Angiotensinogen cleavage by renin: importance of a structurally constrained N-terminus

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Abstract Angiotensinogen, a plasma serpin, functions as a donor of the decapeptide angiotensin I, which is cleaved from the N-terminus by renin. To assess the contribution of the serpin framework to peptide cleavage we produced a chimaeric molecule of α_1-antitrypsin carrying the angiotensinogen N-terminus and determined the kinetic parameters for angiotensin I release. The K_m for plasma angiotensinogen was 18-fold lower than for the chimaeric protein while the catalytic efficiency was four-fold higher. We also show that Cys-18 participates in a disulphide bond and propose that constraints on the N-terminus profoundly affect the interaction with renin.

Key words: Angiotensinogen; Serpin; Hypertension

1. Introduction

The renin-angiotensin system is a major regulator of salt and water homeostasis and has a key role in the control of blood pressure. Epidemiological studies strongly implicate the angiotensinogen gene locus in the pathogenesis of essential hypertension. In particular, a polymorphism in the angiotensinogen promoter region changes the rate of transcription and has been associated with hypertension [1]. Two further polymorphisms affecting residues 174 and 235 within the coding region are linked to hypertension and may cause structural perturbations of angiotensinogen.

Angiotensinogen is the precursor of the angiotensin peptides and is a non-inhibitory member of the serpin (serine protease inhibitor) superfamily of proteins [2]. The core structure of angiotensinogen almost certainly resembles other serpins and can therefore be modelled on family members such as ovalbumin. However, the N-terminus of angiotensinogen is considerably extended and its structure completely unknown. The only function of angiotensinogen identified so far is to act as a donor of the angiotensin I peptide in the first step of the reaction sequence leading to the generation of angiotensin II. In this step, angiotensinogen is cleaved at the N-terminus by renin, an aspartyl protease. Two C-terminal amino acids are then removed from angiotensin I by angiotensin-converting enzyme (ACE) to produce the active octapeptide hormone angiotensin II. This mediates its effect on vascular pressure by binding to cellular receptors and initiating a biochemical cascade which, amongst other effects, results in an increase in vascular tone.

Although it is possible that angiotensinogen could be acting solely as a passive carrier and plasma reservoir of the angiotensin peptide, the complexity of the serpin structure raises the question of whether the remainder of the angiotensinogen molecule has some additional function. Evidence for the involvement of the protein structure in the renin interaction is provided by an increased K_m value for the interaction of renin with the N-terminal tetradecapeptide of angiotensinogen [3,4] compared to the value determined for the reaction of renin with the whole angiotensinogen molecule. This finding suggests that the body of the angiotensinogen molecule facilitates the interaction with renin thereby lowering the K_m. In this report, the role of the serpin body in the interaction between angiotensinogen and renin has been studied by expressing a chimaeric renin substrate in which the major protein framework of angiotensinogen has been replaced with that of the archetypal serpin, α_1-antitrypsin. The 17 N-terminal amino acids of angiotensinogen were added to the N-terminus of α_1-antitrypsin to produce the chimaeric Ang-Antitrypsin (AngAT) protein. The substitution of the angiotensinogen protein framework by the α_1-antitrypsin molecule significantly alters the kinetics of the interaction with renin and release of angiotensin I.

Fig. 1 shows a model of the angiotensinogen structure based on the crystal structure of ovalbumin, another non-inhibitory serpin [5,6], with which angiotensinogen has 21% sequence homology [7]. The extended N-terminus of angiotensinogen contains a cysteine residue at position 18, and in this report we present evidence that this cysteine residue is disulphide linked to the body of the molecule, thereby constraining the N-terminal extension containing the angiotensin peptide.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli (BL21(DE3)pLysS) were grown in 2×TY medium (1.6% w/v bacto-tryptone, 1% w/v bacto-yeast extract, 0.5% w/v NaCl) supplemented with 50 μg/ml ampicillin.

2.2. Construction of the pAngAT expression vector

The pAngAT expression vector was constructed from the α_1-antitrypsin expression vector, pTermAT [8], by insertion of a cassette encoding the sequence of the 17 N-terminal amino acids of angioten-

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Abbreviations: ACE, angiotensin-converting enzyme; AngAT, Ang-Antitrypsin; IPTG, isopropyl-β-D-thiogalactopyronoside; HPLC, high performance liquid chromatography

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sinogen. The cassette was prepared by annealing the two synthetic oligonucleotides: 5′-GAT CGA GTA TAT ATA CAT CCC TTC CAC CTC GTC ATC CAC AAT GAG AGT ACC-3′ and 5′-CT CAT ATA TAT GTA GGG AAG GTG GAG CAG TAG GTG TTA CTC TCA TGG CTA G-3′, and ligating into a *Bam*HI site immediately preceding the α_1-antitrypsin coding sequence. The construct was confirmed by DNA sequencing.

2.3. Expression and purification of the AngAT protein

BL21(DE3)pLysS cells transformed with pAngAT were grown at 37°C to an OD_{600} of 0.4 and induced by the addition of IPTG for the expression of AngAT. Cells were harvested after 3 h, the pellet resuspended in 300 mM NaCl, 50 mM Tris pH 8.0, 10 mM EDTA, 0.5% Triton X-100 and cells lysed by French press. The sample was centrifuged and the pellet washed three times in lysis buffer (30 ml). The final pellet was dissolved in 5 ml (8 M GdHCl, 100 mM DTT, 50 mM Tris pH 8.0) and centrifuged. The soluble denatured protein was refolded by pumping into 800 ml 50 mM Tris pH 8.0, 5 mM DTT at a rate of 0.01 ml/min. Insoluble material was removed by centrifugation and the supernatant passed through a 0.2 μm filter. The refolded protein was applied to a column (1.6×16.5 cm) of Q-Sepharose fast-flow (Pharmacia Biotech, Uppsala, Sweden) and eluted with a gradient of 50–250 mM NaCl in 50 mM Tris pH 8.0. Fractions containing AngAT were identified by rocket immunoelectrophoresis using antibodies to antitrypsin, and the pooled purified AngAT was analysed by SDS-PAGE and Western blotting.

2.4. Characterisation of AngAT

Purified AngAT was characterised by determining its activity as a functional antitrypsin molecule. Inhibition of bovine chymotrypsin was measured as described in Lomas et al., 1993 [9]. N-terminal sequencing of the purified AngAT protein and renin-cleaved N-terminal peptide were performed on an Applied Biosystems 477 Protein Sequencer. The cleaved peptide was additionally confirmed by mass spectrometry using a Kratos Kompact MALDI 4 instrument.

2.5. Renin kinetics

The renin substrates were incubated with recombinant human renin (5 nM for AngAT, 1 nM for plasma angiotensinogen [10] and 0.5 nM for TDP (Calbiochem Novabiochem, UK)) for 15 min at 37°C in 0.2 M sodium phosphate buffer pH 7.4, 0.02% BSA. The reaction was stopped by the addition of 1/10 volume 10% H_3PO_4 and samples kept on ice. Angiotensin I was quantified by reverse phase HPLC of a known volume of reaction product on a Novapak C18 column (Waters Division of Millipore, UK), eluting with an acetonitrile gradient (19–50% in 1% H_3PO_4) over 10 min. A standard curve was prepared using pure angiotensin I peptide (Calbiochem Novabiochem, UK). A single site binding equation (GraphPad Software) was used to construct Michaelis-Menten plots from the experimental data and thus determine K_m and V_{max} values.

2.6. Determination of angiotensinogen disulphide bonds

Two 20 μg samples of pure plasma angiotensinogen were placed in Eppendorf tubes, dried in a vacuum centrifuge and each resuspended in 50 μl 6 M GdHCl, 0.25 M Tris pH 8.5. To one of the protein samples, 2.5 μl 10% v/v β-mercaptoethanol was added, the tube flushed with nitrogen, sealed and left at room temperature (RT) in the dark for 2 h. After this time, 18.6 μmol of the sulphydryl modifying reagent 4-vinyl pyridine (4-VP) was added, the tube again flushed with nitrogen and incubated for a further 2 h in the dark at RT. The second protein sample was treated directly with the 4-VP reagent as described, without the initial reduction step. At the end of the reaction, urea and excess reagents were removed from the samples by repeated dilution with H_2O and reconcentration in a centrifugal concentrator. The final concentrated protein samples were subjected to N-terminal sequence analysis.

3. Results and discussion

Expression of the chimaeric Ang-Antitrypsin (AngAT) in bacteria resulted in the majority of the expressed protein being in the form of insoluble cytoplasmic inclusion bodies which has been observed previously with recombinant antitrypsin [11]. In order to enable functional studies, the insoluble AngAT protein was refolded and purified on Q-Sepharose resin (Fig. 2). N-terminal sequence analysis confirmed the sequence M-D-R-V-Y-I-H-P-F-H-L-V-I-H-N-E-S-T-D-P, corresponding to the 17 N-terminal amino acids of angiotensinogen with the retention of the initiating methionine residue as a result of prokaryotic expression. The angiotensinogen N-terminus is followed by the N-terminal amino acid sequence of antitrypsin, beginning with aspartate and proline. AngAT was shown to be active as an inhibitor of bovine α-chymotrypsin with a specific activity of 46.8% and a K_{ass} of 2.5×10^6 M^{-1} s^{-1}. These figures are comparable with those obtained for purified plasma antitrypsin, indicating that the serpin body of α_1-antitrypsin is correctly refolded. Upon cleavage of AngAT with renin, a peptide of mass 1428.1 ± 1 Da was released,

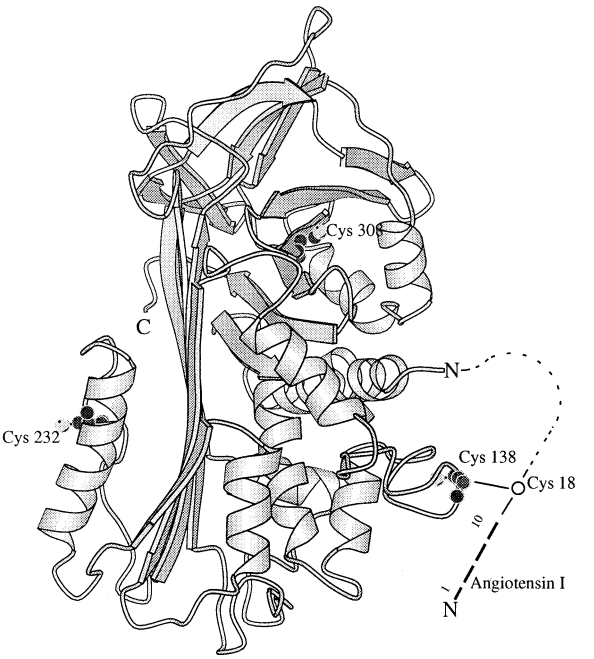

Fig. 1. Model of angiotensinogen structure based on sequence alignment with ovalbumin. The 69 N-terminal amino acids are not included in the model but the diagram illustrates the constraint which may be imposed by a disulphide bond between Cys-18 and Cys-138.

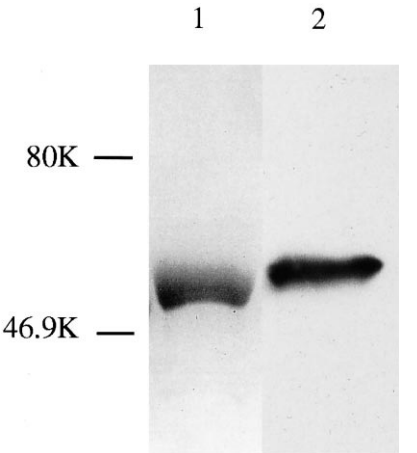

Fig. 2. SDS-PAGE analysis of purified AngAT. Lane 1: Coomassie stained; Lane 2: Western blotted with antibody to antitrypsin. Lane 2 contains 1/10 quantity of protein in lane 1.

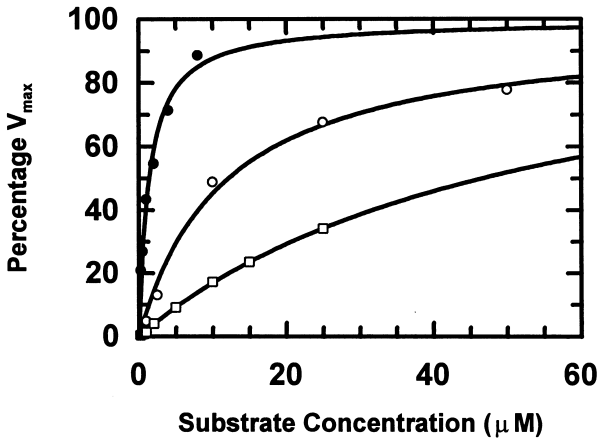

Fig. 3. Michaelis-Menten plots for the release of angiotensin I from renin substrates: ●, plasma angiotensinogen ($n=2$; $V_{max}=24$ nmol/l/min); ○, angiotensinogen N-terminal tetradecapeptide ($n=4$; $V_{max}=69$ nmol/l/min); □, AngAT ($n=2$; $V_{max}=566$ nmol/l/min).

corresponding to the decapeptide angiotensin I, with an additional N-terminal methionine residue and this was confirmed by N-terminal sequence analysis.

In order to determine the values of K_m and V_{max} for the AngAT-renin reaction, increasing concentrations of AngAT were incubated with 5 nM recombinant human renin and a known volume of each reaction mixture was analysed by reverse phase HPLC. The quantity of Met-angiotensin I released was calculated and from the resulting Michaelis-Menten plot (Fig. 3), K_m and V_{max} values for the reaction of AngAT with recombinant human renin were calculated to be 47.5 ± 4.2 μM and 566.2 ± 34.0 nmol/l/min respectively.

The significance of these values of K_m and V_{max} can be assessed by comparison with the kinetic parameters for the reaction of recombinant human renin with other substrates. The K_m for AngAT is considerably higher than the values determined for plasma angiotensinogen ($K_m = 2.6 \pm 0.6$ μM) (Fig. 3). Comparison of K_m values indicate that renin has a higher affinity for angiotensinogen than for AngAT. This implies that the body of the angiotensinogen molecule contributes to the interaction with renin and is not simply a passive carrier of the N-terminal peptide. Renin also cleaves a synthetic angiotensinogen N-terminal tetradecapeptide (TDP) substrate, with a K_m of 8.2 μM (Fig. 3). The fact that the K_m for renin interaction with AngAT is higher than that for the interaction of renin with TDP indicates that the α_1-antitrypsin serpin body of AngAT actually has a negative effect on the association with renin. In order to compare the V_{max} values obtained for plasma angiotensinogen, AngAT and TDP we need to take into consideration the concentration of enzyme used in the assays. Although we have not measured the specific activity of our renin, if we assume it to be fully active then K_{cat} values for the substrate would be 0.4 s^{-1} (plasma angiotensinogen), 1.9 s^{-1} (AngAT) and 2.3 s^{-1} (TDP) and catalytic efficiencies (K_{cat}/K_m) are 0.2 μM^{-1} s^{-1}, 0.04 μM^{-1} s^{-1} and 0.3 μM^{-1} s^{-1} respectively. These figures show that the K_{cat} for AngAT is approximately five-fold higher than for plasma angiotensinogen, indicating that the renin-substrate complex dissociates to form products faster for AngAT than angiotensinogen. By contrast, when catalytic efficiency is calculated, plasma angiotensinogen is approximately four-fold greater. This difference is most notable at the physiological concentration of plasma angiotensinogen where it is a much better substrate for renin than AngAT.

The behaviour of these serpins as substrates may be explained by specific interactions occurring between renin and angiotensinogen at sites away from the scissile bond or by structural constraints imposed upon the N-terminal region by the body of the molecule. The lack of a crystal structure for angiotensinogen makes it difficult to speculate about the conformation adopted by the N-terminus and the nature of its interaction with the serpin body. However, we noted the presence of a cysteine residue at position 18 with the potential to participate in a stabilising intramolecular disulphide bond. To investigate this possibility we assessed the availability of Cys-18 for reaction with the sulphydryl modifying reagent, 4-vinyl pyridine (4-VP) under reducing and non-reducing conditions.

N-terminal sequence analysis of plasma angiotensinogen reacted with 4-VP under reducing conditions detected a pyridylethylcysteine residue while under non-reducing conditions Cys-18 was not detected at all, due to its involvement in a disulphide bond. Taking these data together with the modelled angiotensinogen structure (Fig. 1), we propose that the angiotensinogen N-terminus is linked to the rest of the molecule by a disulphide link between Cys-18 and Cys-138. In support of this proposal, these two residues are conserved in rat, mouse, sheep and human angiotensinogens. The constraint imposed by this disulphide bond may contribute to the N-terminus adopting a favourable conformation for formation of a Michaelis-Menten complex with renin. Since only the 17 N-terminal amino acids of angiotensinogen were attached to α_1-antitrypsin, AngAT does not have the potential to form such an interaction. It is also possible that the lack of N-terminal constraint explains the faster release of angiotensin I from AngAT.

Angiotensinogen interacts with renin to release the decapeptide angiotensin I at a K_m close to the plasma concentration of angiotensinogen [12]. Since the cleavage of angiotensinogen by renin is rate-limiting in the renin-angiotensin system [13] the kinetics of this interaction are of obvious importance and in this report the tertiary structure of angiotensinogen has been shown to profoundly affect the affinity of enzyme for substrate as well as the overall rate of reaction. Furthermore, the demonstration that another serpin molecule can act as a donor of angiotensin I has many more general implications for the design of peptide therapies.

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