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Effect of alanine insertion (P'_5) on the reactive centre of α_1-antitrypsin

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A recombinant mutant of α_1-antitrypsin with an inserted alanine in position P'_5 (362–363) was compared with wild-type recombinant and plasma α_1-antitrypsins. Initial studies showed that contrary to other reports the wild recombinant inhibitor had the same, or even greater, association constants with trypsin and elastases as the plasma inhibitor. The recombinant mutant as predicted had decreased inhibitory activity but no significant alteration in denaturation temperature and it retained the typical serpin S-R change.

α_1-Antitrypsin; Serpin

1. INTRODUCTION

The inhibitory activity of α_1-antitrypsin, as of other serpins, is dependent on its reactive centre loop acting as a pseudosubstrate for the target protease. Cleavage of this loop results in a remarkable change in the thermal stability of the α_1-antitrypsin molecule: the native stressed S form of the molecule being thermoprecipitable at 58°C, the cleaved, relaxed R form remaining in solution at temperatures of 80°C or above [1,2].

It had been proposed that this S-R change was a consequence of the reactive centre loop in the native S form being in a stretched conformation, with cleavage giving a release of the associated stress. We have tested this proposal by the insertion of an extra alanine on the C-terminal (P′) side of the reactive centre to see if the addition of sequence lessens the stress and hence alters the thermolability of α_1-antitrypsin.

2. MATERIALS AND METHODS

2.1. *Preparation of genetic constructs*

An α_1-antitrypsin cDNA isolated by Ciliberto [3] was cloned into pEMBL8. (a gift from Dr R. Foreman, Univ. Southampton). An expression linker was added to the 5′ end and the gene subcloned into M13mp9 cut with *Bam*HI and *Pst*I.

Hind III

Bam HI

AATTCAAGCTTAAAAAAATGGAG
GTTCCAATTTTTTTACCTCCTAG

Eco RI

Expression Linker

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Site directed mutagenesis used the 'In Vitro Mutagenesis' kit (Amersham, UK). Oligonucleotides were provided by Delta Biotechnology (Nottingham, UK). The oligonucleotide, TCTATCCCCCCAGCAGAGGTCAAGTTC, was used to insert an extra alanine between the 362 and the 363 position, which is the P'_5 position. Single-stranded DNA of the mutated clone was then prepared and was sequenced using the dideoxy chain-termination method (Sequenase 2.0, U.S.B. Ohio, USA).

For expression, the *Hind*III–*Hind*III fragment from M13mp9 containing the α_1-antitrypsin was cut out, blunt-ended and ligated into the *Bgl*I-cut and blunt-ended pKV50 (Delta Biotechnology Ltd. Eur. pat. app. no. 87307668.1). This brings the gene under the control of a hybrid promoter consisting of the *Saccharomyces cerevisiae* 3-phosphoglycerate kinase gene under the gal 10 gene regulation. The recombinant pKV50 plasmid was transformed into yeast protoplasts as in [4]. The protease deficient strain of *S. cerevisiae* AB116 was used [5].

The yeast was grown to an $OD_{600} = 1$ in yeast minimal medium (Difco, Detroit, USA) containing uracil, adenine and tryptophan (all at 55 mg/litre) and glucose at 20 g/litre. The cells were then harvested and transferred to new medium containing galactose 20 g/litre instead of glucose. After 24 h the cells were harvested and the intracellular α_1-antitrypsin extracted and purified as in [6]. Plasma α_1-antitrypsin was prepared as in [7].

2.2. *Kinetic measurements*

The association rate constants for the interaction of α_1-antitrypsin with human neutrophil elastase (HNE), porcine pancreatic elastase (PPE) and porcine trypsin were measured as in [8]. The substrates used were N-suc-Ala-Ala-Pro-Val *p*-nitrophenylanilide (200 μM), N-suc-Ala-Ala-Ala-*p*NA (300 μM), and N-α-benzoyl-Arg-*p*NA (550 μM) respectively. The results were analysed using a Uvikon 930 spectrophotometer (Kontron, Zurich, Switzerland) and the software program Enzfitter (R. Leatherbarrow, Imperial College, UK).

2.3. *Heat stability assay*

The heat stabilities of the α_1-antitrypsin variants were tested by incubating samples at 55°C and taking aliquots every twenty minutes [6]. The uncleaved and the cleaved forms were compared by incubating at different temperatures for 2 h [9]; papain was used to cleave the α_1-antitrypsins. The percentage of protein that remained soluble (i.e. non-denatured) was determined by an electroimmunoassay using rabbit anti-human α_1-antitrypsin.

2.4. *Demonstration of protease-inhibitor complex formation*

Samples of inhibitor (10 μg) were reacted with an equimolar activity of proteinase for 15 min at room temperature in 10 mM Tris-HCl pH 8, 50 mM NaCl and then applied to a SDS-PAGE system and stained with Coomassie blue [10].

2.5. *N-terminal sequencing*

Samples of protein to be sequenced (30 μg) were blotted onto PVDF membranes (Immobilon, Millipore) after SDS-PAGE [11]. The blotted proteins were sequenced on an Applied Biosystems protein sequencer by Dr L. Packman (Biochem. Dept., Univ. Cambridge).

3. RESULTS

The association rate constants (Table I) indicate that the recombinant α_1-antitrypsin is as efficient an inhibitor of a range of proteases as that from human plasma, with closely comparable association constants. The mutant α_1-antitrypsin with the inserted alanine remained an efficient inhibitor of HN elastase, though with a reduced association constant, but did not inhibit trypsin. SDS-PAGE analysis of a mixture of mutant inhibitor and trypsin showed that the inhibitor remained intact without the appearance of either a higher molecular weight complex, as for the wild-type protein, or of lower molecular weight degraded material (data not shown). In contrast, transient complex formation was observed with PP elastase using SDS-PAGE, with degradation products being visible as well. Since the calculation of the association constant depends on the irreversible production of an inhibitor–enzyme complex [8] it was not calculable in this case.

The results of the heat stability assays (Fig. 1a) indicate that there is no essential difference in the S-R transition of the mutant, wild-type recombinant and plasma α_1-antitrypsins. However, the wild-type recombinant protein demonstrated a decrease in stability at 55°C (Fig. 1b) as compared to the plasma protein and the alanine mutant.

4. DISCUSSION

In order to clarify the effects of the mutation on the inhibitory kinetics it was first necessary to define an accurate baseline value for the association constant of α_1-antitrypsin. Values reported in the literature [6,8,12] vary and for this reason we made repeated measurements on a series of freshly prepared samples of plasma and recombinant wild α_1-antitrypsin. The integrity of the samples was checked by electrophoresis and N-terminal sequencing. The constants obtained, Table I, were higher, by up to 30-fold, than some of the usually quoted figures [8]. We conclude in agreement with [8] that the association constant of α_1-antitrypsin with HN elastase is about 6.5×10^7 $M^{-1} \cdot s^{-1}$. This is so for both the unmodified inhibitors and also for all the tested N-terminally cleaved derivatives. Identical constants were obtained for both the plasma and recombinant wild-type forms, agreeing with [6] that oligosaccharide does not affect inhibitory function.

The recombinant mutant we report here has had an alanine inserted in position P'$_5$, the region where the C-terminus of the reactive centre loop re-enters the body of the molecule. It has a decrease in inhibitory activity and a slight increase in thermostability as compared to the wild recombinant (Fig. 1b). However, there is no significant change in the temperature of denaturation of the engineered recombinant mutant versus the wild recombinant and plasma α_1-antitrypsins, and the mu-

Table I

Association constant ($M^{-1} \cdot s^{-1}$)

Enzyme	Plasma α_1-antitrypsin	Wild-type recombinant	Alanine recombinant
HNE	$6.6 \pm 0.1 \times 10^7$ (6.5×10^7)	7.0×10^7	$5.8 \pm 0.5 \times 10^6$
PPE	$1.5 \pm 0.5 \times 10^6$ (1.0×10^5)	$2.4 \pm 0.2 \times 10^6$	transient inhibition
Trypsin	$2.8 \pm 0.5 \times 10^5$ (4.2×10^4)	$2.4 \pm 0.5 \times 10^5$	$< 3 \times 10^2$

All values, given with the σ_{n-1} are the average of 3 readings, except those for HNE with plasma and wild-type recombinant which are an average of two. Bracketed values are those given in the literature [8].

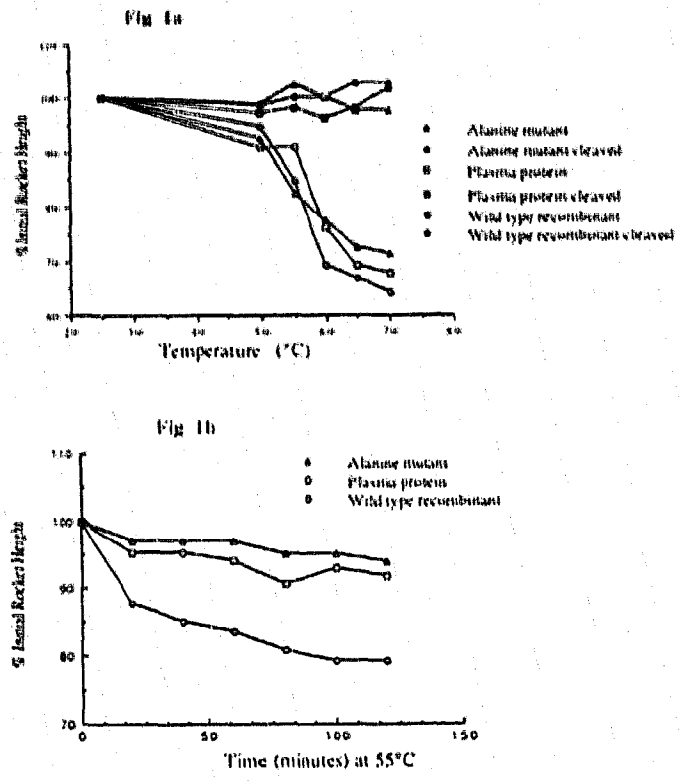

Fig. 1. Measurements of soluble α_1-antitrypsin by rocket electroimmunoassays. (a) Comparison of the heat stability of the cleaved and uncleaved forms of each variant at different temperatures. (b) Comparison of the heat stability of the different (uncleaved) variants at 55°C.

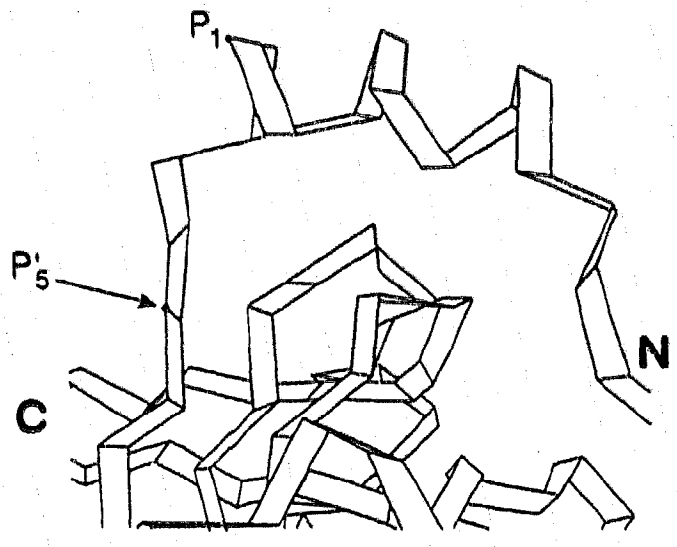

Fig. 2. An expanded view of the prototype reactive centre loop of ovalbumin [13]. The reactive centre is at P'_1 and the alanine in the mutant α_1-antitrypsin is inserted 5 residues C-terminal to this at P'_5 (arrowed). The model for the reactive centre of inhibitory serpins proposes [9] a mobile reactive centre (constrained here in ovalbumin) with partial movement of the N-terminal stalk (N) back into the A sheet to give the stretched prototypical canonical form [14]. In α_1-antitrypsin P_1 is Met-358; P'_5 is the position of insertion of alanine 362-363.

tant undergoes the typical S-R change (Fig. 1a). These results are compatible with our recent proposals as to the function of the reactive centre based on the newly derived crystallographic structure of ovalbumin [13]. Computer graphics using this structure suggest that the insertion at the P'_5 alanine will cause a displacement, i.e. movement at the reactive centre rather than realigning its structure.

The ovalbumin structure provides a model of the intact reactive centre of a serpin in the form of a mobile helix exposed from the pole of the molecule on two peptide stalks (Fig. 2). It is important to emphasise that this structure is mobile in the inhibitory serpins and that the active inhibitory form is likely to be that of the canonical structure [14] in which the N-terminal stalk is partially inserted into the A-sheet. We earlier predicted a stressed, labile state for the native reactive centre [1,2], but from the ovalbumin model it now seems that the stress is likely to be the consequence of two opposing tensions: the attempted formation of a helix on the C-terminal side of the loop and the opposing attempted movement of the N-terminal stalk back into the A-sheet on the other side of the loop. This model is in keeping with the observation that the insertion of an alanine at P'_5 affects inhibitory activity, since there will be displacement of the reactive centre, but does not significantly alter stability, nor the S-R change, since the insertion will predictably neither affect the formation of the helix nor the re-entry of the N-terminal stalk into the A-sheet.

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