their half-lives or FCRs (Fig. 7, Table 1). However, when compared with the complexes with C1INH, only free inactivated kallikrein cleared significantly faster than its complexed forms (all $P < 0{\cdot}03$). There was no significant difference between the clearance of PMSF-inactivated C1s and its complex with C1INH. These observations were true for both human and guinea pig C1INH-complexed proteases.

DISCUSSION

This study compares the turnover rates of human C1INH and guinea pig C1INH in their intact, cleaved and complexed forms in guinea pigs. The plasma half-lives of the intact human and guinea pig inhibitors were 9·7 h and 12·6 h, respectively. Both these values reflect faster plasma clearance rates than the $t_{½}$ of 20 h reported earlier by Quastel *et al.* [22] for the turnover of human C1INH in humans. This discrepancy between the turnover rates of intact human C1INH in humans and in guinea pigs is probably explained on the basis of the host used. Plasma clearance rates of a specific protein have been shown in previous studies to vary in one species in comparison with another [6,23–25]. The plasma clearance rates of human haemoglobin, protein C, protein S and factor IX were found to be faster in rodents than in humans.

On the other hand, the half-life for the plasma clearance of intact human C1INH in rats was significantly shorter than the half-lives of both the human and guinea pig proteins in our study ($t_{½}$ 4·5 h) [16]. This discrepancy between the turnover rates of intact human C1INH in rats and guinea pigs may also be a species difference. However, one other factor could have played a role. The calculations in the above study followed a mono-compartment model, whereas in our study and in that of Quastel *et al.* (and in most other serpin turnover studies) a two-compartment model was followed. In any case, the catabolism of C1INH (and other plasma proteins) is more rapid in rats and guinea pigs than in humans. The important issue is whether or not it is legitimate to use a human protein to study catabolism. Previous data, together with the data presented here, indicate that, at least in short-term studies, the clearance of a protein from one species follows very similar kinetics in the same or a different species [6,26,27]. However, this issue cannot be answered accurately until the precise receptors involved and the clearance pathways for the intact and cleaved/complexed inhibitors have been characterized. The plasma clearance rates of the cleaved human and guinea pig inhibitors were intermediate between their intact and complexed forms. This is in agreement with the data previously reported by de Smet *et al.* in rats [16]. This observation is not unique to the C1INH molecule. In their study of different serpins, Mast *et al.* showed that reactive centre and amino terminal-cleaved human α_1-AT cleared slightly faster than its intact form from the plasma of mice [9]. This was not true, however, for α_1-antichymotrypsin and antithrombin III, which cleared at the same rates in their intact and cleaved forms. The mechanism for the clearance of inactivated serpins is still not well understood. Mast *et al.* have also demonstrated that the removal of modified serpins is not mediated by the hepatic receptors that recognize the inhibitor–protease complexes [9]. Only in the case of α_1-AT were they able to show that the modified form of the protein competes to some extent for the receptor that binds the complex, but with weaker affinity.

There are two known receptors that bind serpin–protease complexes. The SEC I receptor, which has been identified on HepG2 cells and monocytes, binds several serpin–protease complexes, including at least those with α_1-AT, heparin cofactor II and antithrombin III [28]. The SEC II receptor binds the α_2-antiplasmin–plasmin complex [15]. Catabolic turnover studies demonstrated that different serpin–protease complexes compete for the common SEC I receptor [7,8]. *In vitro*, C1INH–C1s complexes were shown to compete with α_1-AT complexes for binding to SEC I on monocytes and HepG2 cells, but to a much lesser extent than the other serpin–protease complexes studied [12]. The SEC I receptor is thought to be responsible for the rapid *in vivo* plasma clearance rates observed with different inhibitor–protease complexes, but not with their respective intact or cleaved inhibitors. The difference in rate of clearance of the cleaved and complexed serpins observed in this, as well as other studies, is compatible with this interpretation. Joslin *et al.* [17] showed that binding of α_1-AT–protease complexes to the SEC I receptor was mediated by a pentapeptide domain that is just carboxy-terminal to the reactive centre loop of the molecule (residues 370–374). This domain is within an area of homology among the serpins, including C1INH [17]. It is contained within the fourth strand of β sheet B and in the crystal structures of intact or cleaved serpins is not exposed [3,29–32]. It had been assumed prior to these studies that this region also would not be exposed in the complex with protease. The data of Joslin *et al.* [17], however, suggest that this domain might be exposed for recognition by the SEC I receptor, after conformational changes of the inhibitor take place during complex formation. In the case of α_1-AT complexes, binding to the SEC I receptor was followed by internalization of the complexes and induction of α_1-AT synthesis [12]. However, this was not shown to be true for C1INH complexes which specifically bound to U937 monocytes, but did not induce inhibitor synthesis [33].

As outlined, many studies indicate that the ligand binding site involved in the clearance of the serpin–protease complexes resides within the inhibitor. Although the data of Mast *et al.* indicate that the protease does not play a role with most serpin complexes, the data here and that of de Smet *et al.* [16] suggest a possible role for protease in C1INH clearance. In the data presented here, the catabolism of complexes with different proteases clearly differed, which suggests that the proteases themselves might be involved in the clearance of the C1INH–protease complexes. Furthermore, the turnover of the human and guinea pig C1INH complexed with human kallikrein, β factor XIIa and C1s was very similar to the turnover rates of the respective proteases. These data confirm and extend the previous observations of de Smet *et al.* [16]. Similarly, tPA has been shown to play a role in clearance of tPA–plasminogen activator inhibitor I (PAI) complexes via the SEC II receptor [34].

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