Identification of a new P1 residue mutation (444Arg→Ser) in a dysfunctional C1 inhibitor protein contained in a type II hereditary angioedema plasma

K.S. Aulak[1], M. Cicardi[2] and R.A. Harrison[1]

[1] *MRC Molecular Immunopathology Unit, MRC Centre, Hill Road, Cambridge CB2 2QH, UK* and [2] *Universita Degli Studi di Milano, Clinica Medica V, Ospedale 'S. Paolo', Via di Rudini 8, 20142 Milano, Italy*

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A new reactive-centre P1 residue mutation (444Arg→Ser), has been identified in a dysfunctional C1 inhibitor protein, C1 inhibitor(Ba), contained in a type II hereditary angioedema plasma. This substitution is compatible with a point mutation of the 444Arg codon (CGC→AGC), and represents the first non-histidine, non-cysteine P1 residue mutant described for C1 inhibitor.

Serpin; C1 inhibitor; Reactive-centre mutation; Dysfunctional protein; Plasma proteinase inhibitor; Hereditary angioedema

1. INTRODUCTION

C1 inhibitor, a member of the serine proteinase inhibitor (serpin) superfamily, is active against proteinases of the complement (C1r, C1s), fibrinolytic (plasmin), coagulation (factor XIa, factor XIIa) and kinin-generation (kallikrein) systems [1–6]. Whilst C1 inhibitor can inactivate other proteinases (e.g. the γ subunit of nerve growth factor [7] and tissue plasminogen activator [8]), the physiological significance of these interactions is unclear. The critical nature of C1 inhibitor in plasma proteinase regulation is highlighted by the disorder hereditary angioedema, which is inherited in an autosomal dominant manner [9,10]. Two forms of the disease occur, type I, in which the plasma level of both functional and antigenic C1 inhibitor is low (5–30% of normal), and type II, where the antigenic level is normal but the functional level is greatly reduced [11]. This latter form is consequent on the synthesis and secretion of a dysfunctional C1 inhibitor protein that is catabolized more slowly than the active protein [12,13].

Proteinase inhibitor members of the serpin family appear to use identical reaction mechanisms, and the inhibited proteinase forms a stable 1:1 complex with the inhibitor. This may be as an acyl-enzyme intermediate, requiring the hydroxyl group of the proteinase active site serine residue and the newly-released carboxyl (of the P1 residue) of the serpin [14,15]. The specificity of inhibition is heavily influenced by the amino acid at the P1 position. The importance of this residue was demonstrated by a naturally-occurring mutant of another member of the serpin superfamily, α_1-antitrypsin Pittsburgh. This had a P1 methionine to arginine substitution, and had lost its inhibitory activity against elastase (the natural substrate for α_1-antitrypsin), but had gained (lethal) inhibitory activity against thrombin [16]. That this effect was due solely to mutation of the P1 residue was confirmed by analysis of genetically-engineered α_1-antitrypsin mutants [17]. It was this demonstrable importance of the P1 residue that provided the initial impetus to search for similar mutations in the dysfunctional C1-inhibitor proteins found in type II HAE plasmas.

Recently, new analytical procedures [18] have allowed rapid analysis of C1-inhibitor proteins from a large number of type II HAE plasmas, and these have shown a preponderance of mutations at the P1 reactive centre residue (approximately 70% of the kindred studied) [19]. Furthermore, while random point mutation of the P1 arginine codon (CGC) potentially gives rise to 6 possible products (Pro, Gly, Leu, Ser, His and Cys), the observed mutations of this residue have, until now, been restricted either to histidine or to cysteine. This limited variability has been explained by the hypermutability of the CpG dinucleotide, generating CpA (hence CAC, histidine) or TpG (hence TGC, cysteine) dinucleotides [19,20].

Correspondence address: R.A. Harrison, MRC Molecular Immunopathology Unit, MRC Centre, Hills Road, Cambridge CB2 2QH, UK

Abbreviations: HAE, hereditary angioedema; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; Ps.a.e., *Pseudomonas aeruginosa* elastase

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We here describe the identification of the first non-histidine, non-cysteine P1 residue mutant of C1-inhibitor, a 444arginine⟶serine substitution in the dysfunctional protein, C1 inhibitor(Ba). This substitution is compatible with a point mutation of the 444arginine codon, CGC⟶AGC which, while it results in loss of the CpG dinucleotide, is not a result of deamination of 5-methyl-cytosine.

2. MATERIALS AND METHODS

Normal plasma was drawn from healthy volunteers and made 5 mM in benzamidine and EDTA. Plasma from the type II HAE patient (Ba) was taken into EDTA only. Samples were then stored at −70°C until required. Normal C1 inhibitor was purified using the method of Harrison [21].

Anti-C1 inhibitor IgG was prepared from a rabbit anti-C1 inhibitor antiserum as described previously [18], and coupled to Sepharose CL-4B using the procedure of March et al. [22].

2.1. *Analysis of the C1 inhibitor proteins*

Plasma C1-inhibitor was analysed as described by Aulak and Harrison [18]. Briefly, 1 vol. of anti-C1 inhibitor-Sepharose CL-4B was incubated with 1 vol. plasma (in 10 mM EDTA, 10 mM benzamidine) and the Sepharose beads maintained in suspension during the incubation period (2 h at 4°C). The loaded beads were then washed with PBS, PBS + 1 M NaCl, and again with PBS before digestion either with trypsin (bovine pancreatic; Tos-Phe-CH_2Cl treated; Sigma) or with *Pseudomonas aeruginosa* elastase (Ps.a.elastase; the kind gift of Dr Morihari, Kyoto Research Laboratories, Tokyo, Japan). Trypsin digestion was performed as follows: 25 μl of the adsorbed resin was incubated with 20 μl trypsin (0.43 μM in 50 mM Tris/HCl, 100 mM NaCl, pH 8.0) for 40 min at 37°C, then digestion stopped by the addition of SDS-PAGE non-reducing sample preparation buffer and heating for 2 min at 100°C. Ps.a.elastase digestion was performed using 350 μl Sepharose beads onto which C1 inhibitor had been adsorbed. After incubation with 5 μl Ps.a.elastase (3.0 μM in 50 mM Tris/HCl, 100 mM NaCl, 5 mM $CaCl_2$, 50 μM $ZnCl_2$, pH 7.0) for 1 h at 37°C, the reaction was terminated by the addition of EDTA to a final concentration of 10 mM; 1 ml HPLC buffer (5% acetonitrile, 0.1% TFA) was added and the mixture was left at 4°C for 20 min. This was then filtered through a 0.2 μm filter (Gelman Sciences) prior to peptide separation by reverse phase HPLC.

Trypsin digestion of purified C1 inhibitor was as described by Aulak et al. [23], and Ps.a.elastase digestion of the purified protein as described by Pemberton et al. [24].

SDS-PAGE was performed using the buffer and sample preparation systems of Laemmli [25] with a 10–20% polyacrylamide gradient gel. Molecular weight marker proteins were purchased from BDH. HPLC separation of Ps.a.elastase digested proteins was performed using a reversed phase polystyrene column (PLRP-S 300 A; 250 mm × 4.6 mm; Polymer Laboratories, Church Stretten, Shropshire, UK) and Waters HPLC instrumentation. After loading, the column was washed with 5% acetonitrile, 0.1% TFA for 10 min, then a linear gradient to 80% acetonitrile/0.1% TFA was developed over 40 min. Finally the column was washed for 10 min with 80% acetonitrile/0.1% TFA before returning to the equilibration conditions. A flow rate of 1 ml/min was used throughout.

Amino-terminal sequence analysis of the reactive centre peptide was performed by Dr L. Packman at the Protein Sequencing Facility of the Department of Biochemistry, University of Cambridge, Cambridge, UK, using Applied Biosystems instrumentation.

3. RESULTS

Fig. 1 shows SDS-PAGE analysis of the trypsin digestion products of purified C1 inhibitor, C1 inhibitor immuno-adsorbed from normal plasma, and C1 inhibitor adsorbed from the variant (Ba) plasma. Both plasmas contain normal-sized (100 kDa) C1 inhibitor (tracks 3 and 5). However, while the normal plasma gives an 85 kDa major fragment after trypsin digestion (track 4), C1 inhibitor from plasma (Ba) gives a 97 kDa major fragment (track 6), showing that it has a P1 residue mutation.

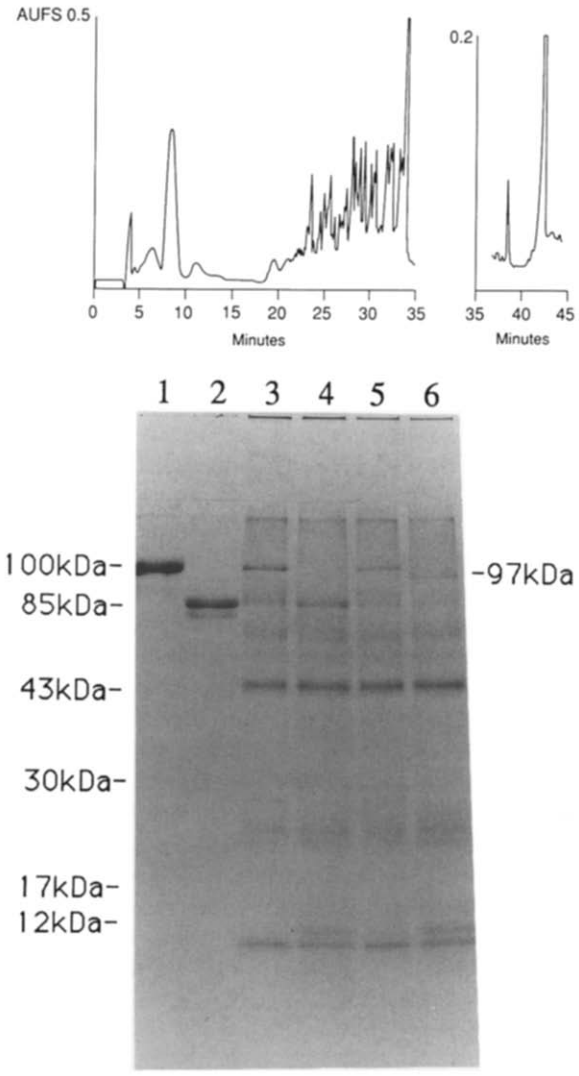

Fig. 1. SDS-PAGE analysis of digestion of C1 inhibitor(Ba) with trypsin. Track 1, normal C1 inhibitor (15 μg). Track 2, normal C1 inhibitor (15 μg) digested with trypsin. Track 3, C1 inhibitor derived from immunoadsorbed normal plasma. Track 4, C1 inhibitor derived from immunoadsorbed normal plasma digested with trypsin. Track 5, C1 inhibitor derived from immunoadsorbed plasma from a type II HAE patient (Ba kindred). Track 6, C1 inhibitor derived from immunoadsorbed plasma from the type II HAE(Ba) patient and digested with trypsin. Molecular mass markers: 100 kDa, C1 inhibitor; 85 kDa, major Ps.a.elastase cleavage fragment of C1 inhibitor; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase; 17 kDa, myoglobin; 12 kDa, cytochrome *c*.

HPLC separation of the Ps.a.elastase digestion products of the immunoadsorbed C1 inhibitor(Ba) is shown in Fig. 2. The major fragment and reactive-

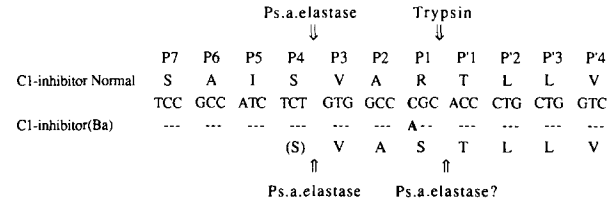

Fig. 2. Chromatogram of HPLC resolution of the Ps.a.elastase digestion products of C1 inhibitor(Ba). Digestion of C1 inhibitor adsorbed onto anti-C1 inhibitor with Ps.a.elastase and HPLC separation was as described in the text.

centre containing carboxy-terminal peptides are well resolved from antibody-derived and C1 inhibitor amino-terminal peptides, eluting at 42 and 38 min, respectively. The 38 min peak was collected and subjected to amino-terminal sequence analysis. This showed two sequences, one generated from the cleavage between the P4 and P3 residues, and the second generated from the cleavage between the P1 and P'1 residues (Fig. 3). The first sequence is analogous to that seen in the reactive centre peptide released by Ps.a.elastase cleavage of the normal, P1 histidine, and P1 cysteine, inhibitors. The second sequence (~25% of the level of the first) could arise either as a consequence of cleaved C1 inhibitor being present in the starting material or as a consequence of generation of a new Ps.a.elastase cleavage site (444Ser-445Thr) by the new P1 residue. (While Fig. 1 appears to suggest that the former explanation is unlikely, a low level of a band similar in size to reactive centre cleaved C1 inhibitor was evident in the original gel.) These sequence data show unequivocally that C1 inhibitor(Ba) has a mutation of the P1 arginine residue to serine.

4. DISCUSSION

A number of C1 inhibitor proteins contained in type II HAE plasmas have now been characterised [18–20,23,26]. Many (about 70%) have P1 residue mutations, justifying our initial concentration on analysis of this residue. However, with the exception of the P1 serine mutant described in this paper, all have been either P1 cysteine or P1 histidine mutants. The high preponderance of cysteine and histidine mutations has been explained by the nature of the P1 arginine codon [19,20]. This (CGC) contains a CG dinucleotide; methylation of the cytosine followed by deamination (to thymine) of the 5-methyl-cytosine thus generated leads to formation of TGC (cysteine; deamination of 5-methyl-cytosine on the coding strand) or CAC (histidine; deamination on the non-coding strand followed by T-A base-pairing during DNA replication) codons. Interestingly, antithrombin III has a similar, high preponderance of P1 cysteine and P1 histidine variants [27–29], although a P1 Arg⟶Pro mutation (antithrombin III Pescara) has recently been described [30]. As antithrombin III also has a P1 arginine encoded by a CG dinucleotide-containing codon (CGT) [31], it seems likely that again it is the CG dinucleotide effect that is responsible for the observed frequency of P1 residue mutations. C1 inhibitor(Ba) represents the first identification of a mutation of the P1 residue to anything other than histidine or cysteine in C1 inhibitor. Furthermore, when considered together with antithrombin III (Pescara), it demonstrates that whilst the hyper-mutation of the CG dinucleotide is the probable reason for the large number and limited diversity of P1 residue mutations in 'argserpins' containing a CG dinucleotide in the P1 residue codon, other mutations of this residue can and do occur.

Identification of this novel mutation is important for the following reason. While the P1 residue is clearly a major determinant of inhibitor specificity, it does not have an over-riding influence. This is best illustrated with reference to the archetypal serpin, α_1-antitrypsin, which has a P1 methionine (and is the physiological inhibitor of leukocyte elastase) rather than a P1 lysine or arginine residue, yet is still a highly effective inhibitor of trypsin [32]. One plausible explanation for the non-identification of P1 mutants other than histidine or cysteine was that other mutations of this residue generated inhibitors that were still effective against the target enzymes of C1 inhibitor, particularly those enzymes that were responsible for generation of the angioedema-causing peptide. Since the P1 serine mutation has been found in a typical type II HAE patient, C1 inhibitor(Ba) must be effectively dysfunctional against the proteinase(s) responsible for the generation of the edema causing agent.

The nature and mechanism of release of the agent(s) (bradykinin or a C2-derived peptide) responsible for edema formation is still controversial, with kallikrein, C1s and plasmin all being implicated in peptide release [33]. The clinical presentation of affected individuals indicates that C1 inhibitor(Ba) is inactive against the complement proteinases C1s and C1r. However, it is possible that it still retains activity against other plasma proteinases (or that it has acquired a novel activity). For example, while antitrypsin Pittsburg had lost inhibitory activity against leukocyte elastase, it had gained activity against thrombin [16]. Similarly, both C1 inhibitor(At), a naturally-occurring P1 arginine to histidine mutant, and an engineered P1 Arg⟶His C1 inhibitor protein have significantly increased inhibitory activity against chymotrypsin ([34], Aulak, unpublished data). We are therefore pursuing full kinetic analysis of C1 inhibitor(Ba) against a spectrum of plasma proteinases in the hope that this might give further insight into the possible molecular mechanism of HAE.

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