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SP-A binds alpha$_1$-antitrypsin *in vitro* and reduces the association rate constant for neutrophil elastase

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Abstract

Background: α1-antitrypsin and surfactant protein-A (SP-A) are major lung defense proteins. With the hypothesis that SP-A could bind α1-antitrypsin, we designed a series of *in vitro* experiments aimed at investigating the nature and consequences of such an interaction.

Methods and results: At an α1-antitrypsin:SP-A molar ratio of 1:1, the interaction resulted in a calcium-dependent decrease of 84.6% in the association rate constant of α1-antitrypsin for neutrophil elastase. The findings were similar when SP-A was coupled with the Z variant of α1-antitrypsin. The carbohydrate recognition domain of SP-A appeared to be a major determinant of the interaction, by recognizing α1-antitrypsin carbohydrate chains. However, binding of SP-A carbohydrate chains to the α1-antitrypsin amino acid backbone and interaction between carbohydrates of both proteins are also possible. Gel filtration chromatography and turnover per inactivation experiments indicated that one part of SP-A binds several molar parts of α1-antitrypsin.

Conclusion: We conclude that the binding of SP-A to α1-antitrypsin results in a decrease of the inhibition of neutrophil elastase. This interaction could have potential implications in the physiologic regulation of α1-antitrypsin activity, in the pathogenesis of pulmonary emphysema, and in the defense against infectious agents.

Background

Alpha$_1$-antitrypsin (α_1-AT) and surfactant protein-A (SP-A) are major defense glycoproteins in the alveolar spaces of human lungs. α_1-AT, a 52,000 D glycoprotein, is secreted mostly by hepatocytes, and, to a lesser extent, by lung epithelial cells and phagocytes. α_1-AT inhibits a variety of serine proteinases by its active site (Met358-Ser359), but its preferential target is human neutrophil

elastase (HNE) as demonstrated by the high association rate constant (K_{ass}) for this proteinase [1]. In the lungs, α_1-AT protects the connective tissue from HNE released by triggered neutrophils; as a result, subjects homozygous for the common deficiency variant Z α_1-AT (associated with 15% of normal plasma α_1-AT levels) develop pulmonary emphysema early in life, especially if they smoke [2].

SP-A, a member of the *collectin* (*col*lagen-*lectin*) family [3], is one of the proteins of surfactant. Structurally, it comprises an N-terminal collagen-like domain connected by a neck to a C-terminal carbohydrate recognition domain (CRD) [4]. Six trimers are linked by disulfide bridges in an octadecamer of 650,000 D, in a "flower bouquet" alignment pattern [4,5]. A complex, predominantly triantennary, carbohydrate chain of ~4,000 D [6] is attached to the asparagine at position 187 of the CRD [7]. SP-A is mainly present in the alveoli in association with phospholipids, only 1% being present in the free form [8,9]. The primary function of surfactant is to reduce alveolar surface tension at end expiration. It is now however clear that SP-A, together with SP-D, another hydrophilic surfactant protein, plays a major role in the innate defenses of lung [5-10]. SP-A, in particular, is able to bind several microorganisms and enhance their uptake by phagocytes, stimulate the production of free oxygen radicals, and induce phagocyte chemotaxis [11].

Most binding to micro-organisms, including influenza and herpes simplex viruses, Gram-positive and Gram-negative bacteria, mycobacteria, fungi, and *Pneumocystis carinii*, occurs via the CRD and is inhibited by sugars or calcium chelators [12].

Since some SP-A is present in the alveoli in the free form, it has a chance of coming into contact with α_1-AT. We hypothesized that, in analogy with what happens with infectious agents, SP-A could bind to α_1-AT, which carries 3 biantennary or triantennary asparagine-linked carbohydrate chains [13].

In this paper we provide *in vitro* evidence that the inhibitory activity of α_1-AT towards HNE is significantly decreased in the presence of SP-A, probably as a consequence of SP-A binding to α_1-AT. Such an interaction would represent a novel mechanism of regulating alveolar α_1-AT. This could have relevance both for the pathogenesis of emphysema in patients with the Z α_1-AT variant and for the lungs' defenses against infectious agents.

Methods

Preparative procedures

All reagents were of analytical grade, unless otherwise specified. The buffer used in all experiments was 0.2 M Na-K phosphate, with 0.5 M NaCl, 2 mM $CaCl_2$, and 0.05% w/w Triton × 100, pH 8.0 (phosphate buffer), unless otherwise specified. Lipopolysaccharide (LPS) from *E. coli* serotype 026:B6 (Sigma) and methyl-α-D-mannopyranoside ($MNOCH_3$) (Sigma) were dissolved in phosphate buffer. HNE and human α chymotrypsin (αChy) (ART, Athens, GA) were dissolved in 50 mM sodium acetate, 150 mM NaCl, pH 5.5 and diluted with phosphate buffer. N-glycocosidase F from *Flavobacterium meningosepticum* (PNGase F; EC 3.5.1.52) was purchased from Roche Diagnostics (Monza, Italy). *Clostridium histolyticum* collagenase type III (EC 3.4.24) came from Calbiochem (La Jolla, CA). The chromogenic substrates MeOSucAlaAlaProValNA (for HNE) and SucAlaAlaProPheNA (for αChy), and the irreversible inhibitors MeOSucAlaAlaProValCMK (for HNE) and TosPheCMK (for αChy) (all from Sigma) were dissolved in $(CH_3)_2SO$. Wild-type α_1-antitryspin (M α_1-AT) was either from ART or purified from human serum by covalent chromatography. Capillary isoelectric focusing (CIEF) with bare fused-silica capillaries filled with polyethylene oxide and carrier ampholyte solutions in the pH 3.5–5.0 range [14] was applied to confirm the presence of the common M α_1-AT variant. Z α_1-AT variant was purified by covalent chromatography from subjects identified within the Italian screening program for α_1-AT deficiency [15]. SP-A was isolated as described [16] from surfactant obtained from 3 patients affected by pulmonary alveolar proteinosis (PAP), subjected to therapeutic whole lung lavage [17] and from adult New Zealand rabbits. To isolate surfactant, the bronchoalveolar lavage fluid was filtered through gauze and centrifuged at 150 g for 10 minutes. The supernatant was centrifuged for 30 minutes at 80,000 × g and the resulting pellet was suspended in 10 mM Tris-HCl pH 7.4, 145 mM NaCl, 1.25 mM $CaCl_2$, 1 mM $MgCl_2$, 2.2 M sucrose (solution A), overlaid with 10 mM Tris-HCl pH 7.4, 145 mM NaCl, 1.25 mM $CaCl_2$, 1 mM $MgCl_2$, 2 M sucrose (solution B) and ultracentrifuged overnight at 85,000 × g in a Ti 60 rotor (Beckman). The floating material was dispersed in water and centrifuged for 30 minutes at 100,000 × g and the pellet recovered was stored at -70°C (purified surfactant). To obtain SP-A, surfactant was injected into a 50-fold excess by volume of 1-butanol and stirred at room temperature for 30 minutes. After centrifugation, the pellet was suspended in 1-butanol and recentrifuged at 4,000 × g for 1 hour at room temperature. The final precipitate was dried under nitrogen and then resuspended in 5 mM Tris-HCl, 145 mM NaCl, 20 mM octyl β-D-glucopyranoside, pH 7.4 (solution C). After centrifugation at 100,000 × g for 1 hour, the pellet was resuspended in 5 mM Tris-HCl pH 7.4 (solution D) and dialyzed against solution D for 48 hours with at least six changes. The final solution was centrifuged at 100,000 × g for 1 hour and the resulting supernatant, containing purified SP-A, was stored. Endotoxin-free SP-A was obtained by treatment with polymyxin-B (Sigma). Small aliquots of

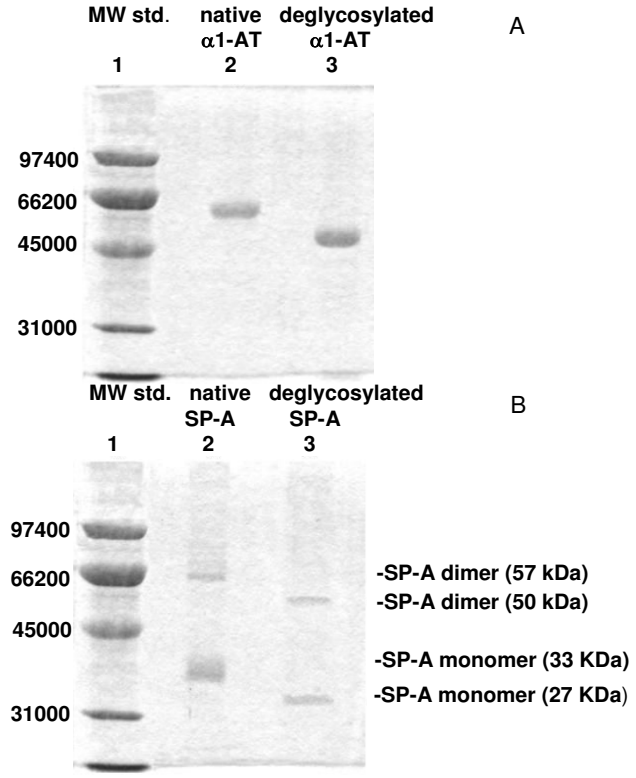

Figure 1
SDS-PAGE under reducing conditions. **A**: α_1-AT; **B**: SP-A. *Lane 1*: molecular weights; *lane 2*: native protein; *lane 3*: deglycosylated protein. The two bands in *gel B, lanes 2 and 3* correspond to dimers (57 and 50 kDa, respectively) and monomers (33 and 27 kDa, respectively) of SP-A.

SP-A in solution D were incubated in a 1:1 ratio for 6 hours at 4°C with polymyxin-agarose previously equilibrated with 5 mM Tris-HCl, 100 mM octyl β-D-glucopyranoside and 2 mM EDTA, pH 7.4. Polymyxin-agarose was removed by centrifugation at 14,000 × g for 15 minutes, and the supernatant was then dialyzed against 5 mM Tris-HCl pH 7.4 for 48 hours with at least six changes and lyophilized [17,18]. For some experiments polymyxin-treated SP-A was further purified by D-mannose sepharose 4B chromatography. SP-A was added to a small column containing D-mannose sepharose 4B (Pharmacia) previously equilibrated with 5 mM HEPES, 0.4% Triton × 100 and 1.5 mM $CaCl_2$, pH 7.2 (solution E), and the column was washed extensively with solution E. SP-A was finally eluted with 5 mM HEPES, 0.4% Triton × 100 and 2.5 mM EDTA, pH 7.2 (solution F).

Modification of the native proteins

Native and modified proteins used in our experiments were at high degree of purification (Figure 1). *See* additional file 1*for more details*.

Identification of the SP-A/ α_1-AT complex

1) Gel filtration HPLC

A mixture of SP-A (1.62 mg/ml) and α_1-AT (1 mg/ml) in a 1:50 molar ratio was incubated for 24 hrs at 37°C in phosphate buffer. The SP-A/α_1-AT mixture and single proteins were loaded in a Jasco PU 980 HPLC system (Japan Spectroscopic, Tokyo, Japan) equipped with two Biosep-SEC-S 4000 columns (300 × 7.80 mm each, Phenomenex, Torrence, CA, USA) connected in series. Samples were eluted with 100 mM Na_2HPO_4, 2 mM $CaCl_2$, pH 6.8 at a flow rate of 0.3 ml/ min, and monitored at 220 nm. The excluded (V_0 = 12.43 ml) and total (V_t = 24.82 ml) volumes were determined using dextran and creatinine, respectively; a calibration curve was obtained by running through the column a set of standard proteins: α_2-macroglobulin (725 kD), aldolase (158 kD), bovine serum albumin (67 kD), chymotrypsinogen (25 kD), and cytochrome C (12.5 kD). The results were reported as mean ± SD of three separate experiments. 2

2) Qualitative immunodetection by ELISA

250 ng of standard α_1-AT, purified SP-A, and SP-A/α_1-AT complex collected from the Size Exclusion Chromatography experiments, were immobilized in 50 mM Na_2CO_3, pH 9.5 overnight at 4°C in a polypropylene plate (Corning, New York, USA). Plates were then brought at room temperature, washed with 150 mM NaCl, 0.1% Tween 20 (ELISA buffer), blocked for 1 h with 50 mM Na_2CO_3, 2% BSA pH 9.5, incubated for 2 hrs in the presence of primary antibodies diluted 1:500 (goat anti-human α_1-AT and rabbit anti-human SP-A; ICN, Aurora, OH, USA), washed and finally reacted for 2 hrs with the appropriate biotinylated secondary antibodies diluted 1:5000 (Chemicon, Temecula, CA, USA). After washing, 100 μL of avidin diluted 1:2000 were added, and samples were incubated for 30 min. Color development was achieved by incubating the samples with 1,2-phenylenediamine dihydrochloride (Dako, Bucks, UK). The reaction was stopped by addition of 100 μl of 0.5 M H_2SO_4 and OD was read at 490 nm with a Bio-Rad 680 Microplate Reader (Bio-Rad Laboratories, CA, USA).

Kinetics studies

Rate constants were derived by competition experiments of HNE and αChy. Kinetic parameters were determined as described [20,21]. The active sites of HNE and αChy were titrated using a procedure based on the measurement of pNa released after enzymatic cleavage of MeOSucAlaAlaProValNA and SucAlaAlaProPheNA, respectively, at 37°C [22]. Product formation was monitored spectro-

Table 1: Association rate constant (K_{ass} $M^{-1}sec^{-1}$) for inhibition of HNE by α_1-AT with SP-A. SP-A employed was both from humans affected by PAP or from rabbit, polymyxin treated and polymyxin-mannose treated. Data are means ± SD of three different experiments.

Reaction conditions		K_{ass}, ($M^{-1}sec^{-1}$) means ± SD			
		Human SP-A			Rabbit SP-A
α_1-AT nM	SP-A nM	*Native*	*Polymyxin-treated*	*Polymyxin/Mannose-treated*	*Native*
7.5	0	3.40 ± 0.0079 × 10^7	3.40 ± 0.0079 × 10^7	3.40 ± 0.0079 × 10^7	3.40 ± 0.0079 × 10^7
7.5	0.15	1.84 ± 0.0577 × 10^7	1.84 ± 0.0580 × 10^7	1.86 ± 0.0565 × 10^7	1.82 ± 0.0585 × 10^7
7.5	1.5	1.70 ± 0.0623 × 10^7	1.70 ± 0.0631 × 10^7	1.72 ± 0.0618 × 10^7	1.68 ± 0.0620 × 10^7
7.5	7.5	5.20 ± 0.0483 × 10^6	5.22 ± 0.0480 × 10^6	5.00 ± 0.0478 × 10^6	5.40 ± 0.0490 × 10^6
7.5	15	4.30 ± 0.0513 × 10^6	4.30 ± 0.0520 × 10^6	4.30 ± 0.0520 × 10^6	4.40 ± 0.0498 × 10^6

photometrically at a wavelength of 405 nm using a Bio Rad Microplate Reader model 3550. To titrate the different forms of α_1-ATs (α_1-AT, deglycosylated α_1-AT and Z α_1-AT), 7.5 nM HNE was incubated for 15 min at 37°C with 0–100 nM inhibitor, in the presence of 2 mM MeO-SucAlaAlaProValNA. All following kinetic experiments were derived from α_1-ATs and SP-A/α_1-ATs complexes (obtained by incubation of α_1-ATs, from 0 to 25 nM, with SP-A 15, 7.5, 1.5, 0.15 mM for 15 min at 37°C). *See* additional file 1*for more details*.

Results

To investigate the interaction between SP-A and α_1-AT we studied whether K_{ass} values, derived from incubating HNE with α_1-AT, were modified by SP-A. Indeed we found a progressive decrease in the K_{ass} as the SP-A concentrations increased (Table 1), irrespective of the animal source of SP-A. To exclude that the observed effect was due to LPS co-purified with SP-A [23], we repeated the assay using endotoxin-free SPA, but found no differences with native SP-A (Table 1). To reinforce this finding, in separate experiments we spiked α_1-AT and SP-A/α_1-AT mixtures with increasing amounts of LPS, without measurable effect on the K_{ass} of α_1-AT or SP-A/α_1-AT mixture (not shown). As expected, [24], we found that the K_{ass} of Z α_1-AT for HNE was 3.5 fold lower than that of the normal, M α_1-AT. When Z α_1-AT was coupled with increasing SP-A concentrations, a further decrease in K_{ass} towards HNE was observed (Table 2).

To exclude that the results were due to non-specific binding, we incubated 7.5 nM HNE with 0–100 nM α_1-AT for 15 min at 37°C in microtiter plates or in glass tubes and then measured the residual HNE activity with 2 mM MeO-SucAlaAlaProValNA, finding no difference between plastics and glass. Furthermore, to exclude binding of SP-A to plastics we incubated 15 nM SP-A with $I^{125}\alpha$1-AT (from 0 to 100 nM) at 37°C. The number of Cpm of the samples with SP-A were the same of wells without proteins. We concluded that our data were compatible with binding of α_1-AT to SP-A.

Gel filtration HPLC was then used to determine the molecular weight of the SP-A/α_1-AT complex. As shown in Figure 2A, profile a, a mixture of SP-A and α_1-AT (1 mg/ml), gave two peaks, one corresponding to free α_1-AT (unreacted α_1-AT) and one, with a theoretical molecular weight of 1,642 kD (α_1-AT/SP-A complex), possibly corresponding to a complex made by one molecule of SP-A (670 kD) and 18 molecules of α_1-AT (54 kD), suggesting that, under the experimental conditions applied, each monomer of SP-A bound one molecule of α_1-AT. Further evidence that the first peak of profile a (Figure 2A) contained the complex SP-A/α_1-AT was obtained by using an immunochemical assay in which a polypropylene plate was probed with antisera anti α_1-AT and anti SP-A. As shown in Figure 2B, the first peak in profile a of Figure 2A contained both α_1-AT and SP-A.

The effect of SP-A on the K_{ass} of α_1-AT for HNE was calcium-dependent, being abrogated by EDTA (Figure 3). Since the calcium-binding domain of SP-A lays at the COOH terminus, next to the CRD [25], we supposed that this part of SP-A could be involved in the binding of SP-A to α_1-AT, *via* the α_1-AT carbohydrate chains. Consistent with these findings, the addition of 1 M mannopyranoside to the SP-A/α_1-AT mixture almost totally reversed the reduction in the K_{ass} (Figure 3), most likely by interfering with the binding of CRD to α_1-AT carbohydrate chains [26,27]. The fact that the lipid recognition domain of SP-A is located in the neck region of the molecule, far from the CRD [23], could explain the lack of influence of LPS on the binding of SP-A to α_1-AT (Table 1).

Table 2: Association rate constant for inhibition of HNE by α_1-AT and Z α_1-AT with SP-A. Data are means ± SD of experiments performed in triplicate with 3 different batches of human SP-A and1batch of rabbit SP-A.

α_1-AT/SPA ratio	α_1-AT				Z α_1-AT			
	Reaction condition		K_{ass}, ($M^{-1}sec^{-1}$)	decrease in K_{ass}, n-fold	Reaction condition		K_{ass}, ($M^{-1}sec^{-1}$)	decrease in K_{ass}, n-fold
	α_1-AT nM	SP-A nM			Z α_1-AT nM	SP-A nM		
	7.5	0	$3.40 \pm 0.0079 \times 10^7$	0	7.5	0	$9.80 \pm 0.0032 \times 10^6$	0
50	7.5	0.15	$1.84 \pm 0.0577 \times 10^7$	1.8	7.5	0.15	$5.20 \pm 0.0314 \times 10^6$	1.9
5	7.5	1.5	$1.70 \pm 0.0623 \times 10^7$	2	7.5	1.5	$4.70 \pm 0.0268 \times 10^6$	2.1
1	7.5	7.5	$5.20 \pm 0.0483 \times 10^6$	6.5	7.5	7.5	$4.30 \pm 0.0240 \times 10^6$	2.3
0.5	7.5	15	$4.30 \pm 0.0513 \times 10^6$	7.9	7.5	15	$3.80 \pm 0.0221 \times 10^6$	2.6

To better clarify the role of the CRD in the binding of SP-A to α_1-AT, we modified both proteins by enzymatic digestion, deglycosylation or boiling and then used them to calculate the K_{ass} of α_1-AT for HNE and to deduce the molar parts of α_1-AT bound to SP-A from the number of turnovers per inactivation of α_1-AT not bound to SP-A. Thus we found that the CRD of SP-A appears to contain all the putative SP-A binding sites for α_1-AT since, when incubated with α_1-AT, it retained the same K_{ass}, as that of native SP-A (Figure 4).

Turnover per inactivation (also referred to as stoichiometry of inhibition (SI) or partition ratio + 1) defines the number of moles of irreversible inhibitor required to completely inhibit 1 mole of target proteinase. The turnover number resulting from the interaction between unmodified SP-A and α_1-AT was 24, i.e. one part of SP-A binds 23 molar parts of α_1-AT and 24 SP-A plus α_1-AT binds inhibit 1 part of enzyme (Figure 5). The same binding pattern emerged when Z α_1-AT was used instead of α_1-AT, suggesting that the difference in the K_{ass} between the two variants of α_1-AT is independent of the number of molar parts of inhibitor bound to SP-A.

Deglycosylated α_1-AT retains its ability to inhibit HNE (K_{ass} 3.38×10^7 $M^{-1}sec^{-1}$). We did, however, find that the inhibitory activity of α_1-AT is greatly decreased in the presence of SP-A (K_{ass} 1.1×10^7 $M^{-1}sec^{-1}$, Figure 4), indicating that binding of SP-A to the carbohydrate moiety of α_1-AT is not the only mechanism involved. The turnover number of the SP-A/deglycosylated α_1-AT is 12, half that displayed by native α_1-AT (Figure 4, 5). To explore other mechanisms of binding between SP-A and α_1-AT, we incubated boiled SP-A and α_1-AT. We found that boiled SP-A/native α_1-AT displayed the same K_{ass} and the same turnover number as native SP-A/deglycosylated α_1-AT (Figures 4, 5). We postulated that SP-A carbohydrate chains could bind α_1-AT, possibly through the amino acid backbone. In fact, carbohydrate chains isolated from SP-A mixed with deglycosylated α_1-AT resulted in the same K_{ass} and turnover number as those of native SP-A/deglycosylated α_1-AT (Figures 4, 5). Besides these mechanisms of binding of SP-A to α_1-AT, a third mechanism, i.e. a carbohydrate/carbohydrate interaction, probably exists since boiled SP-A and native α_1-AT displayed a K_{ass} of 1.9×10^7 $M^{-1}sec^{-1}$ and ~6 turnovers (Figure 4, 5).

Finally, we studied the binding of deglycosylated SP-A to α_1-AT. The K_{ass} of native α_1-AT mixed with deglycosylated SP-A was 1.2×10^7 $M^{-1}sec^{-1}$ and the turnover number 18 (Figure 4, 5). Absence of SP-A/α_1-AT binding, i.e. K_{ass} 3.4×10^7 $M^{-1}sec^{-1}$, and a turnover number of 1, was achieved by two combinations: 1) SP-A deglycosylated and boiled with native α_1-AT, and 2) both proteins deglycosylated (Figures 4, 5). In the former case, absence of SP-A carbohydrates and denaturation of CRDs hindered any possible binding of SP-A to native α_1-AT. In the latter case, the binding was precluded by the absence of carbohydrates on both proteins, in spite of the presence of intact CRDs in the SP-A.

Discussion

The present data provide evidence for an *in vitro* interaction between SP-A and α_1-AT. These glycoproteins belong to two systems of the lung that are supposed to act independently: the surfactant system and the proteinase/proteinase inhibitor system. Nevertheless, evidence for possible links between the two systems does exist. As an example, it has been shown that SP-A may be digested by elastolytic enzymes [28,29], and that inhalation of α_1-AT in patients with cystic fibrosis may result in an increase of SP-A levels in bronchoalveolar lavage fluid (BALf) [30]. In addition, SP-D induces the production of matrix metalloproteinases by human alveolar macrophages [31], whereas the cysteine proteinase cathepsin H is involved in the first N-terminal processing step of SP-C [32]. The two systems may therefore interact in the lungs, both in physiologic and in pathologic pathways. The concentration of

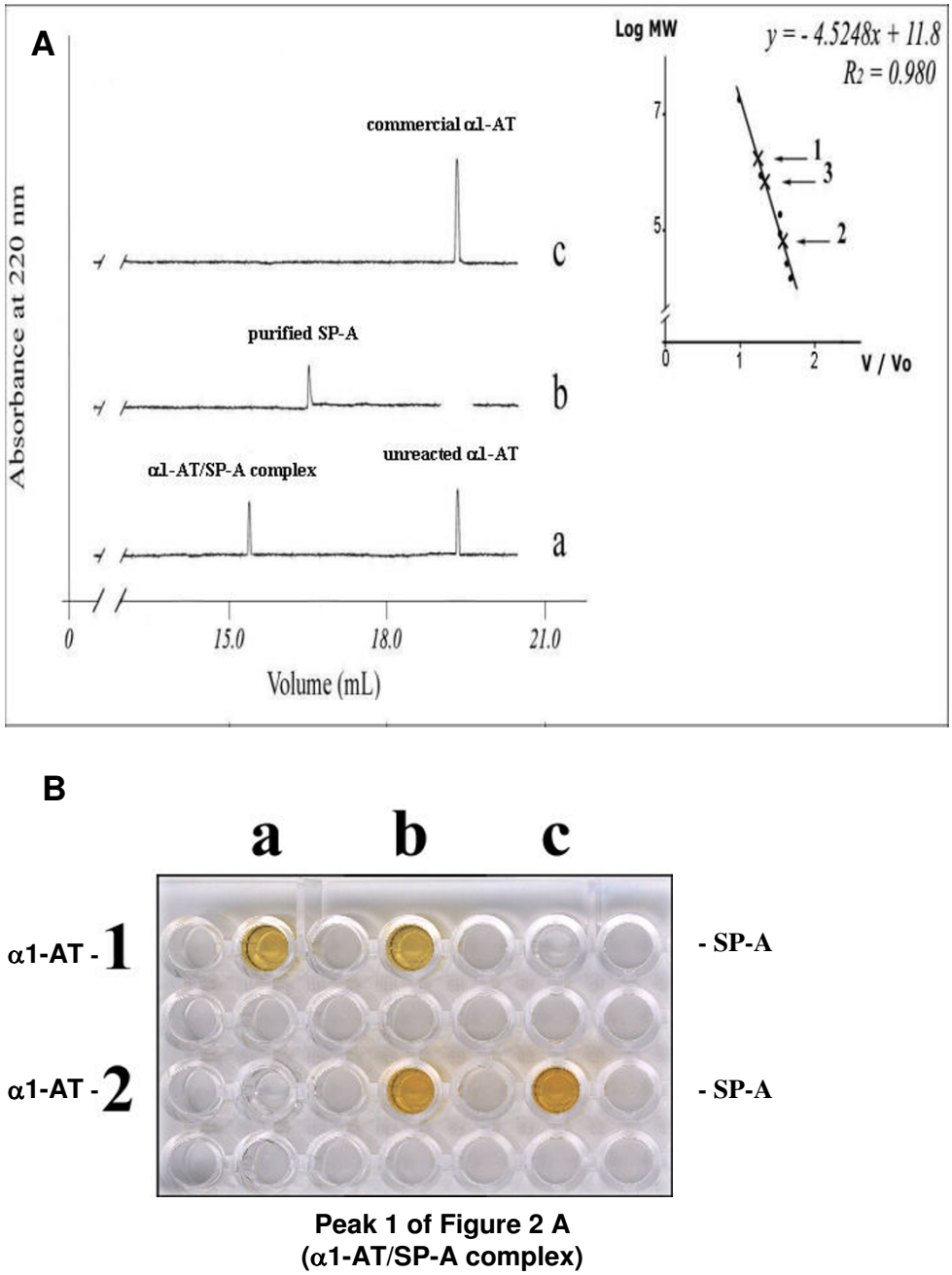

Figure 2

Isolation and immunodetection of the α_1-AT /SP-A complex. **A**: Isolation of the complex by gel filtration chromatography on two Biosep SEC – S 4000 columns connected in series using HPLC. Gel filtration profiles: commercial α_1-AT (*in profile c*; 19.32 ± 0.1 mL); purified SP-A (*in profile b*; 16.49 ± 0.07 mL); α_1-AT /SP-A complex (*in profile a*; 15.31 ± 0.04 mL) and unreacted α_1-AT (*in profile a*; 19.35 ± 0.09 mL). *Inset*: calibration curve obtained using the following standards: A = α_2-macroglobulin (725 kDa), B = aldolase (158 kDa), C = bovine serum albumin (67 kDa), D = chymotrypsinogen (25 kDa), E = cytocrome C (12.5 kDa). **B**: Immunodetection of the complex. α_1-AT was added to wells a1 and a2, peak 1 (α_1-AT /SP-A complex) of Figure 2A was added to wells b1 and b2, and SP-A to wells c1 and c2. Antiserum anti-α_1-AT was added to wells a1, b1 and c1, antiserum anti-SP-A was added to wells a2, b2 and c2. Peak 1 (α_1-AT /SP-A complex) is recognized by both antisera.

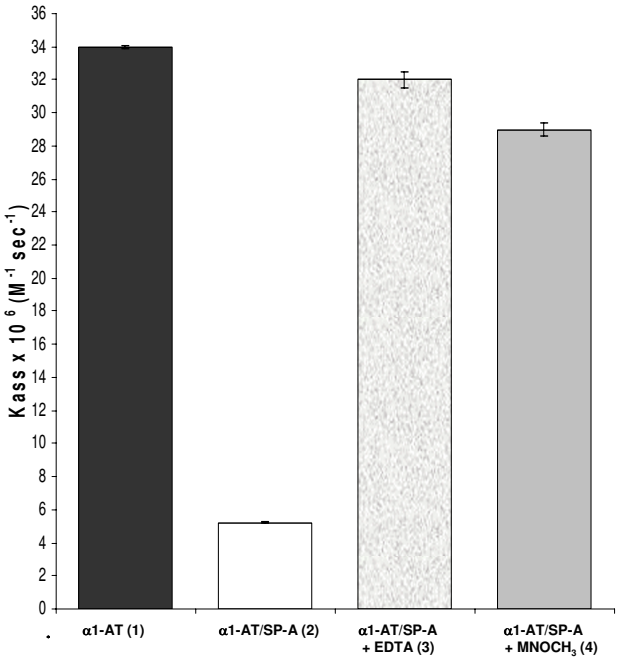

Figure 3
Effects of calcium removal and sugar addition on K_{ass} $M^{-1}sec^{-1}$. Inhibition of HNE by α_1-AT (7.5 nM), alone or coupled with 7.5 nM SP-A: (1) α_1-AT alone, (2) α_1-AT plus SP-A, (3) α_1-AT plus SP-A with 5 mM EDTA and (4) α_1-AT plus SP-A with 1 M $MNOCH_3$. (Data are means ± SD of experiments performed in triplicate).

SP-A in the BALf of normal subjects is estimated to be ~277 nM [33]. Since approximately 1% of total SP-A is present in the free form [8,9], its concentration in BALf would be ~2.8 nM. Given that the concentration of α_1-AT is ~5 μM [34], we reasoned that the two glycoproteins have a good chance of coming into contact during their life cycle.

Indeed our *in vitro* experiments indicate that the interaction between SP-A and α_1-AT results in binding between them. This binding, which is calcium-dependent, appears to be complex since it could involve binding between the CRD of SP-A and carbohydrates on α_1-AT, binding between SP-A carbohydrates and the protein backbone of α_1-AT, and binding between the carbohydrate chains of both proteins.

Turnover per inactivation suggests that one part of SP-A binds 23 molar parts of α_1-AT. Nevertheless, SP-A binds 11 molar parts of deglycosylated, fully active α_1-AT (Figure 4, 5), thus suggesting a possible binding of SP-A carbohydrate chains to the amino acid backbone of α_1-AT. Asn, to which carbohydrates of the native glycoprotein are linked [35], is a likely candidate. This hypothesis was confirmed by the results obtained with boiled SP-A and with isolated SP-A carbohydrate chains (Figure 4, 5). In support of this hypothesis, it has been reported that the binding of SP-A to influenza virus [36], herpes virus type 1 infected cells [37], and *M. tuberculosis* [38], involves N-linked carbohydrate chains on SP-A. Interestingly, there may be multiple binding sites on individual micro-organisms [12].

Our experiments also suggest a possible carbohydrate/carbohydrate interaction between SP-A and α_1-AT. Such a type of linkage has been shown to operate in the calcium-mediated homotypic interaction between two Lewis (Le^x) determinants (Galβ1→4[Fucα$_1$→3]GlcNAc) involved in cell adhesion during murine embryogenesis [39]. Interestingly Le^x-Le^x interactions appear to be calcium-dependent [40], by involving van der Waal forces. The fact that ultra-weak interactions are involved explains why this aspect is often underestimated [39-41].

It is difficult to postulate whether the three proposed mechanisms of binding take place simultaneously between native proteins. It may be that the CRD plays the main role and that the other two mechanisms are less important or take place only as artificial mechanisms once the proteins have been manipulated.

The binding with SP-A results in a decrease in the inhibition of HNE by α_1-AT. There are several known mechanisms that could explain the inactivation of α_1-AT. Beside the physiologic irreversible suicide substrate mechanism by which α_1-AT inhibits HNE [42], α_1-AT may also be inactivated by oxidation of methionine residue(s) located at or near the active site [22,23]. Another mechanism of α_1-AT inactivation is proteolytic degradation at or near the active site by a number of host and non-host, mostly microbial, proteinases [42]. Whether these mechanisms may act *in vivo*, thereby contributing to the imbalance between proteinases and inhibitors in the pathogenesis and progression of pulmonary emphysema, is still a debated issue.

With respect to the inhibitory activity of α_1-AT, that of Z α_1-AT is further impaired by this latter's enhanced tendency to undergo spontaneous polymerization [2]. This phenomenon, also known as loop-sheet polymerization, likely accounts for why Z α_1-AT is less efficient at inhibiting HNE, and has been demonstrated to be present *in vivo*, since Z α_1-AT polymers have been detected in the BALf of Z α_1-AT subjects with emphysema [45]. We found that SP-A binds Z α_1-AT and that the binding further reduces the K_{ass}, which is already impaired with respect to that of α_1-AT. Were this binding to happen *in vivo*, it would further decrease the antiproteinase activity of Z α_1-AT.

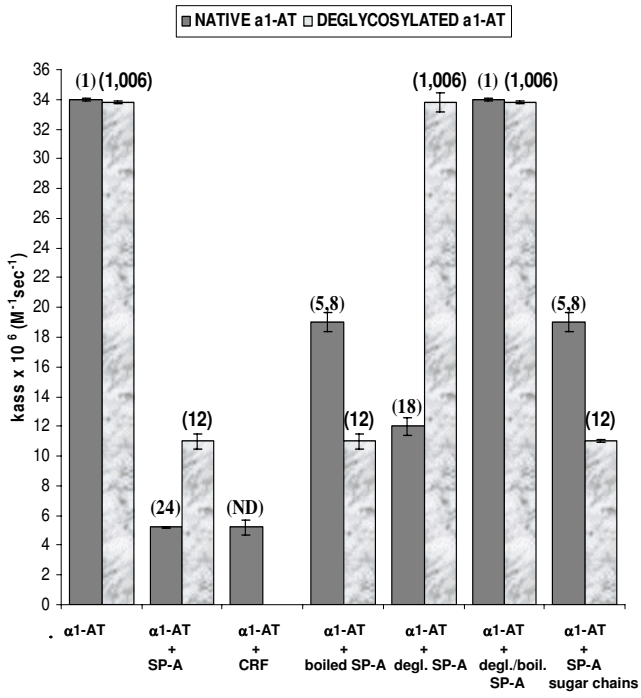

Figure 4
***K*$_{ass}$ M^{-1}sec^{-1} for inhibition of HNE by modified proteins (7.5 nM), alone or in combination**. *K*$_{ass}$ data are means ± SD of experiments performed in triplicate. SI values of the associations in bold at the top of the figure.

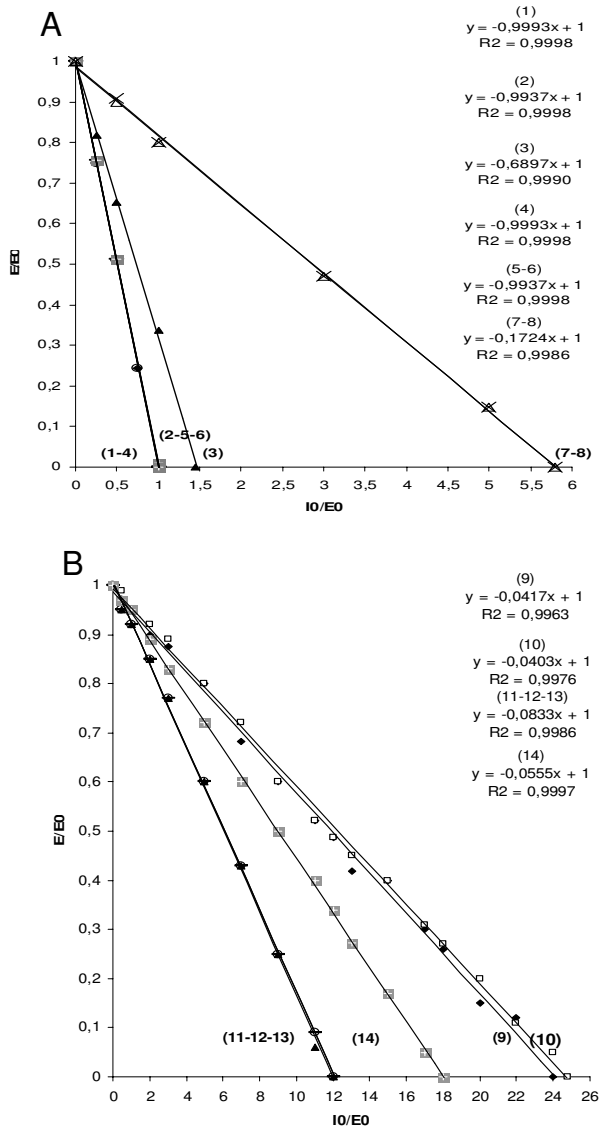

Figure 5
Turnover numbers per inactivation. Turnover numbers were determined plotting residual enzyme activity/initial enzyme activity versus initial inhibitor concentration/initial enzyme activity. **A**: (1) native α_1-AT, (2) deglycosylated α_1-AT, (3) Z α_1-AT, (4) native α_1-AT coupled with deglycosylated and boiled SP-A, (5) deglycosylated α_1-AT coupled with deglycosylated SP-A, (6) deglycosylated α_1-AT coupled with deglycosylated and boiled SP-A, (7) native α_1-AT coupled with boiled SP-A and (8)native α_1-AT coupled with SP-A sugar chains. **B**: (9) native α_1-AT coupled with native SP-A and (10) Z α_1-AT coupled with native SP-A, (11) deglycosylated α_1-AT coupled with native SP-A, (12) deglycosylated α_1-AT coupled with boiled SP-A, (13) deglycosylated α_1-AT coupled with SP-A sugar chains and (14) native α_1-AT coupled with deglycosylated SP-A.

The mechanism by which SP-A binding interferes with the α_1-AT inhibitory mechanism is open to speculation. α_1-AT inactivation taking place *in vitro* upon interaction between the two glycoproteins seems to occur because of the functional slowdown of α_1-AT in the presence of SP-A, the turnover number shifting from 1 to 24. After an initial, non-covalent, Michaelis-like complex, the reaction between α_1-AT and HNE progresses, through an acyl-enzyme intermediate resulting from peptide bond hydrolysis, to either a loop-inserted covalent complex (*inhibitory pathway*) or a cleaved serpin and free proteinase (*non-inhibitory or substrate pathway*) [42]. The number of turnovers for native α_1-AT is 1 (Figure 5), indicating that the reaction inhibitor-HNE progresses towards the inhibitory pathway on the other side (Figure 6A). The number of turnovers after the incubation of native α_1-AT or Z α_1-AT with native SP-A is 24 (Figure 5), thus indicating that for α_1-AT bound to SP-A the inhibitory pathway is precluded, and that the reaction inhibitor – HNE progresses mostly through the substrate pathway (Figure 6B).

In spite of the detailed dissection of the binding mechanism of SP-A to α_1-AT *in vitro*, an obvious limitation of the

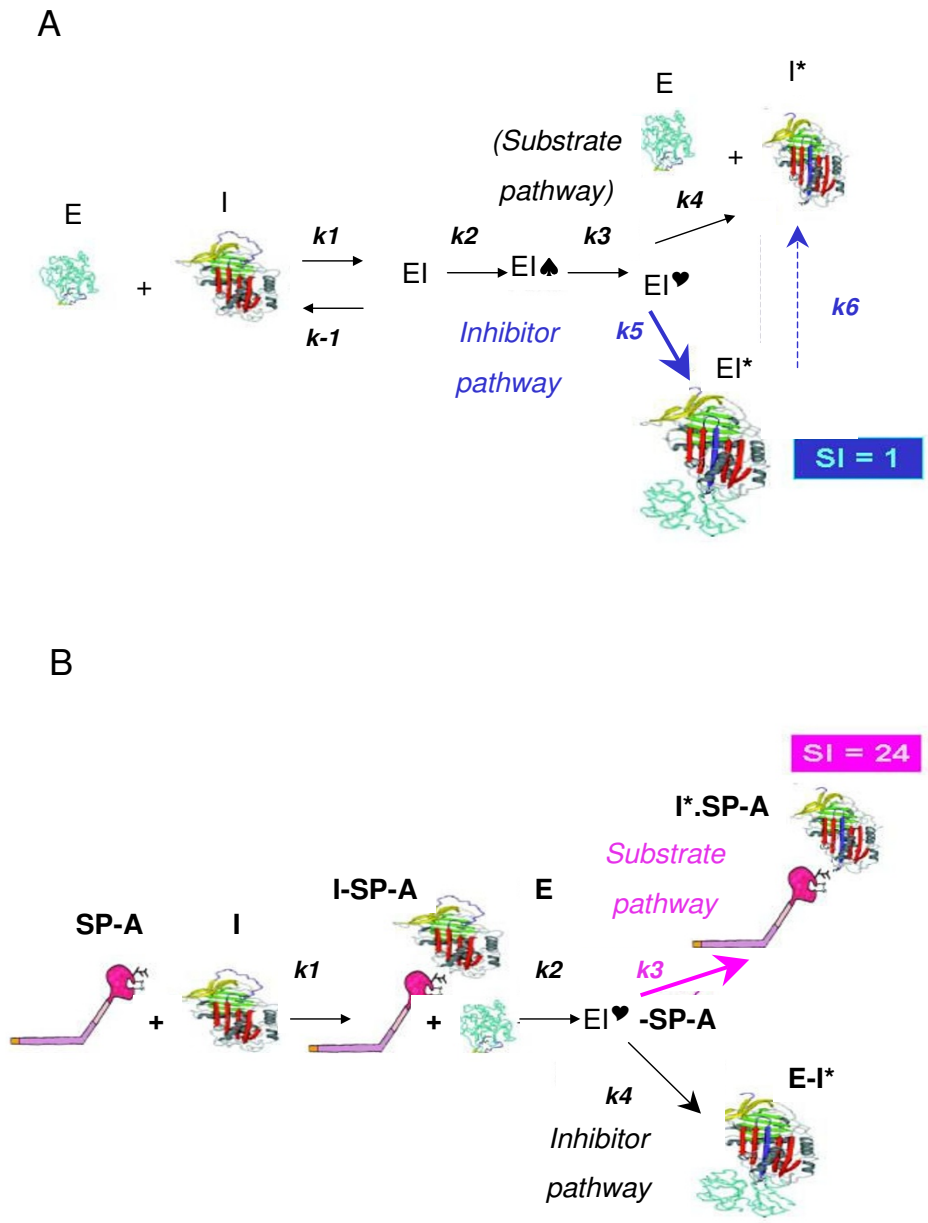

Figure 6

Hypothetical mechanism of SP-A interference with α_1-AT (simplification). **A**: interaction of α_1-AT (I) with HNE (E). After an initial non-covalent Michaelis-like complex (EI), the interaction progresses through a tetrahedral intermediate (EI ♠), forming a covalent acyl-enzyme intermediate (EI ♥). The substrate pathway results in free HNE and cleaved α_1-AT (I*); the inhibitory pathway results in a, about 100%, kinetically trapped loop-inserted covalent complex (E-I*). **B**: the SP-A (here shown as a trimer) interacts with α_1-AT. In the presence of HNE, the formation of a covalent complex E-I* almost precluded (about 4%), and the reaction progresses through the substrate pathway towards free E and I* (cleaved α_1-AT) – SP-A (96%). SI = stoichiometry of inhibition

present paper is the lack of specific studies investigating a possible interaction between SP-A and α_1-AT *in vivo*. Nevertheless, some indirect evidence suggesting that such an interaction might take place is available, although it is not possible to address a plausible expectation of physiologic or pathophysiologic relevance of these findings. For example, a recent report has shown that in human sputum supramolecular complexes with heparan sulfate/Syndecan-1 and proteinase and inhibitors are present [46]. These complexes contain the proteinase inhibitors SLPI and α_1-AT, NE as well, whose proteolytic activity is however not decreased . The large MW of SP-A makes difficult to highlight the occurrence of such supramolecular complexes including α_1-AT by standard techniques [47]. Nevertheless, a report focusing on two-dimensional electrophoretic characteristics of BALf proteins in subjects affected by interstitial lung diseases [48] has intriguingly shown that some α_1-AT fragments were superimposed on spots of SP-A, in its upper, acidic position. These findings, confirmed by mass spectrometric MALDI-TOF analysis, would suggest a possible SP-A/α_1-AT interaction taking place *in vivo*.

Conclusion

We have shown that SP-A binds α_1-AT, and that this binding results in a significant decrease in the association rate constant of α_1-AT for HNE. The mechanism of the binding seems to be predominantly mediated by the SP-A CRDs, as indicated by the calcium dependence and by the turnovers for inactivation, but other mechanisms may be involved, such as an interaction between SP-A carbohydrates and the α_1-AT amino acid backbone or between carbohydrate chains of both glycoproteins. The presence of these complex binding mechanisms would exclude the hypothesis that the α_1-AT inhibition occurred simply due to steric inhibition of the large SP-A molecule, but it would rather suggest a programmed, coordinated mechanism.

The *in vitro* interaction described here, if present *in vivo*, would be a novel mechanism of impairment of α_1-AT inhibitory activity. It might represent a physiologic mechanism of regulating α_1-AT activity, especially in acute conditions (for example during defense against infections agents) [49], in which an excess of α_1-AT would interfere with the physiologic role of proteinases. α_1-AT is indeed a highly specialised proteinase inhibitor [50], but the presence in nature of several, robust mechanisms of α_1-AT downregulation (i.e. inherited deficiency, susceptibility to oxidative stress and proteolysis, polymerization) would imply the occurrence of intrinsic risks related to the overexpression of a nearly perfect and immortal inhibitor. Therefore, the formation of supramolecular complexes SP-A/α_1-AT might be a sort of reserve mechanism, taking place in case of need.

On the other hand, the interaction with SP-A would be of particular relevance in the pathogenesis of pulmonary emphysema associated with α_1-AT deficiency, since it would contribute significantly to the complex mechanisms of imbalance between Z α_1-AT and HNE in the lungs. Obviously, all these speculations need further investigations, first of all to understand whether or not SP-A/α_1-AT binding is a relevant down-regulatory mechanism of α_1-AT inhibitory activity *in vivo*.

Abbreviations

α1-AT, α1-antitrypsin

αChy, α chymotrypsin

$(CH_3)_2SO$, dimethylsulfoxide

CRD, carbohydrate recognition domain

CRF, collagenase-resistant fragment

HNE, human neutrophil elastase

$MNOCH_3$, methyl-α-D-mannopyranoside

PNA, p-nitroanilide

SP-A, surfactant protein-A

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

MG participated in the study design, performed most experiments, and helped to draft the manuscript. AL participated in the deglycosylation experiments and carbohydrate chains isolation. PI designed the experiments for the α1-AT/SP-A complex identification, and helped to draft the manuscript. CDS participated in the kinetic studies. PR performed the experiments for the α1-AT/SP-A complex identification. DD performed the purification of SP-A and CRF. NC took part to some kinetic experiments. EP participated in the coordination of the study. AB performed the purification of SP-A and CRF, helped to draft the manuscript and critically reviewed it. ML conceived the study, participated in its design, and coordinated the manuscript final version. All authors read and approved the final manuscript.

Additional material

Additional File 1

contain portion of Methods' section and include details on modification of native proteins used in the experiments and details of kinetic procedures.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1465-9921-6-146-S1.doc]

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